Universidade do Minho
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Translating Biology into Clinic: New Insights on Prognostic and Predictive Biomarkers for Urothelial Bladder Carcinoma

Da Biologia à Clínica: Evidências de Novos Biomarcadores de Prognóstico e Preditivos de Resposta à Terapêutica no Carcinoma Urotelial da Bexiga

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Resumo | Summary

Urothelial bladder carcinoma (UBC) represents a significant health problem, as a consequence of its heterogeneous natural history and clinical behavior. Most morbidity and mortality associated with UBC is caused by the muscle-invasive (MI) form of the disease, which represents about 20-30% of all newly diagnosed cases. Moreover, an important proportion of high risk non-muscle invasive (NMI) tumours relapse after transurethral resection and progress to MI disease. Despite radical cystectomy, half of the patients with MI tumours develop metastases. Although perioperative and palliative systemic chemotherapy is recommended for locally-advanced or metastatic UBC, survival benefits are impaired in a significant proportion of patients due to inherent or acquired chemoresistance. Currently, prognostication of patients with MI-UBC is severely hampered by the insufficiency of standard clinicopathological risk factors in accurately predicting individual treatment outcomes. This major drawback can potentially be overcome if biomarkers of tumour aggressiveness and response to chemotherapy are routinely evaluated and included in the pathology reports. Current research efforts are directed into the elaboration of nomograms that can combine well-established clinicopathological parameters with novel putative biomarkers. In this line of investigation, we aimed to characterize a phenotype of bladder cancer aggressiveness in a human series of UBC by studying the clinical and prognostic significance of a panel of distinct biomarkers that, although poorly explored in UBC setting, were described as being involved in tumour angiogenesis and lymphangiogenesis, invasion and metastasis, energy metabolism reprogramming and tumour microenvironment. Moreover, we intended to validate potential therapeutic targets in *in vitro* assays.

Angiogenesis, lymphangiogenesis and lymphovascular invasion (LI) occurrence was assessed with the use of immunohistochemical markers, namely the blood endothelial cell marker CD31, the lymphatic endothelial cell marker D2-40, the lymphangiogenic vascular endothelial growth factor (VEGF)-C and its receptor VEGFR-3. The specific staining of blood and lymphatic endothelium significantly contributed to an accurate evaluation of LI occurrence, and to a specific distinction between blood vessel invasion (BVI) and lymphatic vessel invasion (LVI). A correlation among high blood vessel density (BVD), high lymphatic vessel density (LVD), tumour progression and LI occurrence was found. BVI by malignant emboli assessed by CD31 staining, and LVI by isolated malignant cells assessed by D2-40 staining, significantly impaired overall survival, and BVI was identified as an independent prognostic factor. When included in a model of bladder cancer aggressiveness combining classical clinicopathological parameters with biomarkers, BVI and LVI contributed to separate between low and high aggressiveness groups. VEGF-C overexpression was correlated with an aggressive phenotype characterized by increased tumour stage, loss of differentiation, high BVD and LVD counts, and

occurrence of both BVI and LVI. All malignant cells expressed, monotonously, VEGFR-3. Our results endorse the need to establish a reproducible method of LI evaluation that can be incorporated into clinical practice, highlighting the potential role of this biological process in selecting patients who might benefit from adjuvant treatments.

P-mTOR (phospho–mammalian target of rapamycin) levels, as well as their correlation with occurrence of angiogenesis and lymphangiogenesis, were also investigated, aiming to unveil mTOR pathway as a possible mediator of neovasculatization in bladder cancer setting. Tissue sections with tumour and non-tumour regions were selected for analysis. Immunoexpression was observed in umbrella cells from non-tumour urothelium, in all cell layers of malignant NMI urothelium (with a reinforcement in superficial cells), and in spots of cells from MI lesions. P-mTOR expression decreased with increasing stage, but the few pT3/pT4 positive cases had worse survival rates. Conversely, occurrence of angiogenesis was impaired in pT3/pT4 negative tumours. Additional studies directed to the upstream and downstream effectors of this pathway need to be addressed, in order to further explore and clarify our results.

In the scenario of invasion and metastasis, we evaluated the immunoexpression of the endoglycosidase heparanase and of the metastasis suppressor RKIP (Raf kinase inhibitor protein). Heparanase was upregulated in the malignant urothelium, and exhibited a heterogeneous pattern, with the invasion front of the tumours being more intensely stained than the tumour's core, supporting its role in the disassembly of the extracellular matrix as an invasion-promoter mechanism. An opposite pattern was found when evaluating RKIP immunoexpression. This metastasis-supressor biomarker was homogeneously expressed in normal urothelium and in tumour sections with a favourable clinico-pathological profile. Heterogeneous expression, with the tumour centre being more intensely stained than the invasion front, associated with LI occurrence. Low RKIP expression significantly impaired prognosis, remaining as an independent prognostic factor for disease-free survival. Thus, RKIP loss emerges as a novel biomarker of UBC aggressiveness, and additional studies are necessary to validate our results and to further explore therapeutic strategies that can potentially restore RKIP functionality as a suppressor of bladder cancer metastases.

Reprogramming cellular energetics and modeling the tumour microenvironment are inherent traits of malignancy. Among the plethora of biomarkers associated with this hallmark of cancer, we investigated the immunoexpression of CD147, monocarboxylate transporters (MCTs), CD44 and carbonic anhydrase (CA) IX. We observed that MCT1 and MCT4 were overexpressed in malignant urothelial cells, associating with an unfavourable clinicopathological profile. MCT1 expression correlated

with poor prognosis. Significant associations were found between the pattern of expression of CD147, MCT1 and MCT4, supporting the role of CD147 as a chaperone for MCTs. CD147 upregulation clearly associated with UBC aggressiveness and poor prognosis, lowering significantly disease-free and overall survival rates. When included in a scoring system of UBC aggressiveness, CD147 overexpression allowed an accurate discrimination of bladder cancer patients' prognosis. There was a substantial concordance among CD44 and MCTs expressions, and CD44 and CD147, which suggests an interactive scenario where CD44, MCTs and CD147 cooperate in regulating the acidic microenvironment. Moreover, CD44 expression was also associated with UBC aggressiveness. CAIX exhibited a heterogeneous pattern of expression, being stronger at the hypoxic core of MI tumours or at the luminal face of papillary lesions, were its expression was predominant. CAIX expression correlated with MCT4, CD147 and CD44 expressions, supporting hypoxia as a trigger mechanism of the glycolytic phenotype. Importantly, the CD147/MCT1 double-positive profile associated with unfavourable clinico-pathological parameters and poor prognosis, and discriminated a poor-prognosis group within patients who received platinum-based chemotherapy. These interesting results led us to further investigate CD147 as a potential biomarker of aggressiveness and cisplatin resistance in UBC cell lines. CD147 specific downregulation was accompanied by a decrease in MCT1 and MCT4 expressions and, importantly, an increase in chemosensitivity to cisplatin. Our findings shed light into the putative role of CD147 and its interactions in determining progression and resistance to cisplatin-based chemotherapy in UBC setting, unraveling possibilities for target therapeutic intervention that urge to be investigated.

In summary, the results herein reported represent our contribution to a better understanding on biological parameters that seem to influence bladder cancer aggressiveness and chemoresistance, and should be further explored as potential prognosis/theranostics biomarkers and/or therapeutic targets.

O carcinoma urotelial da bexiga (CUB) representa um importante problema de saúde pública, em resultado da heterogeneidade associada à sua histogénese e comportamento clínico. A morbilidade e mortalidade associadas ao CUB são principalmente causadas pela variante músculo-invasora (MI), que representa cerca de 20-30% de todos os casos diagnosticados. Adicionalmente, uma proporção significativa de tumores não-músculo invasivos (NMI) de alto risco recidiva após a ressecção transuretral e progride para formas invasoras. Apesar de submetidos a cistectomia radical, metade dos doentes com tumores MI desenvolvem metástases. Em casos de CUBs localmente avançados ou disseminados, são recomendados esquemas de quimioterapia sistémica peri-operatória e paliativa. No entanto, potenciais benefícios em termos de sobrevivência são francamente diminuídos numa proporção significativa de doentes que apresentam quimio-resistência intrínseca ou adquirida. Atualmente, o prognóstico de doentes com CUBs MI é gravemente prejudicado pela dificuldade que os fatores de risco clínico-patológicos clássicos apresentam em prever, com precisão e por indivíduo, resultados dos tratamentos. Este grande entrave poderá ser potencialmente superado se biomarcadores de agressividade tumoral e resposta à quimioterapia forem rotineiramente avaliados e incluídos nos relatórios de patologia. Os esforços de pesquisa atuais são, cada vez mais, direcionados para a elaboração de nomogramas que combinem parâmetros clínicos padrão com possíveis biomarcadores. Nesta linha de investigação, o projeto descrito nesta tese teve como objetivo principal caracterizar um fenótipo de agressividade do CUB numa série de tumores, estudando o significado clínico e prognóstico de um painel de biomarcadores distintos que, apesar de pouco explorados no âmbito dos CUBs, foram já descritos como mediadores da angiogénese e linfangiogénese tumorais, invasão e metastização, e remodelação do metabolismo energético e do microambiente tumoral. Adicionalmente, pretendeu-se validar potenciais alvos terapêuticos em ensaios *in vitro*.

A ocorrência de angiogénese, linfangiogénese e invasão linfovascular (IL) foi avaliada através de marcação imuno-histoquímica, recorrendo a anticorpos anti- CD31 (marcador de células endoteliais sanguíneas), D2-40 (marcador de células endoteliais linfáticas), VEGF-C (fator linfangiogénico, *vascular endotelial growth factor C*) e VEGFR-3 (recetor de VEGF-C). A marcação específica dos endotélios sanguíneo e linfático contribuiu significativamente para uma avaliação precisa da ocorrência de IL, e para uma distinção específica entre invasão vascular sanguínea (IVS) e invasão vascular linfática (IVL). Foram encontradas correlações entre densidade vascular sanguínea (DVS) e densidade vascular linfática (DVL) elevadas, progressão tumoral e ocorrência de IL. A ocorrência de IVS por êmbolos de células malignas identificada pela marcação específica com CD31, assim como a ocorrência de IVL por células malignas isoladas identificada pela marcação específica com D2-40, diminuíram

significativamente a sobrevivência global. A ocorrência de IVS foi identificada como um fator independente de prognóstico. Quando incluídas num modelo de agressividade do CUB que combinou parâmetros clínico-patológicos clássicos com biomarcadores, a ocorrência de IVS e IVL contribuiu para a distinção entre grupos de baixa e elevada agressividade. O aumento de expressão de VEGF-C associou-se a um fenótipo de agressividade tumoral caracterizado pelo incremento do estádio patológico, perda de diferenciação, contagens de DVS e DVL elevadas, e ocorrência de IVS e IVL. O VEGFR-3 foi expresso, de forma monótona e consistente, pelo urotélio maligno. Tais resultados suportam a necessidade de estabelecer um método reprodutível de avaliação da ocorrência de IL que possa ser incorporado na prática clínica. Destaca-se o potencial papel deste processo biológico na seleção de doentes que poderão beneficiar de tratamentos adjuvantes.

Os níveis de p-mTOR (*phospho–mammalian target of rapamycin*), bem como a possível associação com a ocorrência de angiogénese e linfangiogénese, foram igualmente estudados, na tentativa de clarificar o papel da via mTOR como mediadora de neovascularização no CUB. Foram selecionadas secções tumorais com representação de mucosa não-tumoral adjacente. Observou-se imunoexpressão nas células em guarda-chuva do urotélio não-tumoral, em todas as camadas celulares do urotélio de tumores NMI (de maior intensidade nas células superficiais), e em *spots* de células nas lesões MI. A expressão do p-mTOR diminuiu com o aumento do estádio tumoral, mas os poucos tumores pT3/pT4 positivos associaram-se a piores prognósticos. Por outro lado, a ocorrência de angiogénese ficou comprometida nos tumores pT3/pT4 negativos. Será necessário realizar estudos adicionais direcionados aos restantes membros desta via de sinalização, na tentativa de clarificar os resultados agora obtidos.

Com o objetivo de explorar os fenómenos de invasão e metastização no CUB, avaliou-se a imunoexpressão da endoglicosidase heparanase e do supressor de metástases RKIP (*Raf kinase inhibitor protein*). Observaram-se níveis aumentados de heparanase no urotélio maligno, que exibiu um padrão heterogéneo, onde a frente de invasão tumoral se encontrava mais intensamente marcada do que o centro dos tumores, o que suporta o papel desta enzima na degradação da matriz extracelular, um mecanismo promotor de invasão. Em relação à proteína RKIP, foi encontrado um padrão de expressão oposto. Este biomarcador supressor de metástases foi homogeneamente expresso no urotélio normal e em secções tumorais caracterizadas por um perfil clínico-patológico favorável. Uma expressão heterogénea, com o centro do tumor mais intensamente marcado do que a frente de invasão, associou-se à ocorrência de IL. A diminuição da expressão de RKIP associou-se significativamente a um prognóstico desfavorável, mantendo-se como um fator independente de

prognóstico relativamente à sobrevivência livre de doença. Assim, a perda de expressão de RKIP surge como um novo biomarcador de agressividade do CUB. Estudos adicionais são necessários para validar os resultados aqui apresentados e explorar estratégias terapêuticas que possam potencialmente restaurar a funcionalidade desta proteína como um supressor de metástases no carcinoma da bexiga.

A reprogramação do metabolismo energético e a modelação do microambiente tumoral são características inerentes ao fenótipo de malignidade. Entre a diversidade de biomarcadores associados a tais fenómenos, foi estudada a imunoexpressão de CD147, de transportadores de monocarboxilatos (*monocarboxylate transporters*, MCTs), de CD44 e de anidrase carbónica (*carbonic anhydrase*, CA) IX. Verificou-se o aumento da expressão de MCT1 e MCT4 nas células uroteliais malignas. Os tumores negativos apresentaram perfis clínico-patológicos favoráveis. A expressão de MCT1 associou-se a um prognóstico desfavorável. Foram encontradas associações significativas entre o padrão de expressão de CD147, MCT1 e MCT4, o que fundamenta o papel da proteína CD147 como *chaperone* dos MCTs. O aumento da expressão de CD147 associou-se claramente a um fenótipo de agressividade tumoral e a um prognóstico adverso, reduzindo significativamente as taxas de sobrevivência livre de doença e sobrevivência global. Quando incluída num sistema de discriminação de agressividade tumoral, a expressão de CD147 permitiu distinguir, com rigor, o prognóstico dos doentes com CUB. Verificou-se uma concordância significativa entre a expressão de CD44 e MCTs, e entre a expressão de CD44 e CD147, o que sugere um cenário interativo onde CD44, MCTs e CD147 cooperaram na regulação do microambiente tumoral. Além disso, a expressão de CD44 associou-se igualmente com a agressividade do CUB. A enzima CAIX exibiu um padrão de expressão heterogénea, sendo a marcação mais forte no centro hipóxico dos tumores MI ou na face luminal das lesões papilares, onde a sua expressão se revelou predominante. A expressão de CAIX associou-se com a expressão de MCT4, CD147 e CD44, o que sugere a ocorrência de hipoxia como um mecanismo promotor do fenótipo glicolítico. De salientar que o perfil duplamente-positivo CD147/MCT1 associou-se a parâmetros clínico-patológicos desfavoráveis e a um pior prognóstico, e discriminou um subgrupo de doentes com prognóstico adverso entre um grupo tratado com quimioterapia à base de compostos de platina. Tais resultados encorajaram à realização de estudos adicionais em linhas celulares de CUB, na tentativa de clarificar a função da proteína CD147 como um potencial biomarcador de agressividade tumoral e resistência à cisplatina. O silenciamento específico da CD147 foi acompanhado por uma diminuição da expressão de MCT1 e MCT4 e, notoriamente, por um aumento na quimio-sensibilidade à cisplatina. Estes estudos demonstram o papel provável da CD147 e suas interações na determinação da progressão tumoral e resistência à quimioterapia baseada em cisplatina em doentes com CUB, revelando possibilidades de

intervenção terapêutica dirigida que devem ser exploradas num futuro próximo.

Em resumo, os resultados descritos nesta tese representam o tributo para uma melhor compreensão sobre parâmetros biológicos que parecem influenciar a agressividade do carcinoma urothelial da bexiga, bem como a resistência à quimioterapia, e que devem ser investigados como potenciais biomarcadores de prognóstico e previsão de resposta à terapêutica, bem como alvos terapêuticos.

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a.a.	amino acid
ABC	ATP-binding cassette
ACL	ATP citrate lyase
ADP	adenosine diphosphate
aFGF	acidic fibroblast growth factor
AJCC	American Joint Committee on Cancer
AKT	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
ANG	angiopoietin
Angptl4	angiopoietin-like protein 4
ARE	androgen response elements
ASR	age standardized rate
ATP	adenosine triphosphate
AUM	asymmetric unit membrane
AV	arterial-venous
BCG	*Bacillus* Calmette-Guerin
BCRP	breast cancer resistance protein
BEC	blood endothelial cells
bFGF	basic fibroblast growth factor
BM	basement membrane
BMCD	bone marrow-derived cell
bp	base pairs
BRMS1	breast cancer metastasis-suppressor 1
BSG	basigin
Bv8	Bombina variagata peptide 8
BVD	blood vessel density
BVI	blood vessel invasion
CA	carbonic anhydrase
CAF	cancer-associated fibroblasts
CBP	carboplatin
CCBE1	collagen and calcium-binding EGF domains 1
CDDP	cis-diamminedichloroplatinum (II)
CDK	cyclin-dependent kinase
CDKN2A	cyclin-dependent kinase inhibitor 2A
χ^2	chi-square

CI	confidence interval
CIS	carcinoma *in situ*
CL	collagen
CPT	carnitine palmitoyltransferase
CSCs	cancer-stem cells
CT	computed tomography
CTU	computed tomography urography
CUETO	Club Urológico Español de Tratamiento Oncológico
CXCL12	chemokine, CXC motif, ligand 12
CXCR4	chemokine, CXC motif, receptor 4
DAB	3,3'-diaminobenzidine
DARC	detection of apoptosing retinal cells
DCC	deleted in colorectal carcinoma
DFS	disease-free survival
DLC1	deleted in liver cancer 1
DLL4	delta-like-4
DNA	deoxyribonucleic acid
DRG1	developmentally-regulated GTP-binding protein 1
EAU	European Association of Urology
4EBP1	eukaryotic initiation factor 4E-binding protein 1
EC	endothelial cell
ECM	extracellular matrix
EGFL7	EGF-like domain 7
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGR1	early growth response 1
eIF4E	eukaryotic initiation factor 4E
EL	elastin
EMMPRIN	extracellular matrix metalloproteinase inducer
EMT	epithelial-mesenchymal transition
EORTC	European Organization for Research and Treatment of Cancer
EPC	endothelial progenitor cell
ERK	extracellular signal-regulated kinase
EZH2	enhancer of zeste homolog 2
FADH2	flavin fdenine dinucleotide

FAK	focal adhesion kinase
FASN	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FdG	^{18}fluorodeoxyglucose
FDG-PET	F-fluorodeoxyglucose positron emission tomography
FGF	fibroblast growth factor
FGFR3	fibroblast growth factor receptor 3
Fib	fibrillin
Flk1	mouse foetal liver kinase 1
Flt1	fms-like tyrosine kinase 1
Flt4	fms-like tyrosine kinase 4
FNEIIIA	fibronectin EIIIA
Foxc2	forkhead box C2
GβL	G protein beta subunit like protein
G6P	glucose 6-phosphate
GAS1	growth arrest-specific gene 1
GC	gemcitabine and cisplatin
Gem	gemcitabine
GLUT	glucose transporter
GPCR	G protein coupled receptor
GRK2	G-protein coupled receptor kinase-2
GSH	glutathione
GSK3β	glycogen synthase kinase 3
GSTM1	glutathione S-transferase mu 1
H&E	hematoxylin and eosin
Has	hyaluronan synthase
HER2	human epidermal growth factor receptor 2
HG	high-grade
HIF	hypoxia-inducible factor
HK	hexokinase
HMGA2	high mobility group A
HR	hazard ratio
HRAS	Harvey rat sarcoma viral oncogene homolog
HRP	horseradish peroxidase

HS	heparin sulfate
HSPG	heparan sulfate proteoglycans
HUNK	hormonally up-regulated Neu-associated kinase
IC_{50}	inhibitory concentration 50
IFP	interstitial fluid pressure
IKK	IkB kinase
IL	interleukin
ILV	intratumoural lymphatic vessel
ISUP	International Society of Urological Pathology
JAK	Janus kinase
kb	kilobases
kDa	kilodalton
KDR	human kinase insert domain receptor
KEAP1	kelch like-ECH-associated protein 1
KISS1R	KISS1 receptor
KLF17	krueppel-like factor 17
KSR	kinase suppressor of ras
LAT1	L-type amino acid transporter 1
LDHA	lactate dehydrogenase A
LEC	lymphatic endothelial cells
LG	low-grade
LI	lymphovascular invasion
LKB1	liver kinase B1
LNs	lymph nodes
LOH	loss of heterozygozity
LP	*lamina propria*
LPA	lysophosphatic acid
LSD1	lysine-specific demethylase 1
LVD	lymphatic vessel density
LVI	lymphatic vessel invasion
LYVE-1	lymphatic vessel hyaluran receptor-1
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MCT	monocarboxylate transporter
MDR1	multidrug resistance protein 1

MI	muscle invasive
miRNAs	microRNAs
MK	MAP kinase
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MMC	mitomycin C
MMP	matrix metalloproteinase
mOS	median overall survival
mPFS	median progression-free survival
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRP-1	multi-drug resistance-associated protein-1
MT	membrane-type
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetra-zolium)
mUCC	metastatic urothelial cell carcinoma
MVAC	methotrexate, vinblastine, adriamycin and cisplatin
μg	microgram
μl	microliters
μm	micrometers
MVD	microvessel density
mg	milligram
ml	milliliters
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide (NAD+), reduced
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
NFAT1c	nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	nuclear factor Kappa B
NHE	Na^+/H^+ exchange
NI	non-invasive
NIK	NF-κB inducing kinase
NIP	non invasive papillary
Nm23	nucleoside diphosphate kinase (NDPK)

NMI	non-muscle invasive
NO	nitric oxide
NRARP	Notch-regulated ankyrin repeat protein
NRF2	NF-E2 related factor-2
NRP	neuropilin
OAA	oxaloacetate
OGR1	ovarian cancer G protein-coupled receptor 1
OS	overall survival
OXPHOSP	oxydative phosphorylation
P	pathological
p70S6K	ribosomal p70 S6 kinase
PAI	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PDH	pyruvate dehydrogenase
PDK	phosphoinositide dependent kinase
PDK	pyruvate dehydrogenase kinase
PEBP1	phosphatidylethanolamine-binding protein 1
PET	positron emission tomography
PFK	phosphofructokinase
PG	proteoglycan
PGC	peroxisome proliferator-activated receptor gamma coactivator
PGM	phosphoglycerate mutase
PHD	prolyl hydroxylase domain protein
PHLPP	PH domain and leucine rich repeat protein phosphatases
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIP2	phosphatidylinositol (3,4)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PK	protein kinase
PKM2	pyruvate kinase isoform M2
PLC	phospholipase C
PIGF	placenta growth factor

PLND	pelvic lymph node dissection
PPP	pentose phosphate pathway
Prox-1	prospero related homeobox gene-1
PtdIns(4,5)P2	phosphatidylinositol 4,5-bisphosphate
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RAPTOR	regulatory-associated protein of mTOR
Rb	retinoblastoma
RC	radical cystectomy
Rheb	Ras homologue enriched in brain
RhoGDI2	RhoGTPase dissociation inhibitor 2
RICTOR	rapamycin-insensitive companion of mTOR
RKIP	raf kinase inhibitor protein
ROS	reactive oxygen species
RPM	revolutions per minute
RR	response rate
RRM1	ribonucleotide reductase M1
RTK	receptor tyrosine kinase
RTU	ready-to-use
SDF	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SF	scatter factor
SIN3:HDAC	Sin 3-histone deacetylase
siRNA	small interference RNA
SLC16	solute carrier 16
SLP76	SH2 domain-containing leucocyte protein, 76-kD
SMC	smooth muscle cells
Sox18	SRY (sex determining region Y) box 18
Spred	sprouty-related, EVH1 domain-containing protein
SPSS	Statistical Package for the Social Sciences
SSeCKs	Src-suppressed C kinase substrate
STAT	signal transducer and activator of transcription
SYK	protein-tyrosine kinase SYK
TAK1	TGF-beta activated kinase 1
TAM	tumour-associated macrophages

TCA	tricarboxylic acid
TGF	transforming growth factor
Thr	threonine
TIE	tyrosine kinase with immunoglobulin and EGF homology domains
TIMP	tissue inhibitors of metalloproteinase
TKI	tyrosine kinase inhibitor
TLK	transketolase
TNM	tumour-node-metastases
TP	thymidine phosphorylase
TP53	tumour protein p53
TSC	tuberous sclerosis complex
TSP-1	thrombospondin-1
TUR	transurethral resection
TURBT	transurethral resection of bladder tumour
UBC	urothelial bladder carcinoma
UC	urothelial carcinoma
uPA	urokinase-type plasminogen activator
VDAC	voltage-dependent anion channel
VE	vascular endothelial
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permeability factor
WHO	World Health Organization

Thesis Layout

This thesis is organized into eight chapters and one appendix section.

CHAPTER 1 presents a general introduction divided into two major parts. In the first part, an overview about the current knowledge on urothelial bladder cancer is provided, summarizing the epidemiological and etiological aspects of the disease, its histology, and its natural history and molecular pathogenesis. The management and prognosis of urothelial bladder cancer patients are also addressed, in an attempt to direct the reader's attention into the major drawbacks and concerns in the care of these patients, which will substantiate the translational research reported throughout this thesis. In the second part of the introduction, we review the state of the art about the three cancer hallmarks that were explored in the context of bladder malignancies during the development of the PhD project: tumour angiogenesis and lymphangiogenesis, invasion and metastasis, and energy metabolism reprogramming and the tumour microenvironment. Special emphasis is given to the molecular mechanisms that characterize each of the hallmarks, as well as their contribution to the malignant phenotype, aiming to unveil potential targets that, although poorly explored in bladder cancer setting, may represent promising therapeutic strategies.

CHAPTER 2 presents the rationale of the research that was developed, justifying the need to characterize a phenotype of urothelial bladder cancer aggressiveness, as well as the specific aims that were projected during the PhD time course.

In chapters three to seven we provide our contribution to a better understanding on the clinical, prognostic and/or therapeutic impact of some biological parameters inherent to the three previously mentioned hallmarks of cancer, and that seem to be associated with urothelial bladder cancer progression, metastasis and/or chemoresistance. Therefore:

CHAPTER 3 reports the contribution of molecular markers of blood vessels (like CD31) and lymphatic vessels (like D2-40) to accurately assess the occurrence of blood vessel invasion and/or lymphatic vessel invasion (LVI), also demonstrating the prognostic value of these two parameters.

CHAPTER 4 presents our attempt to further characterize the pattern of expression, the clinical and prognostic significance of phospho-mTOR levels of expression, and its contribution to angiogenesis and lymphangiogenesis occurrence.

CHAPTER 5 demonstrates the significant impact of the loss of expression of RKIP (Raf kinase inhibitor protein) on the aggressive behaviour of the tumours and on patients' outcome.

CHAPTER 6 reports the development of a tumour aggressiveness scoring system where we combined classical clinicopathological parameters, like stage and grade, with biological parameters, like lymphovascular invasion occurrence (specifically highlighted by endothelial markers), as well as CD147 overexpression. CD147 overexpression allowed an accurate discrimination of bladder cancer patients' prognosis.

CHAPTER 7 presents our research on additional microenvironment-related molecules, such as monocarboxylate transporters, CD44 and carbonic anhydrase (CA) IX, that seem to cooperate with CD147 in the establishment of a hyper-glycolytic, acid-resistant phenotype associated with invasion and chemoresistance. We assessed the clinical and prognostic significance of these biomarkers, and further validated the impact of CD147 on chemoresistance in bladder cancer cell lines.

CHAPTER 8 aims to summarize and discuss our main findings on the basis of other relevant published data. We additionally acknowledge some limitations of our studies, and suggest future directions in order to complement the research. Brief concluding remarks are also presented.

The appendix section encloses the book chapter – Angiogenesis, Lymphangiogenesis and Lymphovascular Invasion: Prognostic Impact for Bladder Cancer Patients – published during the research on bladder tumour angiogenesis and lymphangiogenesis, as our contribution to the state of the art on this subject.

CHAPTER 1 | General Introduction

1.1. UROTHELIAL BLADDER CANCER – AN OVERVIEW

The epithelial lining of the urinary tract, named urothelium, extends from the renal pelvis to the proximal urethra [1]. Because it constitutes a strategic permeability barrier between urine and blood, the urothelium is constantly exposed to a variety of potential carcinogens. The bladder is a particularly high risk organ for cancer development, since the carcinogens stagnate in the urine and interact with the urothelium for a few hours before urination [2]. Therefore, it is not surprising that bladder cancer represents a significant epidemiological problem, with an estimated 386,300 new cases and 150,200 deaths occurring in 2008 worldwide [3], and that more than 90% of all bladder cancers are urothelial tumours [4].

Of all newly diagnosed cases of urothelial bladder carcinoma (UBC), 70%-80% are non-muscle invasive (NMI). Even though without aggressive histopathological features, the NMI tumours, particularly high grade lesions, frequently recur and progress to invasive forms. To predict whose tumours will recur and progress remains a challenge. On the other hand, 20%-30% of tumours present as muscle-invasive (MI) disease, for which radical cystectomy (RC) with bilateral pelvic and iliac lymphadenectomy is the gold standard of treatment [4]. The dissemination risk for these neoplasms is high, underlying the need of associating neoadjuvant and adjuvant therapies. However, heterogeneity in treatment response and patient fragility are major problems in the management of MI-UBC patients, and the 5-year overall-survival rate varies from 36% to 48% [5]. Although the formulae based on clinical staging and histopathological parameters are classically used as diagnostic and prognostic tools, they have proven insufficient to characterize the individual biological features and clinical behaviour of the tumours. Understanding the pathobiology of the disease can add important information to these classical criteria, and contribute to accurately predict outcome and individualize therapy for UBC patients.

1.1.1. ANATOMY AND HISTOLOGY OF THE URINARY BLADDER

The urinary bladder constitutes the extraperitoneal muscular urine reservoir that sits on the pelvic floor, behind the pubic symphysis. Urine enters the bladder through the ureters and exits through the urethra. The organ is partly covered on its outside by peritoneal serosa and partly by fascia. Its morphofunctional basis is the detrusor muscle, a muscular wall formed by smooth muscle fibers

arranged in three differently orientated layers (outer and inner layers: longitudinal orientation; middle layer: circular orientation). Internally, a mucous membrane composed of *lamina propria* and urothelium protects the muscular coat from contacting urine [1, 6-7] (Figure 1, A).

The urothelium, from all the urothelial tumours originate, is a specialized stratified epithelium, comprising a single-cell type with three degrees of cellular differentiation that contribute to phenotypic differences between them [1, 8-12] (Figure 1, B):

- the small cubic/cylindrical basal cells (10 μm in diameter) forming a single layer containing the proliferative compartment and stem cells, that contacts the underlying connective tissue and capillary bed of the *lamina propria*. Their mitotic-index is very low, which contributes to the stability of the urothelium;
- the intermediate cells (10-25 μm in diameter) of pyriform shape forming one to five layers thick, depending on the state of bladder filling (one layer in distended bladder to five layers in voided bladder). This seems to result from cell sliding during filling;
- the large polyhedral umbrella cells (25-250 μm in diameter), often bi-nucleated, forming a permeability barrier that accommodates alterations in urine volume while preventing the unregulated exchange between urine and blood. Specializations like high-resistance tight junctions, surface uroplakins (asymmetric unit membrane – AUM) and dynamic apical membrane exocytosis/endocytosis modulate the barrier function of the urothelium.

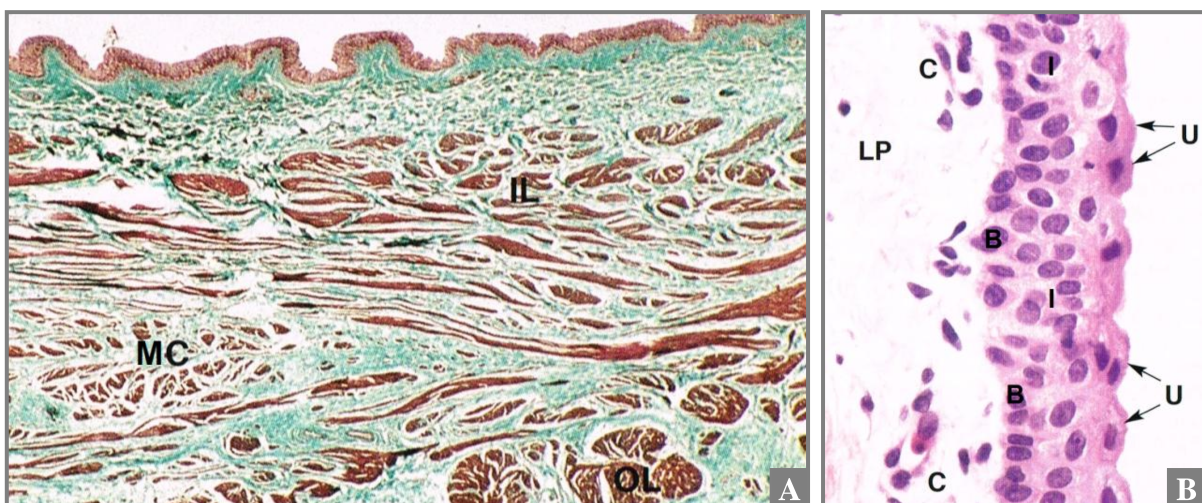


Figure 1 | Histology of the normal urinary bladder. **A**, a microscopic low-magnification of the bladder wall; **B**, a microscopic high-magnification of the mucous layer (adapted from [1]).
Abbreviations: B: basal cells; C: capillaries; I: intermediate cells; IL: inner longitudinal; LP: *lamina propria*; MC: middle circular; OL: outer longitudinal; U: umbrella cells.

The specialized composition of the urothelium makes it a physiologically effective and mechanically flexible barrier. By being one of the slowest cycling epithelia in the human body [9, 12], the urothelium constitutes a unique biological context for carcinogenesis to occur.

1.1.2. EPIDEMIOLOGY AND ETIOLOGY

Urothelial bladder cancer is the second most common malignancy of the genitourinary tract, following prostate cancer [4]. It affects mainly the elderly, peaking between age 50 and 70 years; men are 3-4 times more likely to develop bladder cancer than women, although women present with more aggressive disease and have worse survival rates [4, 13-14]. This gender disparity seems to be the result of the different exposure to carcinogens, also reflecting genetic, anatomic, physiological, environmental and societal factors [14-16] .

An estimated 386,300 new cases and 150,200 deaths from bladder cancer occurred in 2008 worldwide. It was the seventh most common cancer type in men and the eighteenth in women. The highest incidence rates were found in developed countries in Europe, Northern America and Northern Africa [3, 17] (Figure 2).

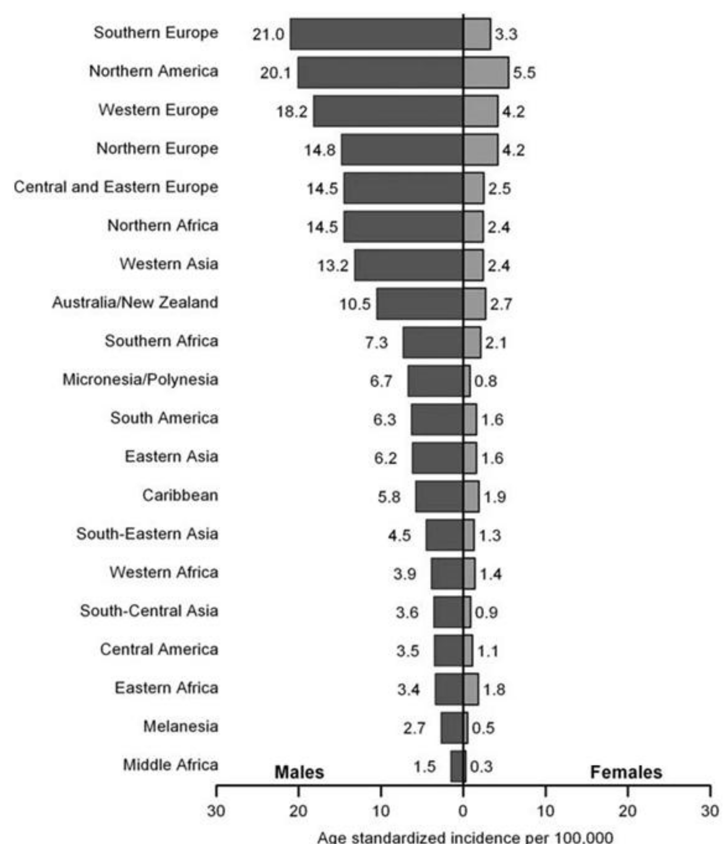


Figure 2 | Age-standardized urinary bladder cancer incidence rates by sex and world area in 2008 (adapted from [3]).

In Portugal, it is estimated that 1935 new cases of bladder cancer occurred in 2008, and that 721 patients died as a consequence of this disease. It was the fifth most common cancer type in men and the thirteenth in women [18] (Figure 3).

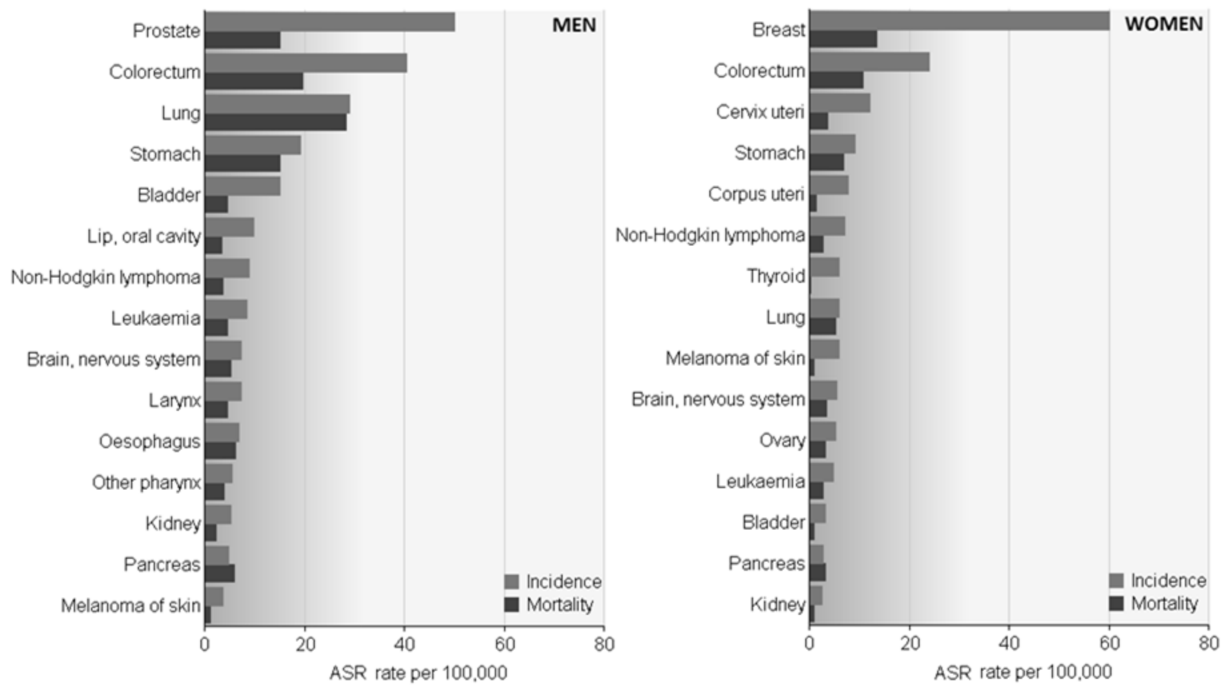


Figure 3 | Age-standardized urinary bladder cancer incidence and mortality rates by sex in Portugal, 2008 (ASR: age standardized rate) (adapted from [18]).

The risk factors for the development of bladder cancer include lifestyle choices, occupations, dietary factors, drugs, urologic pathologies, family histories and genetic polymorphisms. Table 1 summarizes their mechanisms of carcinogenesis induction, the primary cellular processes altered, and the strength of association [13, 19-21].

The most well established risk factor for bladder carcinogenesis is cigarette smoking: it seems to be responsible for 50% of the UBCs [22]. Tobacco smoke is rich in aromatic amines and hydrocarbons that can form highly reactive species and DNA adducts. Differences in the metabolism of these smoking-related carcinogens may modify the risk of smoking-related bladder cancer [23].

Following smoking, occupational exposure is the second most important risk factor for bladder cancer. Workers in industrial areas processing paint, dye, metal and petroleum products are constantly exposed to a variety of aromatic amines, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons. Roughly 20% of all UBCs have been suggested as being related to such exposure, although this percentage tends to decrease with the implementation of safety measures [13, 20-21].

Nutritional factors, particularly those related with fluid intake, have also been attributed to UBC risk. Albeit an adequate fluid intake may reduce exposure to carcinogens by diluting urine and increasing the frequency of micturition, the long-term consumption of water containing arsenic and/or chlorination by-products can increase the risk for bladder cancer [24-25].

Table 1 | Risk factors for bladder cancer development (adapted from [20]).

Risk factor	Mechanism of carcinogenesis	Primary cellular process(es) altered	Strength of association
Lifestyle			
Tobacco smoking	Exposure to carcinogens in tobacco smoke, including aromatic amines, hydrocarbons, and tar	Cell-cycle regulation, gene regulation	Strong
Hair dye use	Exposure to aromatic amines	Cell-cycle regulation	Weak
Occupation			
Dyestuff manufacturing	Exposure to aromatic amines and aniline dyes	Cell-cycle regulation, gene regulation	Strong
Rubber manufacturing	Exposure to aromatic amines, aniline, and *o*-toluidine	Cell-cycle regulation	Strong
Painting	Exposure to aromatic amines and aniline dyes	Cell-cycle regulation, gene regulation	Moderate
Leather processing	Exposure to aromatic amines	Cell-cycle regulation	Moderate
Printing	Exposure to aromatic amines and aniline dyes	Cell-cycle regulation, gene regulation	Weak
Hairdressing	Exposure to aromatic amines from hair dyes and gels	Cell-cycle regulation	Weak
Aluminum smelting	Exposure to polycyclic aromatic hydrocarbons	Cell-cycle regulation	Strong
Asphalt paving	Exposure to polycyclic aromatic hydrocarbons	Cell-cycle regulation	Inadequate
Firefighting	Exposure to aromatic amines and polycyclic aromatic hydrocarbons	Cell-cycle regulation	Weak
Truck driving	Exposure to diesel exhaust	Cell-cycle regulation	Moderate
Diet			
Chlorine and chlorination by-products (in drinking water)	Direct carcinogenic effect	Unconfirmed	Moderate
Arsenic (in drinking water)	Direct carcinogenic effect	Cell-cycle regulation, signal transduction, gene regulation	Strong
Coffee	Carcinogenic metabolites from caffeine in the urine	Unconfirmed	Inadequate
Artificial sweeteners	Unknown in humans	Unconfirmed	Inadequate
Drugs and therapies			
Phenacetin, cyclophosphamide, pelvic irradiation	Induction of DNA fragmentation	Gene regulation	Moderate
Urologic pathologies			
Schistosoma hematobium	Exposure to toxins and N-nitrosamines	Gene regulation	Strong
Cystitis or other urinary tractinfection	Chronic inflammation	Cell-cycle regulation, cell death, gene regulation	Moderate
Urinary calculi	Chronic inflammation	Cell-cycle regulation, cell death, gene regulation	Weak
Ancestry and genetics			
Family history	Genetic predisposition	Depends on the genetic alteration(s)	Strong
NAT2 polymorphism	Inefficient detoxification of aromatic amines	Gene regulation	Strong
NAT1 polymorphism	Promotion of formation of DNA adducts of aromatic amines	Gene regulation	Inadequate
GSTM1 polymorphism	Inefficient detoxification of carcinogens	Gene regulation	Weak

Abbreviations: GSTM1, glutathione S-transferase mu 1; NAT, N-acetyltransferase.

The medical history may also predispose to bladder carcinogenesis, although the cancer type manly associated with the chronic irritation of the urothelium is squamous cell carcinoma. Schistosomiasis (bladder infection caused by the parasite *Schistosoma haematobium*, endemic in some parts of Northern Africa) or recurrent urinary tract infections have direct causative roles on tumourigenesis, while pelvic irradiation and pharmaceutical agents predispose to bladder cancer as a side effect of treatment [13, 20-21, 26].

Variants within genes encoding metabolic enzymes have been associated with susceptibility to bladder cancer, with particular highlight for NAT2 (N-acetyltransferase 2) slow acetylator and GSTM1 (glutathione S-transferase mu 1) null genotypes. While these null genotypes may confer an additional risk to exposure of carcinogens present in tobacco products [23, 27], increasing evidences suggest an intrinsic role of genetic predisposition in bladder cancer incidence [13]. Additionally, there is a two-fold higher risk of bladder cancer in first-degree relatives of UBC patients [28].

1.1.3. Pathological Subtypes, Staging and Grading

Urothelial carcinoma is the most common histological subtype of bladder cancer in developed countries, being responsible for about 90% of all cases. However, UBC has a propensity for divergent differentiation, and it is frequent to observe urothelial variants accompanying, in variable proportions, the typical urothelial carcinoma. Divergent differentiation generally implicates aggressive, high stage or high grade bladder cancer, which portends an unfavorable prognosis. The most common variants are squamous and glandular. Pure squamous cell carcinomas and glandular adenocarcinomas represent 5% and 2% of the bladder cancer cases, respectively, and other rare subtypes comprise the remainder of bladder cancers [4, 29-30] (Figure 4).

Histological staging of UBC is generally performed according to the guidelines of the tumour-node-metastases (TNM) system (Table 2). The latest American Joint Committee on Cancer's (AJCC) Cancer Staging Manual [31] introduced minor alterations to the previous version [32]. Under this staging system, T stage of the primary tumour is based on the extent of invasion into the bladder wall. The non-muscle invasive (NMI) tumours include papillary (Ta) or flat (Tis, *in situ*) carcinomas confined to the urothelium, and lesions infiltrating the *lamina propria* (T1). When the tumour invades the *muscularis propria*, it can be staged according to the depth of muscle infiltration (T2a, T2b). If extension to the surrounding connective tissue occurs, the tumour is staged as T3 (T3a, T3b). T4 tumours (T4a, T4b)

invade adjacent structures to the bladder [31] (Table 2).

It is estimated that approximately 70-80% of the patients with newly diagnosed bladder cancer present with non-muscle invasive disease, while the remaining 20-30% UBCs are muscle invasive or have metastasized at the time of diagnosis. 50-70% of the NMI lesions will recur, and 10-20% will progress to MI tumours [2, 4, 13, 30].

Urothelial tumours
Infiltrating urothelial carcinoma
- with squamous differentiation
- with glandular differentiation
- with trophoblastic differentiation
- Nested
- Microcystic
- Micropapillary
- Lymphoepithelioma-like
- Lymphoma-like
- Plasmacytoid
- Sarcomatoid
- Giant cell
- Undifferentiated

Non-invasive urothelial neoplasias
- Urothelial carcinoma in situ
- Non-invasive papillary urothelial carcinoma, high grade
- Non-invasive papillary urothelial carcinoma, low grade
- Non-invasive papillary urothelial neoplasm of low malignant potential
- Urothelial papilloma
- Inverted urothelial papilloma

Squamous neoplasms
Squamous cell carcinoma
Verrucous carcinoma
Squamous cell papilloma

Glandular neoplasms
Adenocarcinoma
- Enteric
- Mucinous
- Signet-ring cell
- Clear cell

Villous adenoma

Neuroendocrine tumours
Small cell carcinoma
Carcinoid
Paraganglioma

Melanocytic tumours
Malignant melanoma
Nevus

Mesenchymal tumours
Rhabdomyosarcoma
Leiomyosarcoma
Angiosarcoma
Osteosarcoma
Malignant fibrous histiocytoma
Leiomyoma
Haemangioma
Other

Haematopoietic and lymphoid tumours
Lymphoma
Plasmacytoma

Miscellaneous tumours
Carcinoma of Skene, Cowper and Littre glands
Metastatic tumours and tumours extending from other organs

Figure 4 | World Health Organization (WHO) histological classification of tumours of the urinary tract (adapted from [30]).

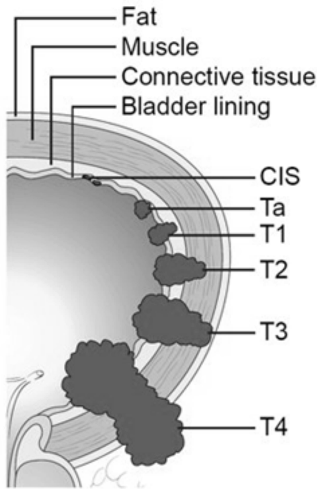
Histological grade is a critical risk factor for progression of NMI disease [33-34]. This variable depends upon the pattern of urothelial cytological alterations, namely the degree of nuclear anaplasia, and some architectural abnormalities [21, 30]. The historical 1973 WHO grading system [35] included urothelial papilloma and grades of well (G1), moderately (G2) or poorly differentiated (G3) carcinomas. In 2004, the WHO [30] adopted the 1998 WHO/ ISUP (International Society of Urological Pathology) revised scheme [36] for urothelial carcinoma, in order to establish a universally acceptable

Table 2 | TNM classification of carcinomas of the urinary bladder (adapted from [31])

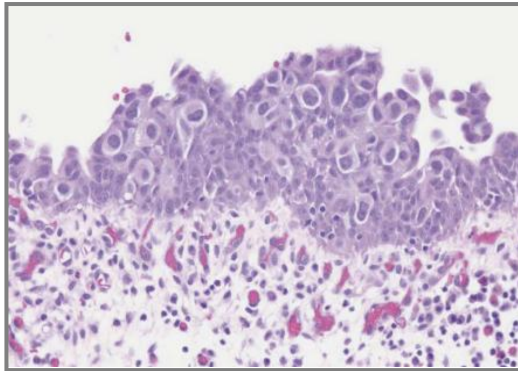
PRIMARY TUMOUR (T)	
TX	Primary tumour cannot be assessed.
T0	No evidence of primary tumour.
Ta	Non-invasive papillary carcinoma.
Tis	Carcinoma *in situ*: "flat tumour."
T1	Tumour invades subepithelial connective tissue.
T2	Tumour invades muscularis propria.
T2a	Tumour invades superficial muscularis propria (inner half).
T2b	Tumour invades deep muscularis propria (outer half).
T3	Tumour invades perivesical tissue.
T3a	Microscopically.
T3b	Macroscopically (extravesical mass).
T4	Tumour invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall.
T4a	Tumour invades prostatic stroma, uterus, vagina.
T4b	Tumour invades pelvic wall, abdominal wall.
REGIONAL LYMPH NODES (N)	
NX	Lymph nodes cannot be assessed.
N0	No lymph node metastasis.
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac or presacral lymph node).
N2	Multiple regional lymph node metastases in the true pelvis (hypogastric, obturator, external iliac or presacral lymph node).
N3	Lymph node metastases to the common iliac lymph nodes.
DISTANT METASTASIS (M)	
M0	No distant metastasis.
M1	Distant metastasis.

ANATOMIC STAGE / PROGNOSTIC GROUPS

STAGE	**T**	**N**	**M**
0a	Ta	N0	M0
0is	Tis	N0	M0
I	T1	N0	M0
II	T2a	N0	M0
	T2b	N0	M0
III	T3a	N0	M0
	T3b	N0	M0
	T4a	N0	M0
IV	T4b	N0	M0
	Any T	N1-3	M0
	Any T	Any N	M1

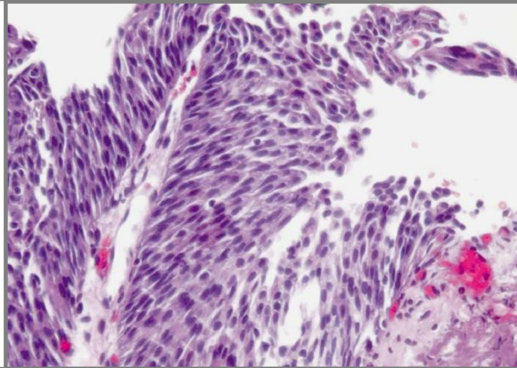


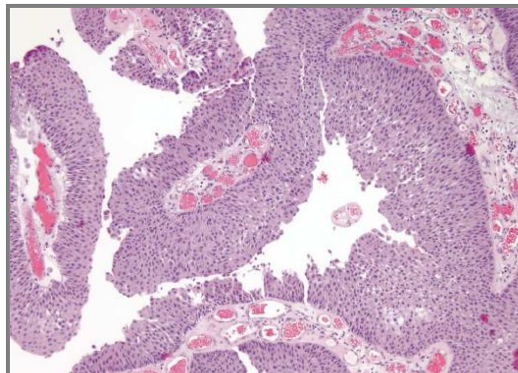
classification system for bladder neoplasias that could be used, with high reproducibility, by pathologists, urologists and oncologists, also stratifying the tumours into prognostically significant categories [21, 29, 37]. This classification system organizes urothelial tumours into infiltrating carcinomas and non-invasive urothelial neoplasias; these last are restricted to the urothelium, and include urothelial carcinoma *in situ* (CIS), and papillary lesions like urothelial papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade and high grade UBC [30] (Figures 4 and 5). Infiltrating urothelial carcinomas invade beyond the basement membrane of the urothelium. Their histology is variable: most of the NMI tumours (pathological T stage pT1) are papillary, low or high grade, whereas most pT2-T4 carcinomas (MI tumours) are non-papillary and high grade [30-31].



Urothelial Carcinoma *In Situ* (CIS)
- Flat urothelial lesion.
- Primary CIS is rare; concomitant CIS is common, being considered as a precursor lesion for MI-UBC.
- Nuclear anaplasia identical to high grade tumours: enlarged, pleomorphic, hyperchromatic nuclei, with condensed chromatin distribution and large nucleoli; atypical mitoses and loss of cell polarity.
- Commonly multifocal; may be diffuse.

Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP)
- Papillary urothelial lesion.
- Resembles the exophytic urothelial papilloma (normal-appearing urothelium lines papillary fronds), but shows increased cellular proliferation.
- Minimal to absent cytological atypia.
- Very low risk of progression.
- Although not labeled as "cancer", it is not an entirely benign lesion.





Papillary Urothelial Carcinoma, Low Grade
- Papillary urothelial lesion.
- Exhibits an overall orderly appearance but has easily recognizable variations in architecture and cytological features: uniformly enlarged nuclei, infrequent mitoses (may occur at any level but are more frequent basally).
- Recurrence is common; progression is rare.

Papillary Urothelial Carcinoma, High Grade
- Papillary urothelial lesion.
- Exhibits a disorderly appearance (papillae are frequently fused and branching) with marked architectural and cytological abnormalities: pleomorphic nuclei, prominent nucleoli, frequent and atypical mitoses (may occur at any level).
- High risk of recurrence and progression.

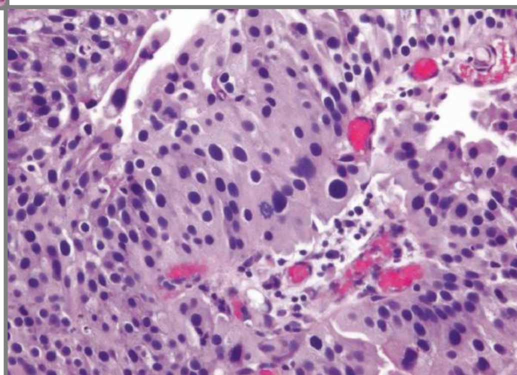


Figure 5 | Features of non-invasive urothelial neoplasias, according to the guidelines of the WHO histological classification ([21, 26, 29-30, 38-43]; microscopic magnifications adapted from [21]).

There is still some debate about the grading system to be used [44-48]. Some have recommended using only the WHO 2004 system [21]; others believe that WHO 1973 and 2004 classifications are complementary and that it is beneficial for clinicians to receive information based on both classifications [49-50]. The European Association of Urology (EAU) advocates the simultaneous use of both systems

for NMI disease until the 2004 grading system is validated in more clinical trials [51]. Importantly, urologists should interact with their pathologists to determine which grading system they are using [52]. Additionally, the risk tables from the European Organization for Research and Treatment of Cancer (EORTC), combining data on previous tumour recurrence rate, number of tumours, tumour diameter, T stage and WHO grade, and the presence or absence of concomitant CIS, are considered reliable tools for estimating recurrence and/or progression of NMI-UBC [33, 53-56].

1.1.4. Natural History and Molecular Pathogenesis

The natural history of bladder cancer encompasses two main phenotypic variants characterized by distinct histopathological and behavioral profiles (Figure 6). The low-grade tumours, always of papillary morphology and usually non-muscle invasive, account for about 80% of UBCs. These tumours are often multifocal and recurrent, but infrequently progress to MI disease. Urothelial hyperplasia (markedly thickened mucosa without cytological atypia) is thought to be a precursor lesion for this variant. Conversely, 20% of UBCs present as high grade cancers, frequently non-papillary and muscle-infiltrating. This variant seems to arise *de novo* or derive from pre-existing CIS, which is, in turn, preceded by urothelial dysplasia (low-grade intraurothelial neoplasia with marked atypia). Additionally, some patients who originally present with low grade superficial papillary tumours may eventually develop CIS in the adjacent mucosa and progress to invasive cancer [2, 21, 26, 29-30, 57-60].

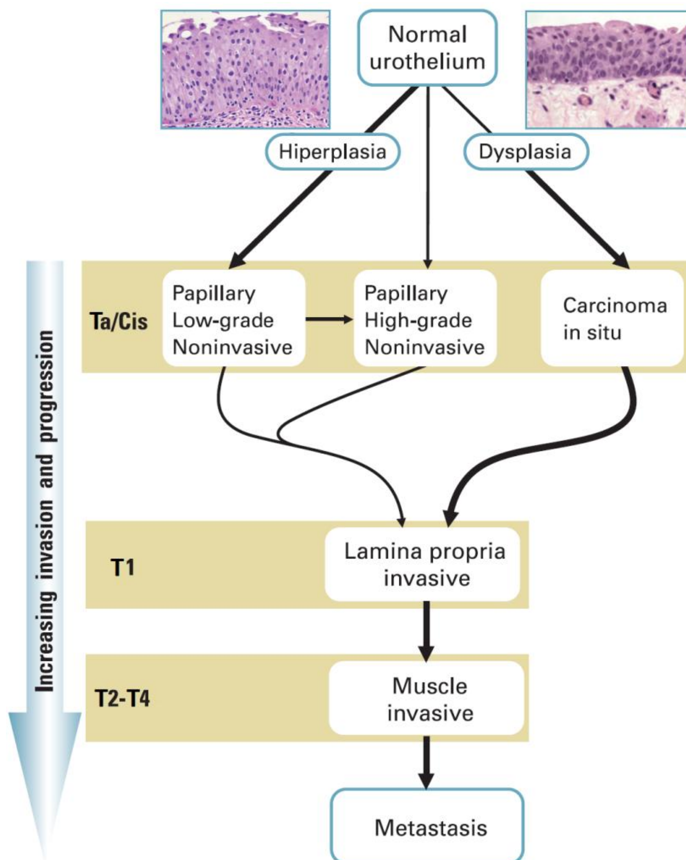


Figure 6 | Natural history of urothelial bladder cancer (the thickness of arrows represents the relative frequency of occurrence) (adapted from [59]; microscopic magnifications adapted from [64]).

Numerous studies concerning pathogenetic pathways, natural history and bladder tumour biology have been

reported, and UBC is a relatively well understood type of cancer. Bladder carcinogenesis, like other carcinogenesis processes, arises due to alterations that disrupt molecular pathways normally responsible for the maintenance of cellular homeostasis. These alterations may occur by numerical and/or structural anomalies of chromosomes, by DNA-sequence or epigenetic modifications, by modulation at the posttranscriptional level or by up- or downregulated protein expression. The disrupted pathways may considerably overlap and include mainly cell cycle regulators and cell growth promoters, cell death modulators, signal transduction factors, gene expression and angiogenesis regulators and invasion modulators [20, 61-63] (Table 3).

The less aggressive and more prevalent phenotypic variant of UBC is characterized by the constitutive activation of the receptor tyrosine kinase (RTK)-Ras cell cycle regulation pathway, exhibiting activating mutations in the oncogenes *HRAS* (Harvey rat sarcoma viral oncogene homolog) and *FGFR3* (fibroblast growth factor receptor 3) [2, 20, 26, 57, 60, 62]. Point mutations on *FGFR3* are the most common genetic alteration identified in bladder cancer, occurring in up to 80% of low-grade pTa tumours, compared with 10% to 20% in invasive tumours. This suggests that *FGFR3* mutation is one of the key events for the genesis of low-grade non-invasive pappilary tumours [64-68] (Figure 7). The overall frequency of *HRAS* mutations is 15%, and these show no association with tumour grade or stage [69]. HRAS seems to be mainly overexpressed in pTa tumours that are not likely to progress [70]. However, activation of FGFR3 may induce signalling via the Ras pathway, and the finding that *FGFR3* and *HRAS* mutations are mutually exclusive in UBC probably reflects activation of the same pathway by either event [69] (Figure 7).

The deletion of chromosome 9 is a common and early event in bladder carcinogenesis, being described as the only alteration in some near-diploid tumours [71]. Additionally, more than half of all bladder cancers harbour chromosome 9 anomalies, independently of stage and grade [72-73]. 9q loss of heterozygozity (LOH) seems to be more frequent in low grade and stage lesions [74], whereas 9p LOH is prevalent in high grade and stage tumours [75]. Efforts have been made to identify possible tumour suppressor genes that drive this common loss. For instance, deletions on the *CDKN2A* (cyclin-dependent kinase inhibitor 2A) *locus* have been found on 9p; this *locus* encodes p16 and $p14^{ARF}$, which are tumour suppressor proteins that induce cell-cycle arrest through the Rb (retinoblastoma) and p53 pathways [76-77]. The homozygous deletion of the $p16^{INK4a}$ gene has been found in high grade pTa tumours [78], although p16 alterations have also been observed in invasive lesions [79] (Figure 7).

CIS and muscle-invasive tumours exhibit a wide range of genomic alterations. The tumour

suppressor genes *RB1* and *TP53* (tumour protein p53) are altered in the vast majority of these lesions [2, 20, 57, 62] (Figure 7). Their proteins are closely associated with the apoptotic, signal transduction and gene regulation processes [20, 80-81].

Table 3 | Molecules and processes that contribute to urothelial carcinogenesis (adapted from [20]).

Marker/ expression in urothelial carcinoma	Normal function	Molecular pathway(s) involved	Prognostic impact
Cell-cycle regulation			
p53[a]	Inhibits G_1-S progression	p53	Increased recurrence; decreased survival; amenable to cisplatin chemotherapy
p21[b]	Cyclin-dependent kinase inhibitor	p53	Increased recurrence; decreased survival
Mdm2[c]	Mediates the proteasomal degradation of p53	p53	Increased with tumor stage and grade
p14[b]	Inhibits *MDM2*	p53	Decreased survival
p16[b]	Cyclin-dependent kinase inhibitor	Rb	Increased recurrence; decreased survival
Rb[d]	Sequesters E2F, inhibits cell-cycle progression	Rb	Increased recurrence; decreased survival
CDK4[c]	Complexes with cyclin D1; involved in G_1-S transition	Rb	Increased with tumor stage and grade
p27[b]	Cyclin-dependent kinase inhibitor	Rb	Decreased survival
Cell death			
Fas[b]	Activation signals formation of death-inducing signaling complex; promotes apoptosis	Extrinsic apoptotic	Decreased cause-specific survival
Bcl-2[c]	Inhibits caspase activation	Intrinsic apoptotic	Decreased survival; poor prognosis with adjuvant therapy
Bax[b]	Releases cytochrome c from mitochondria; promotes apoptosis	Intrinsic apoptotic	Poor prognosis; decreased overall survival
Caspase-3[b]	Promotes apoptosis	Common apoptosis effector	Increased recurrence
Cell growth			
FGFR3[e]	Receptor for fibroblast growth factor; transmits growth signals	Ras-MAPK	Increased recurrence
EGFR[c]	Receptor for epidermal growth factor; transmits growth signals	Ras-MAPK, PI3K-Akt	Increased progression; decreased survival
ErbB-2[c]	Receptor for epidermal growth factor; transmits growth signals	Ras-MAPK, PI3K-Akt	Decreased survival
VEGFR2[c]	Receptor for vascular endothelial growth factor; transmits angiogenic signals	Ras-MAPK, PI3K-Akt	Increased with disease stage, invasion, nodal metastasis
Signal transduction			
HRAS[c]	Activates Raf and PI3K	Ras-MAPK	Increased in nonprogressing Ta tumors
PKC[f]	Activates Raf, c-Fos, NF-κB; inhibits Bad	PLC/PKC	Increased recurrence
PTEN[b]	Dephosphorylates PIP_3; antagonizes PI3K signaling	PI3K-Akt	Decreased with tumor stage and grade
Gene regulation			
STAT3[c]	Regulates gene expression; increases Bcl-2, $Bcl\text{-}X_L$ expression	JAK-STAT	Increased recurrence; decreased survival
NF-κB[g]	Regulates gene expression	NF-κB	Increased recurrence with homozygous insertion
c-Fos[c]	Regulates gene expression	MAPK	Increased with tumor grade
c-Jun[c]	Regulates gene expression	MAPK	Increased recurrence; decreased survival

(continued)

Marker/ expression in urothelial carcinoma	Normal function	Molecular pathway(s) involved	Prognostic impact
Tumor angiogenesis			
HIF[c]	Transcribes genes responsible for angiogenesis		Increased recurrence; decreased survival
VEGF[c]	Promotes angiogenesis through nitric oxide synthase	Ras-MAPK, PI3K-Akt	Increased recurrence and progression; decreased survival
TP[c]	Promotes VEGF and interleukin-8 secretion; induces MMP		Increased recurrence
uPA[c]	Degrades extracellular matrix		Increased progression; decreased survival
bFGF[c]	Growth factor stimulating angiogenesis	Ras-MAPK	Increased risk of local recurrence
aFGF[c]	Growth factor stimulating angiogenesis	Ras-MAPK	Increased with increasing stage
SF[c]	Growth factor stimulating angiogenesis		Increased compared to normal controls
TSP-1[b]	Inhibits angiogenesis	p53	Increased recurrence, decreased survival
Invasion			
E-cadherin[b]	Mediates intercellular adhesion	Cadherin	Increased recurrence and progression; decreased survival
β-catenin[b]	Links cadherins to the actin cytoskeleton	Wnt/β-catenin	Increased progression; decreased survival
α6β4 integrin[h]	Links collagen VII to the actin cytoskeleton; transduces regulatory signals	Cytoskeletal	Decreased survival
MMP-2[c]	Degrades extracellular matrix		Increased recurrence; decreased survival
MMP-9[c]	Degrades extracellular matrix		Increased with tumor stage and grade
TIMP-2[i]	Antagonizes MMP function		Increased recurrence; decreased survival (?)

[a] Altered, [b] underexpressed/lost, [c] overexpressed, [d] lost/hyperphosphorylated, [e] overactivated, [f] overexpressed in membrane, [g] polymorphic insertion/deletion in promoter region, [h] lost/overexpressed, [i] uncertain.

Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; FGFR3, fibroblast growth factor receptor 3; HIF, hypoxia-inducible factor; HRAS, protein of the Harvey rat sarcoma viral oncogene homolog gene; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor–kappa B; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Rb, retinoblastoma protein; SF, scatter factor; STAT, signal transducer and activator of transcription; TIMP-2, tissue inhibitor of metalloproteinase 2; TP, thymidine phosphorylase; TSP-1, thrombospondin-1; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

The p53 protein inhibits cell-cycle progression at the G1-S transition and mediates its control through the transcriptional activation of *p21*$^{WAF1/CIP1}$, which encodes for the CDK (cyclin dependent kinase) inhibitor p21. By being the "guardian of the genome", *TP53* is the most commonly mutated gene in human cancer [82]. In UBC, *TP53* mutations are strongly associated with high-grade CIS or MI disease (Figure 7), and predict recurrence and progression for NMI-UBC patients [83-86]. Consistent with this, p21 expression is downregulated in the majority of urothelial cancers that harbour *TP53* mutations [87].

The Rb protein interacts with multiple regulatory proteins involved in the G1-S transition. In its non-phosphorylated active form, Rb sequesters and inhibits the transcription factor E2F. CDKs phosphorylate Rb, which causes E2F release that, in turn, is able to induce gene transcription for DNA replication [88]. Inactivation mutations in the *RB1* gene have been found in all tumour stages and

grades of UBC [89] (Figure 7). However, UBC patients with pRb-expressing tumours have poorer outcomes than patients demonstrating inactivating mutations [90]. This seems to be the consequence of pRb hyperphosphorylation due to the loss of the CDK inhibitor p16 and/or cyclinD1 overexpression [91]. Alterations in the Rb pathway, either alone or in combination with altered p53 pathway, have high prognostic value for bladder cancer patients [92-94].

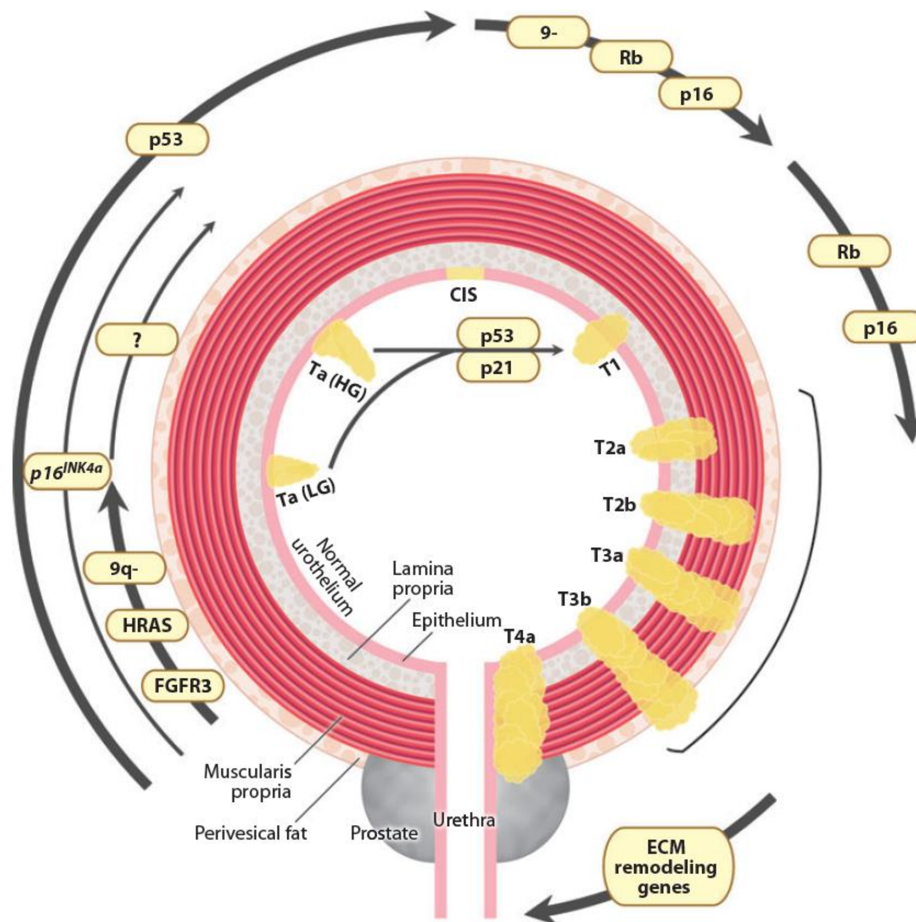


Figure 7 | Model for urothelial carcinogenesis and progression, characterizing distinct molecular alterations in non-invasive and invasive tumours (locations of the molecules indicate characteristic alterations that pose a risk for progression of a particular phenotype; the thickness of the arrows is approximately proportionate to the relative frequency of occurrence) (adapted from [20]).
Abbreviations: ECM, extracellular matrix; FGFR3, fibroblast growth factor receptor 3; HG, high-grade; HRAS, Harvey rat sarcoma viral oncogene homolog; LG, low-grade; Rb, retinoblastoma protein.

The ability of MI bladder tumours to infiltrate the muscular wall and the surrounding connective tissues, to invade adjacent organs and to metastasize, depends not only on the intrinsic genetic factors of the malignant cells, but also on the tumour microenvironment. Therefore, the most aggressive UBC phenotype is accomplished by angiogenesis occurrence, loss of intercellular adhesion and remodelling of the extracellular matrix by matrix metalloproteinases (MMPs), among others [2, 20, 59, 61-62].

Increased VEGF (vascular endothelial growth factor) expression and microvessel density [95-99], decreased E-cadherin and increased N-cadherin expression [100-102], and increased MMPs activity [103-105] are commonly observed in bladder lesions, and associate with recurrence, progression and poor prognosis in UBC patients.

1.1.5. Diagnosis, Management and Prognosis

The most common presenting symptom of bladder cancer is painless haematuria. Additionally, urinary frequency and urgency, irritative voiding and/or dysuria should alert the clinician to a possible diagnosis of a malignant tumour. Symptoms of advanced disease, like flank pain, lower extremity edema, palpable pelvic mass, weight loss and bone pain, almost never occur without a previous history of haematuria. If a tumour is suspected, the initial assessment includes voided urine cytology and cystoscopy, as well as imagiological examination [4, 21, 106] (Figure 8). Cytology is the standard non-invasive test for detecting UBC, but has a poor sensitivity, especially for screening low-grade tumours. A number of soluble and cell based markers have been developed for diagnosing and monitoring UBC patients, some of which are approved by the Food and Drug Administration (FDA) [20, 107-108].

After the first diagnosis, transurethral resection (TUR) is performed. This surgical procedure provides diagnostic information, by allowing local staging and grading, and often achieves therapeutic benefit, by resecting or fulgurating all grossly visible tumours without affecting bladder's function. As the resection should include *muscularis propria*, especially if the tumour infiltrates the *lamina propria*, pTa and pT1 tumours are generally treated by TUR [4, 21, 109]. However, TUR should be repeated in high grade and/or pT1 lesions, due to risk of upstaging or presence of residual tumours [110-114]. Moreover, the elevated rate of recurrence and progression after TUR advocates the use of adjuvant intravesical treatments, particularly in those patients harbouring pathological risk factors – tumour grade and stage, multifocality, tumour size and presence of associated pTis [50, 115-116]. The immunomodulator BCG (*Bacillus* Calmette-Guerin), and chemotherapeutics such as mitomycin C (MMC) and epirubicin, are common agents used for intravesical instillations [4, 109, 116]. A single postoperative instillation of chemotherapy within 24 hours of TUR is currently recommended for all newly diagnosed bladder tumours [51] (Figure 8).

Only a very thin line separates NMI from MI disease, but the management and clinical outcomes for MI disease are completely different (Figure 8). First, MI tumours must be re-staged with cross-sectional imaging of the bladder and sites of possible metastases (frequently in pelvic and non-regional

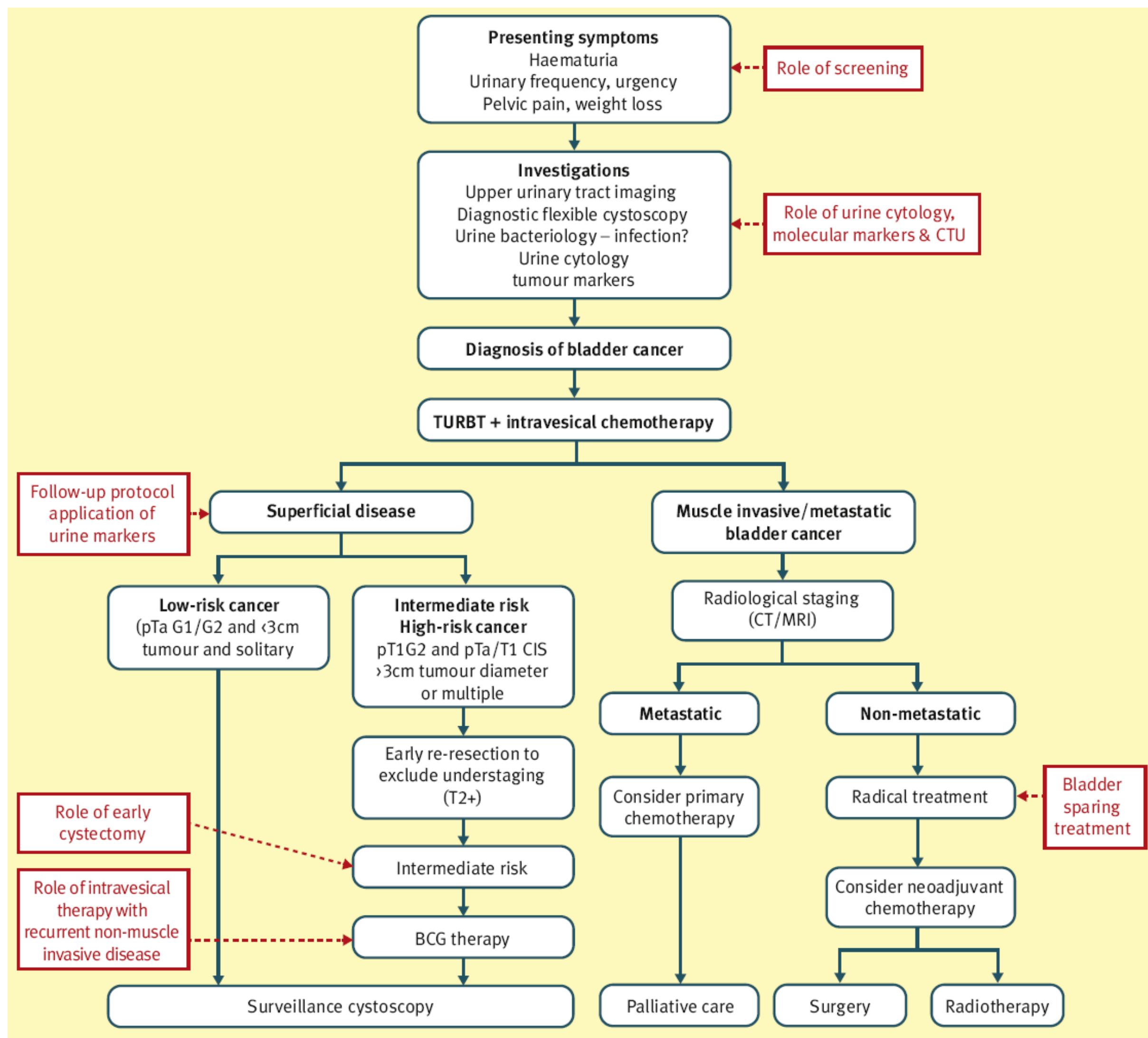


Figure 8 | Presenting symptoms, diagnosis and management of bladder cancer (red boxes represent areas of ongoing investigation and clinical trials) (adapted from [117]).
Abbreviations: BCG, *Bacillus* Calmette-Guerin; CIS, carcinoma *in situ*; CT, computed tomography; CTU, computed tomography urography; MRI, magnetic resonance imaging; TURBT, transurethral resection of bladder tumour.

lymph nodes, liver and lungs) by abdominal and chest computed tomography (CT) and pelvic magnetic resonance imaging (MRI) [4, 49-50]. More sensitive techniques in detecting lymph node metastasis or micrometastasis, like MR lymphangiography [118-119] and (18) F-fluorodeoxyglucose positron emission tomography (FDG-PET) [120-122], have recently been introduced. Second, radical cystectomy (RC) is the standard of treatment in the setting of *muscularis propria* invasive disease. Additionally, in pTis and pT1 tumours that are refractory to BCG instillations, in high grade recurrent pT1 tumours, or in high-volume tumours that cannot be managed by TUR, cystectomy should also be considered [51, 116]. In fact, delaying cystectomy in these patients may lead to decreased disease-specific survival [123]. The standard surgical approach in men is radical cistoprostatectomy, and in women is anterior exenteration,

coupled in both cases with pelvic lymphadenectomy and some form of urinary diversion in either a non-continent or continent-way [4, 50, 124-125]. The boundaries of the lymphadenectomy have been widely discussed, and there is increasing evidence that an extended lymphadenectomy with the cephalad limits of dissection extending up to the aortic bifurcation and including caudally the presacral nodes provide additional data for tumour staging as well as survival improvements [126-129]. Because RC is quite an invasive procedure and exhibits significant complications, optimal methods of urinary diversion and the use of robot-assisted laparoscopic cystectomy are evolving, although requiring further study [116, 130-134]. Alternatively to RC, multimodality bladder-sparing approaches involving chemoradiation may be considered as therapeutic options for eligible patients - patients with small tumours, stage pT2, with visibly and microscopically complete TURs, who have no associated CIS or hydronephrosis, and who are medically fit to receive chemotherapy, and patients who present with severe medical co-morbidities for whom RC represents a too high risk [50, 116, 135-138].

Neoadjuvant, adjuvant and palliative systemic chemotherapy has been explored in patients with MI, locally-advanced or metastatic UBC. Platinum-based compounds are established standards for fit patients, namely MVAC (methotrexate, vinblastine, adriamycin and cisplatin) and GC (gemcitabine and cisplatin) combinations [4, 50, 125, 139-142]. The doublet regimen is becoming preferred over MVAC, due to a comparable survival benefit, coupled with a better safety profile [139, 142-145]. Patients unfit for platinum-based chemotherapy may be palliated with carboplatin-based regimens or single-agent taxane or gemcitabine [50, 139, 144]. The third-generation vinca alkaloid vinflunine is an option for second-line chemotherapy in metastatic patients progressing after first line platinum-based chemotherapy [146-147].

The outcome for bladder cancer patients is very heterogeneous (Figure 9). It is mandatory that risk-stratification tools are developed, in order to accurately classify patients with similar risks of recurrence and progression, and to determine the appropriate treatment modalities for each risk group. The combined analysis of the six risk factors identified by the EORTC allowed to develop risk scores and to classify pTa and pT1 patients into low-, intermediate- and high-risk groups

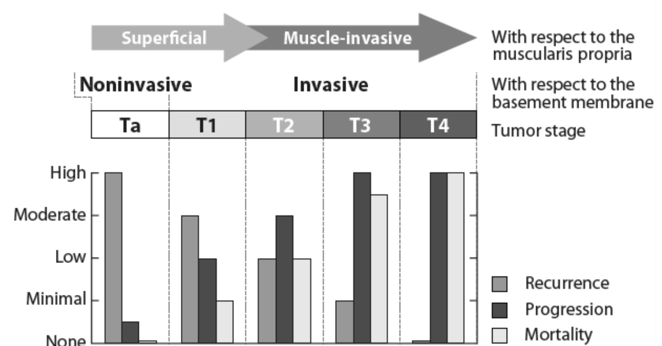


Figure 9 | Recurrence, progression and mortality probabilities for urothelial bladder cancer patients according to tumour stage (adapted from [20]).

Table 4 | EORTC risk factors used to calculate the recurrence and progression scores, and probability of recurrence and progression according to total score (adapted from [33]).

Factor	Recurrence	Progression
Number of tumors		
Single	0	0
2 to 7	3	3
≥8	6	3
Tumor size		
<3 cm	0	0
≥3 cm	3	3
Prior recurrence rate		
Primary	0	0
≤1 rec/yr	2	2
>1 rec/yr	4	2
T category		
Ta	0	0
T1	1	4
CIS		
No	0	0
Yes	1	6
Grade		
G1	0	0
G2	1	0
G3	2	5
Total score	0–17	0–23

Recurrence score	Prob recurrence 1 year (95% CI)	Prob recurrence 5 years (95% CI)
0	15% (10%, 19%)	31% (24%, 37%)
1–4	24% (21%, 26%)	46% (42%, 49%)
5–9	38% (35%, 41%)	62% (58%, 65%)
10–17	61% (55%, 67%)	78% (73%, 84%)

Progression score	Prob progression 1 year (95% CI)	Prob progression 5 years (95% CI)
0	0.2% (0%, 0.7%)	0.8% (0%, 1.7%)
2–6	1.0% (.4%, 1.6%)	6% (5%, 8%)
7–13	5% (4%, 7%)	17% (14%, 20%)
14–23	17% (10%, 24%)	45% (35%, 55%)

for recurrence and progression; these vary from 31% to 78% and from 0.8% to 45% at five years, respectively [33] (Table 4). The main limitation of the EORTC tables is that the risk groups were based on patients who did not have a second TUR or receive maintenance chemo- or immuno-therapy. The Club Urológico Español de Tratamiento Oncológico (CUETO) has recently developed a scoring model for BCG-treated patients that predicts the short- and long-term risks of recurrence and progression. Using these tables, the calculated risk of recurrence is lower than that obtained by the EORTC tables, but the risk of progression is lower only in high-risk patients [148]. A single chemotherapy instillation 24h within TUR reduces the risk of recurrence by 39% [149].

Carcinoma *in situ* is a high risk of progression lesion. In high grade pT1 tumours, the most important prognostic factor is the presence of concomitant CIS [33]. Without any treatment, approximately 54% of patients with CIS progress to MI disease [150]. The EAU recommends at least one year of intravesical BCG for patients with CIS and/or high risk of progression tumours. If BCG therapy fails, RC should be considered [51].

For those patients who progress from NMI to MI disease, or initially present with MI tumours, the prognosis is significantly worse than for NMI disease (Figure 9). The timely performance of RC – within 90 days since diagnosis [151-152]– and the delivery of neoadjuvant and/or adjuvant radiation or chemotherapy provides a cure for most patients with organ-confined tumours or with extravesical tumours that are completely resected: in a large cohort, the disease-free survival (DFS) at 5 years was 89%, 87%, 62% and 50% for pT2, pT3a, pT3b and pT4 lymph node negative disease, respectively. When lymph node involvement occurred, the 5-year DFS lowered to 35% [153]. The presence of visceral metastasis is also a poor prognosis factor: in a randomized trial comparing long-term survival in

patients with locally advanced or metastatic UBC treated with GC or MVAC, the 5-year overall survival rates for patients with and without visceral metastases were 6.8% and 20.9%, respectively; the treated patients achieved a median survival of up to 14-15 months, similar in GC and MVAC arms [154].

1.1.6. Major Drawbacks and Concerns

Urothelial bladder cancer is a complex disease with variable natural history and clinical behaviour, representing an important cause of morbidity and mortality worldwide. The vast majority of the patients present with pTa tumours that, although rarely progress, have high recurrence rates. This demands for long-term follow-up and repeated interventions, making UBC the most expensive cancer to treat [155]. Additionally, the risk of progression to muscle-invasive disease is an important threat for pT1 and pTis patients. MI tumours carry a significant metastatic potential and, despite advances in surgical techniques and perioperative chemotherapy, up to 50% of MI-UBC patients experience recurrence and/or progression and eventually die from the disease [156]. Although cisplatin-containing combinations are the standard of care for UBC patients, no method yet exists that can predict the individual response to the treatment. As a consequence, the non-responder patients will not achieve any survival benefit and will suffer from the typical adverse effects that can limit the application of a second-line scheme [157].

The relapsing and progressive nature of bladder tumours, and the heterogeneity in treatment response, are the major drawbacks and concerns in the care of UBC patients. The conventional clinical and pathological parameters have undeniable diagnostic and prognostic value [51, 158]. However, although several risk-stratification algorithms have been developed [33, 148], they are not sufficient to individually characterize a patients' tumour. This crucial goal may only be accomplished when biomarkers of tumour aggressiveness and response to chemotherapy are routinely evaluated in pathological specimens.

UBC is one of the best understood types of cancer, with relatively well-defined pathogenetic pathways and tumour biology [2, 4, 20]. Moreover, traditional approaches of profiling single molecules or pathways are currently being replaced by medium- to high-throughput gene-expression profiling technologies that perform a multiplexed assessment of molecular alterations responsible by carcinogenesis and tumour progression. The wide range of bladder cancer biomarkers that have been reported may prove valuable in several areas, including molecular diagnostics, prediction of tumour recurrence, detection of lymph node metastasis and detection of circulating malignant cells,

identification of therapeutic targets and individualization of treatment [61-62].

Therefore, it is urgent to bridge the gap between the lab bench and the clinical practice, so that bladder cancer patients can rapidly benefit from the use of molecular tests that may diagnose the disease, predict individual prognosis, and suggest the application of targeted therapies.

1.2. Urothelial Bladder Cancer – Translating Biology into Clinical Practice

Thirteen years ago, Hanahan and Weinberg suggested that, although encompassing variable mechanistic strategies, cancers in general acquire a set of functional biological capabilities during their multistep development. These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [159]. In their recent review, the authors added to their previous model two enabling characteristics and two emerging hallmarks (Figure 10). They considered that genome instability generates the genetic diversity underlying the acquisition of all hallmarks, and that inflammation promotes multiple hallmark functions. Additionally, the establishment of a tumour microenvironment by the malignant cells but also by recruited normal cells importantly contributes to energy metabolism reprogramming and immune destruction evasion in order to effectively support neoplastic proliferation [160]. This molecular knowledge is already being applied into clinical practice, with targeted therapies that interfere with each of the hallmarks being developed and entering in clinical trial phase or, in some cases, being approved for clinical use in treating certain forms of human cancer [161-162].

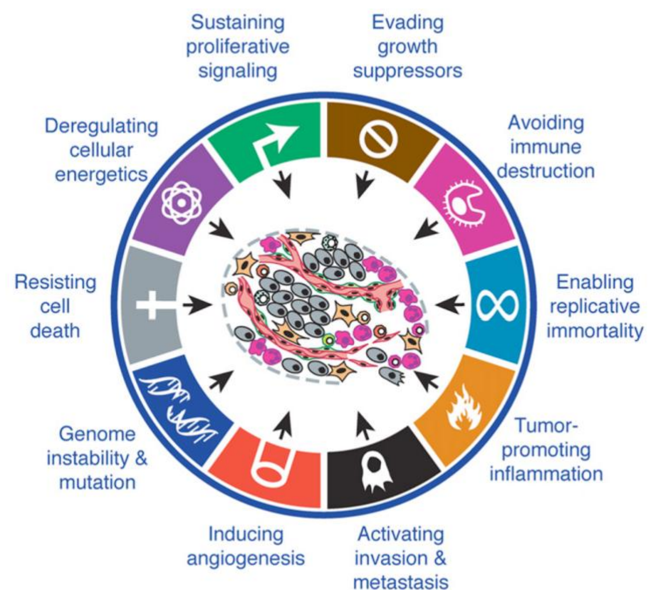


Figure 10 | Capabilities acquired by the malignant cells necessary for tumour growth and progression (adapted from [160]).

In bladder cancer setting, although a reasonable number of biomarkers seem to be prognostically relevant (Table 3), there is a substantial delay in the translation into the clinics, and clinical trials with molecularly targeted agents have been few in number and largely unsuccessful. This is probably due to the unique complexity involved in the dual-track pathway of bladder carcinogenesis, which postulates that UBC develops via two distinct but somewhat overlapping pathways, resulting in the two main phenotypic variants with different biological behaviours and prognoses [163]. Because bladder carcinogenesis involves several genetic and epigenetic alterations, multiple biomarkers must be

integrated into a molecular signature that can accurately predict prognosis and may be suitable for targeted therapy. Inducing angiogenesis (and lymphangiogenesis), activating invasion and metastasis and reprogramming cellular energetics and the tumour microenvironment are considerably overlapping hallmarks that certainly contribute to the acquisition of the ultimate malignant phenotype responsible for the majority of bladder cancer deaths.

1.2.1. Tumour Angiogenesis and Lymphangiogenesis

The dissemination of malignant cells to distant organs from the primary tumour is the leading cause of mortality from cancer and, with few exceptions, all cancers can metastasize [164-165]. Although metastasis can occur by local tissue invasion and direct seeding of body cavities, the main routes of dissemination are the hematogenous and lymphogenous spread. Preclinical and clinical studies suggest that the lymphatic vascular system is preferred over the blood vascular system, and occurrence of lymph node metastasis is an important factor for patients' prognosis and treatment decision-making [166-168]. The malignant cells exploit both vascular systems by expressing growth factors that alter the normal pattern of blood and lymphatic vessel growth, creating conduits for metastasis to occur by tumour-induced angiogenesis and lymphangiogenesis [169-171].

1.2.1.1. Overview of the Vascular Systems

The blood vascular system is the first organic system to develop and reach a functional state in the embryo. In a circular way, it allows that blood leaves the heart, runs through the arteries, arterioles, capillary plexus, venules, and veins, and returns to the heart (Figure 11). This closed circulation is responsible for the cellular inflow of nutrients, outflow of waste products and gas exchanges in most tissues and organs, and also provides gateways for patrolling immune cells [172-173] (Table 5).

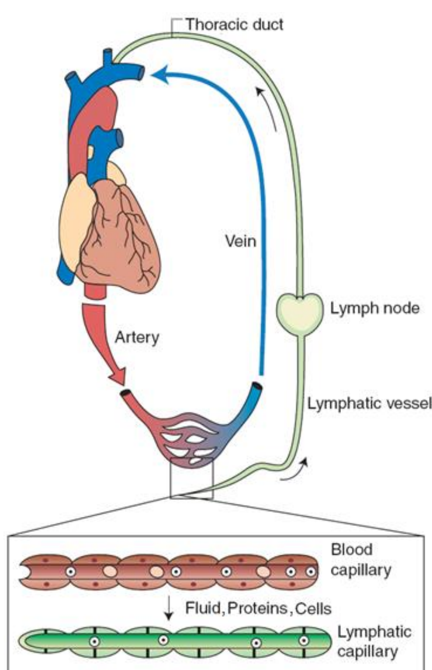


Figure 11 | Macroscopic view of the blood and lymphatic vascular systems (adapted from [172]).

When the cardiovascular system is already functioning, a second vascular network of low-pressure lymphatic vessels and lymphoid organs like lymph nodes develops in order to collect extravasated fluid and macromolecules from tissues and return them to the blood flow via the thoracic duct at the junction of the jugular and subclavian veins (Figure 11). Besides having this essential role in maintaining tissue homeostasis, the lymphatic system also participates in immune surveillance by carrying antigens and antigen-presenting cells from the interstitium to be displayed for B and T cells in the lymph nodes. Moreover, the intestinal villous lacteal lymphatics absorb and transport triglycerides and fat-soluble vitamins [174-176] (Table 5).

Table 5 | Features of blood and lymphatic vascular systems (adapted from [172]).

Feature	Blood vessels/BEC	Lymphatics/LEC
Constituents	Blood, blood cells	Lymph (interstitial fluid rich in protein, fat, and lipids, extravasated immune cells, and large extracellular molecules)
Gross structure	Closed, circular	Open, linear
Start/end	Heart/heart	Tissue/lymph-vein connection of the thoracic duct
Hierarchical division	Arteries, arterioles, capillaries, venules, veins	Capillaries, precollectors, collecting vessels, thoracic duct, lymph nodes
Vessel wall	Adherens and tight junctions, continuous basement membrane, pericytes, or vascular smooth muscle cells	Overlapping LECs, no tight junctions, anchoring filaments, discontinuous basement membrane, few pericytes (collecting lymphatic vessels have both continuous membranes and mural cells)
Development	Vasculogenesis and angiogenesis	Lymphangiogenesis (budding from cardinal vein)
Origin	Mesoderm, endothelial stem/precursor cells from bone marrow for adults	Mesoderm (vein) during development, lymphatic progenitor cells from bone marrow for adults
Examples of cell type–specific markers	CD34, CD105/endoglin	Prox1, LYVE-1, VEGFR-3, and podoplanin
Absence	Cartilage, cornea	Cartilage, brain, bone, spinal cord, and the retina
Functions	Hemostasis, inflammation, leukocyte trafficking, barrier function, delivery for oxygen, nutrients, and tissue wastes	Tissue fluid homeostasis, absorption of large molecules and lipids in the digestive systems, trafficking of lymphocytes and antigen-presenting cells to regional lymph nodes, transport of degraded extracellular molecules, cell debris, and lymph fluid
Heterogeneity	Well-established phenotypic heterogeneity	Comparable LEC heterogeneity was reported. LEC fate is highly plastic in response to genetic and environmental stimuli

Abbreviations: BEC, blood endothelial cells; LEC, lymphatic endothelial cells; LYVE-1, lymphatic vessel hyaluran receptor-1; Prox-1, prospero related homeobox gene-1; VEGFR, vascular endothelial growth factor receptor.

Unlike blood flow, the lymph is not guided by a central pump and flows unidirectionally, initiating in blind-ended lymphatic capillaries. Blood endothelial cells (BEC) are covered by a complete basement

membrane and then encircled by pericytes or smooth muscle cells, which form one or multiple layers increasing in thickness with vessel size, whereas lymphatic capillaries are lined with a single layer of partly overlapping lymphatic endothelial cells (LEC) with a discontinuous basement membrane, and lack vascular mural cells. This structure forms valve-like openings connected to the extracellular matrix by elastic fibers known as anchoring filaments, responsible for maintaining lumen patency during increased interstitial pressure. The collected fluid then drains to precollecting and collecting lymphatics and will eventually be filtered through a series of lymph nodes before re-entering the blood circulation. The lymphatic vessels contain a complete basement membrane, are covered by smooth muscle cells, and form one-way valves (Figure 12). Since the lymphatic network lacks a central driving force, these valves, together with the contractile activity of the vessels' muscular wall, skeletal muscle and respiratory movements, avoid lymph backflow and provide a slow transport under minimal shear stress. Therefore, cell survival conditions inside the lymphatic network are optimal [168, 172, 174, 176-180].

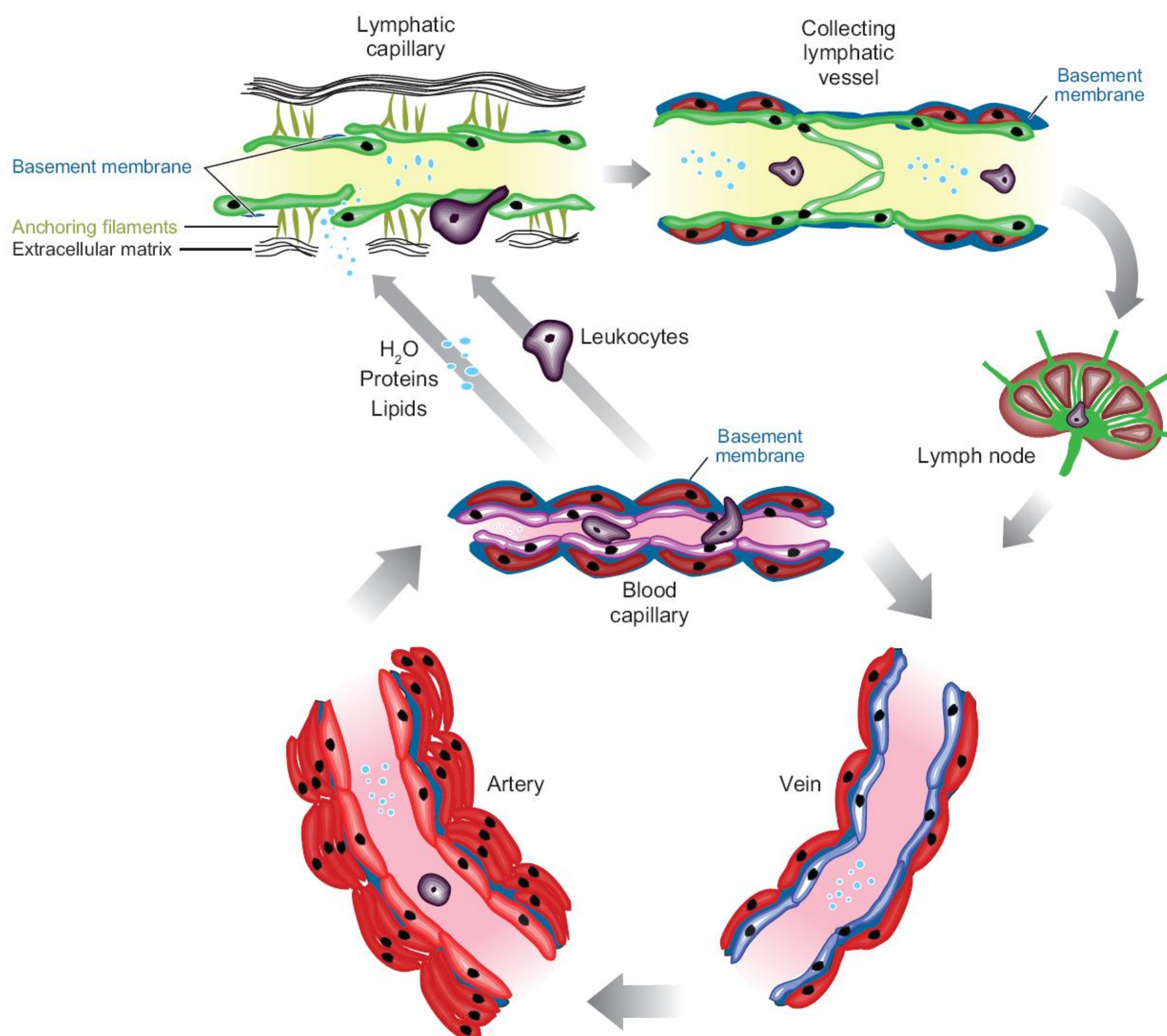


Figure 12 | Structure of blood and lymphatic vessels (adapted from [174]).

1.2.1.2. From Angiogenesis to Lymphangiogenesis

During embryonic development and organogenesis, the formation of the blood vascular system initiates by vasculogenesis: haemangioblast progenitors proliferate, migrate and differentiate into endothelial cells, which in turn will organize a primitive vascular plexus. The plexus serves as a scaffold for angiogenesis, by which sprouting, growth, splitting and pruning remodels the primitive vessels into a hierarchical network of arterial, venous and capillary structures closely interconnecting in a branching pattern. Arteriogenesis forms mature quiescent vessels with the recruitment of mural cells that stabilize the endothelium and control perfusion [173, 181-183] (Figure 13).

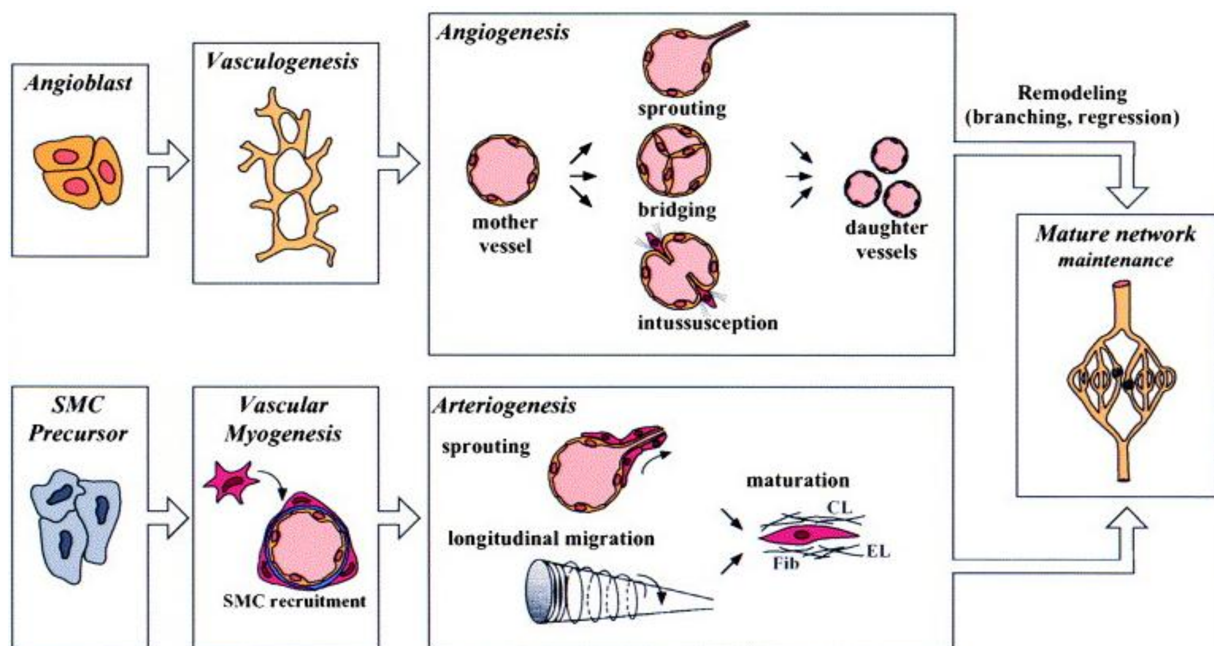


Figure 13 | Overview of vasculogenesis, angiogenesis and arteriogenesis (adapted from [184]).
Abbreviations: CL, collagen; EL, elastin; Fib, fibrillin; SMC, smooth muscle cells.

In the vessel-branching model described above, the sprouting activity of the primitive plexus is initiated by endothelial tip cells. These cells do not proliferate, but are highly polarized and motile, extending filopodia that, in response to an angiogenic stimulus, guide the sprouting at the forefront. Following the tip cells, proliferative endothelial stalk cells elongate a new branch and create a lumen. The fusion between different tip cells connects the branches for initiation of blood flow. Endothelial phalanx cells resume a stable quiescent phenotype, sensing and regulating perfusion in the persistent sprout, and the nascent plexus is then stabilized by arteriogenesis [173, 185-186] (Figure 14).

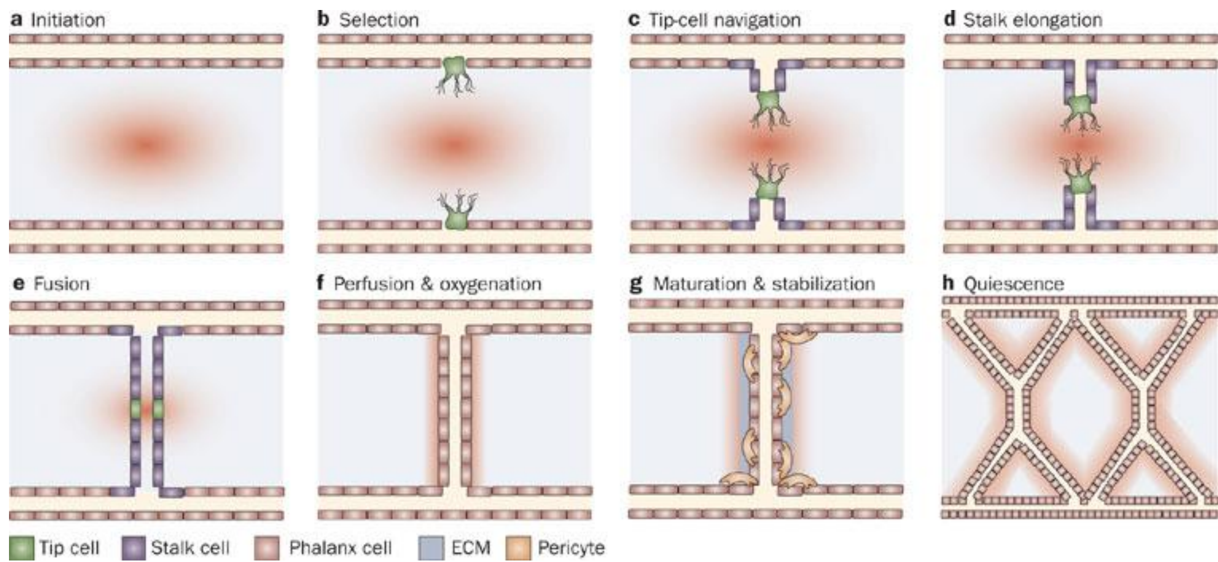


Figure 14 | Formation of novel blood vessel branches by the vessel-branching model (adapted from [185]).

The lymphatic vascular system arises after the cardiovascular system is established and functional. The most widely accepted theory regarding its origin postulates that early in fetal development, a distinct subpopulation of endothelial cells on one side of the anterior cardinal vein responds to lymphatic-inducing signals and commits to the lymphatic lineage, sprouting from the venous endothelium and migrating to form primitive lymph sacs in the jugular region. After several lymph sacs form close to major veins in different regions, centrifugal sprouting of lymphatic vessels from the lymph sacs occurs; these will merge and assemble into separate lymphatic capillary networks that will undergo further remodelling and maturation [172, 174, 178, 180, 187-190] (Figure 15).

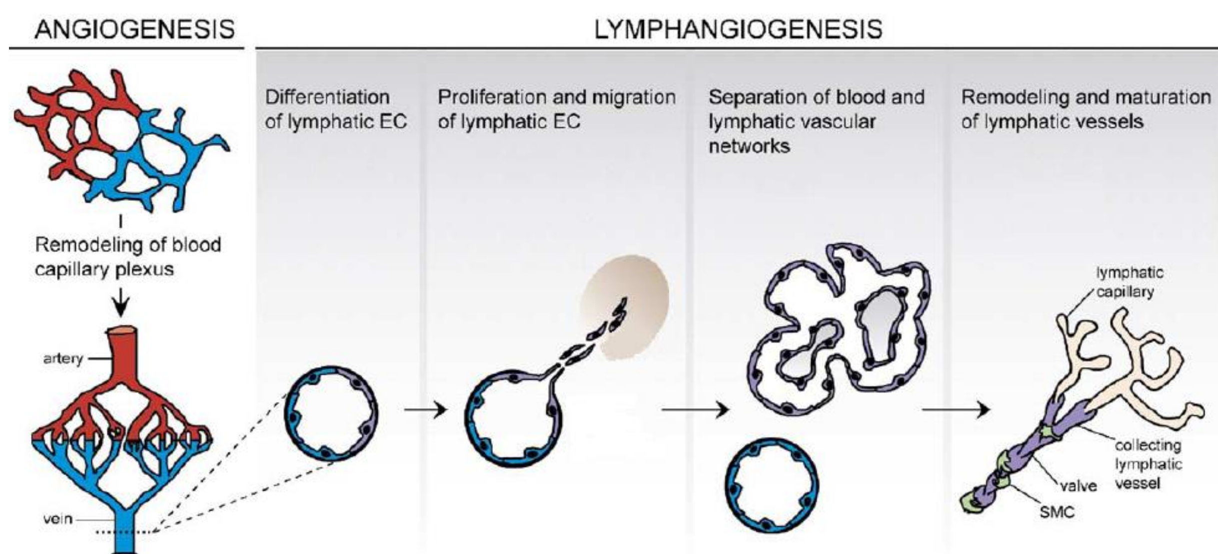


Figure 15 | Development of the lymphatic vasculature during embryogenesis (adapted from [189]).
Abbreviations: SMC, smooth muscle cells.

Angiogenesis and lymphangiogenesis are dynamic processes during embryonic development, but are largely absent in the post-natal period, under normal physiological conditions. In a healthy adult, endothelial cells are quiescent and have long half-lives, although remaining competent to respond to several stimuli in order to maintain or restore tissue integrity, namely during wound healing and the ovarian cycle [170, 173-174, 191]. Once blood and lymphatic vessels nourish and sustain nearly every organ of the body, alterations to the standard pattern of vascular development contribute to numerous diseases. Stroke, myocardial infarction, ulcerative disorders and neurodegeneration may occur as a consequence of insufficient blood vessel growth, and abnormal growth or remodelling of the blood vasculature occurs in inflammatory disorders, pulmonary hypertension and macular degeneration [173, 182, 192]. Congenital or acquired dysfunctions of the lymphatic system result in primary or secondary lymphedema, which impairs fluid balance and immune function [172, 174-175, 178, 193]. Additionally, tumour-induced angiogenesis is critical to the growth and survival of a primary malignant neoplasm by forming a nutrient capillary network, and both vascular systems are exploited by the malignant cells to disseminate and kill patients with cancer. Therefore, the molecular factors involved in the formation of blood and lymphatic vessels during embryogenesis are newly recruited by the growing tumour [170-171].

1.2.1.3. MOLECULAR BASIS OF ANGIOGENESIS AND LYMPHANGIOGENESIS

Cooperative signalling pathways control the proliferation, sprouting and migration of endothelial cells that occur during physiological and pathological blood and lymphatic neovascularization. The main players of this signalling network are vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) [169-170, 180, 194-196].

The VEGF family includes five members in mammals: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF). Moreover, two VEGF homologs, VEGF-E and VEGF-F, have been identified in the genome of the Orf virus, and isolated from snake venom, respectively. All seven VEGFs belong to the platelet-derived growth factor (PDGF)/VEGF supergene family of secreted dimeric glycoprotein growth factors, having a homodimer cysteine knot motif structure with eight conserved cysteine residues in a monomer peptide. The existence of different alternatively spliced isoforms of VEGF-A, VEGF-B and PlGF, and proteolytic processing of VEGF-C and VEGF-D, contribute to further increase the complexity of the VEGF family. Splicing and processing activities regulate the ability of the ligands to bind to specific endothelial transmembrane tyrosine kinase receptors, VEGFR-1/fms-like

tyrosine kinase 1 (Flt1), VEGFR-2/human kinase insert domain receptor (KDR)/mouse foetal liver kinase 1 (Flk1) and VEGFR-3/fms-like tyrosine kinase 4 (Flt4). VEGFRs have a series of immunoglobulin-like domains in the extracellular region, and a conserved intracellular tyrosine kinase domain. In addition, neuropilin-1 (NRP-1) and NRP-2, a few integrins and extracellular matrix components like heparan sulphate function as co-receptors for some members of the VEGF family [169-170, 194, 197-203] (Figure 16).

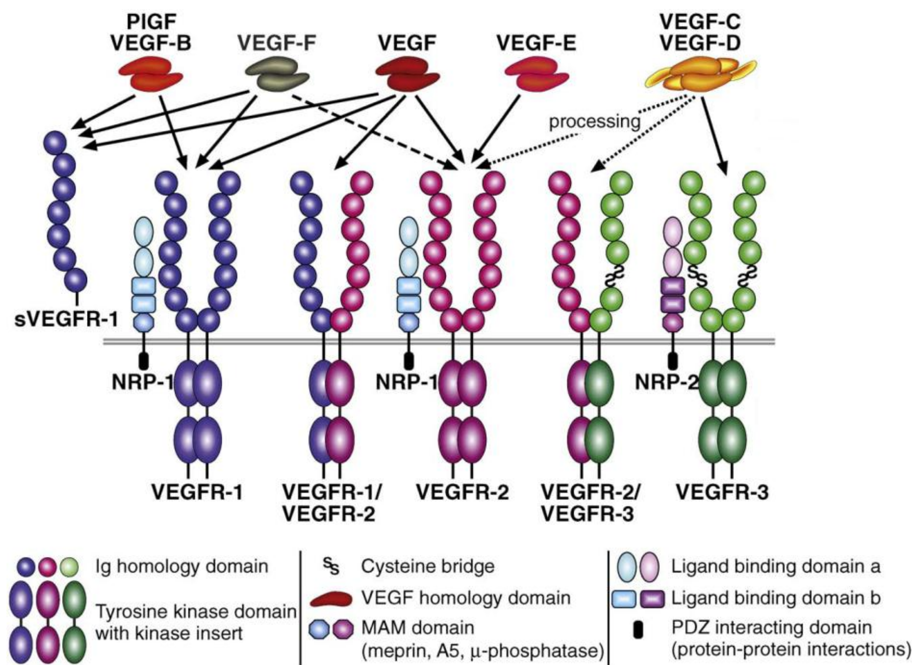


Figure 16 | Structure and interactions of VEGFs, VEGFRs and their NRP co-receptors. (adapted from [170]).

Abbreviations: NRP, neuropilin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

VEGFR-2 is the earliest marker for BEC development [204]. VEGF signalling through VEGFR-2 (Figure 16) is the major mediator of both vasculogenesis and angiogenesis, inducing the proliferation, survival, sprouting and migration of BEC, and also increasing endothelial permeability (VEGF was originally described as vascular permeability factor, VPF [205]). VEGF or VEGFR-2 loss aborts vascular development in the embryo [204, 206]. In response to a VEGF gradient, a quiescent vessel dilates and a tip cell, abundantly expressing VEGFR-2 in filopodia, is selected to sprout. The transmembrane ligand delta-like-4 (DLL4) and its receptor NOTCH are also implicated in the vessel branching model: the tip cell up-regulates DLL4 expression, and the stalk cells up-regulate the expression of the NOTCH receptor and down-regulate VEGFR-2 expression. This renders stalk cells less responsive to VEGF, and warrants that the tip cell takes the lead in the branch formation [170, 173, 185, 195, 207]. Additionally, in order to tip guidance and stalk elongation occur, the basement membrane of the activated endothelium must be degraded and pericytes must detach. These events are mainly guided by the angiopoietin (ANG) and tyrosine kinase with immunoglobulin and EGF homology domains (TIE) family, particularly the ligands

ANG-1 and ANG-2, and the receptor TIE-2. ANG-1 and ANG-2 are expressed by perycites and BECs, respectively. ANG-1/TIE-2 signalling maintains cell quiescence, and stimulates perycite coverage and the deposition of the basement membrane. In the presence of an angiogenic stimulus, sprouting BECs release ANG-2, which antagonizes ANG-1/TIE-2 signalling to enhance perycite detachment, vascular permeability and BEC sprouting [170, 173, 208] (Figure 17).

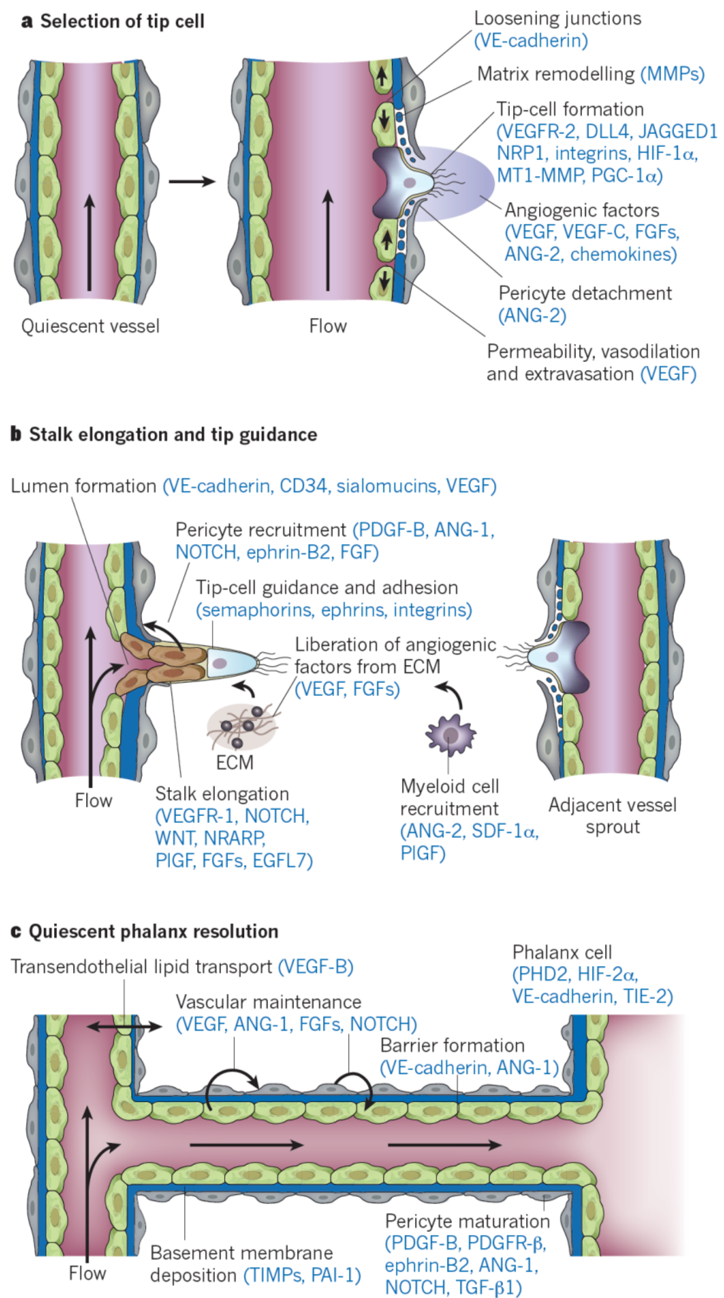


Figure 17 | Molecular basis of the vessel branching model (adapted from [173]). Abbreviations: ANG, angiopoietin; DLL4, delta-like-4; EGFL7, EGF-like domain 7; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; MMP, matrix metalloproteinase; MT, membrane-type; NRARP, Notch-regulated ankyrin repeat protein; NRP, neuropilin; PAI, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PGC, peroxisome proliferator-activated receptor gamma coactivator; PHD, prolyl hydroxylase domain protein; PIGF, placenta growth factor; SDF, stromal cell-derived factor; TGF, transforming growth factor; TIE, tyrosine kinase with immunoglobulin and EGF homology domains; TIMP, tissue inhibitors of metalloproteinase; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endotelial growth factor; VEGFR, VEGF receptor.

After embryogenesis, VEGF/VEGFR-2 axis is downregulated. However, in settings of physiological and pathological angiogenesis, both ligand and receptor become again upregulated. Moreover, although VEGF-B, PIGF and VEGFR-1 do not activate angiogenesis during embryonic development, they have demonstrated angiogenic activity in pathological conditions like ischaemia, inflammation, wound healing and tumour growth [170, 209-210].

In addition to VEGF/VEGFR-2 signalling, other biological factors are involved in the formation of

new blood vessels. Events like remodelling of the extracellular matrix, stalk elongation, tip-cells' guidance and fusion, and quiescent phalanx resolution, are regulated by a crosstalk of several molecular actors, which denotes the complexity of the angiogenic process [170, 173, 185, 195] (Figure 17).

Following the formation of the blood vascular system in the embryo, certain BEC become responsive to lymphatic inducing-signals. The first marker of lymphatic endothelial commitment is the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), a CD44 homologous transmembrane protein. Initially, LYVE-1 is evenly expressed by the blood endothelium of the cardinal vein [211]. Then, the prospero related homeobox gene-1 (Prox-1), activated by the transcription factor Sox18 [SRY (sex determining region Y) box 18], is selectively expressed in a subpopulation of BEC, which determines the establishment of the lymphatic competence and initiates the formation of the lymphatic vascular system [212-214]. Later, Prox1/LYVE-1–positive cells sprout and migrate dorsolaterally from the cardinal vein, forming the first lymphatic structures in regions where surrounding mesenchymal cells express the lymphangiogenic growth factor VEGF-C [215]. The matrix-interacting protein collagen and calcium-binding EGF domains 1 (CCBE1) strongly enhances the pro-lymphangiogenic activity of VEGF-C [216-217] (Figure 18).

VEGF-C signalling through VEGFR-3 and its nonsignalling transmembrane co-receptor neuropilin 2 is required for the proliferation, migration, and survival of LEC until the postnatal maturation of the lymphatic vasculature occurs [215, 218] (Figure 16). In the absence of VEGF-C, the development of the lymphatic system is blocked [215]. VEGF-D, another known ligand for VEGFR-3, seems to be largely dispensable for the embryogenesis of lymph vessels [219]. VEGFR-3 is evenly expressed by all endothelial cells during initial stages of development. In fact, *Vegfr3* deletion leads to defective development of the cardiovascular system and embryonic death at mid-gestation, which postulates an early blood vascular function [220]. As the lymphatic vascular system begins to develop, its expression becomes restricted to LEC, with the exception of the fenestrated blood vessels present in some endocrine organs (thyroid, adrenal glands and pancreas) [221-222]. VEGFR-3 inhibition during later embryonic and early postnatal stages leads to regression of developing lymphatic vessels [223-224].

The specific biologic effects of VEGF-C interaction with VEGFR-3 are critically dependent on the proteolytic processing of the ligand. Upon proteolytic cleavage, VEGF-C affinity toward its receptor increases, and the fully processed forms of VEGF-C/VEGF-D also activate VEGFR-2 and can induce blood vessel growth [225-228]. Additionally, VEGFR-3 can heterodimerize with VEGFR-2, and it has

been suggested that, in adult lymphangiogenesis, VEGFR-2 and VEGFR-3 signalling have cooperative and redundant roles [229] (Figure 16).

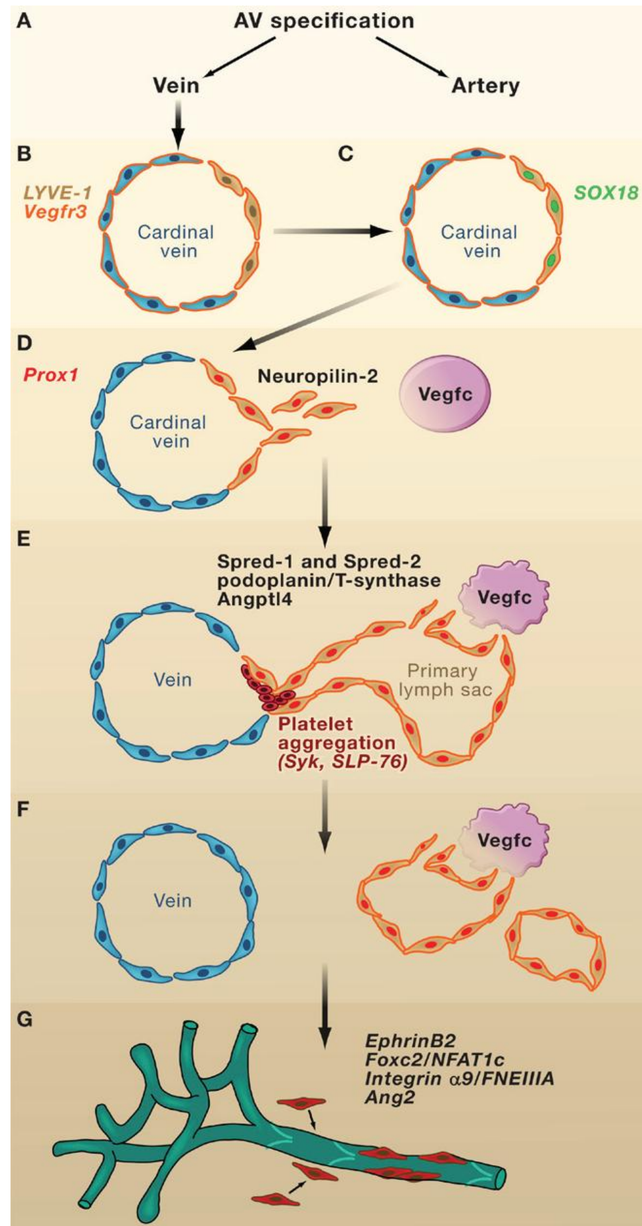


Figure 18 | Molecular basis of the lymphatic vasculature development. **A**, arterial-venous specification; **B**, lymphatic competence; **C**, lymphatic commitment; **D**, budding, migration, and proliferation of lymphatic endothelial cells; **E**, separation of blood and lymphatic vasculature; **F**, centrifugal growth of the lymphatic vessel network; **G**, remodeling and maturation of the lymphatic vasculature (adapted from [230]).

Abbreviations: Ang2, angiopoietin 2; Angptl4, angiopoietin-like protein 4; AV, arterial-venous; FNEIIIA, fibronectin EIIIA; Foxc2, forkhead box C2; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; NFAT1c, nuclear factor of activated T-cells, cytoplasmic 1; Prox-1, prospero related homeobox gene-1; SLP-76, SH2 domain-containing leucocyte protein, 76-kDa; Sox18, SRY (sex determining region Y) box 18; Spred, sprouty-related, EVH1 domain-containing protein; Syk, protein-tyrosine kinase SYK; Vegfc, vascular endothelial growth factor c; Vegfr3, vascular endothelial receptor 3.

After LEC commitment and establishment of the primary lymph sacs, the critical process of separation of the lymphatic vessels from the blood vessels must occur in order to ensure the proper function of the two vascular systems. Expression of the tyrosine kinase SYK and its adaptor protein SLP-76 (SH2 domain-containing leucocyte protein, 76-kDa) by circulating lymphatic endothelial precursors, and platelet activation by podoplanin, seem to be essential for the blood/lymphatic disconnection [231-233]. The sustained VEGF-C/VEGFR-3 signalling assures the centrifugal growth of the lymphatic vessel network, and additional molecular players promote the remodelling and maturation of the final lymphatic vasculature [172, 174, 176, 178-179, 230] (Figure 18).

1.2.1.4. ANGIOGENIC AND LYMPHANGIOGENIC SWITCH IN TUMOURS

More than forty years ago, Judah Folkman articulated the theory that tumour growth depends on the recruitment of new blood vessels, anticipating possible therapeutic implications from this biological event [234]. Later, the term "angiogenic switch" has emerged to describe the transition phase where a pre-vascular hyperplasia evolves to highly vascularised and progressively outgrowing tumours [235]. In fact, once a primary tumour mass reaches a critical size, its growth is impaired by the lack of an appropriate supply of oxygen and nutrients. However, the malignant cells rapidly overcome this growth inhibition and gain additional capabilities of progression and dissemination by inducing the formation of new blood vessels from pre-existing ones [236]. The angiogenic switch is a discrete step in tumour development that can occur at different stages in the progression pathway, depending on the nature of the tumour and its microenvironment [237].

In adult normal tissues, the levels of pro- and anti-angiogenic signals, regulated at the level of gene expression, secretion and proteolytic activation, dictate whether an endothelial cell will be in a quiescent or an angiogenic state (Figure 19). During the angiogenic switch in tumours, the dynamic balance between positive and negative controllers, derived from tumour cells themselves and from tumour-infiltrating stromal cells (pericytes, cancer-associated fibroblasts and cells of the immune system) is lost [235-237] (Figure 20). Overexpression of pro-angiogenic factors is induced by environmental stresses like hypoxia [238-239], nutrient deprivation [240-241], formation of reactive oxygen species (ROS) [242], cellular acidosis [243] or iron deficiency [244], by activation of oncogenes [245] or by loss of function of tumour suppressor genes [246]. Recent evidences highlight the importance of the epigenetic control of angiogenesis, particularly by non-coding microRNAs (miRNAs) that are expressed by BEC in response to hypoxia or VEGF levels [247-249].

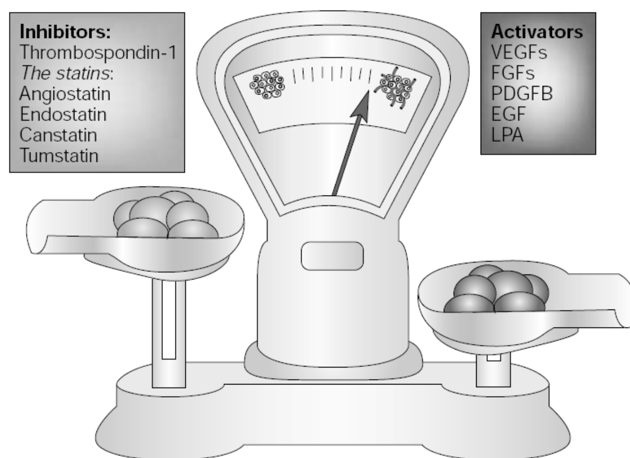


Figure 19 | The angiogenic balance (adapted from [237]). Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; LPA, lysophosphatic acid; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

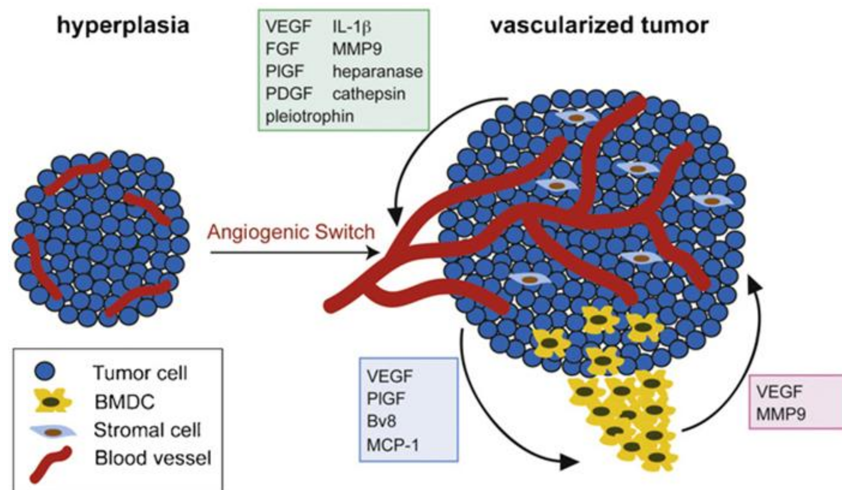


Figure 20 | Molecular and cellular players underlying the angiogenic switch in tumours (green box, pro-angiogenic factors and proteases secreted by the tumor cells; pink box, pro-angiogenic factors and proteases secreted by cells of the immune system recruited to the tumor site; blue box, pro-angiogenic factors secreted by the tumor cells to recruit inflammatory cells) (adapted from [236]).
Abbreviations: BMCD, bone marrow-derived cell; Bv8, Bombina variagata peptide 8; FGF, fibroblast growth factor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PlGF, placenta growth factor; VEGF, vascular endothelial growth factor.

VEGF signalling through VEGFR-2 in the hypoxic microenvironment of the avascular primary tumour seems to be the most ubiquitous molecular mechanism underlying the angiogenic switch. *Vegf* gene expression is upregulated in hypoxia via the oxygen sensor hypoxia-inducible factor (HIF)-1α. Under normoxic conditions, HIF-1α is hydroxylated by the oxygen sensing enzymes prolyl hydroxylases (PHDs) expressed by BEC, and targeted for proteasomal degradation. However, under hypoxic conditions, PHDs become inactivated, and HIF-1α initiates transcriptional responses to increase oxygen supply by angiogenesis, namely by upregulating VEGF expression [239, 250-251]. Tumour, myeloid or other stromal cells release paracrine VEGF, which increases vessel branching and contributes to vessel abnormalization [252]. Additionally, paracrine VEGF induces the expression of plasminogen activators and matrix metalloproteinases, indirectly mediating the degradation of the basement membrane [253]. Autocrine VEGF released by BEC maintains vascular homeostasis [254].

The mechanisms implicated in the embryonic development of the cardiovascular system – vasculogenesis and sprouting angiogenesis – are newly recruited during the formation of the tumour's blood supply. Moreover, the malignant cells can use other modes of vessel formation, namely intussusception (process of vessel splitting that also occurs in normal tissues), vessel co-option (process in which tumour cells hijack the existing vasculature), vascular mimicry (process in which tumour cells line vessels) or differentiation of putative cancer stem cells into BEC [173, 195, 255] (Figure 21). Both tumour and endothelial cells can present distinct phenotypes in particular organs, tumour types and subtypes [256].

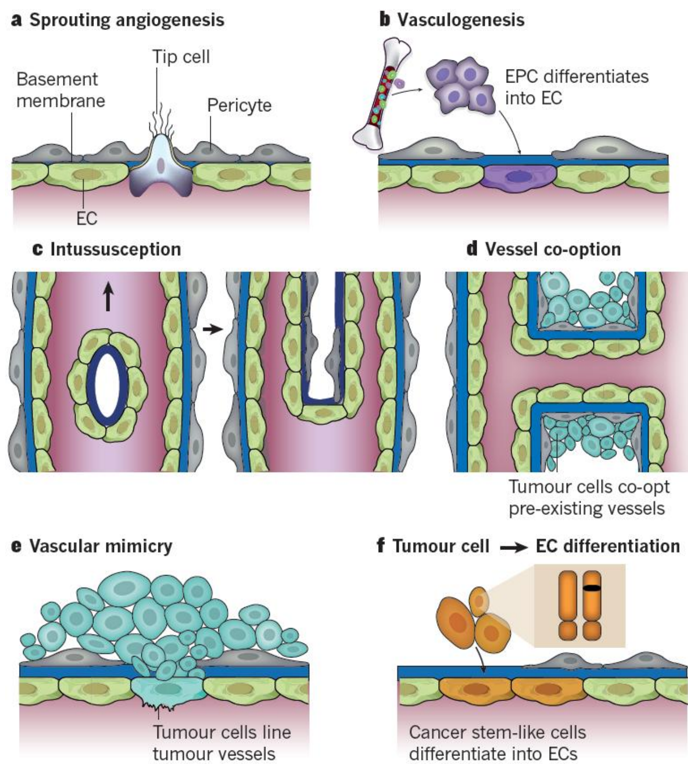


Figure 21 | Possible mechanisms of blood vessel formation in tumours (adapted from [173]).
Abbreviations: EC, endothelial cell; EPC, endothelial progenitor cell.

Although the concept of the "angiogenic switch" is clearly defined, the mechanisms that trigger tumour lymphangiogenesis are not fully understood. Experimental evidence to support a "lymphangiogenic switch" is still lacking. However, as for tumour angiogenesis, it seems probable that the acquisition of new lymphatic vessels is elicited at some point during tumour development. The high interstitial pressure generated inside the tumors due to the excessive production of interstitial fluid has been proposed as a putative trigger mechanism. In fact, a tumour-associated lymphatic neovasculature can potentially collect the interstitial fluid leaked from blood vessels, also establishing the route for lymphatic vessel invasion, lymph node involvement and distant metastasis [167]. Moreover, inflammation seems to promote lymphatic neovascularization: VEGF can indirectly support inflammatory lymphangiogenesis by attracting VEGFR-1 expressing macrophages; these secrete lymphangiogenic growth factors, namely VEGF-C and VEGF-D [257-258]. Interleukins 6 and 17 equally seem to mediate VEGF-C upregulation in some tumours [259-260]. Extracellular matrix signalling can also mediate tumour lymphangiogenesis. Tumour-derived hyaluronan may directly interact with cell surface receptors in LEC, namely LYVE-1, and accelerate tumor lymphangiogenesis [261]. The induction of integrin $\alpha9\beta1$ expression by Prox1 on LEC stimulates migration of these cells towards VEGF-C and VEGF-D gradients [203, 262]. The matrix cell-adhesive glycoprotein fibronection and the endoglycosidase heparanase, important players of the metastatic cascade, have shown to induce VEGF-C secretion by malignant cells [263-264].

VEGF-C and VEGF-D signalling through VEGFR-3 is the key molecular pathway underlying tumour lymphatic neovascularization. Furthermore, proteolytic processed forms of the ligands may be generated in some tumours, which target VEGFR-2 homodimers or VEGFR-2/VEGFR-3 heterodimers, thus contributing to tumour angiogenesis [167, 193, 230, 257, 265]. Conversely, in order to prepare a

pre-metastatic niche and eventually sculpt an immune response permissive to malignant cells' survival, VEGF interaction with VEGFR-2 may also promote distal lymphangiogenesis inside the sentinel lymph nodes before lymph node metastasis occurrence [266]. The molecular properties of the VEGF family of ligands and receptors link blood and lymphatic neovascularization in tumours, and it seems that the ultra-structure of these tumor-associated vessels largely contributes to the success of malignant dissemination [267].

1.2.1.5. Structure of Tumour Neovasculature

During embryonic development and post-natal physiological events that require a new blood and lymphatic supply, angiogenesis and lymphangiogenesis occur under highly coordinated molecular signalling cascades in order to form structured and functional vasculatures. On the other hand, during tumour development, the excessive and disorganized production of angiogenic and lymphangiogenic factors, coupled with an imbalance in the growth of both vascular systems, renders tumour neovessels hyperactive and abnormal in almost all aspects of their structure and function [267].

Tumour blood vessels are tortuous, following a serpentine course and branching irregularly, and have uneven lumens. Heterogeneity is also evident: the vasculature is shaped by different vessel subtypes, including large and hyperpermeable "mother" vessels, capillaries, glomeruloid microvascular proliferations and vascular malformations. BEC express an aberrant molecular signature and may, in some cases, switch their phenotype. These cells lose their polarized alignment, detach from the basement membrane and stack upon each other, forming pseudostratified layers that obstruct the lumen with filipodia-like protrusions, and intercellular gaps that constitute gateways for the entry of tumour cells. In addition, the basement membrane is discontinuous or absent, and the mural pericytes have an abnormal shape, loosely associating with BEC and extending their membrane processes into the surrounding stroma [173, 185, 192, 267-271] (Figure 22). As a consequence of these aberrant features, tumour blood vessels are leaky, which substantially increases interstitial tumour pressure, and blood flow is chaotic and variable, impairing the functional delivery of oxygen, nutrients, immune cells and drugs. A very hostile microenvironment is generated, characterized by hypoxia, low pH and high interstitial pressure. In response, the malignant cells overexpress pro-angiogenic factors as an attempt to overcome oxygen shortage, which produces a vicious cycle of abnormal blood vessels and hypoxia and selects resistant clones of malignant cells, facilitating their escape through the leaky vasculature. Hypoxia also reduces the efficacy of radiation therapy and many chemotherapeutics that rely on the

formation of ROS to eliminate malignant cells [173, 192, 267, 272].

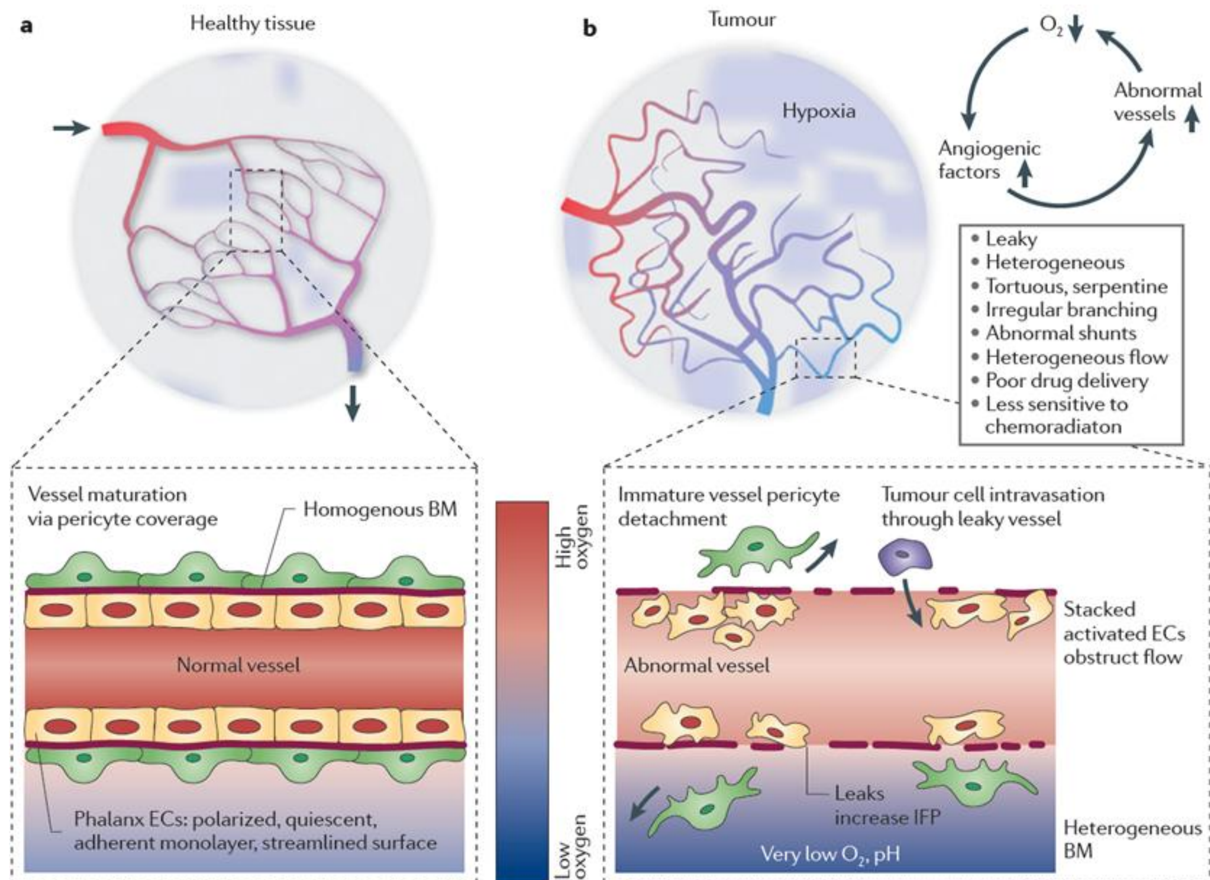


Figure 22 | Structure of blood vessels in normal tissues and in tumours (adapted from [192]).
Abbreviations: BM, basement membrane; EC, endothelial cell; IFP, interstitial fluid pressure.

The intratumoural edema generated by the leaky blood vessels is pernicious to the tumour mass. The formation of a lymphatic neovasculature could potentially drain the excessive amount of fluids, and the pressure gradient, together with the specific structure of the lymphatic capillaries (the lymphatic endothelium has a loose and overlapping structure, Figure 12), facilitates tumour cells' entry into the lymphatic vasculature. Opposing to the bloodstream, where intravased malignant cells or emboli experience serum toxicity, high shear stress and mechanical deformation, the lymph flow is ideal for the survival and dissemination of malignant cells. Lymph has a composition similar to interstitial fluid, and flows slowly, encountering stagnation areas in the lymph nodes that represent optimal "incubators" for the growth of tumour cells. Here, these cells can exit through the efferent channels or high endothelial venules, or can remain entrapped, originating micrometastases [168, 258, 265, 273] (Figure 23).

Tumour-associated lymphatic vessels are, in general, morphologically similar to normal vessels, but display different molecular profiles, thus contributing to the active role of the endothelium in

mediating progression and metastasis of the primary neoplasm, even in an organ-specific context [274-275]. LEC extend long filopodia towards malignant cells secreting VEGF-C/D, which results in the enlargement of the lumen from lymphatic capillaries and collecting vessels, facilitating the transendothelial migration of the malignant cells and the transit of cellular emboli [276] (Figure 23). Chemokines, that under physiological conditions are critical to the homing of hematopoietic cells to specific locations, seem to be involved in the chemotactic migration of tumour cells into the lymphatic vessels [277]. For instance, tumour-associated LEC, but not normal LEC, highly express CXCL12 (chemokine, CXC motif, ligand 12). On the other hand, the receptor CXCR4 (chemokine, CXC motif, receptor 4) is abundantly expressed by numerous types of malignant cells (like breast, prostate or ovarian epithelia), being largely absent in their normal counterparts. Interestingly, isolated LEC and lymphatic endothelium from vessels present at preferential sites of metastasis, such as lung, liver and bone, also express CXCL12. Lymph nodes equally display high concentrations of CXCL12. Therefore, CXCR4-expressing tumour cells disseminate specifically into tissues that express the ligand [168, 278-280]. Moreover, VEGF-C or VEGF-mediated lymph node lymphangiogenesis (Figure 23), occurring prior to the arrival of malignant cells, seems to facilitate the subsequent metastatic spread throughout the lymphatic vasculature [266, 281]. The selective expression of tumour cell adhesion mediators in the lymph node sinus can also contribute to further direct metastasis [282].

Lymphangiogenesis can occur both in peritumoural and intratumoural regions (Figure 23). However, the functionality of intratumoural lymphatic vessels has been highly debated [275, 283]. These vessels are small and often collapsed by the high interstitial pressure or occluded by infiltrating malignant cells, and the new lymphatic vessels that sprout from pre-existing ones at the tumour margin may be more important for malignant dissemination [284-288]. However, other studies have demonstrated that intratumoural lymphatics are vital to the success of lymphatic metastasis in several types of tumours [289-296]. Probably, organ-specific determinants mediate the occurrence of peritumoural and/or intratumoural lymphangiogenesis, as well as the function of the newly formed vessels. Additionally, the specific features of the tumour-associated lymphatics, together with the structure and physiology of the lymphatic system, makes them the preferential routes for malignant cells' intravasion and dissemination: follow-up data have shown that only 20% of the tumours use the blood vascular system to metastasize to distant organs; 80% of the tumors, particularly those of epithelial origin, follow an orderly pattern of dissemination via the lymphatic network [257, 297].

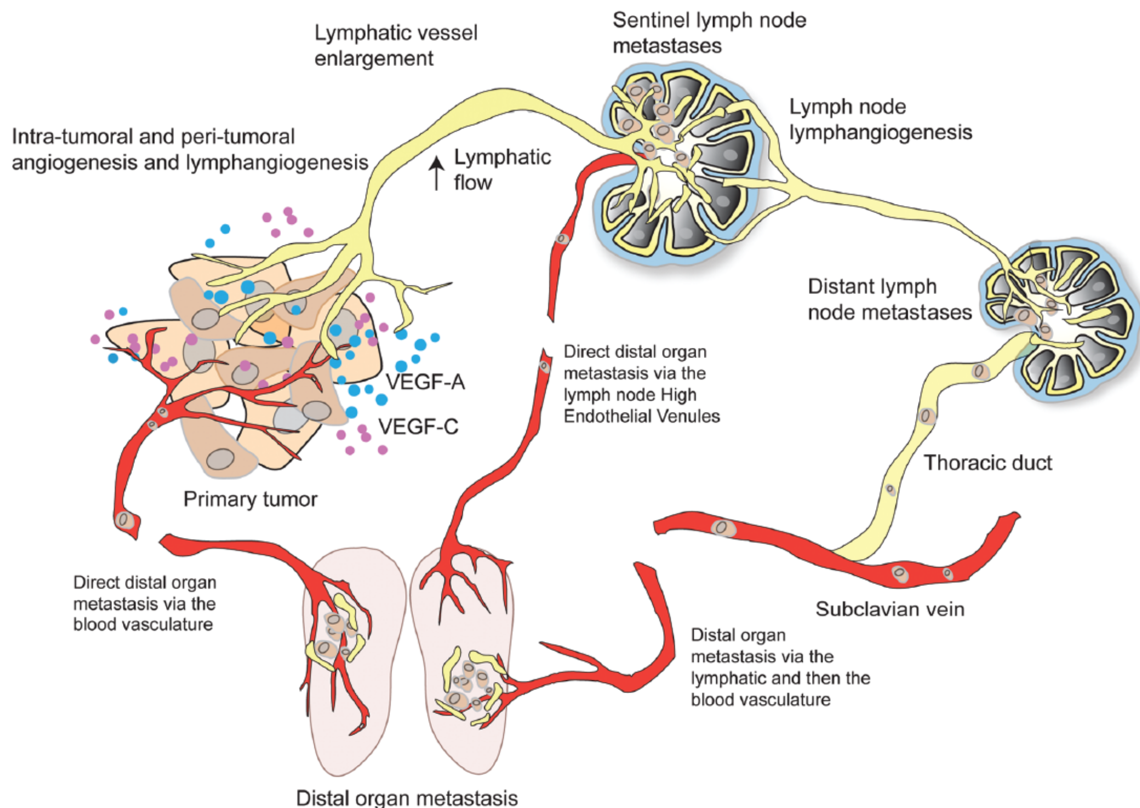


Figure 23 | Pathways for malignant cells' dissemination (adapted from [168]).
Abbreviations: VEGF, vascular endothelial growth factor.

1.2.1.6. Tumour Neovascularization – Impact on Cancer Patients

The overexpression of angiogenic and lymphangiogenic growth factors in tumours significantly increases blood vessel density (BVD) and lymphatic vessel density (LVD), and establishes the routes for blood vessel invasion (BVI) and lymphatic vessel invasion (LVI) by malignant cells. A significant number of retrospective studies have primarily investigated the influence of these parameters on patients' prognosis via immunohistochemical analysis, using specific antibodies that highlight the expression of the growth factors or the expression of specific markers of BEC and LEC. The results generally point out for a significant association between the occurrence of angiogenesis and BVI, lymphangiogenesis and LVI, and the risk of tumour recurrence, progression, lymph node metastasis, distant metastasis and death for patients with non-small cell lung cancer [298-302], breast [303-307] and ovarian [307-309] carcinomas, head and neck cancers [308-313], gastrointestinal tract malignancies [288, 314-321], urological cancers [95-96, 322-327], among others. In accordance with those results, blocking the expression of angiogenic and lymphangiogenic growth factors in preclinical models has inhibited tumour growth and expansion of the tumour-associated vasculature, and reduced malignant spread [328-334].

Therefore, it is not surprising that novel anti-angiogenic/lymphangiogenic agents and combinations including chemotherapeutic drugs, as well as targeted inhibitors, are currently under clinical trial phase or have already obtained the FDA approval for treating cancer patients.

Two types of tumour-associated neovasculature inhibitors have been described. Direct inhibitors are molecules or compounds that block a common pathway of vessel development by acting directly on endothelial cells; indirect inhibitors, being preferred over the direct ones due to their mode of action, are antibodies, soluble receptors or small chemical compounds that target different levels of the growth-factor receptor-activated signalling pathways, from the ligands, their receptors or downstream signalling components [258, 335]. The therapeutic interference with VEGFs/VEGFRs signalling has been the focus of the vast majority of the trials, by testing monoclonal antibodies and soluble versions of receptors that neutralize the ligand-receptor interaction, or molecule tyrosine kinase inhibitors (TKIs) that inhibit the kinase activity of multiple receptors [271, 336]. This last strategy has the advantage of targeting both angiogenic and lymphangiogenic cascades, which might compromise the success of both haematogenous and lymphogenous spread (Figure 24). Regarding specific anti-lymphangiogenic compounds, there is some delay with the translation into the clinics, although several possibilities have been tested in the pre-clinical scenario [337].

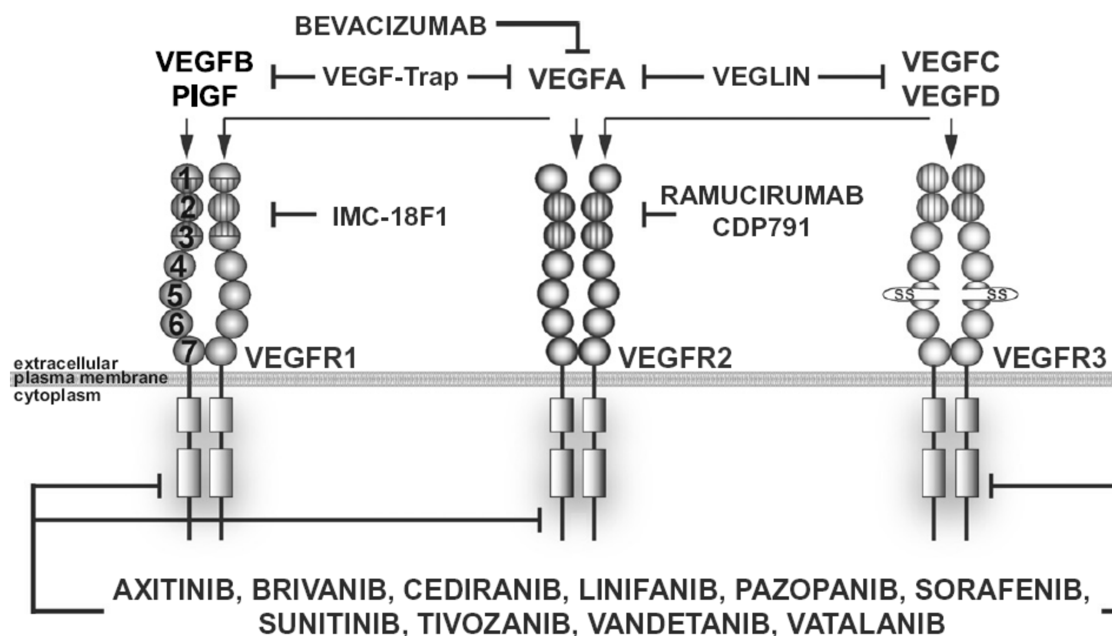


Figure 24 | Inhibitors and targets of vascular endothelial growth factors and receptors (adapted from [200]). Abbreviations: PIGF, placenta growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

In 2004, the VEGF-neutralizing antibody bevacizumab (Avastin®), used in combination with

traditional chemotherapy, became the first anti-angiogenic therapy for cancer [338] (Figure 24). So far, The FDA has approved its use for metastatic colorectal cancer (with chemotherapy), metastatic non-squamous non-small-cell lung cancer (with chemotherapy), metastatic breast cancer (with chemotherapy), recurrent glioblastoma multiform (in monotherapy) and metastatic renal cell carcinoma (with interferon-α) [173]. In addition, several multi-targeted TKIs have also obtained FDA approval, namely sorafenib (Nexavar®, for metastatic renal cell carcinoma and hepatocellular carcinoma) [339-340], sunitinib (Sutent®, for metastatic renal cell carcinoma, pancreatic neuroendocrine tumors and gastrointestinal stromal tumors) [341-343], pazopanib (Votrient®, for metastatic renal cell carcinoma and soft tissue sarcoma) [344-345] and vandetanib (Zactima™, for unresectable or metastatic medullary thyroid cancer) [346].

The strategy to arrest tumour neovascularization – originally proposed by Judah Folkman [234] – has been challenging. The treatment with VEGFs/VEGFRs signalling blockers generally prolongs the survival of responsive cancer patients only a few months, coupled with a plethora of serious side effects (hemorrhage and arterial thromboembolic events, surgery and wound healing complications, gastrointestinal perforation, among others) [347]. Bevacizumab, being a specific anti-VEGF antibody, generally allows survival benefits only when administered in combination with cytotoxic or cytokine drugs [348]. Although multi-targeted TKIs are effective as monotherapy in certain types of cancer, fail in other types or are toxic when combined with chemotherapy. Importantly, a substantial part of the patients with advanced disease do not respond to neovascularization inhibitors, and even develop resistance. Currently, there are no validated predictive biomarkers to appropriately select cancer patients for anti-neovascularization therapy; only a few candidates have been identified, although emerging from small studies and requiring prospective validation [349-350]. There is the need to modify the initial theory of radically starving the tumour by abrogating the blood supply, because aggressive neovascularization inhibition may intensify tumour metabolism and promote malignant dissemination [351].

Several mechanisms have been proposed to explain refractoriness to VEGFs/VEGFRs signalling blockade in advanced malignancies. First, the vicious cycle of hypoxia and upregulation of the production of neovascularization factors may facilitate further tumour progression from hypoxia tolerant cells. Second, the alternative mechanisms of vessel formation beyond angiogenesis (Figure 21) may originate vessels that are less sensitive to VEGF blockade. Third, mural cells may also contribute for the insensitivity to inhibition strategies. Fourth, non-tumoural cells, like bone marrow-derived cells, macrophages and fibroblasts may also produce pro-angiogenic factors and rescue tumour

neovascularization. Importantly, pro-angiogenic and lymphangiogenic molecules alterative to the VEGFs/ VEGFRs signalling may be produced by tumour or stromal cells, namely PlGF, PDGFs, FGFs, chemokines and ephrins, turning neovascularization into a VEGF-independent phenomenon [173, 349-350] (Figure 25).

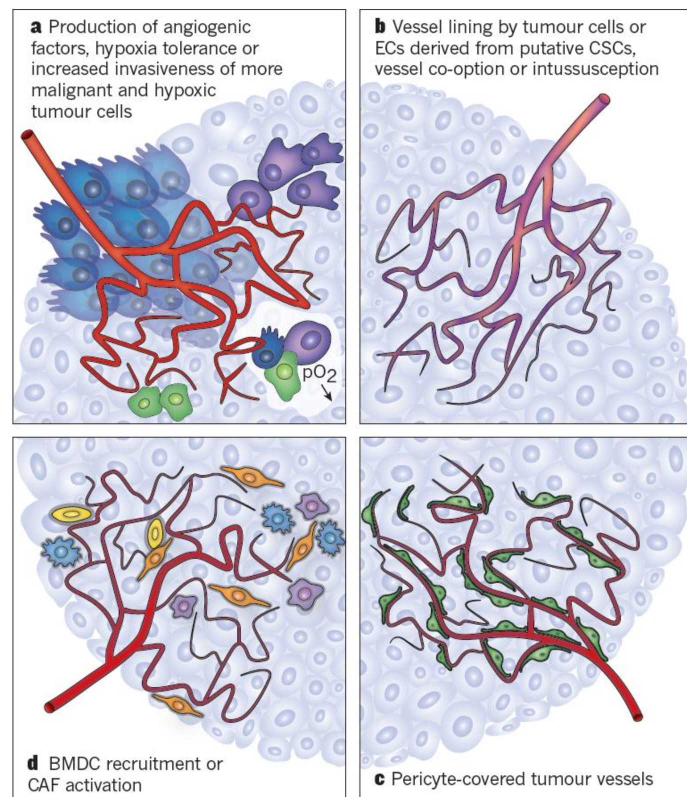


Figure 25 | Potential mechanisms of resistance to targeted VEGF therapy (adapted from [173]).
Abbreviations: BMDC, bone marrow-derived cells; CAF, cancer-associated fibroblasts; CSCs, cancer-stem cells; ECs, endothelial cells.

Although the original therapeutic goal of traditional anti-angiogenic agents was to inhibit neovascularization and/or to eliminate existing vessels, conflicting clinical evidences have confirmed the occurrence of vessel normalization in cancer patients receiving those agents [270]. Importantly, in pre-clinical models, vessel normalization does not have an effect on the growth of the primary tumour, but improves perfusion and oxygenation, reduces interstitial fluid pressure and, more importantly, decreases BVI and metastasis, and increases the efficacy of cytotoxic drugs during the transient window of normalization [352-354]. Therefore, vessel normalization is emerging as a promising target to complement current anti-angiogenic strategies. However, many challenges remain to be solved until those insights can be translated into daily clinical practice. Moreover, predictive biomarkers are desperately needed [173, 192, 349, 355].

An alternative approach to inhibit tumour neovascularization and metastasis is to target VEGFs' upstream signalling pathways that indirectly promote angiogenesis and/or lymphangiogenesis in physiological and malignant scenarios. The mammalian target of rapamycin (mTOR) intracellular pathway (Figure 26) represents a potential target. It is a family of large proteins (~ 290 kDa) that share 40%–60% identity, belonging to the phosphoinositide-3-kinase-related kinase family, which controls signal transduction from several growth factors and upstream proteins to the level of mRNA and

ribosome, lying at the interface of two major signalling pathways. One is initiated by phosphatidylinositol 3-kinase (PI3K), and its accumulation is antagonized by the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10). The other is initiated by an energy-sensing pathway that involves LKB1 (liver kinase B1) [356-357].

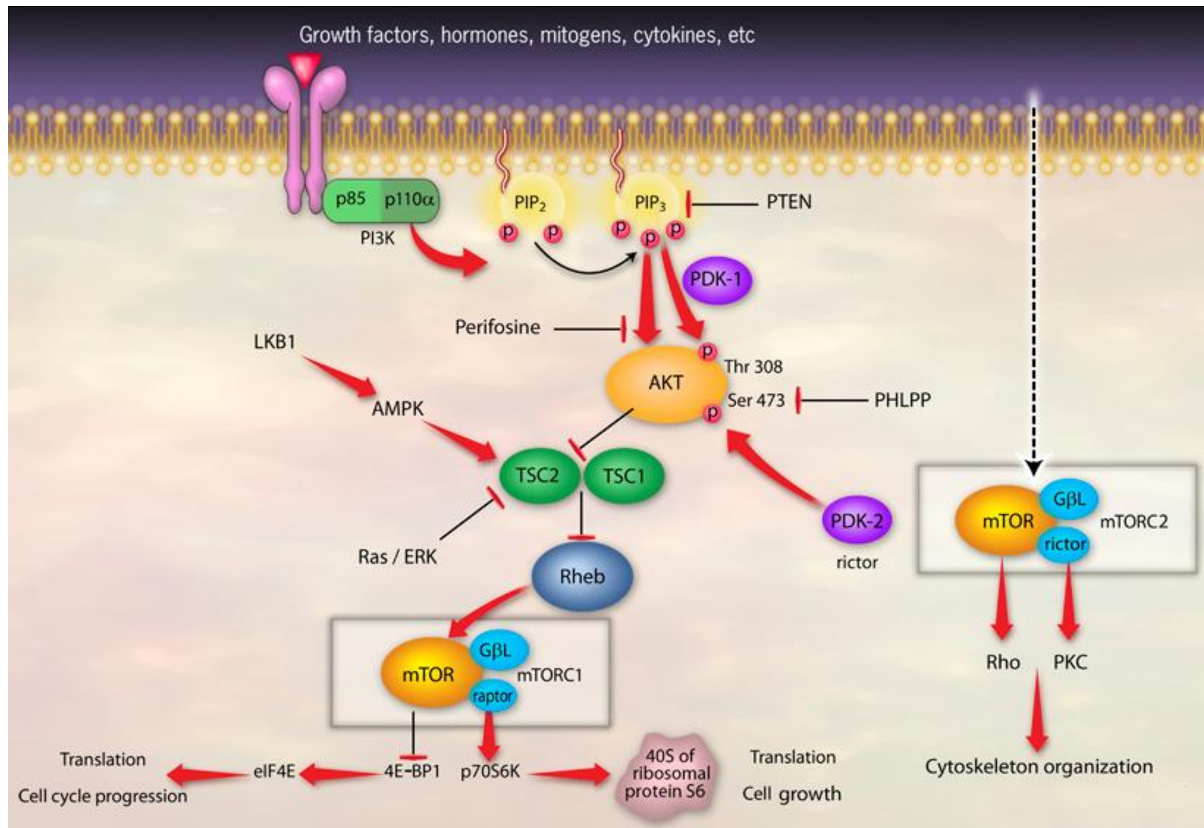


Figure 26 | Schematic representation of the mTOR-signalling pathway. Arrows represent activation, and bars represent inhibition (adapted from [358]).

Abbreviations: AKT, protein kinase B; AMPK, AMP-activated protein kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular-signal-regulated kinase; GβL, G protein beta subunit like protein; LKB1, liver kinase B1; mTOR, mammalian target of rapamycin; p70S6K, ribosomal p70 S6 kinase; PHLPP, PH domain and leucine rich repeat protein phosphatases; PDK, phosphoinositide dependent kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol (3,4)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKC, protein kinase C; PTEN, phosphatase and tensin homolog deleted on chromosome 10; raptor, regulatory-associated protein of mTOR; rheb, Ras homologue enriched in brain; rictor, rapamycin-insensitive companion of mTOR; Ser, serine; Thr, threonine; TSC, tuberous sclerosis complex.

Although a single mTOR gene exists in mammals (*mTOR*, located at the 1p36.2 chromosomal position), its product functions as the catalytic subunit of two distinct complexes, mTORC1 and mTORC2, composed by accessory proteins that function as scaffolds for assembling the complexes and for binding substrates and regulators. Regulatory-associated protein of mTOR (RAPTOR), and rapamycin-insensitive companion of mTOR (RICTOR) define mTORC1 and mTORC2, respectively (Figure

26). mTOR pathway is activated by nutrients, mitogens, growth factors and other extracellular molecules, being centrally involved in protein synthesis, cell cycle regulation, cellular proliferation and cancer cell metabolism. Additionally, mTOR plays important roles in interplays between tumour and stromal cells, including endothelial cells, and is also an important signalling mediator in hypoxia-induced angiogenesis [359-365].

The major substrates of the mTORC1 known so far are 4EBP1 (initiation factor 4E-binding protein 1) and p70S6K (ribosomal p70S6 kinase, S6K). Through its interactions with the partners RAPTOR and GβL (G protein beta subunit like protein), mTOR regulates protein translation and cell cycle progression, by phosphorylation of 4EBP1 and S6K, and by the subsequent phosphorylation of the downstream molecule 40S ribosomal protein S6. mTORC1 responds to mitogen, energy and nutrient signals in part through the upstream regulators tuberous sclerosis complex 1/2 (TSC1/2) and Rheb. mTORC2, although less explored than mTORC1, seems to promote actin cytoskeleton organization, cell migration and survival via the phosphorylation of PKC (protein kinase C), activation of Rho GTPases and phosphorylation of AKT. The regulation of mTORC2 is beginning to be unravelled, but evidences point out that only growth factors directly regulate this complex [360, 366-368] (Figure 26).

The signalling network upstream of mTORC1 comprises numerous oncogenes and tumour suppressor genes that frequently underlie tumourigenesis and tumour progression. Therefore, increased mTORC1 activity (generally detected by phosphorylation at S2448), as well as the phosphorylation levels of its downstream targets, have been detected in a considerable percentage of human tumours [359, 361, 369-370] (Table 6).

Table 6 | Studies reporting activation of mTORC1 signalling in malignancies (adapted from [370]).

Cancer	*p4E-BP1*	*pS6K (T389) (%)*	*pS6*	*P-mTOR (S2448) (%)*	N
Breast		71.9	58.5% (S235/236)	44.9	89
	41.2% (S65)			42.4	165
	87.3% (T70)	77.7	77.7% (S240/244)		103
Colorectal	82.1% (T37/46)	66.1		60.7	56
		40			69
Endometrial			61% (S235/236)		75
Glioblastoma		56			56
		94		75	268
Hepatocellular carcinoma			47.7% (S240/244)		86
			88.3% (S235/236)		528
Lung adenocarcinoma			84% (S235/236)		77
			100%		37
			54% high (S235/236)		
Lymphoma	66% (T70)	66	66% (S240/244)	66	29
Melanoma			73% (S235/236)		107
Ovarian	41.1% (T70)	26.4	15.5% (S240/244)		129
Prostate	90.6% (T70)		71.7% (S235/236)	96.2	84
Renal cell carcinoma			59% (S235/236)		29
			85% (S235/236)		375

Abbreviations: 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; mTOR, mammalian target of rapamycin; p, phosphorylated; S6K, S6 kinase.

Rapamycin (sirolimus) is a classical immunosuppressant drug used to prevent rejection in organ transplantation, and a known inhibitor of the mTOR signalling, particularly mTORC1 [371]. Recent data suggested that prolonged treatment with rapamycin may also affect the mTORC2 assembly and AKT mediated-signalling [372]. Sirolimus and derivative compounds (everolimus and tensirolimus, among others) have demonstrated potent anti-tumour effects by targeting mTOR signalling in endothelial cells, inhibiting their proliferation and migration, inducing apoptosis, and impairing angiogenesis, lymphangiogenesis and lymphatic metastasis [363, 373-375]. Some of these compounds have already obtained the FDA approval for the treatment of human malignancies [376].

1.2.1.7. NEOVASCULARIZATION IN UROTHELIAL BLADDER CANCER

Urothelial bladder carcinoma, similarly to the majority of tumours with epithelial origin, disseminates preferentially through the lymphatic vasculature, and the occurrence of regional lymph node metastasis is an early event in progression. The extensive lymphatic drainage network of the urinary bladder clearly contributes to that preference. It is accomplished by a system of lymphatic channels and lymph nodes (LNs) separated into six distinct areas: (1) a visceral lymphatic plexus within the submucosa and extending into the muscular layer of the bladder wall; (2) juxtavesical LNs located within the perivesical fat (anterior, lateral, and posterior groups); (3) pelvic collecting trunks, (4) regional pelvic LNs (external iliac, hypogastric, and presacral groups); (5) lymphatic trunks leading from the regional pelvic LNs; (6) common iliac LNs on the common iliac vessels. The primary drainage initiates at the external iliac, hypogastric and obturator regions; secondary drainage is from the common iliac regions; tertiary drainage occurs from the trigone and posterior bladder wall into the presacral LNs [126-128] (Figure 27).

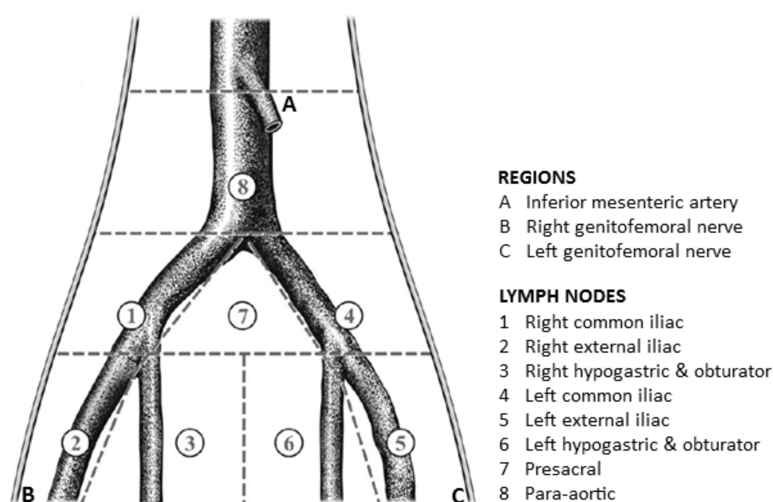


Figure 27 | Anatomy of the lymphatic drainage of the bladder (adapted from [377]).

In muscle-invasive disease setting, the gold standard of treatment is radical cystectomy with pelvic lymph node dissection (PLND) [116, 158, 378]. PLND has irrefutable diagnostic and prognostic value, but its optimal boundaries remain a highly controversial issue, mainly because of the lack of prospective trials and the limitations of the retrospective mapping studies performed so far (for instance, inter-institutional comparisons are difficult due to overlap between the various PLND areas). The idea of defining a minimum number of LNs required to be removed during the course of PLND is also a debated question [126-128, 379-380]. Nevertheless, increasing evidences suggest that an extended lymphadenectomy, with an increased number of LNs removed, improves survival in patients with both node-positive and node-negative UBC, when compared with limited approaches. The procedure potentially guarantees a complete removal of the primary, secondary, and tertiary lymph node drainage, and provides accurate staging. Although it increases the surgery time, it does not alter overall morbidity. Importantly, the removal of LNs with undetected micrometastases (the "false" LN-negative cases) clearly decreases the likelihood of leaving residual cancer, and thus affects outcome [381-388].

Lymph node density – the ratio of the number of positive LNs to the total number of LNs removed – can be considered a simple measure of the efficacy of the lymphadenectomy. This concept has been identified as a significant prognostic factor in several cystectomy series [377, 389-393]. Some authors suggested that it is superior to the TNM staging system in predicting disease-specific survival, namely in node-positive patients treated with adjuvant chemotherapy [394-396]. In the future, when a validated model for PLND is defined, lymph node density could be used as a criterion to treat patients with adjuvant therapy. In addition, assessment of lymphovascular invasion (LI) – defined as the presence of malignant cells in an endothelium-lined space – in the primary tumour has also been proposed as critical for stratification of risk groups, namely in identifying patients with occult micrometastases that might benefit from adjuvant treatment [382, 397-401]. In this context, angiogenesis and lymphangiogenesis occurrence should be considered when exploring biomarkers that predict extravesical dissemination. As in other types of malignancies, tumour neovascularization is implicated in bladder cancer progression, lymphovascular invasion, lymph node metastasis and visceral metastasis, representing a potential diagnostic and prognostic factor, and a target for guided therapy [95-99].

Angiogenesis occurrence in UBC seems to have an impact in both non-muscle invasive and muscle-invasive disease. High levels of VEGF have been found in tumour tissue [402-404] and in urine [405-407] of patients with NMI carcinomas. These results were significantly associated with the occurrence of recurrence and progression. Similar associations were found in the case of high BVD

counts in this group of tumours [99, 408-410].

In the subset of MI tumours, overexpression of VEGF [411-412] correlates with high BVD [95], and both parameters have been identified as predictors of progression and lymph node metastasis, significantly impairing prognosis [98-99, 413-415]. A large-scale approach on angiogenic pathways in UBC, studying the expression levels of 40 genes involved in angiogenesis, identified VEGF as a major independent prognostic marker [416]. A distinct large-scale evaluation of single nucleotide polymorphisms in candidate genes for cancer identified several VEGF polymorphisms that could be associated with bladder cancer risk [417]. VEGF urinary levels have been proposed as a potential biomarker in the non-invasive evaluation of UBC patients [418-419]. Moreover, other proangiogenic (matrix metalloproteinases, fibroblast growth factors, platelet derived-growth factors, integrins, angiopoietins, Notch signalling) and antiangiogenic (thrombospondin-1, angiostatin-endostatin) factors alternative to the VEGF signalling have also been implicated in the angiogenic cascade in UBC, associating with tumour recurrence, progression, metastasis and overall outcome [96-97, 420-423]. These important findings, together with promising results obtained from pre-clinical *in vitro* [424-429] and *in vivo* [430-433] bladder tumour models, make UBC angiogenesis a suitable therapeutic target.

Studies reporting lymphangiogenesis occurrence in UBC, as well as its relevance for the outcome of bladder cancer patients, are fewer in number when compared with angiogenesis. In a UBC transgenic mouse model, a significant increase in LVD was found concomitantly with bladder cancer progression, and the labelling of the tissue sections with specific antibodies for proliferating LEC indicated cancer-induced lymphangiogenesis [434]. The results obtained with patients point out for a significant impact of lymphangiogenesis occurrence on lymph node metastasis, recurrence and poor prognosis [95, 435-438]. VEGF-C and VEGF-D expressions associate with high LVD, both peritumourally and intratumourally [95, 438-439], and VEGF-C seems to be an important predictor of lymph node metastasis [435-436]. VEGF-C overexpression also promotes angiogenesis, probably by interacting with the fully processed form of VEGFR-2 [95]. In an *in vitro* study, RNA interference-mediated VEGF-C reduction suppressed malignant progression and enhanced mitomycin C sensitivity of bladder cancer cells [440]. Moreover, in an orthotopic urinary bladder cancer model, tumour lymphangiogenesis occurrence was accompanied by a massive infiltration of VEGF-C/D expressing tumour-associated macrophages (TAM) in the primary tumor and in lymphatic metastasis in LNs. These TAM were flocking near lymphatic vessels, possibly assisting lymphangiogenesis in the bladder tumour by paracrine signalling. A soluble VEGFR-3 blocked VEGF-C/D and markedly inhibited lymphangiogenesis and

lymphatic metastasis. TAM depletion exerted similar effects [441].

As already mentioned, the malignant cells explore the unique physiological and structural features of the tumour neovasculature in order to intravasate and disseminate through the blood and lymphatic flows. In UBC setting, lymphovascular invasion has been identified as an independent prognostic factor for recurrence and survival by several authors [400, 442-445]. It was demonstrated that LI independently associates with poor outcome for patients with MI tumours that were treated with bladder-conserving therapies [446]. There is significant agreement of the LI status at transurethral bladder tumor resection and at subsequent cystectomy [447-448]. Importantly, LI helps to stratify N0 UBC patients who are at increased risk of bladder cancer recurrence and death, both in the case of NMI [399, 449] and MI disease [397, 400, 442, 444, 450]. The expectable association between LI and lymph node occult micrometastasis advocates the application of adjuvant treatments in those patients.

Although LI seems to be a significant prognostic factor for UBC patients, it is not routinely described on the pathology reports. Diagnosis reproducibility has not been achieved yet, mostly due to two reasons: first, it is difficult to distinguish between LI and peritumoural stromal retraction, a common finding in hematoxylin and eosin (H&E) stained sections; second, it is difficult to differentiate BEC and LEC, which compromises the separation between BVI and LVI [451-452]. In fact, the vast majority of the aforementioned studies did not distinguish between blood and lymphatic vessels invasion in the H&E slides. Some authors endorsed that BVI and LVI should be commented on separately in the pathology report, and attempts were made by considering BVI occurrence when tumour cells were present in vessels with a thick vascular wall and blood cells within the lumen [453-455]. The role of immunohistochemical markers of BEC and LEC in the differentiation of BVI, LVI and retraction artifacts, in UBC setting, remains to be defined. In other cancer types, it has been demonstrated that immunohistochemical staining allows proving blood and/or lymphatic vessels invasion, increasing its detection rate and avoiding false-positive reports due to the common stromal retraction artifacts [456-460]. It is urgent to establish a consensus on strict diagnostic criteria, so that LVI evaluation can be rapidly incorporated into the clinical care of UBC patients [451].

Anti-neovascularization target therapies in UBC setting are still in a very preliminary phase of clinical research (Table 7) [96, 461-463]. Bevacizumab, the first anti-angiogenic to obtain FDA approval [338], has entered in a phase II clinical trial in combination with cisplatin and gemcitabine for metastatic UBC. The overall response rate was 72%, and the median overall survival was 19.1 months

[464]. A phase III trial (NCT00942331) is currently recruiting participants to further investigate these important results [461-462]. Bevacizumab is also under phase II testing in the neoadjuvant scenario (NCT00506155 and NCT00268450) [96]. Importantly, in a pre-clinical *in vitro* study, it was demonstrated that, at clinical bevacizumab concentrations, the malignant cells compensate the VEGF-A blockade, by improving the expression of VEGF and related genes. This highlights the need to follow the patient's adaptation response to bevacizumab treatment [465]. Regarding multi-targeted TKIs, several compounds are under evaluation in phase II clinical trials [96, 461-462, 466]. In a trial with sunitinib use as a single agent as first-line treatment in cisplatin ineligible patients, a clinical benefit of 58% was obtained, with median overall survival of 8.1 months [467]. In another trial combining sunitinib with gemcitabine and cisplatin in the first-line setting for patients with metastatic disease and as neoadjuvant therapy for patients with MI disease, the delivery of the treatment was hampered by severe toxicity [468]. Two sorafenib trials completed so far did not show sufficient activity of this agent [469-470]. Pazopanib was studied as a single agent in advanced and platinum-resistant UBC patients, and demonstrated a 17% response rate [471].

Table 7 | Preliminary/final results from clinical trials exploring anti-neovascularization therapies in urothelial bladder carcinoma (adapted from [462]).

Author	Phase/setting	Treatment schedule	*N*	RR (%)	mPFS (months)	mOS (months)
Anti-VEGF						
Monoclonal antibodies						
Hahn	II/1st line mUCC	GC + bevacizumab	43	72	8.2	19.1
Balar	II/1st line mUCC	CBP – Gem + bevacizumab	51	46	N.R.	N.R.
Small molecules						
Dreicer	II/2nd line mUCC	Sorafenib	22	0	2.2	6.8
Sridhar	II/1st line mUCC	Sorafenib	17	0	1.9*	5.9
Menhert	II/1st line mUCC	CBP – Gem + sorafenib	17	30	N.R.	N.R.
Krege	II/1st line mUCC	CBP – Gem + sorafenib/placebo	40	52.5	6.3	11.3
Gallagher	II/CDDP-refractory	Sunitinib 50 mg/d 4/2	45	7	2.4*	7.1
		Sunitinib 37.5 mg/d	32	3	2.3*	6.0
Bellmunt	II/1st line unfit mUCC	Sunitinib	38	8	4.8*	8.1
Galsky	II/1st line mUCC	GC + sunitinib	15	53.3	N.R.	N.R.
Pili	II/2nd line mUCC	Pazopanib	19	0	1.9	N.R.
Necchi	II/CDDP refractory	Pazopanib	38	11	3	5
Choueri	II/CDDP refractory	Docetaxel + vandetanib	142	N.R.	2.5	5.8
		vandetanib			1.5	7.0

Abreviattions: CBP, carboplatin; Gem, gemcitabine. GC, gemcitabine and cisplatin; mOS, median overall survival; mPFS, median progression-free survival; mUCC, metastatic urothelial cell carcinoma; N, number of patients; N.R., not reported; RR, response rate; VEGF, vascular endothelial growth factor.

* These clinical trials reported Time to Treatment Progression.

The mTOR pathway also seems to be a potential therapeutic target in bladder tumours [358]. In fact, mutations in the members of the signalling cascade, like PI3K and PTEN, are relatively frequent in MI disease [358, 472-473]. However, the levels of mTORC1 activation in tumour tissue have been

poorly explored. A few studies reported the increased expression of p-mTOR in muscle-invasive and metastatic UBC [474-475]. Despite this, promising results have been obtained with mTOR inhibitors in preclinical trials [475-480], and several clinical trials are ongoing [462]. Interestingly, in an *in vitro* study, rapamycin decreased hypoxia-induced synthesis of VEGF [476]. In a phase II study of everolimus in patients with locally advanced or metastatic UBC, clinical activity was demonstrated, and the profile of plasma angiogenesis-related proteins suggested a possible role of everolimus antiangiogenic properties in the control of the disease [481].

While anti-neovascularization agents are currently approved for the treatment of several solid malignancies, having significantly changed the outcome of numerous cancer patients, in UBC setting there is a substantial delay in the translation into the clinics. The majority of the current clinical investigation in MI and metastatic UBC corresponds to small phase II nonrandomized trials involving one to three institutions [482]. It is urgent to promote cooperation among the bladder cancer community, in order to facilitate the design and conduct of trials capable of expedite the translation of important pre-clinical results achieved so far into the care of bladder cancer patients.

1.2.2. INVASION AND METASTASIS

Tumour metastasis, the most fearsome aspect of cancer, is a multistage process during which malignant cells separate from the primary tumour and invade discontiguous organs. Angiogenesis and lymphangiogenesis, as already mentioned, are essential for invasion and metastasis to occur, but numerous additional events are also necessary for the success of the metastatic spread. In fact, a long series of sequential, rate limiting, interrelated steps must occur, and the final result depends not only on the intrinsic properties of the tumour cells, but also on the host responses [165, 483].

The succession of biological alterations that characterizes invasion and metastasis can be summarized into two main phases: in the first one, the physical translocation of a malignant cell to a distant organ occurs; the second one involves the ability of that cell to develop into a metastatic lesion at the distant site [484]. The multistep process has been schematized in the “invasion-metastasis cascade” (Figure 28). After the initial transforming event, the continuous growth of the primary tumour relies on the establishment of a neovasculature that supports its metabolic demands. Local invasion then begins, which requires that the malignant cells breach the basement membrane and infiltrate locally into the surrounding extracellular matrix (ECM). They can migrate collectively, or individually in a

mesenchymal or in an amoeboid type of movement, and then intravasate the blood or lymphatic vasculature. The thin-walled tumour-associated blood and lymphatic capillaries offer little resistance to the entry of malignant cells. The intravasated cells must resist to the rigors of the subsequent transport, especially in the blood flow. The formation of large emboli via interactions with blood platelets allows tumour cells to protect themselves from shear forces and to evade immune surveillance. In order to colonize a secondary organ, the tumour cells first arrest in a capillary bed and then extravasate into the new host tissue. Once there, and so that survival and proliferation in the foreign microenvironment can be assured, the malignant cells reactivate their proliferative and defensive programs, initially originating pre-angiogenic micrometastasis that will further develop a new blood supply. This will allow the growth of a macroscopic, clinically detectable tumour. Metastasis of metastases may then occur [160, 164-165, 483, 485-487].

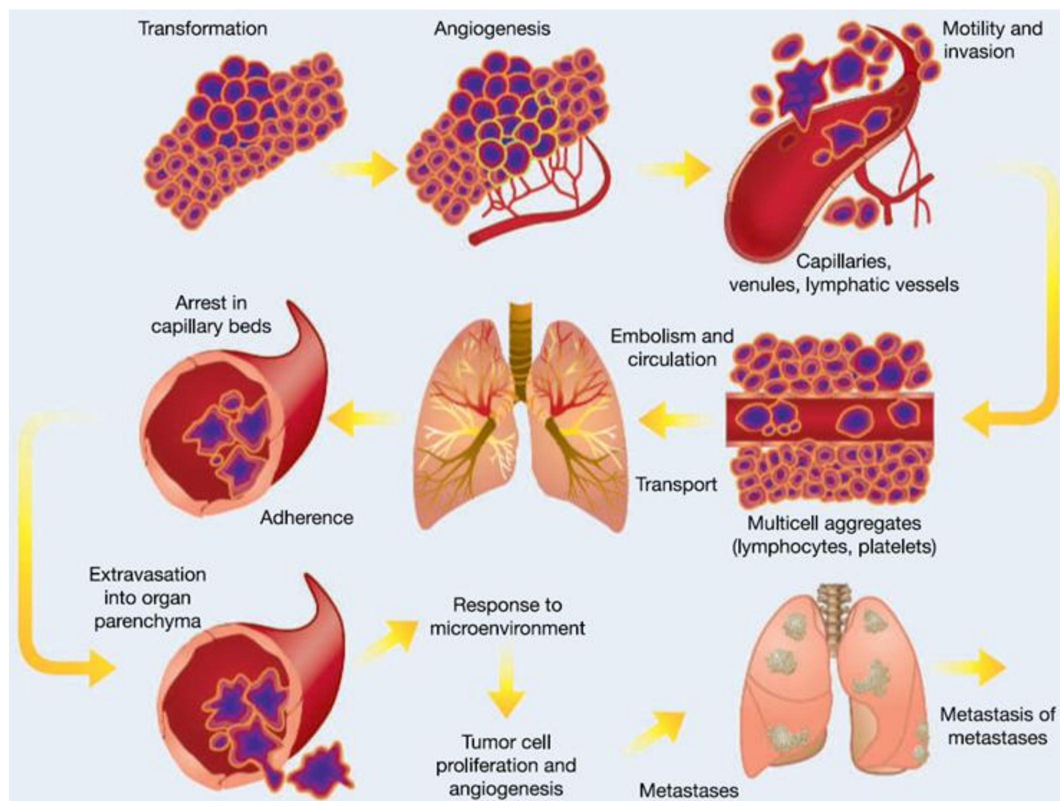


Figure 28 | The invasion-metastasis cascade (adapted from [165]).

1.2.2.1. Heparanase - A Molecular Player of Invasion and Metastasis

The migratory and invasive skills of the malignant cells are the critical parameters of the metastatic cascade, being strongly dependent on the permissive action of the microenvironment [487-488]. The

production of proteolytic enzymes involved in the degradation and remodelling of the ECM is a crucial event, and classically involves the activity of the large family of matrix metalloproteinases (MMPs). In fact, MMPs' expression is upregulated in almost every type of malignancies, associating with promotion of cell proliferation and migration, angiogenesis and metastasis occurrence, and poor outcome. Tumour cells, tip cells of collective cell clusters, fibroblasts and immune cells secret MMPs. These act manly on cleaving cell adhesion molecules, degrading ECM proteins, and processing and activating cytokines and growth factors. Moreover, they co-regulate inflammation and contribute to the generation of the metastatic niche [489-493].

Although MMPs have attracted most of the attention on the "local invasion" scenario, many other proteases can be found in the ECM. An additional large family consists of lysosomal cysteine proteases named cathepsins. This family includes endo- and exopeptidases synthesized as inactive precursors, and sharing a conserved active site formed by cysteine and histidine residues. Besides being capable of cleaving a wide variety of substrates in the lysosome, some cathepsins also act at distinct locations, namely the nucleus, the cytosol, the cell membrane and the ECM; in these last two locations, cathepsins breakdown important constituents of the ECM and the basement membrane, namely laminin, fibronectin, and type IV collagen, thus mediating local invasion [494-496]. Moreover, cathepsin L is responsible for processing and activating heparanase [497], the only functional endo-β-glycosidase capable of cleaving heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPG) in mammals [498].

HSPG are ubiquitous macromolecules consisting of protein cores to which several linear HS chains (units of N-acetylglucosamide and glucuronic/iduronic acid) are covalently O-linked. HS clusters provide numerous docking sites for a variety of protein ligands, establishing an interface for cytokines, growth factors, enzymes, protease inhibitors and ECM proteins to bind the cell surface and the ECM, thereby acting in the control of several physiological and pathological processes. The enzymatic degradation of HS chains leads to disassembly of the ECM, being involved in biological processes associated with tissue remodelling and cell migration, namely inflammation, angiogenesis and metastasis [499-501].

The heparanase gene (*HRP1*) is located on chromosome 4q.21, being expressed as 5 kb and 1.7 kb mRNA species that are generated by alternative splicing. The two mRNA transcripts have the same open reading frame and encode the same polypeptide of 543 amino acids with a molecular weight of 61.2 kDa [502-503]. Transcriptional activation of the heparanase promoter is stimulated by demethylation, early growth response 1 (EGR1) transcription factor, estrogen, inflammatory cytokines

and p53 inactivation. The 61.2 kDa pro-enzyme is post-translationally cleaved by cathepsin L, in late endosomes/lysosomes, into 8 and 50 kDa subunits that non-covalently associate to form the active heparanase. Normally, its expression is restricted to platelets, mast cells, placental trophoblasts, keratinocytes and leukocytes, with little or no expression in connective tissue cells and normal epithelia. Conversely, heparanase is preferentially overexpressed in malignant tumours [504-506].

Heparanase activity in malignancies was first investigated in B16 melanoma [507] and T-lymphoma [508] cells that demonstrated great metastatic potential. A succession of overexpression and silencing studies provided important insights regarding the pro-metastatic and pro-angiogenic abilities of heparanase [501]. In fact, besides the direct involvement in basement membrane and ECM degradation, heparanase activity releases HS-bound angiogenic growth factors such as VEGF and FGF-2 [509] (Figure 29). Its enzymatically inactive form phosphorylates signalling molecules such as AKT and Src. AKT mediates invasion and migration of primary endothelial cells [510], and Src up-regulates VEGF expression [511]. Heparanase is also involved in lymphangiogenesis, stimulating VEGF-C expression and facilitating the formation of lymphatic vessels [264]. Heparanase released from activated platelets and cells of the immune system mediates extravasation of inflammatory and tumor cells [512].

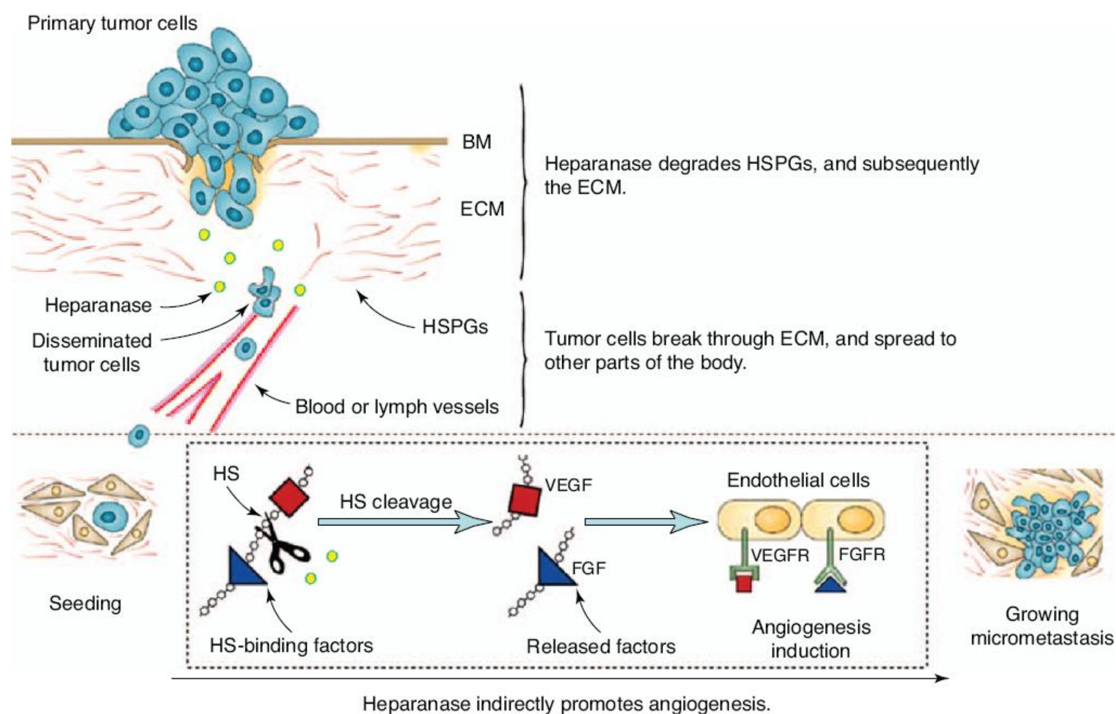


Figure 29 | Heparanase enhancement of tumor metastasis by degradation of the extracellular matrix and release of pro-angiogenic factors (adapted from [513]).

Abbreviations: BM, basement membrane; ECM, extracellular matrix; FGF, fibroblast growth factor; FGF-R, FGF receptor; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor.

The impact of heparanase expression on cancer patients was revealed from its systematic evaluation in primary human tumours. It was demonstrated that heparanase is up-regulated in carcinomas, sarcomas and hematological malignancies, associating with high blood vessel density counts, occurrence of metastasis and adverse prognosis [504-505, 514] (Table 8). These results encourage the development of heparanase inhibitors to target malignant tumours [513, 515-517]. In fact, and due to its pleiotropic effects, targeting heparanase may potentially impair multiple signalling pathways involved in progression, invasion and metastasis. Some phase I and phase II clinical trials have already been developed, and evidence of anti-tumour efficacy supports further evaluation [518-522].

Table 8 | Correlation between heparanase expression and clinical parameters in malignancy (adapted from [504]).

Author	Carcinoma	Patient number	% Positive	Positive correlation with			
Gohji et al., 2001	Bladder	40	85 (17/20)	MVD		Metastasis	Survival
Gohji et al., 2001	Bladder	67	48 (32/67)			Metastasis	
Maxhimer et al., 2002	Breast	53	36 (19/53)		Tumor size		
Shinyo et al., 2003	Cervical	92	49 (45/92)	MVD			Survival
Friedman et al., 2000	Colon	17	100				
Nobuhisa et al., 2005	Colon	54	69 (37/54)			Metastasis	Survival
Sato et al., 2004	Colorectal	130	25 (33/130)	MVD		Metastasis	Survival
Watanabe et al., 2003	Endometrial	40	50 (20/40)	MVD			
Endo et al., 2001	Gastric	63	49 (31/63)			Metastasis	
Tang et al., 2002	Gastric	116	83 (96/116)		Tumor size	Metastasis	Survival
Takaoka et al., 2003	Gastric	44	80 (35/44)			Metastasis	Survival
Doweck et al., 2006	Head & Neck	74	86 (64/74)		Tumor size		Survival
El-Assal et al., 2001	Hepatocellular	55	47 (26/55)	MVD	Tumor size		
Takahasi et al., 2004	Lung	76				Metastasis	Survival
Cohen et al., 2008	Lung	114	75 (85/114)			Metastsis	Survival
Kelly et al., 2003	Multiple Myloma	100	86 (86/100)	MVD			
Bar-Sela et al., 2006	Nasopharyngeal	46	35 (16/46)				Survival
Zheng et al., 2009	Neuroblastoma	42	62 (26/42)				Survival
Koliopanos et al., 2001	Pancreatic	33	75 (25/33)				Survival
Kim et al., 2002	Pancreatic	89	78 (69/89)				Survival
Rohloff et al., 2002	Pancreatic	50	76 (38/50)			Metastasis	Survival
Mikami et al., 2008	Renal	70				Metastasis	Survival
Ben-Izhak et al., 2006	Salivary gland	60	70 (42/60)				Survival
Shafat et al., 2010	Ewing's Sarcoma	69	100		Tumor size		
Nagler et al., 2007	Tongue	60	92 (55/60)		Tumor size		Survival

Abbreviations: MVD, microvessel density.

The potential role of heparanase on UBC biological behaviour is still poorly understood. A few studies evaluated its expression on primary tumours, and found that heparanase overexpression associates with tumour progression, high BVD, invasion and metastasis, and is an independent prognostic factor for disease-free and overall survival [523-525]. Urine heparanase levels are also

elevated during bladder cancer progression [525-526]. The increased heparanase expression during UBC pathogenesis seems to be mediated by promoter hypomethylation and by the transcription factor EGR1 [527]. In two *in vitro* studies, heparanase gene silencing significantly suppressed tumor growth, angiogenesis, invasion and metastasis of bladder cancer cells [528-529]. Additional studies are necessary to further explore the potential impact of heparanase as a diagnostic and prognostic marker, and as a therapeutic target in bladder tumours.

1.2.2.2. Raf Kinase Inhibitor Protein – A Metastasis Suppressor

Oncogenes and tumour suppressor genes have been classically implicated in malignant transformation and tumour formation, positively or negatively regulating the multistep process of carcinogenesis [159-160]. Additionally, and in order to similarly control the development of secondary tumours, molecular promoters and suppressors of metastasis have also been described. Genes that inhibit metastasis without blocking the ability of the transformed cells to develop a primary tumour are included in the group of metastasis suppressors [530-534].

Metastasis is an extremely inefficient process, with only small fractions of cells from a primary tumour mass actually overcoming the many hurdles to grow at a distant site. In fact, it was demonstrated that 24 hours after entry into the circulation, less than 0.1% of the migrating malignant cells are still viable, and less than 0.01% will survive to produce metastases [483]. A malignant cell must express particular genetic programs that enable it to interact with distinct microenvironments, in order to metastatic colonization at the second tissue site may successfully occur. Understanding those genetic programs is critical to unravel the complex process of metastasis. Obviously, loss of expression of metastasis suppressor genes is part of the metastatic genetic program, and a mandatory requisite for the success of the cascade. This loss occurs during cancer progression, and not during transformation [532, 534].

The hypothesis for the existence of metastasis suppressors was first described in 1988, with the discovery of the gene *Nm23* [535]. Although initially received with scepticism, this finding was followed by multiple investigations, using variable model systems that demonstrated the existence of more than thirty protein coding/noncoding genes that significantly reduce the onset of metastasis without affecting the formation of the primary tumor. It seems that metastasis suppressors can be found within cells and in the extracellular space, acting through diverse mechanisms, and regulating diverse steps of the metastatic cascade [532-534] (Table 9).

Table 9 | Metastasis suppressor proteins (adapted from [534]).

Metastasis suppressor	Chromosomal location	Proposed mechanism(s) of action	Cellular localization	Step(s) in metastasis inhibited
BRMS1	11q13.1–ql3.2	Transcriptional regulation via interaction with SIN3:HDAC complexes; downregulates PtdIns(4,5)P_2	N, some C	Multiple; colonization
Caspase 8	2q33–q45	Induction of apoptosis if cells bind to unliganded integrins	C	Transport
E-cadherin	16q22	Cell:cell interactions	M	EMT; invasion
N-cadherin	8q11.2	Cell:cell interactions	M	EMT; invasion
Cadherin-11	16q22.1	Cell:cell, cell:matrix interactions	M	EMT; invasion
CD44	11p13	Hyaluronic acid receptor; osteopontin receptor stem cell marker (selected)	M	Migration
DCC	18q21.3	Regulates cytoskeletal organization; regulates MAPK signaling	C	Transport; migration
DLC1	8p22–p21.3	RhoGTPase activating protein; regulates cytoskeletal structure	C	Motility; migration; invasion
DRG1	8q24.3	Unknown	C, some N	Angiogenesis; colonization (?); intravasation (?)
GAS1	9q21.3–q22	Inhibit cell cycle	N, some C	Unknown
Gelsolin	9q33	Regulates cytoskeletal structure; reduces motility	C	Motility; migration
HUNK	21q22.1	Protein kinase	C	Migration; invasion
KAI1	11p11.2	Interacts with endothelial DARC to induce apoptosis	M	Intravasation; transport
KISS1 (kisspeptins)	1q32	Maintains dormancy at secondary sites	S	Colonization
KISS1R	19p13.3	G-protein coupled receptor	M	Colonizaton
KLF17	1p34.1	Transcription	N	Invasion; EMT
LSD1	1p36.12	Chromatin remodeling	N	Invasion
MKK4	17p11.2	Stress-activated MAPK signaling	C	Colonization; migration
MKK7	19p13.3–p13.2	Stress-activated MAPK signaling	C	Colonization; migration
p38	6p21.3–p21.2	Stress-activated MAPK signaling	C	Colonization; migration
Nm23	17q22	Phosphorylates KSR to prevent downstream activation of MAPK pathways	C, some N	Migration; colonization
OGR1	14q31	GPCR signaling	M	Migration
RhoGDI2	12p12.3	Regulates Rho; negatively alters endothelin 1 and neuromedin U expression	C	Migration; colonization
RKIP	12q24.23	Competitive inhibitor of RAF1–MEK interactions	C	Migration; invasion
RRM1	11p15.5	Increases PTEN expression; decreases FAK phosphorylation	C	Motility; invasion
SSeCKS	6q24–q25.1	Scaffold protein for PKA and PKC; inhibits osteopontin, VEGF expression; up regulates vasostatin	C	Angiogenesis; migration
TIMPs	Multiple	Inhibit metalloproteinases; signaling	C, S, M	Angiogenesis; migration; invasion; transport

Abbreviations: BRMS1, breast cancer metastasis-suppressor 1; C, cytoplasmic; DARC, detection of apoptosing retinal cells; DCC, deleted in colorectal carcinoma; DLC1, deleted in liver cancer 1; DRG1, developmentally-regulated GTP-binding protein 1; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; GAS1, growth arrest-specific gene 1; GPCR, G protein coupled receptors; HUNK, hormonally up-regulated Neu-associated kinase; KISS1R, KISS1 receptor; KLF17, krueppel-like factor 17; KSR, kinase suppressor of ras; LSD1, lysine-specific demethylase 1; M, membrane; MAPK, mitogen-activated protein kinase; MKK4, mitogen-activated protein kinase kinase; N, nuclear; Nm23, nucleoside diphosphate kinase (NDPK); OGR1, ovarian cancer G protein-coupled receptor 1; PK, protein kinase; PtdIns(4,5)P_2, phosphatidylinositol 4,5-

bisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RhoGDI2, RhoGTPase dissociation inhibitor 2; RKIP, raf kinase inhibitor protein; RRM1, ribonucleotide reductase M1; S, secreted; SIN3:HDAC, Sin 3-histone deacetylase; SSeCKs, Src-suppressed C kinase substrate; TIMPs, tissue inhibitor of metalloproteinases; VEGF, vascular endothelial growth factor.

The impact of the loss of metastasis suppressors in the success of the metastatic cascade highlights the potential benefits of functionally reconstituting these proteins. Several strategies have been proposed, including the re-expression of the gene by induction of the endogenous *locus* or by exogenous gene therapy, the direct administration of the protein itself, or by targeting critical downstream pathways that are concomitantly induced when metastasis suppressor losses occur [532].

Raf kinase inhibitor protein (RKIP), a described metastasis suppressor, was originally characterized as a phospholipid binding protein in bovine brain, and named as PEBP1 (phosphatidylethanolamine-binding protein 1) [536]. Later, RKIP was identified by a yeast two-hybrid screen for proteins that bind the RAF-1 kinase domain; this revealed its function in the competitive inhibition of RAF1-MEK interaction and downstream signalling, being then coined as raf kinase inhibitor protein [537]. However, both names are insufficient to fully characterize the plethora of functions and interactions that can be attributed to this protein, implicating it in neurodegenerative processes, emotions, reproduction and the suppression of metastasis [538-541]. Table 10 summarizes the genetic and protein information for RKIP.

RKIP is a widely expressed and highly conserved protein that does not share any significant homology with any known protein family; being a member of the PEBP family, it has two critical features that enable it as a regulator of cell homeostasis: a ligand binding pocket, and a compact globular structure that provides ample surface area for interaction with other proteins [542].

The landmark study elucidating a role for RKIP in a pivotal cellular signalling cascade demonstrated its involvement in the MAPK (mitogen-activated protein kinase) pathway (or RAF-MEK-ERK cascade) [537]. MAP kinase is a highly preserved signalling pathway that can influence cell growth, differentiation, migration and apoptosis in response to extracellular stimuli, being frequently activated in cancer. Structurally, it is a three component kinase module comprising a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK) and a MAP kinase (MAPK). The RAF kinases (A-RAF, B-RAF and RAF-1) belong to the family of MKKK [543]. RAF has the ability to interact with a large number of proteins, but RKIP, in its non-phosphorylated form, is the only known inhibitor of the MAPK pathway. RKIP also binds, although with weaker affinity, to MEK and ERK, interfering with downstream phosphorylation steps [537]. Besides inhibiting the MAPK pathway, phosphorylated RKIP inhibits NF-κB

(nuclear factor Kappa B) by negatively regulating IKK (IkB kinase), an activator of NF-κB transcription, and therefore abrogates the antiapoptotic properties of this signalling pathway [544-545]. Moreover, RKIP controls GPCRs (G-protein coupled receptors) by inhibiting GRK2 (G-protein coupled receptor kinase-2), thereby influencing neurotransmission, inflammation, and regulation of blood pressure [546]. RKIP also binds to centrosomal and kinetochore regions of prometaphase chromosomes, possibly influencing the Aurora B kinase and spindle checkpoint proteins, and thus regulates the progression of the cell cycle [547]. Conversely, besides acting as an inhibitor, blocking the access of kinases to their substrates, RKIP binds and maintains GSK3β (glycogen synthase kinase 3) levels, and prevents its inhibitory p38-mediated phosphorylation [548], avoiding the stabilization of cyclin D1 and the subsequent expression of β-catenin, SNAIL and SLUG, important mediators of epithelial-mesenchymal transition (EMT) and invasion [549]. Altogether, the multiple RKIP interactions implicate this protein in cell differentiation, cell cycle kinetics, apoptosis, EMT and cell migration [538-539, 541] (Figure 30).

Table 10 | Genetic and protein information for RKIP (adapted from [541]).

PROTEIN	Phosphatidylethanolamine-binding protein 1.
NAMES	HCNPpp; neuropolypeptide h3; prostatic-binding protein; raf kinase inhibitor protein.
GENE NAMES	*PEBP1; PEBP; PBP.*
EPIGENETICS	EZH2-targeted inhibition of RKIP through SNAIL; CpG islands methylation.
GENOMICS	*Chromosomal location*: 12q24.23. *Length*: 9728 bp. *Gene Layout*: 4 exons, 3 introns. *PEBP1 gene promoter*: houses multiple CpG islands, E1 and E2-box, ARE, p53 binding site.
PROTEOMICS	*Subcellular location*: cytoplasm and occasionally nuclear. *Length*: 187 a.a. *Mass*: 21-23 kDa. *Subunit*: interacts with RAF-1 and enhanced by the phosphorylation of RAF-1 'S338', 'S339', 'Y340' and 'Y341'.
FUNCTIONS	Scavenger protein (binds nucleotides, opioids and phosphatidylethanolamine); inhibits the kinase activity of RAF-1 by inhibiting its activation and by dissociating the RAF-1/MEK complex and acting as a competitive inhibitor of MEK phosphorylation; modulates behavioral responses and circadian rhythms; participates in the organization of phospholipids in myelin sheath; influences memory and learning; endocrine factor in cardiac physiology; involved in spermatogenesis.
SIGNALING PATHWAYS	MAPK; GPCR; NFkB; GSK3β.
DOWNSTREAM EFFECTOR MOLECULES	RAF-1; MEK; ERK; GRK2; TAK1; NIK; IKK; P38; GSK3β; NRF2; KEAP1; Aurora B.
PHYSIOLOGICAL BEHAVIOR INFLUENCED BY PEBP1	Growth and differentiation; proliferation; migration; motility; cell cycle; genomic stability; apoptosis; drug resistance.
ORTHOLOGS	Human; mouse; chicken; rat; fruit fly; dog; cow; chimpanzee; yeast; bacteria.
DISEASES ASSOCIATED WITH PEBP1 PERTURBATION	Metastasis; Alzheimer's disease; diabetic neuropathy; prostate cancer; gastric cancer; melanoma; breast cancer; colorectal cancer; ovarian cancer; gastrointestinal stromal tumors; hepatocellular carcinoma; nasopharyngeal carcinoma; lung cancer; gliomas.
EXPRESSION IN NORMAL TISSUES	*Protein*: expressed in almost all tissues to variable extent (neurons, neuroendocrine cells, liver, testes, prostate, glandular epithelia of breast, salivary glands and pancreas, kidney, bladder, endothelia of lymph and blood vessel, milk duct epithelial cells, primary melanocytes, blood plasma and urine, HEK-293 cell lines, bronchoalveolar lavage cells). *mRNA*: the highest expression levels were reported in the testis (epididymis, seminal vesicle), adrenal cortex, brain, thyroid, liver, thymus, bone marrow, heart, lung, prostate, pancreas, kidney and spleen.

Abbreviations: a.a., amino acid; ARE, Androgen Response Elements; bp, base pairs; EZH2, enhancer of zeste homolog 2; GPCR, G-protein coupled receptor; GRK2, G-protein coupled receptor kinase-2; GSK3β, glycogen synthase kinase 3; IKK, IkB kinase; kDa, kilodalton; KEAP1, Kelch like-ECH-associated protein 1; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor Kappa B; NIK, NF-κB inducing kinase; NRF2, NF-E2 related factor-2; TAK1, TGF-beta activated kinase 1.

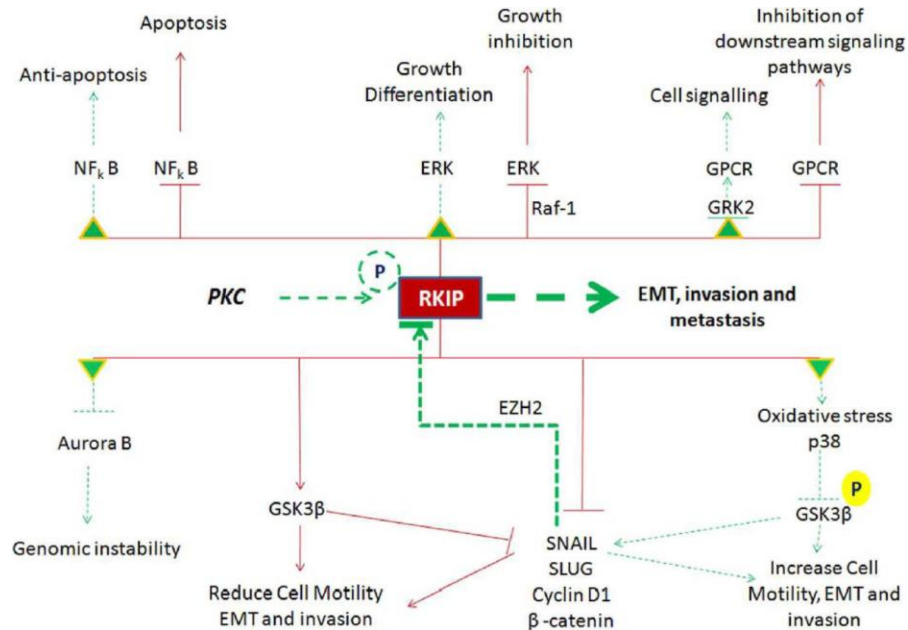


Figure 30 | RKIP interactions with signalling pathways (red colored lines and arrows denote functions under basal conditions; green triangles, broken green lines and arrows denote RKIP phosphorylation or loss/diminution of function, resulting in pathological processes) (adapted from [541]).
Abbreviations: EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinases; EZH2, enhancer of zeste homolog 2; GPCR, G-protein coupled receptor; GRK2, G-protein coupled receptor kinase-2; GSK3β, glycogen synthase kinase 3; NFκB, nuclear factor Kappa B; NIK, NF-κB inducing kinase; PKC, protein kinase C.

The role of RKIP in the suppression of the metastasic cascade seems to arise from several mechanisms. It has been suggested that RKIP expression inhibits metastasis by decreasing angiogenesis and lymphovascular invasion [550-551]. By influencing MAPK and NF-κB signalling pathways, RKIP may potentiate apoptosis induced by chemotherapeutic agents [552]. The role of RKIP in preventing chromosomal abnormalities could contribute to its function as a metastasis suppressor [547], and the absence of RKIP may increase the rate of cell division [553], accelerating DNA synthesis and downregulating cell cycle checkpoints [549]. Recent reports have proposed that RKIP inhibits the migration and invasion abilities of the malignant cells by negatively regulating the expression of specific matrix metalloproteinases [554]. RKIP expression inversely correlates with the expression of SNAIL, a key modulator of normal and neoplastic epithelial-mesenchymal transition program [555].

Given its multifaceted abilities in maintaining cellular homeostasis, it is expected that RKIP downregulation favours metastasis. This was first demonstrated in a metastatic prostate cancer cell line expressing low RKIP mRNA and protein levels [550]. Since then, increasing evidences with multiple types of solid tumours point out an important biological role of this molecule in preventing malignant dissemination. Several authors demonstrated that RKIP depletion associates with metastatic events in prostate [550], breast [556] and colorectal [557] cancers, as well as in melanoma [558], insulinoma [559], ovarian [560], gastric [561], hepatocellular [553], cervical [562] and thyroid [563] carcinomas,

among others. Moreover, RKIP expression status was identified as an independent prognostic marker in colorectal [557], prostate [564] and gastric [565] carcinomas, glioma [566], carcinoma of the ampulla of Vatter [567], esophageal carcinoma [568], pancreatic ductal adenocarcinoma [569], gallbladder, nasopharyngeal [570] and renal cell [571] carcinomas. These promising results are the gateway for exploring therapeutic strategies that can potentially restore RKIP functionality as a metastasis suppressor. Moreover, those strategies could also re-sensitise the malignant cells to chemotherapy and radiotherapy, since RKIP ablation seems to be associated with drug resistance [552, 562, 570, 572].

RKIP function in UBC has been preliminarily investigated. Only one study examined *PEBP1* mRNA levels, revealing a significant reduction in NMI tumours, when compared with normal urothelium [573]. It is urgent to perform tumour tissue immunostaining to validate these results.

1.2.3. Energy Metabolism Reprogramming and the Tumour Microenvironment

The performance of cellular functions relies primarily on energy production, and our cells are equipped with a pair of engines that act in tandem to generate the energy necessary to metabolic reactions. Under aerobic conditions, normal differentiated cells metabolize glucose to pyruvate via glycolysis in the cytosol; glycolytic pyruvate is then oxidized to carbon dioxide in the mitochondria through the tricarboxylic acid (TCA) cycle, which generates NADH [nicotinamide adenine dinucleotide (NAD+), reduced] molecules that will fuel oxydative phosphorylation (OXPHOSP). This is an efficient process of energy production, generating more adenosine triphosphate (ATP) than glycolysis. It is only under anaerobic conditions that differentiated cells favour glycolysis, producing large amounts of lactate that allows glycolysis to persist (by cycling NADH back to NAD+), although generating much less ATP molecules than OXPHOSP [574-575]. Conversely, the uncontrolled cell proliferation inherent to the malignant phenotype necessarily involves adjustments of energy metabolism. Otto Warburg first observed that tumour cells reprogram their glucose metabolism by producing large amounts of lactate, even under aerobic conditions [576]. This metabolic switch was termed “aerobic glycolysis” or “the Warburg effect”. By being a less efficient process of ATP production, aerobic glycolysis demands that tumour cells avidly uptake glucose to maintain bioenergetics, biosynthesis and redox status [577-580] (Figure 31). Although not applicable to all malignant tumours, this enhanced glucose uptake is sufficiently prevalent and allowed the widespread clinical application of the imaging technique positron

emission tomography (PET) using the glucose analogue [18]fluorodeoxyglucose (FdG). FdG-PET, combined with computed tomography, has a specificity and sensitivity of near 90% to identify primary and metastatic lesions of most epithelial malignancies [581].

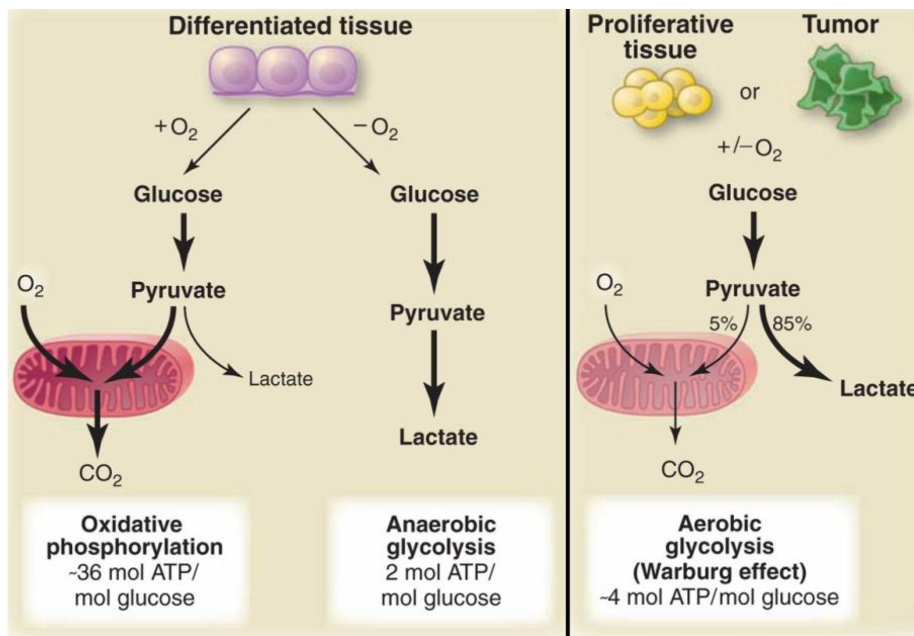


Figure 31 | Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (adapted from [541]).
Abbreviations: ATP, adenosine triphosphate.

1.2.3.1. Aerobic Glycolysis in Tumours – How and Why?

Warburg originally hypothesised that aerobic glycolysis occurs in tumours due to primary injuries in mitochondrial OXPHOS [576]. However, his theory has been challenged by studies indicating that most of the malignant cells do not harbour mitochondrial defects, retaining the capacity for OXPHOS and consuming oxygen at similar rates to those observed in normal tissues [582-583]. Additionally, although aerobic glycolysis has been recently proposed as a hallmark of cancer, it does not seem to be a hallmark of all cancer cells, since some tumours do not reprogram energy metabolism, obtaining their ATP mainly by OXPHOS [584]; other tumours, depending on the environmental conditions, can reversibly switch from aerobic glycolysis to OXPHOS [585]. Interestingly, the existence of a “metabolic symbiosis” through lactate shuttling between populations of hypoxic and aerobic cells within the tumour has been proposed [586]. These evidences point out that the metabolic plasticity inherent to malignant cells is more an effect than a cause. In spite of some uncertainties remaining, the considerable efforts to elucidate the mechanisms responsible for the Warburg effect have allowed substantial progress in the field. A recent review summarizes eight possible trigger events: i) HIF-1α activation and stabilization during hypoxic stress; ii) oncogene activation (e.g. AKT), and loss of tumor suppressor genes (e.g. p53); iii) mitochondrial dysfunction in malignant cells; iv) nuclear DNA mutations in genes that encode

mitochondrial proteins; v) epigenetic deregulation of enzymatic activities during glycolysis; vi) miRNAs targeting genes directly involved in aerobic glycolysis and regulating oncogenes and tumor suppressor genes indirectly involved in modulating glucose metabolism; vii) glutaminolysis and truncated citric acid cycle occurrence in glucose-deprived conditions; viii) post-translational modifications of metabolic proteins linked to the Warburg effect [580].

Whatever is the mechanism (or the combination of mechanisms) triggering the glycolytic phenotype in tumours, it is currently accepted that the enhanced glucose uptake for glycolytic ATP generation confers an advantage to tumour growth during the somatic evolution of cancer. At first glance, this proliferative advantage is not clear, because aerobic glycolysis is far less efficient than OXPHOS in generating ATP molecules, and the metabolic products of glycolysis cause a consistent acidification of the extracellular milieu, which might result in serious toxicity [587]. However, what seems to be a harmful trait represents, in fact, a selective advantage for tumours, with several reasons supporting this theory. First, the high proliferative rate of the tumour cells advocates not only energy demands, but also metabolic intermediates for the biosynthesis of macromolecules, such as nucleic acids, lipids and proteins, that can be obtained from the glycolytic pathway [588]. Second, the high concentration of ATP generated by mitochondrial OXPHOS could exert a negative feedback effect in glycolysis, which is unfavourable for tumour proliferation. In fact, not only tumour cells but also normal proliferative cells rely on aerobic glycolysis for energy production (Figure 31), because ATP molecules, although less in number, are generated at a higher rate than in OXPHOS [589]. Additionally, OXPHOS would generate reactive oxygen species, potentially deleterious for tumour cells [590]. Third, glucose can be metabolized through the pentose phosphate pathway, generating nicotinamide adenine dinucleotide phosphate (NADPH) that ensures an antioxidant defense against a hostile microenvironment and chemotherapeutic drugs, and can also contribute to fatty acid synthesis [577]. Fourth, glycolytic tumour cells are able to survive in a microenvironment where oxygen tension is variable [591]. Fifth, aerobic glycolysis produces lactate which is released in the extracellular space, creating an acidic microenvironment that favours tumour growth, invasion and metastasis [587, 592-593], and suppresses host immune response [594]. Stromal cells can collect the extracellular lactate to regenerate pyruvate that can be used again by glycolytic tumour cells, thus contributing to sustain tumour survival and growth [595]. Therefore, persistent aerobic glycolysis alters the local microenvironment in a way that is harmless to itself, but severely harmful to the competing populations of unadapted normal and tumour cells.

1.2.3.2. Tumour Metabolism – Role of the Hypoxic Microenvironment

Hypoxia is considered to be one of the most important mechanisms leading to the acquisition of the glycolytic phenotype in tumours. In fact, hypoxia is present since pre-malignancy, when proliferating epithelial layers with intact basement membranes become thickened and develop hypoxic regions near the oxygen diffusion limit. Oxygen seems to be the first limiting substrate for cell growth. In this scenario, microenvironnmental forces arise to select cell populations that adapt to hypoxia by switching their metabolism to aerobic glycolysis, competing for nutrient resources and resisting acid-induced toxicity. Therefore, tumours seem to acquire the glycolytic phenotype as an adaptation to local hypoxia (Figure 32). As tumour growth proceeds, persistent or cyclical hypoxia continues to exert a selective pressure that will eventually lead to the constitutive upregulation of glycolysis, even in the presence of oxygen [587, 596-597].

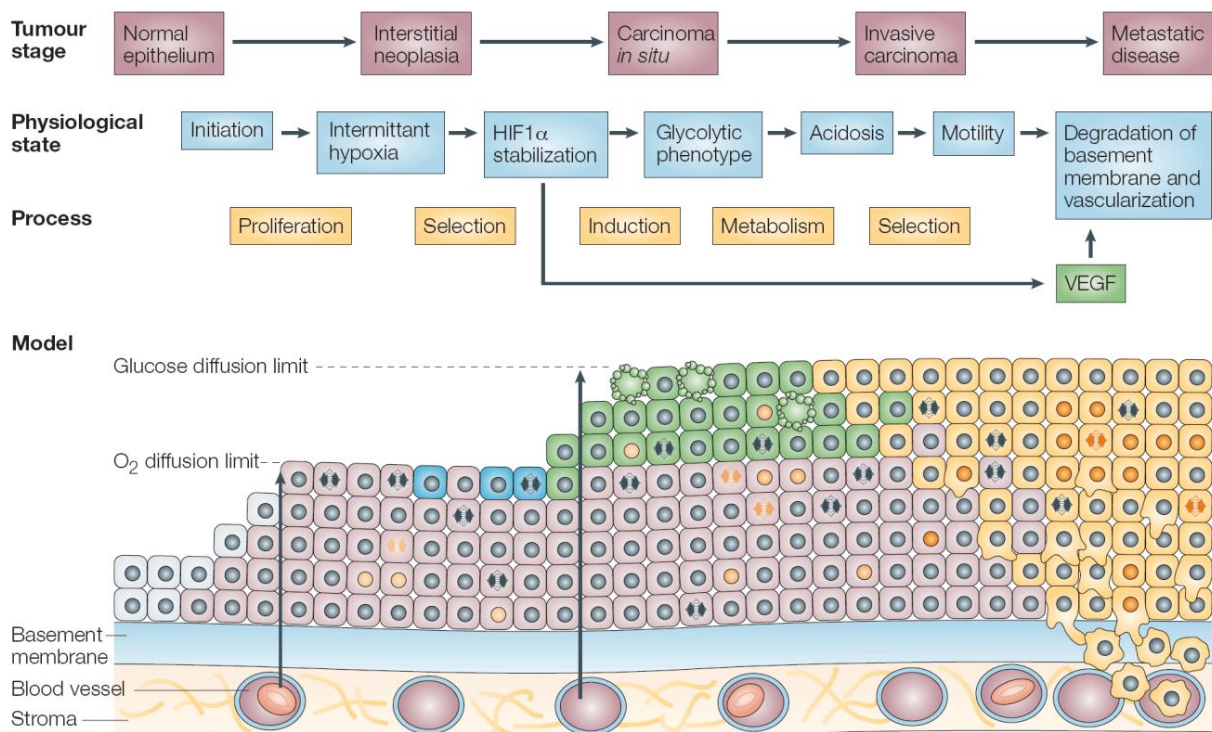


Figure 32 | Model for cell-environment interactions in carcinogenesis [cell colours represent different cell types (grey, normal epithelial cells; pink, hyperproliferative cells; blue, hypoxic cells; green, cells adapted to the glycolytic phenotype; blebbing green, apoptotic cells; yellow, motile cells); altered nuclei represent mutations (light orange, one mutation; dark orange, more than one mutation)] (adapted from [587]).
Abbreviations: HIF1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

Tumour cells adapt to the hypoxic microenvironment via the ubiquitously expressed hypoxia-inducible factor (HIF)-1α. As already mentioned, under hypoxic stress (but also under oncogenic,

inflammatory, metabolic and oxidative stress), HIF-1α is not targeted for proteasomal degradation and becomes stabilized [239, 250-251]. Once activated, HIF-1α amplifies the transcription of genes encoding glucose transporters (GLUTs), glycolytic enzymes (e.g. hexokinases, HK1 and HK2) and lactate dehydrogenase A (LDHA), stimulating the conversion of glucose to pyruvate and lactate [598]. Moreover, HIF-1α activates the pyruvate dehydrogenase kinases (PDKs), which inactivate the mitochondrial pyruvate dehydrogenase (PDH) complex, decreasing the conversion of pyruvate to acetyl-CoA, therefore compromising OXPHOS [599-600]. To ensure intracellular pH homeostasis, HIF-1α induces the expression of pH regulators, such as the hypoxia-inducible carbonic anhydrase IX (CAIX) and the lactate-extruders monocarboxylate transporters (MCTs), which will further contribute to the acidification of the microenvironment [601-602] (Figure 33).

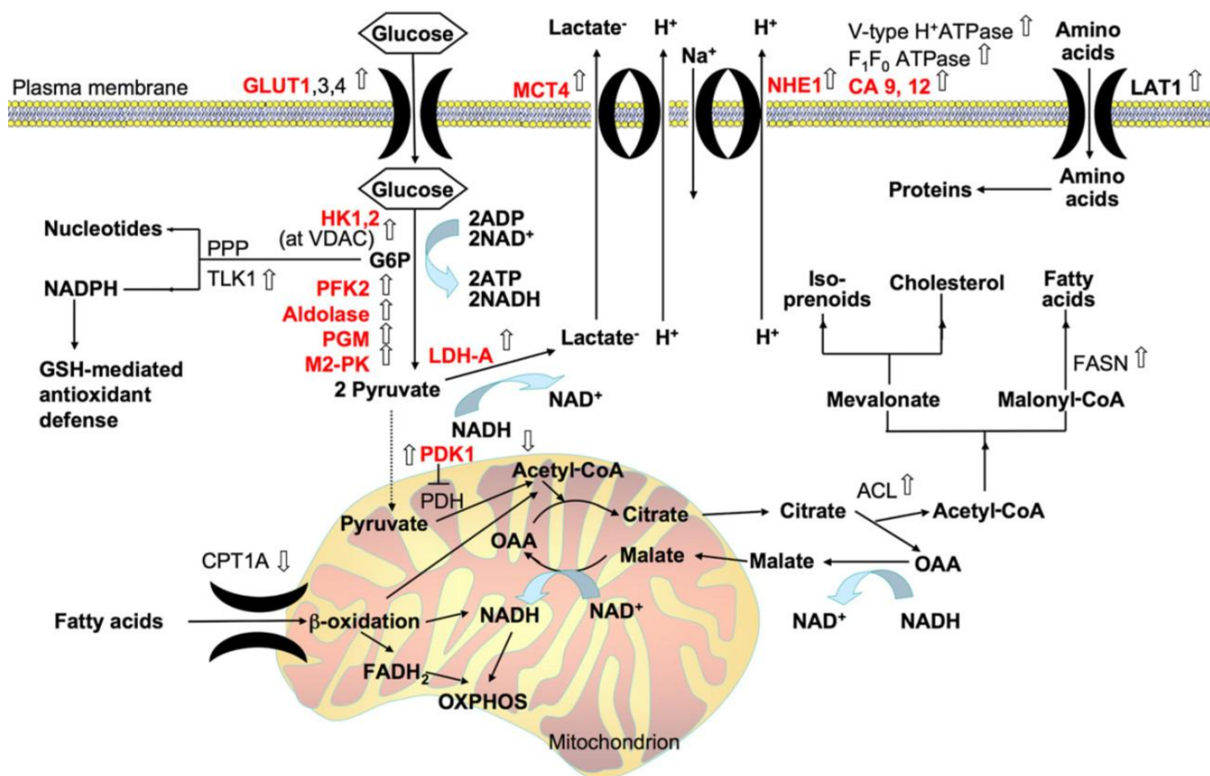


Figure 33 | Metabolic reprogramming in malignant cells – Contribution of hypoxia-inducible factor (HIF)-1α (small arrows pointing up or down indicate cancer-associated upregulation/activation or downregulation/inhibition of enzymes, respectively; alterations indicated in red can be caused by HIF-1α activation) (adapted from [583]).

Abbreviations: ACL, ATP citrate lyase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CA9 and CA12, carbonic anhydrases 9 and 12; CPT, carnitine palmitoyltransferase; $FADH_2$, flavin fdenine dinucleotide; FASN, fatty acid synthase; G6P, glucose 6-phosphate; GLUT, glucose transporter; GSH, glutathione; HK, hexokinase; LAT1, L-type amino acid transporter 1; LDH-A, lactate dehydrogenase A; MCT, monocarboxylate transporter; NAD^+, nicotinamide adenine dinucleotide; NADH, NAD^+ reduced; NADPH, nicotinamide adenine dinucleotide phosphate; NHE, Na^+/H^+ exchange; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PGM, phosphoglycerate mutase; PKM2, pyruvate kinase isoform M2; PPP, pentose phosphate pathway; TLK, transketolase; VDAC, voltage-dependent anion channel.

Carbonic anhydrases (CA) are a group of ubiquitously expressed metalloenzymes. There are at least five distinct CA families, but only the α-CAs are found in humans. α-CAs comprise 16 isoforms, which differ in their subcellular localization (cytosolic, membrane-bound, mitochondrial and secreted) catalytic activity, and susceptibility to different classes of inhibitors [603]. CAIX (*CA9* chromosomal location, 9p13.3), a target for HIF-1α, is a multidomain protein containing a short intracytosolic tail, one transmembrane segment, an extracellular CA domain, and a unique proteoglycan (PG)-like domain composed of 68 amino acid residues [604]. Like other α-CAs, CAIX is a catalyst involved in the hydration of cell-generated carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). This activity promotes the extracellular trapping of acid, which will favor the malignant phenotype [593, 605]. Interestingly, CAIX only controls acidification of the tumoural extracellular pH under hypoxic conditions, and its expression dramatically increases by a direct HIF-1α-mediated transcriptional activation of the *CA9* gene [606-607]. Therefore, overexpression of this hypoxic marker is a frequent trait of malignancies, and has been correlated with tumour progression, invasion, metastasis and poor prognosis in a considerable number of tumours [603, 608-612]. This consistent upregulation has implicated CAIX as a target for tumor therapy with respect to pH disruption. Numerous inhibitors are being tested *in vitro* and *in vivo*, in pursuit of designing high-affinity compounds that specifically bind to CAIX and other isoforms, reducing side effects caused by off-target binding [610, 613].

CAIX expression has been reported in bladder cancer [614-622], being identified as an independent prognostic factor for recurrence-free and overall survival [614, 617]. This surrogate marker of hypoxia is predominant on the luminal surface of the tumours, and surrounding areas of necrosis [620-621]. Interestingly, several authors reported a higher expression in NMI than in MI tumours [617, 619-620], ant it has been suggested that CAIX urinary levels might complement cytology as a noninvasive marker to monitor for UBC, because it seems to be able to differentiate between normal urothelial cells and low-grade tumours [618], and may also be useful for the early detection of relapse in patients following transurethral resection [622]. These intriguing results demand for further investigation.

1.2.3.3. Microenvironmental Acidosis – Contribution of Lactate and Monocarboxylate Transporters

Monocarboxylic acids, namely lactate, play a key role in maintaining metabolic homeostasis in the

majority of cells [623]. Some glycolytic cells, such as white skeletal muscle fibers, erythrocytes and many malignant cells, rely on glycolysis for rapid ATP generation, and the end product – lactate – must be effectively exported so that glycolysis may proceed. Conversely, in other tissues, lactate must enter the cells, being oxidized to become a respiratory fuel (in brain, heart and red skeletal muscle) or the dominant gluconeogenic substrate in the Cori cycle (in liver) [624-626].

Lactate is the main source of tumour microenvironmental acidosis, thus contributing to the acid-resistant phenotype. Extracellular acidity supports increased migration and invasion abilities of cancer cells, favouring the metastatic cascade. This is thought to occur through pH-dependent activation of matrix metalloproteinases and/or cathepsins, loss of the adhesion mediator E-cadherin and upregulation of hyaluronan, an important structural component of the extracellular matrix, and its receptor CD44 [587, 592, 596, 627]. Moreover, VEGF overexpression promotes angiogenesis, which further contributes to tumour dissemination [628]. Conversely, immune defences are impaired, whereas infiltrating inflammatory cells, like tumour-associated macrophages, enhance the aggressive behaviour of the growing tumour [594, 629]. Acidosis itself can be mutagenic or clastogenic, can promote radioresistance and resistance to anthracyclines, and can induce apoptosis in cells that lack acidosis-adapting mechanisms [587]. Altogether, the pleiotropic effects of increased lactate concentrations contribute to the success of tumour progression and dissemination, impairing therapeutic response and overall prognosis in cancer patients [630].

Lactate export to the tumour microenvironment is mediated by the membrane-bound proton-coupled monocarboxylate transporters (MCTs). MCTs belong to the SLC16 (solute carrier 16) gene family, comprising fourteen members that share the same basic structure: twelve transmembrane helices, intracellular C and N termini and a large cytosolic loop between transmembrane domains 6 and 7 [623, 631]. Table 11 summarises the proposed function (when known), alternative names, tissue distribution, gene location and potential involvement in disease of the SLC16 family members [632]. Of the fourteen MCTs, only MCT1, MCT2, MCT3 and MCT4 – the proton-linked MCTs – transport monocarboxylates [633]. Lactate is not the only monocarboxylate to be transported – pyruvate, oxoacids, ketone bodies transport is also mediated by MCTs, which denotes their important role in cellular metabolism [623, 634]. MCTs facilitate unidirectional proton-linked transport of monocarboxylates across the plasma membrane, mediating either influx or efflux, depending of the prevailing substrate and pH gradients [631, 633].

Table 11 | Features of the monocarboxylate transporter family (adapted from [632]).

Human gene name	Protein name	Aliases	Predominant substrates	Transport type/ coupling ions	Tissue distribution and cellular/subcellular expression	Link to disease	Human gene locus	Sequence Accession ID	Splice variants and their specific features
SLC16A1	MCT1	MOT1	Lactate, pyruvate, ketone bodies	C/H^+ or E/ monocarboxylate	Ubiquitous except β cell of endocrine pancreas	Exercise-induced hyperinsulin-aemia hypo-glycaemia	1p12	NM_003051	Splice variants in non-coding region
SLC16A2	MCT8	MOT8 XPCT MCT7	T2, rT3, T3, T4	F	Most tissues including liver, heart, brain, thymus, intestine, ovary, prostate, pancreas, placenta, lung kidney, skeletal muscle	Allan-Herndon-Dudley syndrome	Xq13.2	NM_006517	
SLC16A3	MCT4	MOT4 MCT3	Lactate, ketone bodies	C/H^+	Skeletal muscle, chondrocytes, leucocytes, testis, lung, ovary, placenta, heart		17q25.3	NM_004207	Splice variants in non-coding region
SLC16A4	MCT5	MOT5 MCT4	O		Brain, muscle, liver, kidney, lung, ovary, placenta, heart		1p13.3	NM_004696	Multiple splice variants listed in ENSG00000168679
SLC16A5	MCT6	MOT6 MCT5	? bumetanide probenecid nateglinide		Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta		17q25.1	NM_004695	Several splice variants listed in ENSG00000170190
SLC16A6	MCT7	MOT7 MCT6	O		Brain, pancreas, muscle, prostate		17q24.2	NM_004694	
SLC16A7	MCT2	MOT2	Pyruvate, lactate, ketone bodies	C/H^+	High expression in testis, moderate to low in spleen, heart, kidney, pancreas, skeletal muscle, brain and leucocyte		12q13	NM_004731	Multiple splice variants listed in ENSG00000118596
SLC16A8	MCT3	MOT3 REMP	Lactate	C/H^+ (pH dependent but cotransport not confirmed experimentally)	Retinal pigment epithelium, choroid plexus		22q112.3-q13.2	NM_013356	Several splice variants listed in ENSG00000100156
SLC16A9	MCT9	MOT9		O	Endometrium, testis, ovary, breast, brain, kidney, spleen adrenal, retina		10q21.1	NM_194298	Several splice variants listed in ENSG00000165449
SLC16A10	TAT1, MCT10	MOT10	Aromatic amino acids, T3,T4	F	Kidney (basolateral), intestine, muscle, placenta, heart		6q21-q22	NM_018593	Several splice variants listed in ENSG00000112394
SLC16A11	MCT11	MOT11		O	Skin, lung, ovary, breast, lung, pancreas, retinal pigment epithelium, choroid plexus		17p13.1	NM_153357	Two splice variants listed in ENSG00000174326
SLC16A12	MCT12	MOT12		O	Kidney, retina, lung testis	Juvenile cataracts with microcornea and renal glucosuria	10q23.31	NM_213606	Several splice variants listed in ENSG00000152779
SLC16A13	MCT13	MOT13		O	Breast, bone marrow stem cells		17p13.1	NM_201566	
SLC16A14	MCT14	MOT14		O	Brain, heart, muscle, ovary, prostate, breast, lung, pancreas liver, spleen, thymus		2q36.3	NM_152257	Several splice variants listed in ENSG00000163053

Abbreviations: C, cotransporter; E, exchanger; F, facilitated transporter; MCT/MOT, monocarboxylate transporter; O, orphan transporter.

MCT1 and MCT4 are the best characterized MCTs in human tissue. MCT1 has the most ubiquitous tissue expression (Table 11), with no evidence for splice variants. It seems to function mainly in the uptake or efflux of monocarboxylates across the plasma membrane, depending on the metabolic demands of the cell, having a high affinity for L-lactate, propionate, D,L-β-hydroxybutirate and

acetoacetate. MCT1 preferentially exports lactate when anaerobic glycolysis occurs. Conversely, it primarily mediates lactate and ketone bodies uptake in heart and red skeletal muscle, where these molecules are important respiratory substrates. MCT4 seems to be the dominant lactate exporter under conditions of aerobic glycolysis, being highly expressed by lymphocytes, astrocytes, white muscle fibres and malignant cells [633, 635-636]. Interestingly, MCT1 and MCT4 also facilitate the shuttling of lactate between cells that produce it and those that use it within the same tissue. This occurs, for instance, in skeletal muscle (between red and white fibers) and in brain (between astrocytes and neurons) [635, 637]. MCT1 has also been reported to mediate the transport of some drugs [633].

The functions of MCT1 and MCT4 are dependent upon interaction with other proteins, namely the chaperone CD147 (EMMPRIN) (Figure 34), to ensure the correct expression of the transporters at the plasma membrane and to maintain their activity. In the absence of the mature, glycosylated form of CD147, MCT1 and MCT4 fail to reach the plasma membrane and are accumulated in the Golgi apparatus [638-639]. CD44 also seems to contribute to the localization and function of MCT1 and MCT4 at the plasma membrane [640].

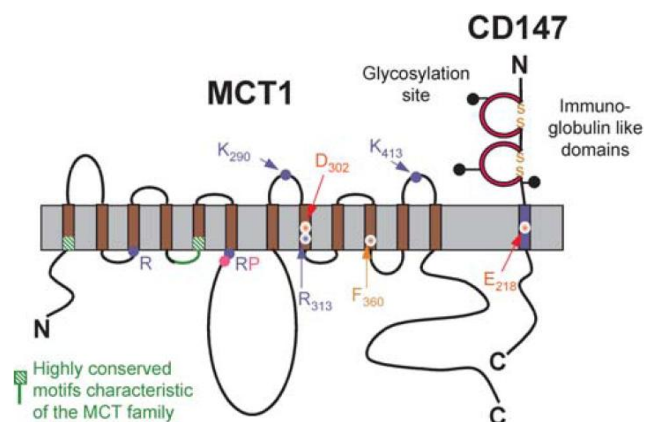


Figure 34 | MCT1 and CD147 proposed typology and interaction (adapted from [633]).

Besides MCTs regulation by chaperone proteins at the transporter activity level, numerous factors regulate protein amounts at the transcriptional and post-transcriptional levels, conditioning their expression in different physiological and pathological conditions. The regulatory mechanisms vary among the MCT isoforms, denoting the cell's abilities to adapt to special energy demands [632, 636, 641] (Figure 35).

As already mentioned, high lactate levels are a common trait of malignant tumours, and its dependence on MCTs for the transport across the plasma membrane directly implicates MCTs on tumour behavior. The pioneering studies on MCT expression in human tumour samples reported a decrease on MCT1 levels in the colonic transition from normality to malignancy [642-643]. These intriguing results were later contradicted by evidences indicating increased MCT1 levels in colon adenocarcinoma, when compared to normal colonic samples [595, 644]. Moreover, MCT1 plasma

membrane expression was associated with lymphovascular invasion, highlighting its important role in the transport of lactate, the most important player of microenvironmental acidosis, an angiogenesis-promoting condition [644]. For other malignant tumours, although some controversial results are reported in the literature, the main observed tendency is a general upregulation of MCTs, particularly MCT1 [641, 645] (Table 12). Additional studies, with standardized immunohistochemistry protocols and evaluation methods, are necessary to further elucidate the role and impact of monocarboxylate transporters in cancer patients. On the other hand, inhibition of MCTs would necessarily have a major effect on lactate transport, pH balance and tumour homeostasis, by compromising aerobic glycolysis and microenvironmental acidosis, and the cell-cell lactate shuttle between aerobic and hypoxic cell populations, with these last undergoing hypoxic cell death (Figure 36). The invasive abilities of the tumour mass could potentially be decreased, host immune response could potentially be reactivated, and response to therapy could potentially be enhanced [586]. This appealing scenario has already been demonstrated *in vitro* and *in vivo*, using different approaches to disrupt MCTs, namely the inhibitors CHC (α-cyano-4-hydroxycinnamate) and lonidamine, and specific small-interfering RNAs (siRNAs) [243, 586, 646-652]. Efforts are being taken to find adequate compounds for clinical use. Numerous agents targeting metabolic pathways are currently under clinical trial phase for several human malignancies [653-655], but MCTs are not yet included in the list of metabolic targets.

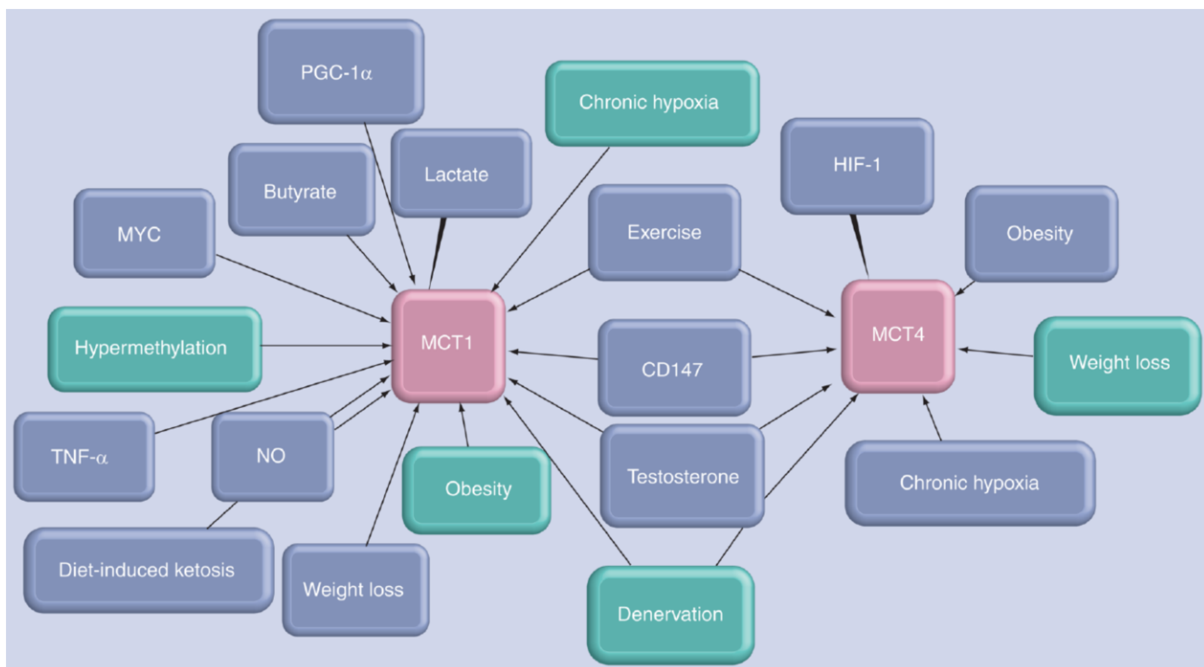


Figure 35 | Regulation of monocarboxylate transporters 1 and 4 at the transcriptional and translational levels (blue boxes, factors that induce upregulation; green boxes, factors that induce downregulation (adapted from [641]).
Abbreviations: HIF, hypoxia-inducible transcription factor; MCT, monocarboxylate transporter; NO, Nitric oxide.

Table 12 | Overview on MCT1 and MCT4 expression and impact on prognosis in different tumour types (adapted from [645]).

	MCT1 expression	MCT4 expression
Colon	↓ from normality to malignancy (Ritzhaupt et al. 1998; Lambert et al. 2002)	Not detected in either normal or tumor tissues (Lambert et al. 2002)
	(+) in tumor cells but (−) normal epithelium (Koukourakis et al. 2006)	Cytoplasm of cancer cells (Koukourakis et al. 2006)
	↑ in tumor cells, compared to normal epithelium/associated with vascular invasion (Pinheiro et al. 2008a)	↑ in tumor cells, compared to normal epithelium (Pinheiro et al. 2008a)
	(+) in tumor cells (Pinheiro et al. 2010a)	(+) in tumor cells (Pinheiro et al. 2010a)
Stomach	(+) with no change along progression/associated with advanced gastric cancer, Lauren's intestinal type, stage III+IV and lymph-node metastases when (co-expressed with CD147) (Pinheiro et al. 2009b)	↓ from normal tissue, to primary tumor, to lymph-node metastases/associated with early gastric cancer and Lauren's intestinal type (Pinheiro et al. 2009b)
Breast	↓ due to gene hypermethylation (Asada et al. 2003)	Tendency to be ↑ in tumor cells, compared to normal epithelium (Pinheiro et al. 2010b)
	↑ in tumor cells, compared to normal epithelium/associated with basal-like subtype, high histological grade, estrogen and progesterone receptors, cytokeratins 5 and 14 and vimentin (alone or co-expressed with CD147) (Pinheiro et al. 2010b)	↑ in tumor cells, compared to normal epithelium (Pinheiro et al. 2010a)
Lung	Cytoplasmic accumulation in alveolar soft-part sarcoma (Ladanyi et al. 2002)	(+) in tumor cells but (−) normal epithelium (Koukourakis et al. 2007)
	(+) in tumor cells but (−) normal epithelium (Koukourakis et al. 2007)	↓ in tumor cells, compared to normal epithelium (Pinheiro et al. 2010a)
	(+) in tumor cells and normal epithelium (Pinheiro et al. 2010a)	
Gynecologic tract	↑ from preinvasive to invasive cervical cancer/associated with metastases in AC (when co-expressed with CD147) (Pinheiro et al. 2008b)	↑ from preinvasive to invasive cervical cancer/↑ AC (Pinheiro et al. 2008b)
	(+) in ovarian tumor cells (Pinheiro et al. 2010a; Chen et al. 2010), but (−) in normal and benign epithelium (Chen et al. 2010)/associated with low grade, high FIGO stage, residual tumor, lack of tumor relapse and presence of ascites (Chen et al. 2010)	(+) in ovarian tumor cells (Pinheiro et al. 2010a; Chen et al. 2010), but (−) in normal and benign epithelium (Chen et al. 2010)/associated with high grade, high FIGO stage, residual tumor, tumor relapse and presence of ascites (Chen et al. 2010)
Prostate	(+) in tumor cells but (−) normal epithelium and PIN lesions/associated with high pretreatment PSA, high Gleason score, high pathological grade and nodal involvement (Hao et al. 2010)	(+) in tumor cells but (−) normal epithelium and PIN lesions/associated with high pretreatment PSA, high Gleason score, high pathological grade and nodal involvement (Hao et al. 2010)
	↓ in tumor cells, compared to normal epithelium/associated with high PSA, absence of perineural invasion and presence of biochemical recurrence (Pertega-Gomes et al. 2011)	↑ in tumor cells, compared to normal epithelium/high PSA levels, advanced tumor stage, higher Gleason score, presence of perineural invasion, and presence of biochemical recurrence (Pertega-Gomes et al. 2011)
Central nervous system	Strongest in high grade glial neoplasms, compared to low grade glial neoplasms (Froberg et al. 2001)	(-) in glioblastoma (Mathupala et al. 2004)
	(+) in glioblastoma and (−) in normal tissue (Mathupala et al. 2004)	
	(+) in neuroblastoma/associated with age >1 year at diagnosis, stage 4 disease, unfavorable Shimada histopathology, DNA diploid index, *n-myc* amplification and high-risk clinical group (COG criteria) (Fang et al. 2006)	

↓ downregulation; ↑ upregulation; (+) positive expression; (-) negative expression.

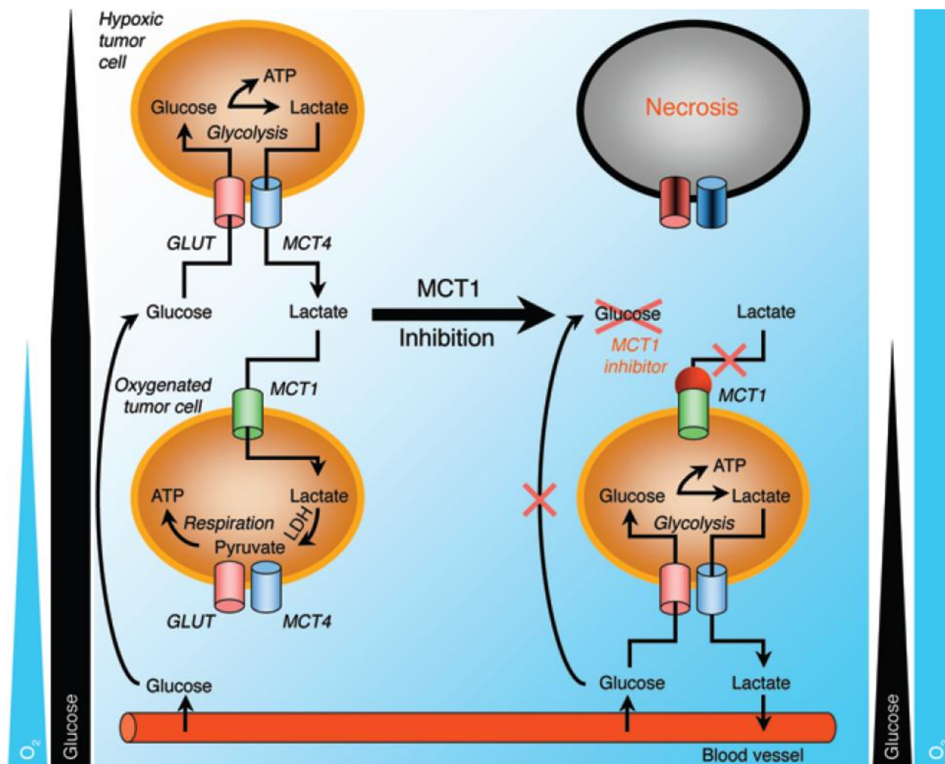


Figure 36 | Model for therapeutic targeting of lactate-based metabolic symbiosis in tumors (adapted from [586]). Abbreviations: ATP, adenosine triphosphate; GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter.

The biological role of MCTs in UBC is largely unknown. In a study investigating the hypoxia transcriptome in primary UBC, 32 of 6000 genes were hypoxia-inducible. Among them, MCT4 was upregulated in tumour cell lines and in tumour tissue [656]. In another study trying to establish a method for predicting response to MVAC therapy using a cDNA microarray consisting of 27,648 genes, *SLC16A3* gene was found to be upregulated in non-responder patients. The authors suggested that MCT4 upregulation might influence resistance to MVAC neoadjuvant chemotherapy via its association with CD147 [157]. This important finding highlights the need to investigate, in UBC setting, not only MCTs, but also their cooperation with CD147 and other chaperones, in an attempt to further elucidate the biological mechanisms of the life-threatening chemotherapy resistance.

1.2.3.4. CD147 and CD44 – Chaperones for MCTs

As already mentioned, CD147 is necessary for the expression of MCTs at the plasma membrane. However, the functions of this immunoglobulin superfamily member extend far beyond its role as a chaperone, being involved in fetal, neuronal, lymphocyte and extracellular matrix development, and in pathological conditions like heart disease, Alzheimer's disease, stroke and cancer [657-659].

CD147 (or EMMPRIN, extracellular matrix metalloproteinase inducer) was initially described as an inducer of MMPs production and expression [660]. The gene name for CD147/EMMPRIN is basigin

(*BSG*, chromosomal location at 19p13.3), consisting of seven exons and six introns spanning 7.5 kb [661-662]. *BSG* encodes a 29 kDa protein, but the mature, glycosylated form of CD147 ranges between 25 and 65 kDa, depending on the degree of glycosylation, which is necessary for its MMP stimulating activity. CD147 is a transmembrane glycoprotein composed of two immunoglobulin-like extracellular domains (where three glycosylation sites have been identified), a single transmembrane domain and a short cytoplasmic tail (Figure 34). The transmembrane and cytoplasmic domains are critical for protein-protein interactions within the plasma membrane. Besides interacting with MCT1 and MCT4, CD147 also appears to interact with integrin, caveolin-1 and cyclophilins [638, 657-658, 663]. Interestingly, while proton-coupled MCTs (MCT1, MCT3 and MCT4) depend on the association with the glycosylated form of CD147 to be expressed and functional on either plasma or mitochondrial membranes, it appears that CD147 maturation is affected by MCT expression [664].

CD147 is ubiquitously expressed on hematopoietic and non-hematopoietic cells such as monocytes, granulocytes, activated T lymphocytes, epithelial and endothelial cells [659]. Moreover, increased CD147 expression occurs in several types of malignancies [641, 645, 658]. Together with its ability to induce MMPs expression in adjacent stromal cells (e.g. fibroblasts and endothelial cells) [665], this evidence suggests that CD147 must be connected with one or more signalling pathways, being a key regulator of tumourigenesis and tumour progression. In fact, a link to the MAPK cascade has been demonstrated, with CD147 expression strongly correlating with activated ERK concomitantly with increased MMP-2 production [666]. CD147 stimulates its own expression through a positive feedback mechanism and induces the production of a soluble form, enhancing the potential for MMP stimulation from neighboring stromal cells to distant sites [667]. CD147 is also able to upregulate VEGF production via the PI3K/AKT pathway [668]. It associates with the laminin-interacting $\alpha3\beta1$ and $\alpha6\beta1$ integrins, major receptors for the cellular anchoring to the ECM [669], and stimulates hyaluronan production [670], co-localizing with the hyaluronan receptor CD44 [640]. Constitutive interactions between hyaluronan, CD44, and CD147 contribute to the regulation of MCT localization and function in the plasma membrane, and ultimately affect lactate transport [640]. Altogether, the pleiotropic effects of CD147 promote tumour growth, ECM degradation, angiogenesis, migration and invasion, enhancing the metastatic potential of CD147-expressing tumour cells [657-658, 665]. Importantly, CD147, through hyaluronan-CD44 interaction, crosstalks with various multidrug transporters of the ABC (ATP-binding cassette) family classically associated with anti-apoptotic signalling and chemotherapy resistance [671] (Figure 37).

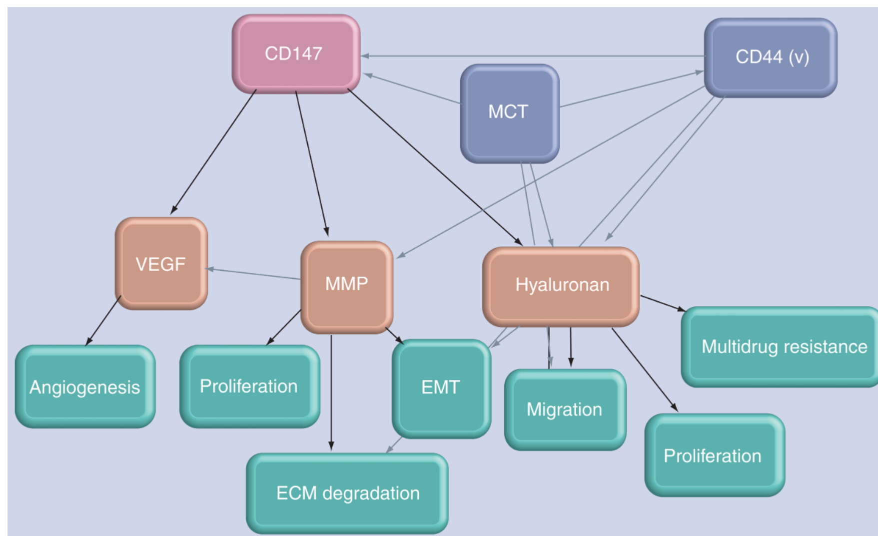


Figure 37 | CD147 signal-ling and interactions (black arrows, stimulation/ activati-on; gray arrows, asso-ciated molecules and their additional signalling or au-gmentation of effect) (adapted from [641]). Abbreviations: ECM, extracel-lular matrix; EMT, epithelial-mesenchymal transition; MCT, monocarboxylate transporter; MMP, matrix metalloproteina-se; VEGF, vascular endothelial growth factor.

CD44 is a ubiquitous single chain transmembrane glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is encoded by a single gene (*CD44*, 11p13, twenty exons), but their transcripts undergo complex alternative splicing that, together with variations in N-glycosylation, O-glycosylation, and glycosaminoglycanation (by heparan sulfate or chondroitin sulfate), generate multiple isoforms of different molecular sizes (85-230 kDa) [672]. Normal cells (and also malignant cells) abundantly express the smallest, standard CD44 (CD44s, 85-95 kDa) isoform (lacks variant exons). The variant CD44 (CD44v) isoforms contain a variable number of exon insertions (v1–v10) and are expressed predominantly by malignant cells. All forms of CD44 include an N-terminal, membrane-distal, hyaluronan-binding domain, and hyaluronan is its principal ligand (among other partner proteins like osteopontin, fibronectin, collagens, and MMPs). The glycosaminoglycanation pattern of the CD44 ectodomain enables it to additionally bind to growth factors (e.g. VEGF, FGF). The short cytoplasmic tail mediates interactions with the cytoskeleton. This protein participates in a wide range of cellular functions, namely lymphocyte activation, recirculation and homing, hematopoiesis, and tumour dissemination. Most of the multiple cellular functions of CD44 rely on its association with hyaluronan [673-676].

Hyaluronan (also hyaluronic acid or hyaluronate) is a very large, linear, negatively charged glycosaminoglycan composed of 2,000–25,000 disaccharides of glucuronic acid and N-acetylglucosamine, with molecular weights ranging from 105 to 107 Da. It is produced by three integral plasma membrane hyaluronan synthases (Has1/Has2/Has3), being extruded as it is elongated, and then targeted to the cell surface or to pericellular and extracellular matrices [674, 677]. Hyaluronan is distributed ubiquitously and, in addition to its structural role, strongly dependent on its remarkable hydrodynamic characteristics and its interactions with other ECM components, has an instructive role in

signalling via binding to specific cell-surface receptors. CD44 is its major cell-surface receptor, and it is clear that the effects of the hyaluronan-CD44 interactions are activated during the dynamic cell processes involved in tumourigenesis and tumour progression [674, 676, 678]. CD147 stimulates hyaluronan production and many of its signalling actions [670].

Hyaluronan-CD44 interactions in malignant cells promote resistance to growth arrest and apoptosis under anchorage-independent growth [679]. The adherence of malignant cells on capillary beds prior to extravasion into metastatic sites seems to involve the pericellular hyaluronan that surrounds the metastatic cells; this adherence probably involves CD44 expression by endothelial cells [680]. By forming highly hydrated, malleable matrices, by regulating the production and presentation of proteases, and by inducing cytoskeletal rearrangements, hyaluronan also mediates invasion [681]. Its breakdown products seem to be angiogenesis promoters, possibly by interacting with CD44-expressing endothelial cells [682]. Hyaluronan-CD44 binding influences the activity of several downstream signalling pathways, namely the anti-apoptotic MAPK and PI3K-AKT pathways, consequently promoting tumour cell proliferation, survival, motility, invasiveness, and chemoresistance [676, 683]. In fact, in addition to its pro-malignant and anti-apoptotic properties, these pathways seem to mediate the increased expression of multidrug membrane efflux pumps of the ABC family, such as MDR1 (multidrug resistance protein 1), MRP-1 (multi-drug resistance-associated protein-1) and BCRP (breast cancer resistance protein) [684-686]. Hyaluronan-CD44 interaction regulates MDR-1 and BCRP in malignant cells, possibly due to the stabilization of the transporter at the plasma membrane through the co-localization with CD44 [685, 687-688]. Once CD147 induces hyaluronan production, its enhanced expression probably mediates increased drug-resistance in a hyaluronan-dependent manner [689]. Moreover, this also indicates a possible association of hyaluronan to the hyper-glycolytic phenotype. Hyaluronan synthesis and expression of CD44v in tumour and tumour-associated stromal cells is also stimulated by lactate [627, 690]. CD44 co-localizes with MCT1 and MCT4 at the plasma membrane of breast cancer cells, and has been proposed as an additional chaperone for MCTs. Disruption of hyaluronan-CD44 signalling led to MCTs internalization and attenuation of their function [640]. These evidences point out for a probable partnership between hyaluronan, CD44 and CD147 in regulating the hyper-glycolytic and acid-resistant phenotype, and also chemotherapy resistance. Moreover, expression levels of CD147, CD44 and hyaluronan are consistently increased in tumour tissues, correlating with cancer progression, invasion, metastasis and recurrence [641, 645, 658-659, 691]. Therefore, antagonists of these molecules are promising candidates for targeted therapy. Several hyaluronan-CD44 signalling disrupting methods have been tested *in vitro* and *in vivo*, namely the use of small hyaluronan

oligosacharides that compete with the endogenous hyaluronan polymer, soluble CD44, blocking antibodies against the hyaluronan binding site of CD44 and CD44 siRNAs [675-676]. Interestingly, hyaluronan has the potential to be used as a drug transport vehicle. Since activated CD44 is overexpressed in solid tumors but not on their normal counterparts, and since CD44 can internalize hyaluronan, hyaluronan-drug conjugates are internalized via CD44, and the drug is released inside the malignant cells [692-694]. Monoclonal antibodies and siRNAs directed against CD147 have also been developed [659]. However, these strategies are still in a very preliminary phase of basic and translational research.

The initial studies investigating CD147 in bladder cancer patients reported its possible usefulness as a sensitive urinary marker [695-696]. Positive CD147 staining in UBC tissue sections was significantly associated with TNM stage, grade and histological subtype, and with poor prognosis [697-701], being identified as an independent prognostic factor for disease-free and overall survival [698-699, 702]. Importantly, CD147 positivity was able to predict response and survival following cisplatin-containing chemotherapy in patients with advanced UBC [702]. In UBC cell lines, CD147 downregulation with siRNA significantly decreased proliferation, migration and invasion, and also reduced secretion of MMP-2 and MMP-9, and expression of VEGF [697, 699]. Cisplatin response was not investigated.

Hyaluronan levels are increased in tissue and urine from UBC patients, and seem to be an accurate diagnostic marker [703-706]. Moreover, hydrosoluble drug-hyaluronan bioconjugates are being tested as a strategy of efficient drug delivery [707-709]. In accordance, a few studies reported CD44 overexpression in tissue samples [703, 710-712]. In an *in vitro* study, Has-1 expression regulated bladder cancer growth, invasion and angiogenesis through CD44 [713]. However, the complexity of hyaluronan-CD44 interactions, as well as their impact for UBC patients, are far from being clarified. Associations among CD44, MCTs and CD147, in an attempt to unravel a possible crosstalk between these molecules in mediating the hyper-glycolytic phenotype, were also not investigated.

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CHAPTER 2 | Rationale and Aims

Urothelial bladder cancer (UBC) represents an important epidemiological problem mostly due to its heterogeneous, relapsing and progressive nature. Although the majority of the tumours present as non-muscle invasive, in a significant proportion of patients the disease recurs and develops progression, underlying the need of radical surgical approaches and chemotherapy treatments. Half of the muscle-invasive tumour patients face the fearsome drawback of inherent or acquired chemoresistance. To predict whose tumours will recur, progress and/or develop resistance to chemotherapy is a major challenge, and the conventional clinical and pathological parameters, although representing pivotal diagnostic and prognostic tools, are far from being sufficient to individually differentiate UBCs. Research efforts need to be urgently directed into the molecular characterization of biological phenotypes of bladder cancer aggressiveness, in an attempt to find biomarkers that might allow more detailed prognostication and optimization of the treatments, with the main goal of improving patient outcome and quality of life. Therefore, based on the question "Is it possible to predict the prognosis and to personalize the treatment for UBC patients?", the central aim of this thesis was to characterize a phenotype of UBC aggressiveness in order to unveil potential prognostic and predictive biomarkers. The laboratory work was planned with the initial study on the clinical and prognostic significance of several biomarkers encompassing three hallmarks of cancer – tumour angiogenesis and lymphangiogenesis, invasion and metastasis, and energy metabolism reprogramming and the tumour microenvironment – in a population of UBC patients with known clinicopathological parameters and follow-up data. Subsequently, we intended to validate potential therapeutic targets in *in vitro* assays. In the pursuit of these general achievements, specific objectives were addressed regarding each of the explored hallmarks of cancer, as follows.

(i) <u>To characterize the clinical and prognostic impact of angiogenesis, lymphangiogenesis and lymphovascular invasion occurrence in UBC patients.</u>

Aiming to address the need of using specific antibodies in the establishment of a consensus concerning lymphovascular invasion detection, applicable to routine pathological evaluation, immunohistochemical biomarkers of blood (CD31) and lymphatic (D2-40) endothelial cells were used to quantify blood and lymphatic vessels density, both in peritumoural and intratumoural regions, and to assess the occurrence of blood and lymphatic vessels invasion. Different evaluation methods were performed and compared (classical hematoxylin and eosin staining *versus* specific highlighting of endothelial cells). The immunoexpression of the lymphangiogenic vascular endothelial growth factor (VEGF)-C and its receptor VEGFR-3 were also assessed.

As a secondary objective, we also aimed to evaluate the levels of expression of the mammalian target of rapamycin (mTOR), and to assess its contribution on the promotion of angiogenesis and lymphangiogenesis in the malignant context.

(ii) To characterize the clinical and prognostic impact of the expression of biomarkers of invasion and metastasis in UBC patients.

To achieve this objective, we evaluated the immunoexpression of the endo-β-glycosidase heparanase and of the metastasis suppressor RKIP (Raf kinase inhibitor protein).

(iii) To characterize the clinical and prognostic impact of the expression of microenvironment-related biomarkers in UBC patients.

In order to shed some light on the contribution of the tumour microenvironment and the inherent metabolic reprogramming of the malignant cells for the phenotype of UBC aggressiveness, we performed immunohistochemistry studies to assess the expression of CD147, monocarboxylate transporters (MCTs) 1 and 4, CD44 and carbonic anhydrase (CA) IX. Due to the apparent role of CD147 as a chemoresistance mediator, we also aimed to evaluate the discriminatory value of this biomarker when included in a tumour aggressiveness scoring system.

(iv) To assess the therapeutic impact of downregulation of a microenvironment-related biomarker, CD147, *in vitro*.

To further explore the preponderance of CD147 in mediating chemoresistance in bladder tumours, we intended to characterize the chemosensitivity of parental and CD147-silenced UBC cell lines to cisplatin.

CHAPTER 3 |

The aggressiveness of urothelial carcinoma depends to a large extent on lymphovascular invasion – the prognostic contribution of related molecular markers

The results presented in this chapter were:

(i) Published as an original article in an international peer reviewed journal

Afonso J, Santos LL, Amaro T, Lobo F, Longatto-Filho A: **The aggressiveness of urothelial carcinoma depends to a large extent on lymphovascular invasion – the prognostic contribution of related molecular markers**. *Histopathology* 2009; **55**(5): 514-524.

(ii) Discussed in an indexed book chapter edited by an international open access publisher

(in **APPENDIX**)

Afonso J, Santos LL, Longatto-Filho A: **Angiogenesis, Lymphangiogenesis and Lymphovascular Invasion: Prognostic Impact for Bladder Cancer Patients**, In: Bladder Cancer – From Basic Science to Robotic Surgery, Abdullah Canda. Croatia: INTECH Open Access Publisher, ISBN 978-953-307-839-7; 2012.

(iii) Selected for publication as an abstract in an international scientific website on Urology

Afonso J, Santos LL, Amaro T, Lobo F, Longatto-Filho A: **The aggressiveness of urothelial carcinoma depends to a large extent on lymphovascular invasion – the prognostic contribution of related molecular markers**. UroToday.com Bladder Cancer Session, ISSN 1939-4810; 2009.

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The aggressiveness of urothelial carcinoma depends to a large extent on lymphovascular invasion – the prognostic contribution of related molecular markers

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The aggressiveness of urothelial carcinoma depends to a large extent on lymphovascular invasion – the prognostic contribution of related molecular markers

Aims: Bladder cancer is the second most common malignancy of the urogenital region. The majority of bladder cancer deaths occur as a consequence of metastatic disease. Blood vessel density (BVD), a surrogate marker for angiogenesis, has been shown to be predictive of progression and poor prognosis, as well as lymphatic vessel density (LVD). The aim of this study was to evaluate, in human urothelial bladder cancer (UBC), the clinical and prognostic significance of angiogenesis, lymphangiogenesis and lymphovascular invasion, assessed with the use of specific immunohistochemical markers.

Methods and results: Immunohistochemistry for CD31 (a blood vessel endothelial cell marker), D2-40 (a lymphatic vessel endothelial cell marker), vascular endothelial growth factor (VEGF)-C and VEGF-receptor 3 antibodies was performed in 83 patients with urothelial carcinoma who underwent radical cystectomy. The classic histopathological characteristics, associated with lymphovascular invasion and loco-regional dissemination, had a negative influence on 5-year overall survival (OS) rates. BVD and LVD were correlated with advanced and poorly differentiated UBC with lymphovascular invasion. Blood vessel invasion (BVI) by malignant emboli assessed by CD31 staining, and lymphatic vessel invasion (LVI) by isolated malignant cells assessed by D2-40 staining significantly affected OS. VEGF-C overexpression was correlated with both BVI and LVI by single malignant cells assessed by CD31 and D2-40, respectively. BVI by malignant emboli assessed by CD31 staining remained as an independent prognostic factor.

Conclusions: Patients with UBC with embolic BVI assessed by CD31 and LVI by isolated malignant cells assessed by D2-40 have a worse prognosis and may benefit from adjuvant therapies.

Keywords: blood vessel density, CD-31, lymphatic vessel density, urothelial carcinoma, VEGFR-C, VEGFR-3

Abbreviations: BVD, blood vessel density; BVI, blood vessel invasion; CI, confidence interval; DAB, 3,3′-diaminobenzidine; DFS, disease-free survival; H&E, haematoxylin and eosin; HR, hazard ratio; ILV, intratumoral lymphatic vessel; LVD, lymphatic vessel density; LVI, lymphatic vessel invasion; OS, overall survival; RC, radical cystectomy; RTU, ready-to-use; UBC, urothelial bladder cancer; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor-receptor

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Introduction

Bladder cancer represents a significant health problem. An estimated 357 000 bladder cancer cases occurred worldwide in 2002, making this the ninth most common cause of cancer for both sexes combined.[1] Urothelial carcinoma is the most frequent histological type. Approximately 70–80% of initial tumours are superficial lesions confined to the bladder mucosa. Although without aggressive histopathological features, these tumours frequently recur and progress to invasive forms. Twenty percent to 40% of all patients will have or develop muscle invasive disease for which radical cystectomy (RC) is indicated. Such neoplasms are associated with a high risk of metastasis.[2]

The majority of bladder cancer deaths occur as a consequence of metastatic disease. Angiogenesis is crucially involved in cancer development and metastasis. Malignant cells of bladder tumours produce high levels of several stimulatory factors, including vascular endothelial growth factor (VEGF).[3] Blood vessel density (BVD), a surrogate marker for angiogenesis, has been shown to be predictive of progression and poor prognosis in invasive urothelial bladder cancer (UBC).[4,5] These tumours can penetrate deeply through the bladder wall and demonstrate a high propensity for lymphatic and distant metastasis.

In addition to angiogenesis, the tumoral microenvironment also exhibits *de novo* formation of lymphatic vessels (lymphangiogenesis). This represents a potential route to facilitate metastatic spread. However, lymphangiogenesis has only recently been demonstrated as a useful parameter for cancer prognosis, mostly due to the discovery of specific markers to recognize lymphatic vessels, and the efforts to elaborate a consensual methodology to quantify and interpret lymphangiogenesis in solid human tumours.[6] Tumoral lymphangiogenesis is not fully understood, especially in relation to the mechanisms that control the activation of the molecular cascade involved in lymphatic endothelial cell proliferation. Nevertheless, it is implicit that lymph node metastasis is a decisive event in determining prognosis and therapy.[7] Lymphatic molecular players from the VEGF family are believed to be valuable targets for anticancer therapy, and the regulation of VEGF-C and VEGF-D may represent a useful method for inhibition of lymphangiogenesis.[8,9] Actually, augmented lymphatic vessel density (LVD) during cancer progression has been demonstrated in an experimental model with co-localization of Ki67 (a cell cycle marker) with LYVE-1+ lymphatics.[10] Accordingly, lymphangiogenesis may make an important contribution to UBC dissemination. Fernandez and colleagues[11] have reported that higher intratumoral LVD significantly correlates with poor histological differentiation, and higher peritumoral LVD shows a significant association with lymph node metastasis. Algaba [12] has emphasized that, in this field, it will be necessary to reach a consensus on strict diagnostic criteria as soon as possible, to be able to incorporate this prognostic factor in clinical practice.

The aim of the current study was to clarify the clinical and prognostic significance of LVD and BVD in bladder cancer, and to assess the need to use specific antibodies in the establishment of a consensus concerning lymphovascular invasion, applicable to clinical practice.

Materials and methods

PATIENTS AND TUMOUR SAMPLES

The study included 83 urothelial carcinomas from patients who underwent RC at the Portuguese Institute of Oncology, Porto, from August 1992 to September 2005. Each cystectomy specimen was examined according to the College of American Pathologists.[13] Tissue samples were reviewed according to standard histopathological methods. Staging and grading were conducted according to the American Joint Committee on Cancer[14] and to World Health Organization classification systems.[15] For statistical analysis, tumours were divided into three groups based on T stage: group 1 (high risk of progression non-muscle invasive bladder tumours, including T1 and Tis stages); group 2 (T2 a and b); and group 3 (T3 and T4). Table 1 summarizes the clinicopathological parameters.

Tissue sections were analysed for CD31 expression [to determine BVD and blood vessel invasion (BVI)], D2-40 expression [to determine LVD and lymphatic vessel invasion (LVI)], and VEGF-C and vascular endothelial growth factor-receptor (VEGFR)-3 expression.

Histopathological examination was attempted to identify BVI and LVI by routine haematoxylin and eosin (H&E) staining (Table 1). These data were correlated with those obtained with the use of CD31 and D2-40.

All immunohistochemical reactions were correlated with the clinical data and the outcome variables [overall survival (OS) and disease-free survival (DFS)].

IMMUNOHISTOCHEMICAL PROCEDURES

Immunohistochemistry was carried out with the streptavidin–biotin–peroxidase complex technique, to detect CD31 (blood endothelial cell marker) and

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Table 1. Clinicopathological parameters

Gender (%)	Male	67 (80.7)
	Female	16 (19.3)
Age (years)	Median (range)	70 (41–83)
Tumour stage (%)	Group 1	20 (24.1)
	Group 2	14 (16.9)
	Group 3	49 (59.0)
Grade (%)	II	25 (30.1)
	III	58 (69.9)
Morphological type of lesion (%)	Non-invasive papillary	16 (19.3)
	Urothelial carcinoma *in situ*	4 (4.8)
	Infiltrating urothelial carcinoma	49 (59.0)
	Infiltrating mix carcinoma	14 (16.9)
Vascular invasion (H&E) (%)	Blood vessel	19 (22.9)
	Lymphatic vessel	18 (21.7)
	None	46 (55.4)
Loco-regional metastasis (%)	Yes	22 (26.5)
	No	61 (73.5)

VEGFR-3, and with the avidin–biotin–peroxidase complex assay, to detect D2-40 (lymphatic endothelial cell marker) and VEGF-C. The primary antibodies were obtained from DakoCytomation (CD31 and D2-40; Carpinteria, CA, USA), Zymed Laboratories (VEGF-C; San Francisco, CA, USA) and Santa Cruz Biotechnology (VEGFR-3; Santa Cruz, CA, USA). Briefly, 4-μm tumour tissue sections were dewaxed and rehydrated. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol. The slides were incubated with normal horse serum block solution at room temperature [10 min in Large Volume Ultra V Block (Labvision, Fremont CA, USA) for CD31 and VEGFR-3 detection; 20 min in ready-to-use (RTU) normal horse serum (Vector, Burlingame CA, USA) for D2-40 and VEGF-C detection]. Primary antibodies were diluted at 1:100 (CD31 and D2-40), 1:70 (VEGF-C) and 1:200 (VEGFR-3), and incubated on the sections (60 min incubation for anti-CD31 and anti-VEGFR-3, at room temperature; overnight incubation for anti-D2-40 and anti-VEGF-C, at 4°C). This step was followed by extensive washes with phosphate-buffered saline. Subsquently, sections were incubated with the secondary biotinylated antibody at room temperature [10 min in Goat Anti-Polyvalent (Labvision) for CD31 and VEGFR-3 detection; 30 min in RTU Biotinylated Universal Antibody (Vector) for D2-40 and VEGF-C detection] and with the streptavidin/avidin–biotin–peroxidase complex solution [10 min at room temperature in Large Volume Streptavidin Peroxidase (Labvision) for CD31 and VEGFR-3 detection; 45 min at 37°C in Vectastain RTU Elite ABC (avidin–biotin complex) Reagent (Vector) for D2-40 and VEGF-C detection]. Staining was developed using a liquid 3,3′-diaminobenzidine (DAB) substrate kit (10 min at room temperature in Liquid DAB+ substrate chromogen system; DakoCytomation). Sections were counterstained with Mayer's haematoxylin. Negative controls were carried out by omitting the primary antibodies. Sections of positive controls were used as indicated by the manufacturers (invasive ductal breast carcinoma for CD31 and VEGFR-3 detection, tonsil for D2-40 detection and colonic carcinoma for VEGF-C detection).

EVALUATION OF IMMUNOPOSITIVE REACTIONS

The expression of CD31 and D2-40 was evaluated in the cytoplasm and membrane of blood vessel and lymphatic endothelial cells, respectively. Evaluation was performed blindly and both LVD and BVD were assessed as proposed by Weidner *et al.*,[16] with slight modifications. A blood or lymphatic microvessel was defined as a single endothelial cell or cluster of endothelial cells positive for CD31 or D2-40, respectively, located around a visible lumen clearly separate from adjacent microvessels and from other connective tissue components. Furthermore, as lymphatic vessels can appear as distorted and overlapped structures in a cancer setting, the packed vessels were assumed to represent one lymphatic unit. In contrast, blood vessels commonly do not display a distorted and packaged appearance. The number of vessels was quantified at ×200 (×20 objective lens and ×10 ocular lens) magnification. A median of 10 hotspot fields was defined for the purpose of vessel density. The examination of each hotspot corresponds to the number of vessels confined to an area of 0.15 mm^2. Both CD31 and D2-40 immunopositive reactions were independently counted in blood and lymphatic vessels from intratumoral and peritumoral areas. The intratumoral area was defined as the stromal tissue within two or more neoplastic aggregates, and the peritumoral area was defined as

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the stroma tissue surrounding this neoplastic mass. CD31 and D2-40 positivity in tumour cells was classified as negative (negative or weak immunoreactivity) and positive (moderate to strong immunoreactivity). For evaluation of BVI and LVI, only CD31+ and D2-40+ vessels occupied by neoplastic cells, respectively, were considered.

The positive expression of VEGF-C and VEGFR-3 was semiquantitatively assessed using ×200 magnification, considering membrane and cytoplasmic staining of urothelial malignant cells (VEGFR-3 expression was also assessed in blood and lymphatic vessel endothelial cells). Positive reactions were assessed in hotspot areas where urothelial malignant cells and proliferating vascular structures were present and stained. For each case, 10 fields with approximately 100 malignant cells each were evaluated. The following grading system was used: negative (–), absence of expression; slightly positive staining (+), expression in ≤10% of cells; moderately positive (++), expression in >10% up to 50% of cells; strongly positive (+++), expression in >50% of cells.

EVALUATION OF VASCULAR INVASION

Evaluation of vascular invasion was performed by three different methods:

1) Traditional H&E method (method 1): positive invasion was defined as BVI or LVI by at least one well-characterized malignant cell surrounded by endothelial cells. Distinction between BVI and LVI was based on the presence of red blood cells in the lumen of blood vessels.

2) Immunohistochemical marker and isolated malignant cells (method 2): BVI or LVI was recorded if at least one well-characterized malignant cell was surrounded by endothelial cells highlighted by specific positive immunohistochemical expression for CD31 or D2-40, respectively.

3) Immunohistochemical marker and emboli of malignant cells (method 3): BVI or LVI was assumed if an embolus of well-characterized malignant cells was surrounded by endothelial cells highlighted by specific positive immunohistochemical expression for CD31 or D2-40, respectively.

STATISTICAL ANALYSIS

The relationship between the expression of immunohistochemical markers and clinicopathological parameters was examined for statistical significance using Pearson's χ^2 test and Fisher's exact test (when $n < 5$). The Mann–Whitney test was used for continuous variables. For BVD and LVD analysis, data were expressed as the median, and this value was used as a cut-off point for statistical analysis. Additionally, the cut-off values that were better correlated with tumour aggressiveness were determined by receiver–operating characteristic curve analysis. Five-year DFS and OS were evaluated using Kaplan–Meier curves and differences were analyzed by log rank or Breslow tests. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered in a multivariate analysis using Cox proportional hazards analysis. The hazard ratios (HR) were estimated with their 95% confidence intervals (CI). Data were stored as excel files and analysed using the Statistical Package for Social Sciences (SPSS) software, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

CLINICOPATHOLOGICAL PARAMETERS

Table 2 shows the significant correlation of vascular invasion (evaluated by the traditional H&E method) with T3/4 pathological stage ($P < 0.001$), grade III ($P = 0.001$) and infiltrating urothelial carcinoma histopathological type ($P < 0.001$). Loco-regional metastasis development was associated with advanced stage ($P < 0.001$), grade ($P = 0.009$) and infiltrating urothelial carcinoma ($P = 0.020$). The analysis of 5-year DFS rate showed a positive correlation with T3/4 stage ($P = 0.006$), grade III ($P < 0.001$) and infiltrating urothelial carcinoma histopathological type ($P = 0.002$). Table 3 exhibits additional information. Importantly, the classic histopathological characteristics, associated with vascular invasion and loco-regional dissemination, had a negative influence on the 5-year OS rate.

BLOOD AND LYMPHATIC VESSELS DENSITIES USING SPECIFIC ANTIBODIES

The median value of BVD was 17.6 (range 5.7–31.4) (Figure 1A). This value was used as a reference for analytical evaluation with the other clinicopathological parameters and survival rates. However, no significant correlation was found, but the existence of intratumoral blood vessels (in 81.9% of tumours, $n = 68$) was prevalent in infiltrating urothelial carcinoma cases with deeper muscular invasion.

The LVD median value was 8.8 (range 0–22.6). In 47.3% ($n = 35$) of cases, lymphatic vessels were observed in the peri- and intratumoral areas; 37.8% ($n = 28$) showed only peritumoral lymphatic vessels

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Table 2. Association between vascular invasion (H&E stain) and loco-regional metastasis, and clinicopathological variables

		Vascular invasion			Loco-regional metastasis		
		Negative, %	Positive, %	P*	Negative, %	Positive, %	P*
Tumour stage	Group 1	20 (43.5)	0 (0.0)	<0.001	20 (32.8)	0 (0.0)	<0.001
	Group 2	9 (19.6)	5 (13.5)		13 (21.3)	1 (4.5)	
	Group 3	17 (37.0)	32 (86.5)		28 (45.9)	21 (95.5)	
Grade	II	21 (45.7)	4 (10.8)	0.001	23 (37.7)	2 (9.1)	0.009
	III	25 (54.3)	33 (89.2)		38 (62.8)	20 (90.9)	
Morphological type of lesion	Non-invasive papillary	16 (34.8)	0 (0.0)	<0.001	16 (26.2)	0 (0.0)	0.02
	Urothelial carcinoma *in situ*	4 (8.7)	0 (0.0)		4 (6.6)	0 (0.0)	
	Infiltrating urothelial carcinoma	20 (43.5)	29 (78.4)		31 (50.8)	18 (81.8)	
	Infiltrating mix carcinoma	6 (13.0)	8 (21.6)		10 (16.4)	4 (18.2)	

*χ^2 or Fisher exact tests.

and 14.9% ($n = 11$) only intratumoral lymphatic vessels (ILV) (Figure 1B). ILV had visible lumens in 41.3% of cases, and no oedema was observed. Similarly to BVD, the cut-off for statistical analysis of LVD was the median value (8.8 vessels). Notably, an overall LVD (intra- and peritumoral LVD) >8.8 was correlated with T3/4 stage ($P < 0.001$), grade III ($P = 0.004$), infiltrating urothelial carcinoma ($P = 0.005$) and with the occurrence of vascular invasion ($P = 0.015$) (assessed by method 1). Peritumoral (but not intratumoral) LVD was correlated with higher T stage ($P = 0.040$) and infiltrating urothelial carcinoma ($P = 0.038$).

COMPARISON OF DIFFERENT METHODS TO COUNT VESSEL INVASION

The comparison between the three different methods used to identify vascular invasion revealed interesting results. Overall agreement was observed in 42.2% ($n = 35$) of cases. We observed significant differences between the classical method to recognize invasion (H&E stain, method 1) versus invasion detection with the use of specific antibodies (CD31 and D2-40 stain, method 2, vascular invasion by isolated malignant cells) ($P = 0.008$). However, no significant difference was observed between the classical and the immunohistochemical methods using CD31 and D2-40 antibodies, if only malignant emboli were considered (method 3). No significant differences were found between the three methods if only BVI was considered. However, the identification of isolated malignant cells invading lymphatic vessels was significantly improved with the use of D2-40 (method 2) in comparison with the classical method (method 1) ($P = 0.001$).

BVI by malignant emboli (CD31 stain, method 3) (Figure 2A) was correlated with the 5-year OS rate ($P = 0.001$). Furthermore, the traditional BVI diagnosis method (H&E stain, method 1) was also useful to identify patients with a low 5-year OS rate ($P = 0.002$). Conversely, LVI by isolated malignant cells assessed by D2-40 staining (method 2) (Figure 2B) showed a significant correlation with the 5-year OS rate ($P = 0.013$) (Table 4).

VASCULAR INVASION VERSUS VASCULAR DENSITY

BVI occurred more frequently in cases with BVD >17.6 vessels. The correlation between intratumoral BVD and BVI was significant in cases stained with CD31 highlighting single malignant cells ($P < 0.001$).

There was a significant correlation among high LVD and LVI identified by the three methods used for counting lymphatic vessels and lymphatic invasion ($P = 0.035$ for method 1, $P < 0.001$ for method 2 and $P = 0.001$ for method 3). Table 5 illustrates the comparison between the existence of peritumoral/intratumoral lymphatic vessels (and additional analysis of ILV structure) and the occurrence of LVI, also demonstrating the occurrence of lymphatic invasion identified by the three methods of vessel recognition.

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Table 3. Correlation between 5-year disease-free survival (DFS) and overall survival (OS) rates, and clinicopatological variables (univariate analysis)

		n	5-year DFS rate, %	*P**	5-year OS rate, %	*P**
Gender	Male	67	25.9	NS	36.4	NS
	Female	16	53.3		38.1	
Age	≤70 years	42	29.3	NS	31.1	NS
	>70 years	41	39.2		31.2	
Stage	Group 1	20	69.1	0.006	53.3	0.001
	Group 2	14	18.1		43.0	
	Group 3	49	31.9		19.7	
Grade	II	25	75.0	<0.001	51.5	0.002
	III	58	0.0		17.8	
Morphological type of lesion	Non-invasive papillary	16	73.8	0.002	47.2	0.008
	Urothelial carcinoma *in situ*	4	50.0		100	
	Infiltrating urothelial carcinoma	49	17.0		24.8	
	Infiltrating mix carcinoma	14	18.0		14.2	
Vascular invasion (H&E stain)	Negative	46	42.5	NS	43,6	<0.001
	Positive	37	28.5		21.2	
Loco-regional metastasis	Negative	61	41.3	NS	38.3	0.007
	Positive	22	19.3		11.5	

NS, Not significant.
*Log rank or Breslow tests.

VASCULAR ENDOTHELIAL GROWTH FACTOR-C

VEGF-C expression was frequently observed in macrophages (Figure 3), but the vast majority of cases showed immunopositivity also for VEGF-C in malignant cells (Figure 4): 38 (45.8%) cases had 10–50% of malignant cells stained and 22 (26.5%) cases had >50% of immunopositivity.

The importance of a positive reaction for VEGF-C was tested with a dichotomous strategy: all cases <50% were grouped (group A) and compared with cases with >50% (group B). Carcinomas displaying a less differentiated phenotype showed more pronounced VEGF-C overexpression (>50% of positive malignant cells) than those well-differentiated. Grade III and stage T3/4 carcinomas were more strongly immunoreactive for VEGF-C ($P = 0.002$ and $P = 0.023$, respectively). VEGF-C overexpression did not correlate with overall BVD, but intratumoral BVD was considerably enhanced by VEGF-C overexpression ($P = 0.008$). Conversely, both peri- and intratumoral LVD were significantly correlated with VEGF-C+ reaction >50% ($P = 0.049$). VEGF-C overexpression was also correlated with both BVI and LVI by single malignant cells assessed by CD31 ($P = 0.042$) and D2-40 ($P = 0.020$) imunopositivity, respectively (method 2). Although VEGF-C overexpression was more well-defined in the group of patients with poor clinical prognosis, we found no significant association with survival rates.

VASCULAR ENDOTHELIAL GROWTH FACTOR-RECEPTOR-3

VEGFR-3+ immunoreactivity was observed in both malignant and endothelial cells from blood and lymphatic vessels (Figure 5). Remarkably, all tumours showed immunopositivity for VEGFR-3 in up to 50% of malignant cells. The huge preponderance of VEGFR-3

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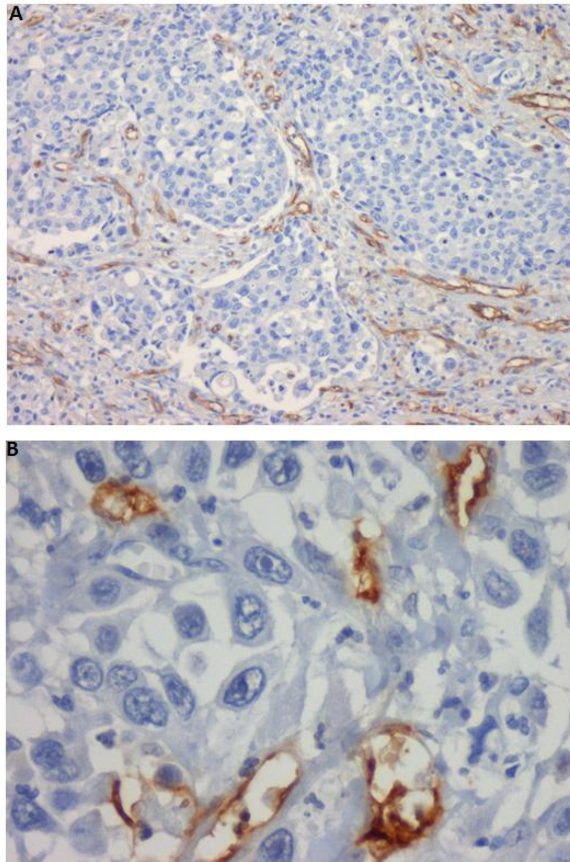

Figure 1. Intratumoral blood vessels highlighted by CD31 (**A**), and intratumoral lymphatic vessels highlighted by D2-40, in invasive urothelial carcinoma (**B**). Evidence of internal negative control in **AB** (D2-40– blood vessel).

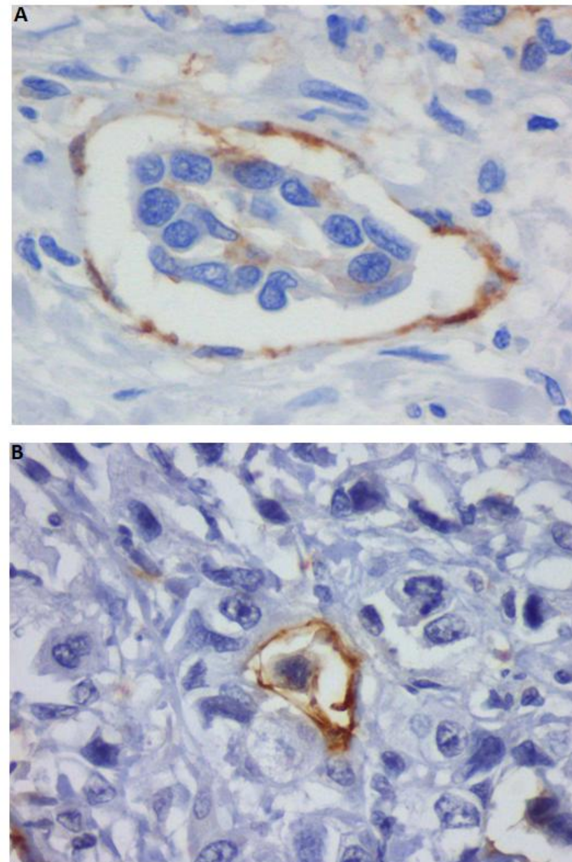

Figure 2. Intratumoral blood vessel highlighted by CD31 invaded by a small malignant embolus (**A**), and intratumoral lymphatic vessel highlighted by D2-40 invaded by an isolated malignant cell (**B**), in invasive urothelial carcinoma.

strong (>50%) immunopositivity (94%) was synchronously observed in both blood and lymphatic vessels. These findings obviously limited the statistical correlation of this marker with other clinicopathological parameters.

MULTIVARIATE ANALYSIS

On univariate analysis, the 5-year OS rate was significantly influenced by tumour stage ($P = 0.001$), grade of tumour cell differentiation ($P = 0.002$), type of lesion ($P = 0.008$), occurrence of vascular invasion (assessed by classic H&E stain, method 1) ($P < 0.001$) and loco-regional metastasis ($P = 0.007$). Additionally, patients with BVI demonstrated by CD31 staining highlighting tumoral emboli (method 3, $P = 0.001$) and/or the traditional H&E method (method 1, $P = 0.002$) had significantly worse 5-year OS rates. Occurrence of LVI invaded by single malignant cells (D2-40 stain, method 2) was also significantly correlated with the 5-year OS rate ($P = 0.013$). On multivariate analysis of these variables, only BVI (CD31 stain, method 3) remained an independent prognostic factor (HR 3.187, 95% CI 1.240–8.195; $P = 0.016$).

Discussion

In this study we aimed to investigate different ways to count vessel invasion. We observed significant differences between the daily routine method to identify vasculature invasion using H&E stain versus the use of specific antibodies (CD31 and D2-40) to highlight both blood and lymphatic vessels. The use of the specific markers significantly improved the recognition of vascular invasion for both lymphatic and blood vessels. Furthermore, the use of endothelial markers is

Table 4. Correlation between 5-year overall survival (OS) rate and occurrence of blood vessel invasion and/or lymphatic vessel invasion (H&E stain, method 1, and staining with specific immunohistochemical markers, methods 2 and 3)

			n	5-year OS rate, %	*P**
BVI	Classical H&E method (method 1)	Non-occurrence	64	36.5	0.002
		Occurrence	19	13.6	
	CD31 with single cancer cells (method 2)	Non-occurrence	50	32.3	NS
		Occurrence	33	28.7	
	CD31 with malignant emboli (method 3)	Non-occurrence	72	33.7	0.001
		Occurrence	11	15.2	
LVI	Classical H&E method (method 1)	Non-occurrence	65	33.9	0.045
		Occurrence	18	28.6	
	D2-40 with single cancer cells (method 2)	Non-occurrence	52	38.3	0.013
		Occurrence	31	22.6	
	D2-40 with malignant emboli (method 3)	Non-occurrence	67	32.4	0.068
		Occurrence	16	24.2	

*Log rank or Breslow tests.

Table 5. Association between the existence of peritumoral and intratumoral lymphatic vessels (additional analysis of intratumoral lymphatic vessel structure) and the occurrence of lymphatic vessel invasion (LVI) identified by classic H&E stain (method 1) and by D2-40 immunostaining (methods 2 and 3)

		PLV			ILV			ILV structure		
LVI assessing method		Negative	Positive	Total	Negative	Positive	Total	Well-preserved	Collapsed	Total
Classical H&E method (method 1)	No	17	48	65	34	31	65	13	18	31
	Yes	3	15	18	3	15	18	6	9	15
	Total	20	63	83	37	46	83	19	27	46
	*P**			NS			0.008			NS
D2-40 with single cancer cells (method 2)	No	18	34	52	30	22	52	3	19	22
	Yes	2	29	31	7	24	31	16	8	24
	Total	20	63	83	37	46	83	19	27	46
	*P**			0.003			0.003			<0.001
D2-40 with malignant emboli (method 3)	No	19	48	67	32	35	67	11	24	35
	Yes	1	15	16	5	11	16	8	3	11
	Total	20	63	83	37	46	83	19	27	46
	*P**			NS			NS			0.032

*Fisher's exact test.

encouraged because they facilitate discrimination between BVI and LVI, which could help to understand the biology of tumour spread.[17]

In spite of the sample size, these results reveal that BVD and LVD are correlated with advanced and poorly differentiated UBC with lymphovascular invasion, but

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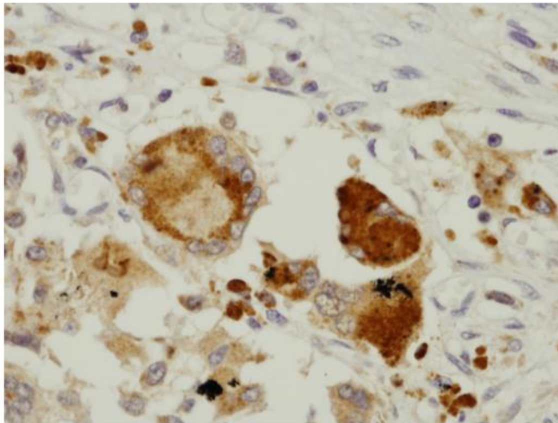

Figure 3. Vascular endothelial growth factor-C expression observed in single and multinucleated giant macrophages.

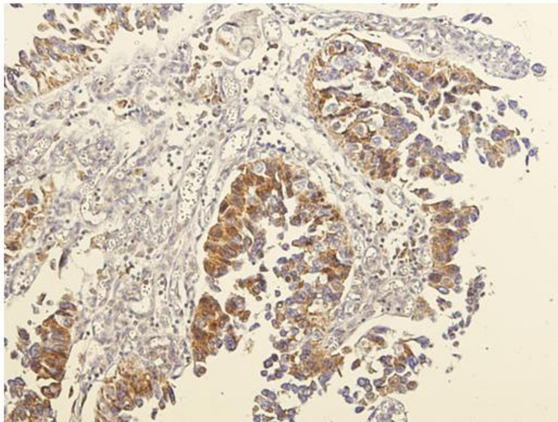

Figure 4. Papillary urothelial tumour immunopositive for vascular endothelial growth factor-C.

do not significantly influence 5-year DFS and OS rates. ILV had visible lumens in 41.3% of cases and, notably, no oedema was observed, which is consistent with the occurrence of *de novo* and efficient lymphangiogenesis. BVI by malignant emboli assessed by CD31 staining, and LVI by isolated malignant cells assessed by D2-40 staining, appear to be important and significant prognostic variables in patients with UBC treated with RC. This is important, because the risk evaluation based on vascular invasion status and pathological analysis could be helpful for selecting patients at high risk who would be appropriate candidates for clinical trials.[18] Moreover, vascular invasion and tumour dissemination are significantly associated with a reduction in survival rates. This ratifies the inclusion of patients with vascular invasion (notably LVI) in appropriate integrated therapy.[19]

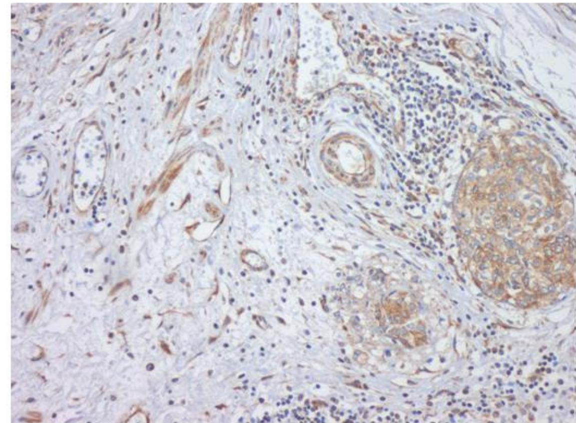

Figure 5. Vascular endothelial growth factor-receptor-3 immunopositivity observed in malignant cells and endothelial cells from blood and lymphatic vessels in invasive urothelial carcinoma.

As we reported previously, BVD and LVD have different significance in so far as their potential to identify patients with a worse prognosis is concerned. No significant values from the BVD analysis were correlated with prognosis, except that intratumoral BVD is indeed higher in cases with deeper muscular invasion. This result is contentious because, in the literature, BVD, assessed by CD34 or CD31 immunohistochemistry, is believed to be a useful parameter for prognosis.[9,20] On the other hand, overall LVD (intra- and peritumoral LVD) was correlated with T3/4 stage, tumour grade III, non-papillary type of carcinoma and with the occurrence of vessel invasion. Furthermore, peritumoral (but not intratumoral) LVD was also correlated with higher T stage and non-papillary type of lesion. These results endorse recent reports that have highlighted the value of LVD as a prognostic factor in an experimental setting [10] as well as under routine conditions.[11]

One of the strongest results observed in the present study was the identification of vascular invasion as a prognostic factor. It has been clearly demonstrated that both LVI and BVI significantly predict worse disease behaviour. Most of the recent reports also confirm the prognostic value of the assessment of LVI and BVI as prognostic factors for bladder cancer.[12,21,22]

The optional arm evaluated in this study considered 'embolic' invasion as an isolated entity; however, this particular condition did not show significant differences between the classical method (H&E) and identification made with CD31 and D2-40. This sounds reasonable, since an embolus is more easily demonstrable than single malignant cells. Conversely, the identification of isolated malignant cells invading lymphatic vessels was

significantly improved with the use of D2-40, in comparison with the classical H&E method. This result supports the use of this lymphatic marker for purposes of counting involved lymphatic vessels.[23]

BVI showed a significant correlation with lower 5-year OS rates, when evaluated with the classical method and with CD31 staining associated with malignant emboli, but not with single malignant cells. BVI is generally associated with distant recurrence and lymph node metastasis.[9,22] On the other hand, LVI showed a significant correlation with the 5-year OS rate only when single malignant cells invaded lymphatic vessels highlighted by D2-40 staining. Although LVD was not strongly associated with poor prognosis, LVI was correlated with high LVD values, mainly in the intratumoral area. As mentioned previously, most of the invaded lymphatic vessels were distorted and collapsed. However, single malignant cells were significantly observed in the well-preserved lymphatic vessels. Therefore, one can hypothesize that the malignant cells are able to spread to lymph nodes only when the intratumoral lymphatic vessels are well-preserved. This may explain the lower OS rates observed in these cases. Moreover, the absence of intratumoral oedema is a surrogate marker of efficient lymphatic flow. In contrast, some authors maintain that malignant cells are able to enter but not to escape from the vascular space. The mechanical stress produced within the tumour mass applies pressure on or restrains lymphatic proliferation and development, reducing the capacity of the confined malignant cells to escape.[24]

The expression of VEGF-C was highly positive in malignant cells, principally in less differentiated carcinomas. Not surprisingly, VEGF-C immunopositivity was importantly correlated with grade III and advanced stage carcinomas.

The overexpression of VEGF-C has been associated with poor prognosis of bladder carcinoma. Several reports have endorsed this statement and highlighted that VEGF-C expression is positively associated with both LVD and BVD,[9] and with lymph node metastasis,[25] being an independent predictive factor for poor prognosis if associated with high BVD.[26] The results presented here corroborate these findings in part, because both LVD and BVD, and blood and lymphatic invasion, were significantly correlated with VEGF-C positivity and poor prognosis. Nevertheless, VEGF-C overexpression did not correlate with worse OS rates. This apparently contradictory result still remains to be clarified, as previously observed.[27] In contrast, overexpression of VEGFR-3, the receptor of VEGF-C, is believed to be related to more aggressive tumour phenotypes associated with a shorter DFS.[8] Conversely, our results showed remarkable preponderance of VEGFR-3 expression in malignant cells and in both blood and lymphatic vessels, which seriously limited the statistical evaluation. In spite of some understandable enthusiasm for these molecular markers, caution is recommended, because the available data are still insufficient to provide supportive evidence for their incorporation into clinical management.[28,29]

Finally, multivariate analysis provided some important information regarding the survival rates of the patients. Most of the traditional parameters to predict outcome (grade of tumour differentiation, stage, lymph node metastasis) are presently associated with lymphovascular invasion.[30] In our series, we found that the 5-year OS rate is indeed influenced by most of these parameters, but we identified that patients with BVI and/or LVI had worse survival rates. Furthermore, multivariate analysis also showed that BVI (by malignant emboli) is an independent factor to predict 5-year poor OS rate. These data are important, because the identification of molecular markers associated with vascular sprouting might help to develop specific treatments tailored to the molecular pattern of each tumour. BVI and/or LVI seem to be important prognostic factors that may facilitate better selection of patients likely to benefit from chemotherapy and/or targeted adjuvant therapies.

Acknowledgement

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CHAPTER 4 |

Phospho-mTOR in Non-tumour and Tumour Bladder Urothelium: Pattern of expression and Impact on Urothelial Bladder Cancer Patients

The results presented in this chapter were:

(i) Submitted for publication as an original article in an international peer reviewed journal

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Phospho-mTOR in Non-tumour and Tumour Bladder Urothelium: Pattern of expression and Impact on Urothelial Bladder Cancer Patients

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BACKGROUNG: Urothelial bladder carcinoma (UBC) represents a significant health problem, due to its heterogeneous natural history and clinical behavior. Evaluation on biomarkers of aggressiveness and response to treatment needs to be added to classical diagnostic and prognostic tools, in an attempt to personalize management, improving survival and quality of life. We aimed to evaluate the pattern of expression, and the clinical and prognostic significance of phospho-mammalian target of rapamycin (p-mTOR) in UBC patients.
METHODS: UBC sections with tumour and non-tumour representative areas from 76 patients were stained by immunohistochemistry for detection of p-mTOR (Ser2448), CD31 (blood vessels identification) and D2-40 (lymphatic vessels indentification). Immunohistochemical reactions were statistically correlated with the clinicopathological and the outcome parameters. 5-year disease-free survival (DFS) and overall survival (OS) rates were estimated using the Kaplan-Meier method. p values < 0.05 were considered significant.
RESULTS: 36% of the non-tumour sections and 20% of the tumour sections were scored positive for p-mTOR expression. Immunoexpression was observed in umbrella cells from non-tumour urothelium, in all urothelial cell layers from non-muscle invasive (NMI) tumours (with a reinforcement in superficial cells), and in spots of cells from muscle invasive (MI) tumours. Positive expression decreased from non-tumour to tumour urothelium, and from pT1/pTis to pT3/pT4 tumours, but the few pT3/pT4 positive cases had worse survival rates, with 5-year DFS being significantly lower (p=0.004). Angiogenesis occurrence was impaired in pT3/pT4 tumours that did not express p-mTOR.
CONCLUSIONS: p-mTOR expression in non-tumour umbrella cells probably reflects their metabolic plasticity, and extension of expression to the inner layers of the urothelium in NMI tumours is consistent with an enhanced malignant potential. Expression in cell spots in a few MI tumours, and absence of expression in the remaining, is intriguing and demands further research. Additional studies directed to the upstream and downstream effectors of the mTOR pathway need to be addressed.

KEYWORDS: P-mTOR, urothelial bladder cancer, pattern of expression, umbrella cells.

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INTRODUCTION

Bladder cancer, the second most common urological malignancy, represents a significant epidemiological problem. An estimated 386,300 new cases and 150,200 deaths occurred in 2008 worldwide [1]. Urothelial carcinoma is the most common histological subtype in developed countries [2]. The majority of the patients present with non-muscle invasive (NMI) tumours that, although without aggressive histopathological features, frequently recur, which demands for long-term follow-up and repeated interventions. High grade NMI lesions harbor an enhanced risk of progression to muscle-invasive (MI) disease. MI tumours carry a significant metastatic potential [3]. Radical cystectomy (RC) with bilateral pelvic and iliac lymphadenectomy is the gold standard of treatment for MI disease [4-5], and provides a cure for most of the patients with organ-confined lesions [6]. However, regional lymph node and visceral metastasis are common findings, advocating the association of neoadjuvant and adjuvant therapies. Cisplatin-containing combinations are the standard of care for UBC patients, but

heterogeneity in the response to the treatment and patient fragility significantly impair survival benefits [7]. Up to 50% of MI-UBC patients will eventually die from metastatic disease [6].
Current investigational strategies have turned attention into the molecular pathogenesis of bladder tumours, trying to find biomarkers of aggressiveness and response to chemotherapy, and potential therapeutic targets. The mammalian target of rapamycin (mTOR) intracellular pathway represents a potential target. mTOR belongs to the phosphoinositide-3-kinase-related kinase family, being centrally involved in the transduction of proliferative factors induced by the phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) signalling pathway, to the level of mRNA and ribosome [8-11]. The *mTOR* gene encodes a protein product that functions as a component of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [10]. The main players downstream of mTORC1 are 4EBP1 (eucaryotic initiation factor 4E binding protein-1) and p70S6K (ribosomal p70S6 kinase, S6K). 4EBP1 negatively regulates eIF4E (eucaryotic initiation factor 4E), but phosphorylation of 4EBP1 by mTORC1 leads to its dissociation from eIF4E, allowing the assembly of the initiation complex of translation at the 5′ terminal of mRNAs. On the other hand, mTORC1 activates p70S6K, which in turn phosphorylates the ribosomal protein S6, promoting translation initiation and elongation [12]. Regarding mTORC2, its best characterized substrate is Akt. Akt is phosphorylated on its hydrophobic motif (Ser473) by mTORC2, and this is required to its fully activation. The ultimate result of Akt activation is the phosphorylation and upregulation of mTORC1 [13]. Through its interactions with Raptor (regulatory-associated protein of mTOR, contained in mTORC1) and Rictor (rapamycin-insensitive companion of mTOR, contained in mTORC2) proteins, activated mTOR regulates protein translation, cell cycle progression, actin cytoskeleton organization, cell migration and survival [8-11]. Moreover, mTOR signalling can increase vascular endothelial growth factor (VEGF) secretion, thus mediating angiogenesis and lymphangiogenesis. It also seems to play an important role in the crosstalk between tumour and endothelial cells [14-16]. Increased mTOR activity, as well as increased phosphorylation levels of its downstream targets, 4EBP1 and p70S6K, have been detected in a significant percentage of human tumours [17-24]. Rapamycin (sirolimus) and rapamycin analogs (e.g. temsirolimus, everolimus) selectively inhibit the mTOR pathway, and have demonstrated potent anti-tumour effects both *in vitro* and *in vivo* [25-28]. Some of these compounds have already obtained the FDA approval for the treatment of human malignancies [29], and numerous clinical trials are ongoing [30-31], including trials with UBC patients [32]. However, the levels of mTOR activation in bladder tumour tissue have been poorly explored, and the existing results are inconsistent. For instance, Hansel et al. reported the expression of phosphorylated mTOR (p-mTOR) in 74% (90/121) MI UBCs, and a significant association with increased pathological stage and reduced disease-specific survival was noted [33]. In the study by Makhlin et al., p-mTOR expression was increased in malignant *versus* normal urothelium in only 32.0% (65/203) of tumours, and no association with clinicopathological and outcome parameters was found [34].
In the present study, we aimed to evaluate, in 76 patients with high risk of progression UBC, the pattern of expression, and the clinical and prognostic significance of p-mTOR, assessed by immunohistochemistry. Angiogenesis and lymphangiogenesis occurrence was also evaluated by immunohistochemistry, in order to correlate blood vessel density (BVD) and lymphatic vessel density (LVD), with p-mTOR expression.

METHODS

- Patients and Tumour Samples

We retrospectively reviewed the records from patients who were clinically diagnosed with high risk of progression UBC (high grade NMI and MI tumours) and treated by RC and limited lymphadenectomy at the Portuguese Institute of Oncology, Porto, from January 1996 to December 2005. Prior approval was obtained from the Ethics Committee of the institution. During this period, 223 RCs were performed. After considering some exclusion criteria, namely the diagnosis of urothelial carcinomas with variant histology, squamous cell or adenocarcinomas, prior radiation, neoadjuvant or adjuvant chemotherapy treatments, insufficient follow-up time and/or tumour samples inadequate for further study (e.g. samples without adjacent non-tumour urothelium), a final cohort of 76 patients were eligible for the study. Each cystectomy specimen was examined following the guidelines of the College of American Pathologists [35]. Two independent pathologists reviewed hematoxylin-eosin (H&E)-stained sections according to standard histo-

pathological examination, considering the American Joint Committee on Cancer [36] and the World Health Organization – WHO (WHO 1999 and WHO 2004) [37-38] classification systems. Table 1 summarizes the clinicopathological parameters.

Sixty-one patients had RC as their first treatment, while the NMI tumours (15, 20%) had had previous therapeutic transurethral resection and BCG instillation; when disease recurrence occurred, or when multiple carcinoma *in situ* (CIS) lesions were observed in the surgical specimen, these patients were then treated by RC. Mean and median follow-up were 35 and 20 months (range 1–132), respectively. Recurrence, disease-free survival (DFS) and overall survival (OS) rates were defined as the reappearance of UBC (loco-regional metastasis or distant metastasis) more than 3 months after RC, the time from RC until recurrence, and the time from RC until death by cancer or the last clinical assessment, respectively.

- Immunohistochemistry and Evaluation of Staining

Immunohistochemical staining to detect p-mTOR was performed on paraffin-embedded 4 μm UBC tissue sections according to the two-step peroxidase conjugated polymer technique (EnVision™+ System, HRP, Dako), following the manufacturer's instructions. The primary antibody [phospho-mTOR (Ser2448), Cell Signalling Technology®] was used in a 1:500 dilution, and incubated on the sections overnight at 4°C. Negative controls were carried out by omitting the primary antibody. A breast tumour with known immunorreactivity for p-mTOR was used as a positive control. Blood and lymphatic endothelial cells were immunohistochemically stained by anti-CD31 and anti-D2-40 (Dakocytomation) antibodies, as previously described [39].

The immunostained sections were examined by light microscopy by two independent observers who had no knowledge of the clinical status; discordant cases were discussed together in a double-headed microscope. p-mTOR expression was semiquantitatively assessed at x200 magnification, considering the cytoplasmic staining of tumour and adjacent, non-tumour urothelial cells. The following grading system was used: negative (-), expression in less than 10% of cells; and positive (+) expression in over 10% of cells. CD31 and D2-40 immunohistochemical positive reactions were assessed as previously described, in order to quantify overall BVD and LVD (peritumoural and intratumoural) [39].

Table 1. Clinicopathological parameters

Gender	Male	63
	Female	13
Age	Median (range)	71 (41-83)
Stage	pT1 and pTis	15
	pT2	12
	pT3 and pT4	49
Grade	II	19
	III	57
Morphological type of lesion	Non-muscle invasive papillary	11
	In situ	4
	Muscle-invasive	61
Lymphovascular invasion	Yes	37
	No	39
Loco-regional dissemination	Yes	22
	No	54
Recurrence	Yes	57
	No	19
Clinical Outcome	Dead, bladder cancer	53
	Alive, lost to follow-up, or dead, other causes	23

- Statistical Analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0. Associations between p-mTOR expression and the clinicopathological parameters were examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when n<5). For BVD and LVD analysis, data were expressed as the median, and this value was used as a cut-off point for statistical analysis. Five-year DFS and OS rates were evaluated using Kaplan-Meier curves, and differences were analysed by Log-Rank or Breslow tests. p values < 0.05 were considered significant.

RESULTS

- Prognostic Significance of the Clinicopathological Parameters

The 5-year DFS and OS rates were significantly lower in patients with tumours invading beyond the muscular layer, with grade III tumours, with occurrence of lymphovascular invasion or with the presence of regional metastases (Table 2). High vascular density did not have an impact on outcome. However, high LVD was predominant in pT3/pT4 (81%, 33/41, p=0.006), grade III (85%, 35/41, p=0.034) or muscle-invasive (37/41, 90%, p=0.033) tumours (data not shown).

		n	5-year DFS rate (%)	p*	5-year OS rate (%)	p*
Gender	Male	63	22.0	0.608	31.3	0.780
	Female	13	34.6		34.2	
Age	≤ 71 years	40	25.6	0.288	33.5	0.317
	> 71 years	36	22.4		30.3	
Tumour stage	pT1 and pTis	15	36.1	0.011	46.5	0.005
	pT2	12	27.8		45.8	
	pT3 and pT4	49	20.4		23.7	
Grade	II	19	45.5	0.007	61.2	0.001
	III	57	17.2		22.3	
Morphological type of lesion	Non-muscle invasive papillary	11	30.7	0.059	48.5	0.048
	In situ	4	50.0		50.0	
	Muscle-invasive	61	21.5		28.1	
Lymphovascular invasion	Negative	39	30.2	0.040	42.9	0.004
	Positive	37	18.9		21.0	
Loco-regional metastasis	Negative	54	28.8	0.043	41.1	0.001
	Positive	22	13.6		10.0	

* Log-Rank or Breslow tests.
Abbreviations: DFS, disease-free survival; OS, overall survival.

Table 2. Correlation between 5-year disease-free survival and overall survival rates, and clinicopathological variables

- Immunoexpression Pattern of p-mTOR

A total of 76 UBC samples with representative tumour and non-tumour (normal-like or hyperplasic urothelium) sections were evaluated for p-mTOR immunoexpression. 20% (15/76) of the tumour sections were scored positive. Regarding NMI

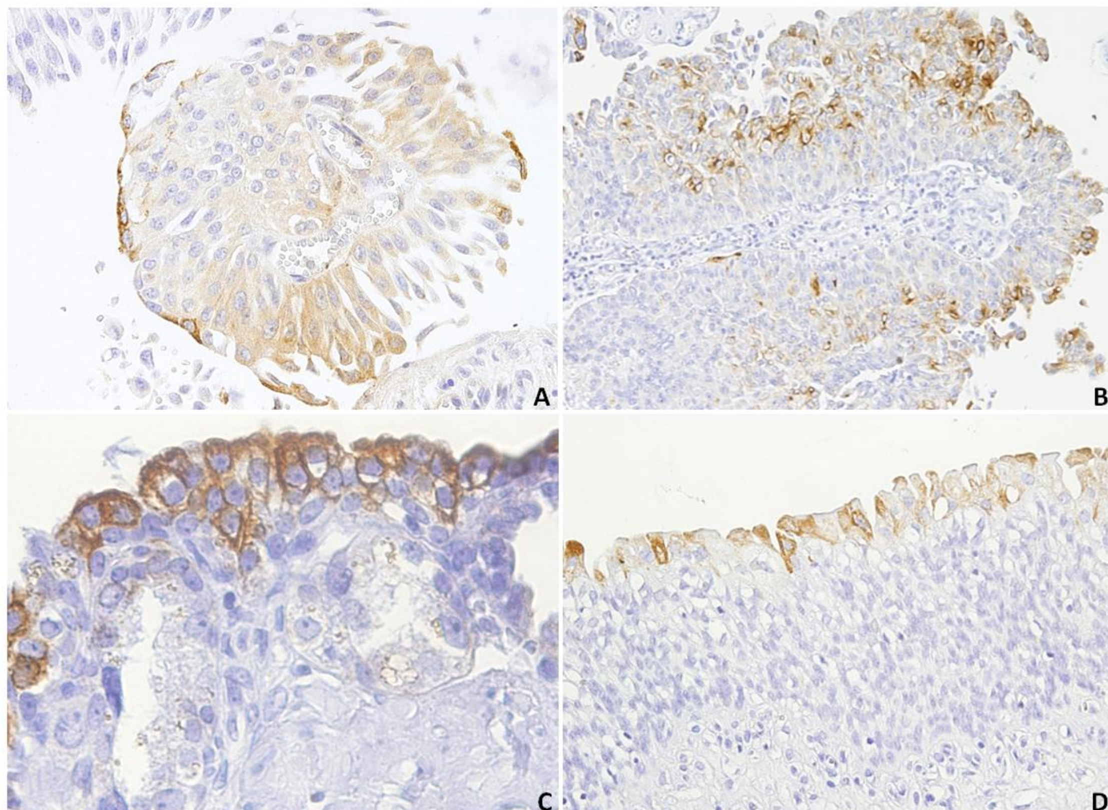

Figure 1: Immunohistochemical positive reactions for p-mTOR, showing different expression patterns in urothelial cells. Non-muscle invasive papillary tumours (x200 amplification) expressing cytoplasmic p-mTOR in near homogeneous **(A)** and heterogeneous **(B)** patterns, with the luminal and intermediate cell layers being more intensely stained than the layer of basal cells. Normal (x400 amplification) **(C)** and hyperplasic (x200 amplification) **(D)** urothelium exhibiting cytoplasmic p-mTOR immunoexpression restricted to the superficial layers.

papillary lesions, p-mTOR expression was frequently evenly distributed in the several layers of urothelial cells, although a more intense staining was noted in the superficial layers (Figure 1A). In some NMI cases, this superficial preponderance of p-mTOR was more evident (Figure 1B). MI positive cases were rare, and p-mTOR was only expressed in a few spots of cells. When non-tumour urothelium [with apparent normal histology (Figure 1C) or hyperplasic (Figure 1D)] samples were scored positive (36%, 27/76), p-mTOR expression was completely restricted to the superficial cell layers, namely the umbrella cells.

Table 3. Correlation between p-mTOR expression status in tumour sections and clinicopathological variables

			p-mTOR expression		
		n	Negative (%)	Positive (%)	p^a
Gender	Male	63	48 (76.2)	15 (23.8)	0.060
	Female	13	13 (100)	0 (0.0)	
Age	≤ 71 years	40	31 (77.5)	9 (22.5)	0.534
	> 71 years	36	30 (83.3)	6 (16.7)	
Stage	Group 1	15	9 (60.0)	6 (40.0)	0.087
	Group 2	12	10 (83.3)	2 (16.7)	
	Group 3	49	42 (85.7)	7 (14.3)	
Grade	II	19	14 (73.7)	5 (26.3)	0.507
	III	57	47 (82.5)	10 (17.5)	
Morphological type of lesion	Non-muscle invasive papillary	11	7 (63.6)	4 (36.4)	0.075
	In situ	4	2 (50.0)	2 (50.0)	
	Muscle-invasive	61	52 (85.2)	9 (14.8)	
Lymphovascular invasion	Negative	39	29 (74.4)	10 (25.6)	0.252
	Positive	37	32 (86.5)	5 (13.5)	
Loco-regional dissemination	Negative	54	42 (77.8)	12 (22,2)	0.532
	Positive	22	19 (86.4)	3 (13.6)	
Median BVD (CD31 stain)	< 17.6	40	33 (82.5)	7 (17.5)	0.774
	≥ 17.6	36	28 (77.8)	8 (22.2)	
Median LVD (D2-40 stain)	< 8.8	35	26 (74.3)	9 (25.7)	0.259
	≥ 8.8	41	35 (85.4)	6 (14.6)	

^{a} χ^2 or Fisher exact tests.
Abbreviations: BVD, blood vessel density; LVD, lymphatic vessel density.

- Clinical and Prognostic Significance of p-mTOR Immunoexpression

P-mTOR expression decreased with increasing stage: 40% (6/15) of pT1 and pTis tumours were positively stained, while only 14.3% (7/49) of pT3/pT4 expressed p-mTOR (p=0.087) (Table 3). Similar correlations were found when considering the morphological type of lesion (p=0.075) (Table 3). When comparing positive tumour and non-tumour sections, concordance among p-mTOR expression was lost with enhanced tumour aggressiveness: 17 pT3/pT4 cases presented positive normal-like mucous regions adjacent to the tumour sections, but p-mTOR expression was only observed in 6 (35.3%) of those cases (p=0.005, data not shown). Angiogenesis and lymphangiogenesis occurrence did not correlate with overall p-mTOR expression results. Even so, in the group of low blood vessel density count, 65% (26/40) of the cases did not express p-mTOR both in the tumour and non-tumour sections (p=0.003, data not shown). No significant associations were found regarding p-mTOR status and survival rates. However, when selecting the group of patients with pT3/pT4 tumours, those with negative expression had a median 5-year OS of 15.7 months (95% CI 6.757-24.643), which was reduced to 3.5 months (95% CI 1.000-8.514) if the tumours were p-mTOR positive, although the differences were not statistically significant (Figure 2A). Accordingly, 5-year DFS was reduced from 8.7 months (95% CI 3.974-13.359) in the negative cases to 1.8 months (95% CI 1.030-2.570) in the positive cases (p=0.004, Figure 2B).

DISCUSSION

The interplay between both mTOR complexes and the PI3K/Akt signalling pathway justifies the consistent upregulation of the mTOR network in cancer. Activating mutations in the mTOR gene have been identified in a few malignancies, although not clearly linked to tumour development [40]. Conversely, upstream components of the mTOR pathway are frequently altered in human tumours [8, 17], and UBC is not an exception, with reported mutations of *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), *AKT1* and *TSC1* (tuberous sclerosis protein 1, hamartin), and loss of heterozygosity, homozygous deletion and inactivating mutations of *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) [41-42]. These observations strongly suggest that mTOR signalling may be activated in bladder tumours. In accordance with this hypothesis, mTOR inhibition via rapamycin or rapamycin analogs reduced proliferation in *in vitro* and *in vivo* UBC models, with correspondent

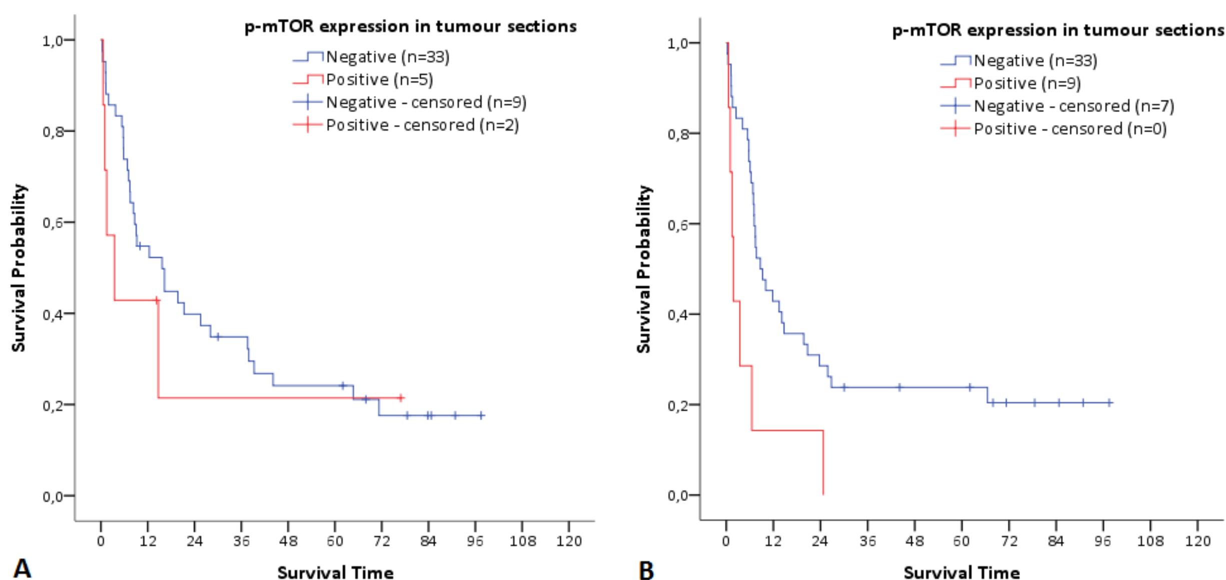

Figure 2: Kaplan-Meier curves demonstrating 5-year overall survival (A) (p>0.05) and 5-year disease-free survival (B) (p=0.004) based on p-mTOR immunoexpression status in pT3/pT4 urothelial bladder tumour sections (n=49).

diminished p-S6 levels [33-34, 43]. Importantly, treatment with mTOR inhibitors enhanced the therapeutic efficacy of cisplatin and gemcitabine in bladder cancer cell lines [34, 44-45], and impaired tumour progression when administered intravesically in a bladder cancer mouse model [46]. In a phase II study of everolimus in patients with locally advanced or metastatic UBC, clinical activity was demonstrated, and the profile of plasma angiogenesis-related proteins suggested that everolimus exhibits antiangiogenic properties that play a significant role in disease control [47]. In spite of these promising results, little is known about the prevalence and clinical relevance of p-mTOR expression in UBC tissue. A better understanding on this subject could be important to appropriately identify UBC patients that can achieve benefits from the molecularly targeted therapies.

Phosphorylation of mTOR at Ser2448 is often used as indicative of its activity [17, 48]. In three studies using the same p-mTOR antibody (with slightly different protocols and quantification methods), the percentage of bladder tumour samples with activated mTOR ranged from 32% to 88% [33-34, 49]. While some authors identified p-mTOR upregulation as an important prognostic factor [33, 50], others found an overall downregulation of the mTOR pathway in UBC [51]. Comprehensive immunohistochemical and molecular approaches encompassing several mTOR upstream and downstream players are better suited for investigating the potential impact of this pathway in UBC patients. Even so, inconsistent results have been described. For instance, reports on p-Akt [49-50] and p-S6K/p-S6 [33, 49-50] upregulation in tumour *versus* non-tumour urothelium contradict those reporting p-Akt [51] and p-S6 [51-52] downregulation. A few studies demonstrated positive associations between mTOR pathway activation and the clinicopathological parameters of bladder tumours [50, 52], while others failed to do so [34] or even reported inverse associations [51]. One can argue that heterogeneity among patient selection criteria and relative proportions of differently staged and graded tumours, immunohistochemistry protocols or evaluation of staining methods may significantly contribute to the conflicting results described so far. However, the unique biological features that define bladder tumourigenesis and tumour progression, together with the intrinsic complexity of the PI3K/Akt/mTOR pathway, are probably the main actors of this puzzling scenario.

In our study, we only evaluated p-mTOR expression in a cohort of 76 urothelial bladder tumours, which constitutes a limitation, but together with markers of blood and lymphatic endothelium. Only 20% of the tumour samples were scored positive for p-mTOR expression; the adjacent non-tumour urothelium (apparently normal or hyperplasic) was immunostained in 36% of the tissue sections, although only the superficial layers, including umbrella cells, were stained. Regarding the malignant urothelium of NMI lesions, an evenly distributed pattern of expression was frequently observed, but the stronger intensity of staining at the superficial layers was maintained. P-mTOR expression decreased with increasing stage, and MI tumours were mainly negative; when

positive, only small clusters of cells were stained. Interestingly, normal-like mucosa of MI lesions preserved p-mTOR expression in a significant proportion of cases that had lost it in the tumour sections. No significant association was found between p-mTOR positivity and neovascularization. Nevertheless, when tumour and non-tumour sections were simultaneously negative, angiogenesis occurrence seemed to be compromised. In the group of pT3/pT4 tumours, p-mTOR expression associated with worse survival rates, although the differences were only significant for 5-year disease-free survival.

The pattern of p-mTOR immunoexpression that was observed in our UBC series has been similarly described in other studies [34, 50]. We may speculate that the restriction of p-mTOR expression to the superficial layers of the normal-like urothelium reflects the biological plasticity inherent to the epithelial cells, namely the umbrella cells. These cells exhibit unique structural and biochemical features that enable them to form an effective permeability barrier while supporting mechanical deformation due to bladder filling [53-54]. Probably, mTOR constitutive expression is necessary as a part of their normal metabolic activities. In fact, it has been described that mTORC1, besides being a master regulator of cell growth and proliferation in non-tumour and tumour conditions, additionally controls specific aspects of cellular metabolism through the induction of metabolic gene expression [55-57]. Moreover, and accordingly to our results, NMI tumours may extend and upregulate mTOR expression up to the basal urothelial layer, which is consistent with an enhanced malignant potential that will guide growth and progression of the primary tumour. Fahmy et al. have recently reported that activation of the mTOR pathway might be used as a predictor of recurrence among patients with high-risk NMI [58]. Interestingly, Pinto-Leite et al. [45], when studying the effect of everolimus, alone or in combination with gemcitabine treatment, in bladder cancer cell lines, observed a significant antiproliferative effect for everolimus in a NMI cell line, while a MI cell line demonstrated marked resistance. These results, together with the results from our study, suggest that interfering with the mTOR pathway may represent an appealing approach for therapeutic intervention in patients with non-muscle invasive tumours.

In the group of muscle-invasive tumours, occurrence of two p-mTOR phenotypes is intriguing. On one hand, positive pT3/pT4 tumours had worse outcome, which is in accordance with findings from several authors that reported mTOR pathway upregulation as an important prognostic factor [33, 50]. On the other hand, p-mTOR positivity was rare and restricted to cell spots, and in the majority of MI tumour sections, immunoexpression was lost in a *de novo* fashion, as supported by the maintenance of p-mTOR expression in the normal-like adjacent mucosa. Probably, unknown biological determinants are acting in the promotion of this unique malignant scenario. Schultz et al. [51] reported the apparent downregulation of the mTOR pathway, as demonstrated by the low expression levels of p-Akt and p-S6 in invasive UBC, when compared to benign urothelium, hypothesizing that the downregulation of p-S6 in MI-UBC could be related to the HIF-1α (hypoxia-inducible factor)-activating hypoxia-resistant microenvironment. Müller et al. [59], when comparing between normal and tumour prostate tissues, also found that p-mTOR expression was reduced in the tumour, correlating with adverse clinicopathological features. These and our results may reflect the occurrence of alternative mTOR signalling mechanisms that lie behind the classical PI3K/Akt activation pathway. Additional studies with larger and more comprehensive UBC series and panels of mTOR upstream and downstream effectors, together with reproducible immunohistochemical and molecular approaches, and with *in vivo* and *in vitro* bladder tumour models, are urgently needed to clarify the backstage of the mTOR pathway in human urothelial bladder cancer, in order to expedite the research on potential target therapeutic approaches.

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CHAPTER 5 |

Low RKIP expression associates with poor prognosis in bladder cancer patients

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ORIGINAL ARTICLE

Low RKIP expression associates with poor prognosis in bladder cancer patients

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Abstract Urothelial bladder cancer (UBC) is a heterogeneous type of disease. It is urgent to screen biomarkers of tumour aggressiveness in order to clarify the clinical behaviour and to personalize therapy in UBC patients. Raf kinase inhibitory protein (RKIP) is a metastasis suppressor, and its downregulation is associated with metastatic events in an increasing number of solid tumours. We evaluated the clinical and prognostic significance of RKIP expression in patients with high risk of progression UBC. Using immunohistochemistry, we determined RKIP expression levels in a series of 81 patients with high-grade pT1/pTis or muscle-invasive UBC. Staining of CD31 and D2-40 was used to assess blood and lymphatic vessels, in order to distinguish between blood and lymphatic vessel invasion (LVI). We found that 90 % of pT1/pTis tumours, 94 % of non-muscle invasive papillary tumours and 76 % of the cases without LVI occurrence expressed RKIP in >10 % of cells. In this group, we observed a subgroup of tumours (42 %) in which the tumour centre was significantly more intensely stained than the invasion front. This heterogeneous pattern was observed in 63 % of the cases with LVI. Low RKIP expression was associated with poorer 5-year disease-free and overall survival rates, and remained as an independent prognostic factor for disease-free survival. Loss of RKIP expression may be an important prognostic factor for patients with high risk of progression bladder cancer.

Keywords Bladder cancer · Biomarkers · RKIP · Lymphovascular invasion · Prognosis

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Introduction

Urothelial bladder carcinoma (UBC), the most frequent type (90 %) of bladder cancer, is the second most common malignancy of the urogenital region. The heterogeneity of UBC in terms of histopathology, clinical behaviour and response to treatment is the key problem in its management. At presentation, 70–80 % of tumours are non-muscle invasive (stages Ta, T1 and Tis). High-grade and Tis lesions frequently recur and progress to invasive forms. The remaining 20–30 % of UBC present as muscle-invasive disease (stages T2, T3 and T4) for which radical cystectomy (RC) with pelvic lymph node dissection is indicated. Such

neoplasms have a high risk of dissemination, underpinning the need for neoadjuvant and adjuvant therapy [1]. Cisplatin-containing combination chemotherapy is the standard treatment [2], but patients with the same disease stage unpredictably respond differently [3]. In order to clarify the clinical behaviour of UBC and to personalize therapy, new molecular markers of tumour aggressiveness need to be identified.

MAP kinase (RAS/RAF/MEK/ERK) is a highly preserved signalling pathway that, in response to extracellular stimuli, can influence cell growth, differentiation, migration and apoptosis [4]. Mutational activation of the MAP kinase pathway has been described in several cancer types [5]. This event is infrequent in bladder cancer [6–8]. However, Karlou *et al.* found that ERK is overexpressed in UBC along with a more aggressive phenotype [8]. More recently, Zaravinos et al. found that *B-RAF* mRNA levels are significantly increased in pT1 grade III bladder tumours [9].

Raf kinase inhibitory protein (RKIP; also known as phosphatidylethanolamine-binding protein or PEBP) is a widely expressed and highly conserved protein initially described as being implicated in physiological activities like reproduction and neurophysiology (reviewed in [10]). More recently, its role in cancer has been highlighted due to its ability to modulate several intracellular signalling cascades involved in cell differentiation [11], cell cycle kinetics [12, 13], apoptosis [14, 15], epithelial to mesenchymal transition (EMT) [16, 17] and cell migration [13, 18]. In its non-phosphorylated form, RKIP has been shown to negatively regulate the RAS/RAF/MEK/ERK pathway by inhibiting Raf-1 (also known as C-RAF); it also binds, although with weaker affinity, to MEK and ERK, interfering with downstream phosphorylation steps [19]. Besides inhibiting the MAP kinase pathway, RKIP inhibits nuclear factor Kappa B (NF-κB) [14, 20] and G-protein coupled receptor kinase-2 [21], and enhances glycogen synthase kinase-3β activity [22]. Moreover, it binds to centrosomal and kinetochore regions of prometaphase chromosomes, possibly regulating spindle checkpoint proteins [12, 13]. Given its pleiotropic abilities in maintaining cellular equilibrium, it is not surprising that downregulation of RKIP expression associates with metastatic events in an increasing number of solid tumours, namely in cancers of the prostate [23], breast [24], colorectal [25] and melanoma [26]. In bladder cancer, Zaravinos et al. detected low *RKIP* mRNA levels, compared with normal bladder tissue [9]. However, immunohistochemical assessment of expression of RKIP was not investigated as a potential toll for treatment decision making.

In the present study, we evaluated in 81 patients with high risk of progression UBC, the clinical and prognostic significance of RKIP expression, assessed by immunohistochemistry. Blood and lymphatic vessels were also stained by immunohistochemistry, in order to correlate lymphovascular

Table 1 Correlation between 5-year disease-free survival and overall survival rates, and clinicopathological variables

		n	5-year DFS rate (%)	*p*[a]	5-year OS rate (%)	*p*[a]
Gender	Male	66	24.0	ns	33.1	ns
	Female	15	44.0		43.6	
Age	≤71 years	42	26.8	ns	34.3	ns
	>71 years	39	28.9		36.1	
Stage	Group 1	20	42.4	0.001	50.3	0.001
	Group 2	12	37.0		55.0	
	Group 3	49	20.4		23.7	
Grade	II	25	50.4	0.001	62.1	0.001
	III	56	17.6		22.9	
Morphological type of lesion	Non-muscle-invasive papillary	16	40.4	0.015	51.6	0.014
	In situ	4	50.0		50.0	
	Muscle-invasive	61	23.2		30.0	
Lymphovascular invasion (H&E stain)	Negative	44	36.2	0.008	47.6	<0.001
	Positive	37	18.9		21.0	
Loco-regional metastasis	Negative	59	33.3	0.015	44.6	<0.001
	Positive	22	13.6		10.0	
Embolic BVI (CD31 stain)	Negative	70	30.9	0.002	39.3	0.001
	Positive	11	9.1		9.1	
LVI by isolated malignant cells (D2-40 stain)	Negative	50	36.2	0.011	42.0	0.018
	Positive	31	14.0		24.3	

DFS disease-free survival, *OS* overall survival, *ns* not significant, *BVI* blood vessels invasion, *LVI* lymphatic vessels invasion, *Group 1* pT1 and pTis stages, *Group 2* pT2 stages, *Group 3* pT3 and pT4 stages

[a] Log-rank or Breslow tests

invasion, which has been previously reported as a prognostic factor for bladder cancer patients [27], with RKIP expression.

Methods

Patients and tumour samples

We retrospectively studied the records from patients who were diagnosed with high-grade UBC and treated by RC and limited lymphadenectomy at the Portuguese Institute of Oncology, Porto from January 1996 to December 2005. Prior approval was obtained from the Ethics Committee of the institution. During this period, 223 RCs were performed. Patients diagnosed with urothelial carcinomas with variant histology, squamous cell or adenocarcinomas, patients who received radiation, neoadjuvant or adjuvant chemotherapy and patients who had an insufficient follow-up time and/or whose tumour samples were inadequate for further study were excluded from the cohort. Thus, 81 patients were eligible for the study. The median age of the patients was 71 years (range, 41–83); sixty-six (81.5 %) were male and 15 (18.5 %) were female. Additionally, tissue sections of the urinary bladder were obtained from apparently normal areas of the bladder of eight autopsy patients without history of bladder cancer.

Each cystectomy specimen was examined according to the guidelines of the College of American Pathologists [28]. Haematoxylin–eosin (H&E)-stained sections were reviewed according to standard histopathological examination by two independent pathologists. The lesions were classified according to the American Joint Committee on Cancer [29] and to the World Health Organization (WHO 1999 and WHO 2004) [30, 31] classification systems: 16 (20 %) were non-muscle-invasive papillary tumours, 4 (5 %) were urothelial carcinomas

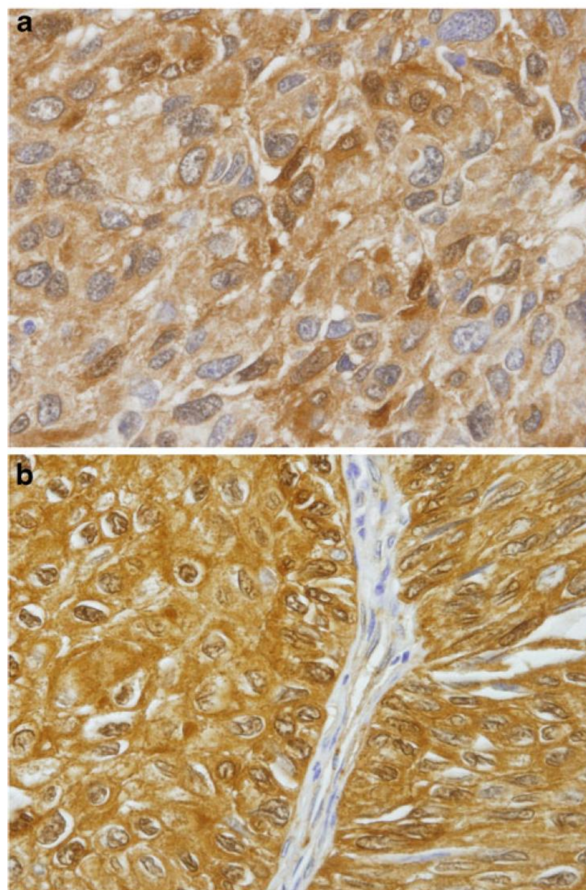

Fig. 1 Immunohistochemical positive reactions for RKIP, showing different expression areas in urothelial bladder carcinoma cells. **a** A muscle-invasive tumour exhibiting cytoplasmic expression (×400 amplification). **b** A non-muscle-invasive papillary tumour showing nuclear and cytoplasmic expression (×400 amplification)

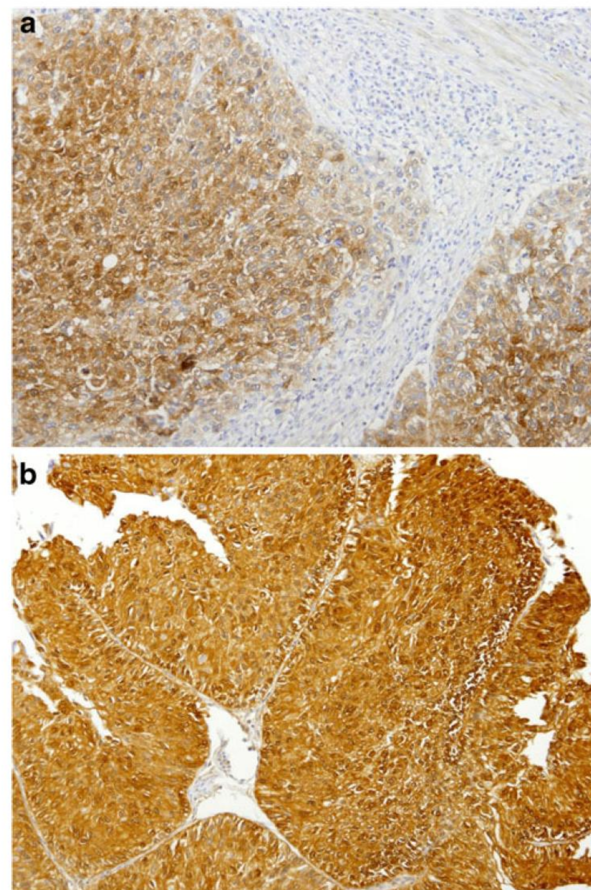

Fig. 2 Immunohistochemical positive reactions for RKIP, showing different patterns of expression in urothelial bladder carcinoma cells. **a** A muscle-invasive tumour exhibiting a heterogeneous pattern, with the centre of the tumour being more intensely stained than the invasion front (×100 amplification). **b** A non-muscle-invasive papillary tumour showing a homogeneous pattern of expression (×100 amplification)

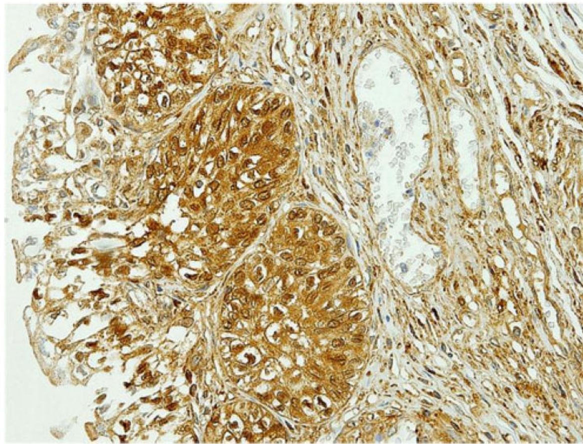

Fig. 3 Immunohistochemical positive reaction (nuclear and cytoplasmic) for RKIP in normal urothelium (×200 amplification)

in situ and 61 (75 %) were muscle-invasive UBCs; all tumours were high grade [31], 25 (31 %) were grade II and 56 (69 %) were grade III [30]. Regarding the clinical presentation of the tumours (T stage), three groups were composed for statistical analysis: group 1 [20 (24.7 %) high risk of progression non-muscle-invasive bladder tumours, including pT1 and pTis stages], group 2 [12 (14.8 %) pT2 tumours] and group 3 [49 (60.5 %) pT3 or pT4 tumours]. Lymphovascular invasion was identified in 37 (46 %) UBC samples.

Sixty-one patients had RC as their first treatment, while the non-muscle-invasive tumours (20, 25 %) had had previous therapeutic transurethral resection and BCG instillation; these patients were treated by RC following disease recurrence or when multiple carcinoma in situ lesions were observed in the surgical specimen. Twenty-two (27 %) patients presented loco-regional metastases at the time of RC. Mean and median follow-up were 38 and 24 months (range, 1–132), respectively. Recurrence [reappearance of UBC (loco-regional metastasis or distant metastasis) more than 3 months after RC] occurred in 61 (75 %) cases. Disease-free survival (DFS) was defined as the time from RC until recurrence. Overall survival (OS) was defined as the time from RC until death by cancer or the last clinical assessment.

Immunohistochemistry

Immunohistochemical staining was performed according to the streptavidin–biotin–peroxidase complex technique (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation) to detect RKIP, as previously described [32–34]. The primary antibody [anti-RKIP, Upstate (Millipore)] was used in a 1:1,000 dilution,

Table 2 Correlation between RKIP expression status and clinicopathological variables

		RKIP expression			
		n	≤10 % of cells (%)	>10 % of cells (%)	p^a
Gender	Male	66	21 (31.8)	45 (68.2)	ns
	Female	15	6 (40.0)	9 (60.0)	
Age	≤71 years	42	16 (38.1)	26 (61.9)	ns
	>71 years	39	11 (28.2)	28 (71.8)	
Stage	Group 1	20	2 (10.0)	18 (90.0)	0.032
	Group 2	12	4 (33.3)	8 (66.7)	
	Group 3	49	21 (42.9)	28 (57.1)	
Grade	II	25	5 (20.0)	20 (80.0)	ns
	III	56	22 (39.3)	34 (60.7)	
Morphological type of lesion	Non-muscle-invasive papillary	16	1 (6.2)	15 (93.8)	0.030
	In situ	4	1 (25.0)	3 (75.0)	
	Muscle-invasive	61	25 (41.0)	36 (59.0)	
Lymphovascular invasion (H&E stain)	Negative	44	12 (27.3)	32 (72.7)	ns
	Positive	37	15 (40.5)	22 (59.5)	
Loco-regional metastasis	Negative	59	20 (33.9)	39 (66.1)	ns
	Positive	22	7 (31.8)	15 (68.2)	
Embolic BVI (CD31 stain)	Negative	70	23 (32.9)	47 (67.1)	ns
	Positive	11	4 (36.4)	7 (63.6)	
LVI by isolated malignant cells (D2-40 stain)	Negative	50	12 (24.0)	38 (76.0)	0.030
	Positive	31	15 (48.4)	16 (56.6)	

[a] χ^2 or Fisher exact tests

ns not significant, *BVI* blood vessels invasion, *LVI* lymphatic vessels invasion, *Group 1* pT1 and pTis stages, *Group 2* pT2 stages, *Group 3* pT3 and pT4 stages

and incubated on the sections for 120 min at room temperature. Negative controls were carried out by omitting the primary antibody. A gastrointestinal stromal tumour with known immunoreactivity for RKIP was used as a positive control. To distinguish between invasion in blood and lymphatic vessels, these were immunohistochemically stained by anti-CD31 and anti-D2-40 (DakoCytomation) antibodies, as previously described [27].

Evaluation of staining

The immunostained sections were examined by light microscopy and digital images were captured using a digital camera. All sections were evaluated without knowledge of clinical status by two independent observers; discordant cases were discussed around a double-headed microscope in order to obtain a consensus classification.

RKIP expression was semiquantitatively assessed at ×200 magnification, considering nuclear and/or cytoplasmic staining of carcinoma cells. The following grading system was used: negative (−), absence of expression; slightly positive staining (+), expression in ≤10 % of cells; moderately positive (++), expression in >10 % up to 50 % of cells; strongly positive (+++), expression in >50 % of cells. In the positive cases (+, ++, +++), the localisation of the expression was taken onto account (cytoplasm, nucleus and cytoplasm). The moderately (++) and strongly positive (+++) cases were also assessed for staining intensity at the invasion front of the tumours and in the tumour centre, in order to establish whether the pattern of expression was homogeneous (equivalent intensity of staining in both regions) or heterogeneous (different intensity of staining in each region). Tumour sections were additionally examined for the occurrence of blood vessel invasion (BVI, highlighted by CD31 staining) and lymphatic vessel invasion (LVI, highlighted by D2-40 staining) by isolated malignant cells, as previously described [27].

Statistical analysis

Data were analysed using the Statistical Package for Social Sciences software for Windows, version 18.0. Associations between RKIP expression and the clinicopathological parameters were examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when $n<5$). Five-year DFS and OS rates were evaluated using Kaplan–Meier curves, and differences were analysed by log-rank or Breslow tests. p values <0.05 were considered significant. Variables that achieved statistical significance in the univariate analysis were entered in multivariate analysis using Cox proportional hazards analysis. The hazard ratios (HR) were estimated with their 95 % confidence intervals (95 % CI).

Results

Prognostic significance of clinicopathological parameters

The 5-year DFS and OS rates were significantly associated with T3/T4 pathological stage, grade III, muscle-invasive type of lesion, lymphovascular invasion (identified in H&E-

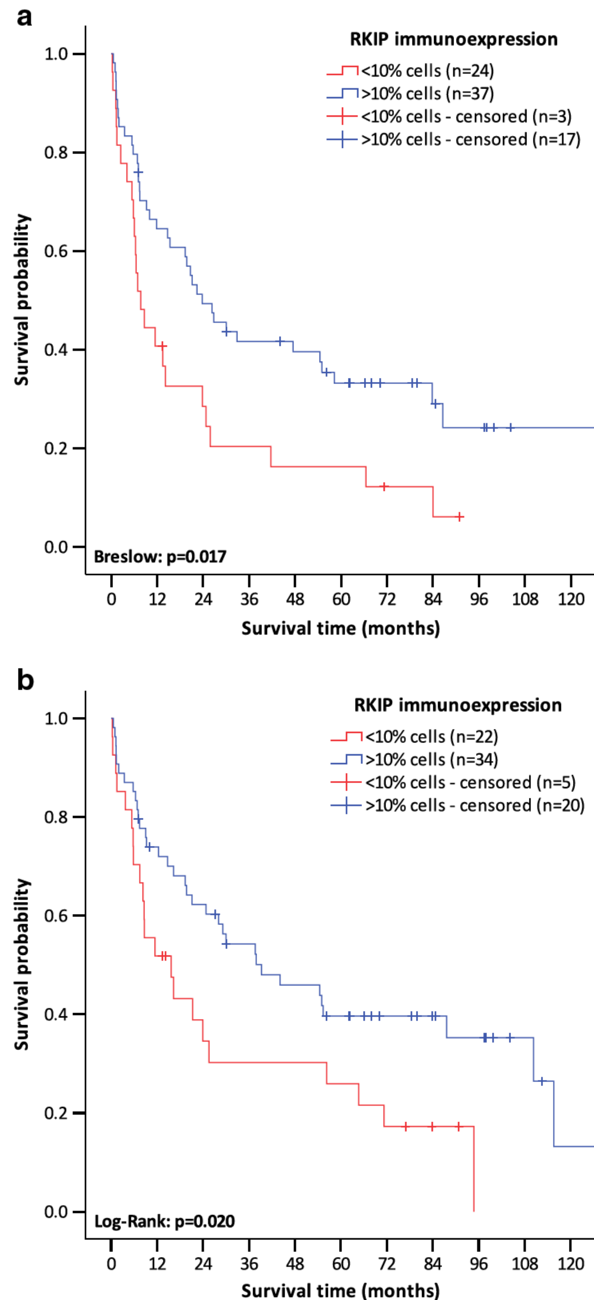

Fig. 4 Kaplan–Meier curves based on RKIP immunoexpression status (n=81). **a** Five-year disease-free survival. **b** Five-year overall survival

stained sections) and loco-regional dissemination (Table 1). Moreover, blood vessel invasion (highlighted by CD31 stain) and lymphatic vessels invasion by isolated malignant cells (highlighted by D2-40 stain) were also associated with worse prognosis (Table 1).

RKIP immunoexpression

According to the grading system used for assessment of RKIP expression, 14 (17 %) cases were negative, 13 (16 %) were slightly positive, 28 (35 %) were moderately positive and 26 (32 %) were strongly positive. The majority of the positive cases (42, 63 %) exhibited cytoplasmic expression (Fig. 1a); 25 (37 %) cases showed nuclear and cytoplasmic immunoreactivity (Fig. 1b). Two different patterns of expression were observed among the moderately and strongly positive cases: in 21 (39 %) cases, the centre of the tumour was significantly more intensely stained than the invasion front (heterogeneous pattern; Fig. 2a); the intensity of staining in the remaining cases (33, 61 %) was homogeneous (Fig. 2b). The normal urothelium showed moderate or strong RKIP expression (Fig. 3).

Clinical and prognostic significance of RKIP immunoexpression

Regarding RKIP expression levels, we compared group 1 (low or no expression) with group 2 (moderate and high expression). Group 2 tumours had more favourable clinico-pathological parameters: 90 % were pT1/pTis (p=0.032), 94 % were non-muscle-invasive papillary tumours (p=0.030) and 76 % were without LVI (p= 0.030; Table 2). In this group, RKIP expression was heterogeneous in 63 % of the cases with LVI occurrence (p=0.032). Low or no RKIP expression was associated with poorer 5-year DFS (p=0.017, Fig. 4a) and OS (p=0.020, Fig. 4b) rates. No statistical relevance was found when considering RKIP expression localisation (cytoplasm, nucleus and cytoplasm).

Multivariate analysis

In univariate analysis, T3/4 pathological stage, grade III, muscle-invasive histopathological type of lesion, occurrence of lymphovascular invasion, occurrence of loco-regional metastasis and low or no RKIP expression were significantly associated with poor 5-year DFS and OS rates. In multivariate analysis, grade III persisted as an independent prognostic factor for DFS (HR, 3.492; 95 % CI, 1.206–10.108, p=0.021) and OS (HR, 3.971; 95 % CI, 1.162–13.567, p=0.028); low or no RKIP expression remained as an independent prognostic factor for DFS (HR, 0.525; 95 % CI, 0.295–0.932, p=0.028), and occurrence of loco-regional metastasis remained as an independent prognostic factor for OS (HR, 2.151; 95 % CI, 1.071–4.319, p=0.031; Table 3).

Table 3 Multivariate survival-time regression for predictors of 5-year disease-free survival and overall survival after radical cystectomy for urothelial bladder cancer

		5-year DFS rate			5-year OS rate		
		HR	95 % CI	p	HR	95 % CI	p
Stage	Group 1	1.000	–	–	1.000	–	–
	Group 2	0.373	0.111–1.250	ns	0.335	0.084–1.331	ns
	Group 3	0.336	0.084–1.349	ns	0.290	0.058–1.451	ns
Grade	II	1.000	–	–	1.000	–	–
	III	3.492	1.206–10.108	0.021	3.971	1.162–13.567	0.028
Lymphovascular invasion (H&E stain)	Negative	1.000	–	–	1.000	–	–
	Positive	1.266	0.671–2.390	ns	1.763	0.898–3.464	ns
Loco-regional metastasis	Negative	1.000	–	–	1.000	–	–
	Positive	1.500	0.774–2.907	ns	2.151	1.071–4.319	0.031
Embolic BVI (CD31 stain)	Negative	1.000	–	–	1.000	–	–
	Positive	1.777	0.844–3.743	ns	1.544	0.736–3.238	ns
LVI by isolated malignant cells (D2-40 stain)	Negative	1.000	–	–	1.000	–	–
	Positive	1.298	0.710–2.375	ns	1.158	0.616–2.179	ns
RKIP expression	≤10 % of cells	1.000	–	–	1.000	–	–
	>10 % of cells	0.525	0.295–0.932	0.028	0.553	0.299–1.024	ns

DFS disease-free survival, *OS* overall survival, *HR* hazard ratios, *CI* confidence interval, *ns* not significant, *BVI* blood vessels invasion, *LVI* lymphatic vessels invasion, *Group 1* pT1 and pTis stages, *Group 2* pT2 stages, *Group 3* pT3 and pT4 stages

Discussion

The mechanisms by which RKIP acts as a metastasis suppressor are not fully understood. It has been suggested that RKIP expression might inhibit metastasis by decreasing angiogenesis and vascular invasion [23]. Recent reports have proposed that RKIP inhibits the migration and invasion abilities of malignant cells by regulating the extracellular matrix [35–39]. Beshir et al. suggested that the role of RKIP in inhibiting malignant dissemination might be associated with its ability to negatively regulate expression of specific matrix metalloproteinases (MMP), particularly MMP-1 and MMP-2 [38]. Beach et al. showed that the expression of RKIP inversely correlates with the expression of Snail, a key modulator of the normal and neoplastic EMT program [39]. This zinc-transcriptional repressor is induced by the chromatin remodelling factor high mobility group A (HMGA2) [40], which is negatively regulated by the *let-7/miR-98* family of microRNAs [41]. Dangi-Garimella et al. demonstrated that RKIP inhibits breast tumour metastasis in part via *let-7* [42]. The same group described an RKIP pathway metastasis signature involving *let-7* targets (HMGA2, BACH1) that, in turn, upregulate bone metastasis genes (*MMP1*, *OPN*, *CXCR4*) [35, 43]. These studies indicate that the biological function of RKIP in cancer can only be elucidated when its interactions with multiple signalling pathways are simultaneously addressed, which might be attained through medium- to high-throughput gene-expression profiling technologies probing into molecular alterations responsible for metastasis.

We only studied RKIP expression in bladder cancer tissue by immunohistochemistry, which represents a limitation, but together with markers for blood and lymphatic endothelium. We found low RKIP expression to be associated with significantly poorer 5-year DFS and OS rates which remained significant by multivariate analysis as an independent prognostic factor for DFS. Our results are in accordance with previous published data from Zaravinos et al., who reported low *RKIP* mRNA levels in comparison with normal bladder tissue [9]. Moreover, other authors demonstrated that RKIP depletion associates with metastatic events [23–26, 44–48] and is an independent prognostic marker [25, 34, 49–55] for several malignancies.

An important role for RKIP in cancer could be in modulating sensitivity of malignant cells for chemo- and radiotherapy. By inhibiting MAP kinase [19] and NF-κB [14, 20] signalling pathways, RKIP expression may potentiate apoptosis induced by chemotherapeutic agents. This has been demonstrated both in vitro [17, 56] and in vivo [57, 58], and could be useful in defining therapy response profiles. Some attempts have been made with drug-induced strategies of RKIP expression modulation, using the histone deacetylase inhibitor Trichostatin A [39] since RKIP promoter methylation has been proposed as a possible RKIP silencing event [59–61]. Additional pathways have been explored as possible targets for personalized therapeutic intervention in RKIP depleted cancers [62].

In summary, we show that loss of RKIP expression is associated with progression in bladder cancer, and is an independent prognostic factor for DFS. Additional studies with larger and more comprehensive series, including patients that undergo radical cystectomy with adequate lymphadenectomy, and with in vivo and in vitro bladder tumour models, are urgently needed to clarify the role of RKIP as metastasis suppressor in bladder cancer.

Conflict of interest The authors declare that they have no conflict of interest.

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CHAPTER 6 |

CD147 overexpression allows an accurate discrimination of bladder cancer patients' prognosis

The results presented in this chapter were:

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CD147 overexpression allows an accurate discrimination of bladder cancer patients' prognosis

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Abstract

Background: Urothelial bladder carcinoma (UBC) is a chemo-sensitive tumour, but the response to treatment is heterogeneous. CD147 has been associated with chemotherapy resistance. We aimed to define tumours with an aggressive phenotype by the combined analysis of clinicopathological and biological parameters.
Methods: 77 patients with T1G3 or muscle-invasive UBC treated by radical cystectomy were studied. Immunohistochemistry was performed to detect CD147, heparanase, CD31 (blood vessels identification) and D2-40 (lymphatic vessels identification) expressions. The immunohistochemical reactions were correlated with the clinicopathological and the outcome parameters. 5-year disease-free survival (DFS) and overall survival (OS) rates were estimated using the Kaplan–Meier method. Multivariate analysis was performed by Cox proportional hazards analysis.
Results: The 5-year DFS and OS rates were significantly influenced by the classical clinicopathological parameters, and by the occurrence of lymphovascular invasion. CD147 and heparanase immunoexpression did not affect patients' outcome. However, patients with pT3/pT4 tumours had a median OS time of 14.7 months (95% CI 7.1–22.3, $p = 0.003$), which was reduced to 9.2 months (95% CI 1.5–17.0, $p = 0.008$) if the tumours were CD147 positive. We developed a model of tumour aggressiveness using parameters as stage, grade, lymphovascular invasion and CD147 immunoexpression, which separated a low aggressiveness from a high aggressiveness group, remaining as an independent prognostic factor of DFS (HR 3.746; 95% CI 1.244–11.285; $p = 0.019$) and OS (HR 3.247; 95% CI 1.015–10.388, $p = 0.047$).
Conclusion: CD147 overexpression, included in a model of UBC aggressiveness, may help surgeons to identify patients who could benefit from a personalized therapeutic regimen. Additional validation is needed.
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Keywords: Bladder cancer; CD147; Heparanase; Lymphovascular invasion; Scoring system

Introduction

Bladder cancer is the second most common tumour of the urogenital tract; urothelial bladder carcinoma (UBC) comprises about 90% of all primary bladder malignancies.[1,2] The debate about the best treatment approach for T1G3 and advanced urothelial carcinoma continually challenges all urologic surgeons and oncologists.[3,4] Chemoresponsiveness of UBC to several drugs has been proved.[5] However, adjuvant systemic chemotherapy does not reveal benefits[6] and neoadjuvant chemotherapy is not yet accepted as the best approach in invasive bladder cancer.[7,8] The

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treatment of UBC may be improved if we understand the molecular events that occur in tumour progression, identifying potential targets to, ultimately, achieve "personalized therapy". In this line of investigation, Takata and colleagues[9] observed that *SLC16A3* (solute carrier family 16 – monocarboxylic acid transporter 4 – MCT4, member 3) is upregulated in patients that do not respond to neoadjuvant M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) therapy. SLC16A3 (MCT4) is closely associated with CD147,[10] a highly glycosylated cell surface transmembrane protein which stimulates matrix metalloproteinases synthesis and angiogenesis in tumour local environment.[11] CD147 seems to be related with cisplatin resistance of bladder cancer.[12] Overexpression of CD147 in patients with bladder cancer associates with poor prognosis.[13,14]

Heparanase is an endoglycosidase that can selectively degrade heparan sulphate glycosaminoglycans and has been shown to play a role in tumour angiogenesis and metastasis.[15] Previous studies have demonstrated that overexpression of heparanase in human tumours, including bladder cancer, facilitates their invasive activity[16]; heparanase and VEGF-C (vascular endothelial growth factor-C, a lymphatic molecular player) co-expression is related with the occurrence of lymphangiogenesis.[17]

Angiogenesis and lymphangiogenesis are essential for tumour progression and metastasis, by promoting oxygenation and fluid drainage, and establishing potential routes of dissemination.[18] Lymphovascular invasion has been suggested as a prognostic factor in several malignancies, including bladder cancer.[19–21]

In order to define tumours with an aggressive phenotype, we evaluated the expression of CD147, heparanase, and lymphovascular invasion in 77 UBC patients admitted in our Institution and treated by radical cystectomy (RC).

Materials and methods

Patients and tumour samples

Data from patients who were clinically diagnosed with high risk of progression non-muscle invasive, cT2 and cT3 (M0) bladder tumours, and treated by RC at the Portuguese Institute of Oncology, Porto, from January 1996 to December 2005, were reviewed retrospectively. During this period, 223 RCs were performed in our institution. For our study we excluded patients diagnosed with squamous cell or adenocarcinomas, patients who received radiation, neoadjuvant or adjuvant chemotherapy, and patients who had an insufficient follow-up time and/or whose tumour samples were inadequate for preparation purposes. Thus, the definitive analysis was based on 77 patients. Each cystectomy specimen was examined according to the College of American Pathologists.[22] Haematoxylin-eosin (H&E)-stained sections were reviewed according to standard histopathological examination by two independent pathologists. Staging and grading were conducted according to the American Joint Committee on Cancer[23] and to the World Health Organization[24] classification systems, respectively. For statistical analysis, tumours were divided into three groups based on T stage: group 1 (high risk of progression non-muscle invasive bladder tumours, including pT1 and pTis stages), group 2 (pT2 a and b) and group 3 (pT3 and pT4). Table 1 summarizes the clinicopathological parameters.

Sixty-one patients had RC as their first treatment, while the non-muscle invasive tumours ($n = 16$) had previous therapeutic TUR and BCG; these patients were treated by RC following disease progression. Mean and median follow-up were 35.5 and 21.1 months (range 1–132), respectively. During this period, seven cases were lost to follow-up. Recurrence was defined as the reappearance of UBC (loco-regional dissemination or distant metastasis) more than 3 months after RC. Disease-free survival (DFS) was defined as the time from the RC until the recurrence. Overall survival (OS) was defined as the time from the RC until death by cancer or the last clinical assessment.

Tumour samples were analysed for CD147 and heparanase expression, and for occurrence of embolic blood vessel (highlighted by CD31 staining) invasion and lymphatic vessel (highlighted by D2-40 staining) invasion by isolated malignant cells, as previously described.[21] All immunohistochemical reactions were correlated with the clinicopathological parameters and the outcome variables (5-year DFS and OS).

Immunohistochemistry

Immunohistochemical staining was carried out with the streptavidin-biotin-peroxidase complex technique to detect CD147, heparanase and CD31, as previously described for CD31 expression analysis,[21] and with the avidin-biotin-peroxidase complex assay to detect D2-40, as previously

Table 1
Clinicopathological parameters.

Gender	Male	64
	Female	13
Age	Median (range)	71 (41–83)
Tumour stage	Group 1	16
	Group 2	12
	Group 3	49
Grade	II	20
	III	57
Morphological type of lesion	Non-invasive papillary	12
	In situ	4
	Infiltrating	61
Lymphovascular invasion (H&E stain)	Yes	37
	No	40
Loco-regional dissemination	Yes	22
	No	55
Recurrence	Yes	58
	No	19
Clinical Outcome	Dead, bladder cancer	54
	Alive, lost to follow-up, or dead, other causes	23

described.[21] The primary antibodies were obtained from Zymed® Laboratories (CD147), Santa Cruz Biotechnology® (heparanase) and DakoCytomation® (CD31 and D2-40). These were used in 1:500 dilution (CD147), 1:100 dilution (CD31 and D2-40) and 1:75 dilution (heparanase), and incubated on the sections for 120 min (CD147) or 60 min (heparanase and CD31) at room temperature, or overnight (D2-40) at 4 °C.

Negative controls were carried out by omitting the primary antibodies. Sections of positive controls were used as indicated by the manufacturers (invase ductal breast carcinoma for CD147 and CD31 detection, gastric mucosa for heparanase detection and tonsil for D2-40 detection).

Evaluation of staining

The immunostained sections were examined by light microscopy and all sections were evaluated without knowledge of clinical status by two independent observers (T.A. and A.L.-F.).

The positive expressions of CD147 and heparanase were semi-quantitatively assessed using ×200 amplification, considering membrane and cytoplasmic staining of urothelial malignant cells. The positive reactions were assessed in hotspot areas were urothelial malignant cells were present and stained. For each case, 10 fields with at least 100 malignant cells each were evaluated. The following grading system was used for CD147 assessment: negative (−), expression in less than 5% of cells; and positive (+) expression in over 5% of cells. For heparanase detection, samples were scored as negative (−), expression in less than 50% of cells; and positive (+), expression in over 50% of cells.

The occurrence of BVI and LVI was evaluated based on CD31 (blood endothelial cells marker) and D2-40 (lymphatic endothelial cells marker) positive vessels' assessment, as previously described.[21] We only considered BVI by emboli of well-characterized malignant cells surrounded by endothelial cells highlighted by specific positive immunohistochemical expression for CD31; LVI was considered when at least one well-characterized malignant cell was surrounded by endothelial cells highlighted by specific positive immunohistochemical expression for D2-40.

Statistical analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) software, version 16.0. The relationship between the immunohistochemical markers expression and the clinicopathological parameters was examined for statistical significance using Pearson's chi-square (χ^2) test and

Table 2
Correlation between 5-year disease-free survival and overall survival rates, and clinicopathological variables, biological parameters and tumour aggressiveness scoring system.

		n	5-year DFS rate	*p*[a]	5-year OS rate	*p*[a]
Gender	Male	64	21.5%	ns	30.7%	ns
	Female	13	34.6%		34.2%	
Age	≤71 years	41	27.0%	ns	35.5%	ns
	>71 years	36	20.6%		30.3%	
Stage	Group 1	16	33.7%	0.008	43.0%	0.004
	Group 2	12	27.8%		45.8%	
	Group 3	49	20.4%		23.7%	
Grade	II	20	42.7%	0.006	57.6%	0.001
	III	57	17.2%		22.3%	
Morphological type of lesion	Non-invasive papillary	12	27.8%	0.046	42.8%	0.039
	In situ	4	50.0%		50.0%	
	Infiltrating	61	21.5%		28.1%	
Lymphovascular invasion	Negative	40	29.4%	0.031	41.5%	0.003
(H&E stain)	Positive	37	18.9%		21.0%	
Loco-regional dissemination	Negative	55	28.1%	0.042	40.1%	0.001
	Positive	22	13.6%		10.0%	
CD147 expression	Negative	18	22.2%	ns	23.9%	ns
	Positive	59	24.3%		34.2%	
Heparanase expression	Negative	42	20.0%	ns	34.4%	ns
	Positive	35	28.6%		28.6%	
Blood vessel invasion (CD31 stain)	Negative	66	26.5%	0.004	35.2%	0.002
	Positive	11	9.1%		9.1%	
Lymphatic vessel invasion	Negative	46	30.3%	0.038	36.2%	0.044
(D2-40 stain)	Positive	31	14.0%		24.3%	
Tumour	0–2 positive parameters	30	43.3%	<0.001	54.8%	<0.001
Aggressiveness Scoring System[b]	3–5 positive parameters	47	11.9%		17.0%	

DFS- disease-free survival, OS- overall survival, ns- not significant.
[a] Log-Rank or Breslow tests.
[b] Includes T3/T4 pathologic stage, grade III, occurrence of blood vessel invasion by malignant emboli, occurrence of lymphatic vessel invasion by isolated malignant cells and CD147 immunoexpression.

Fisher's exact test (when $n < 5$). The Mann–Whitney test was used for continuous variables. 5-year DFS and OS were evaluated using Kaplan–Meier curves and differences were analysed by Log-Rank or Breslow tests. Variables that achieved statistical significance ($p < 0.05$) in the univariate analysis were entered in a multivariate analysis using Cox proportional hazards analysis. The hazard ratios (HR) were estimated with their 95% confidence intervals (95% CI).

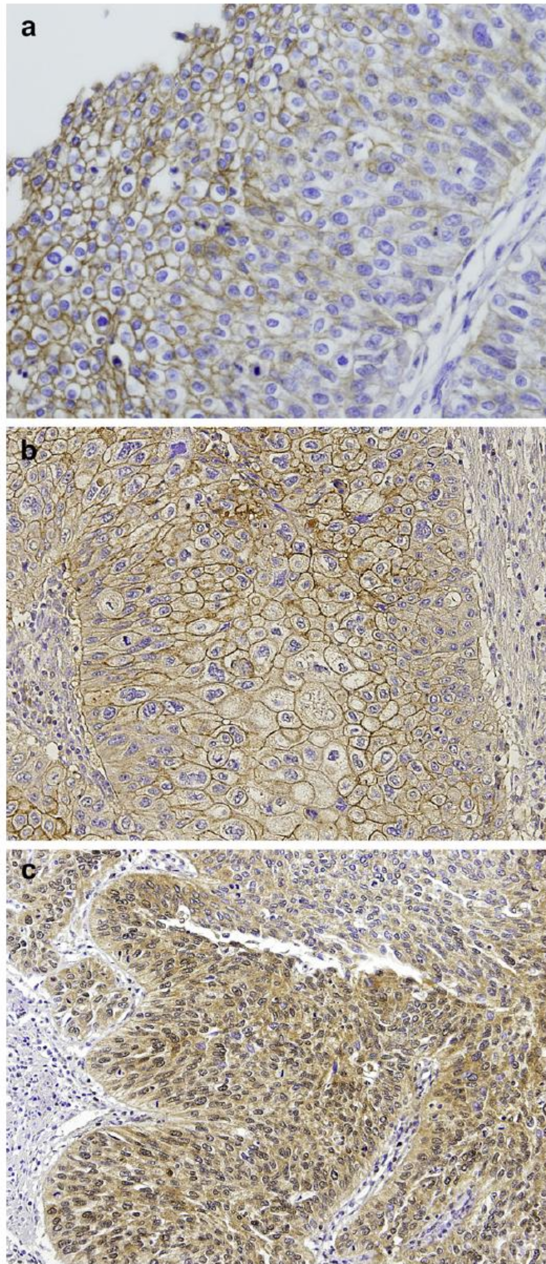

Figure 1. Immunohistochemical positive reactions for CD147 [(a), ×200 amplification; (b), ×100 amplification] and heparanase [(c), ×100 amplification] in urothelial bladder carcinoma cells. (a) A non-invasive case showing superficial malignant cells positive for CD147; (b) an invasive case showing the cytoplasmic membranes of the inner layers of malignant cells stained for CD147; (c) an invasive case showing the strong immunoreaction for heparanase in the invasive front of the tumour.

Results

Prognostic significance of clinicopathological parameters

Table 2 shows the prognostic significance of the clinicopathological parameters. T3/T4 pathologic stage, grade III, infiltrating type of lesion and occurrence of lymphovascular invasion and/or loco-regional dissemination lowered significantly the 5-year DFS and OS rates.

Clinical and prognostic significance of biological parameters

According to the grading system used for CD147 assessment, 18 cases were negative and 59 were positive. A different pattern of expression was observed between non-muscle invasive and invasive tumours (considering membrane staining) (Fig. 1). In the first group, the superficial malignant cells were preferentially stained; in the second group, the inner layers were stained. CD147 immunoexpression did not correlate with the clinicopathological parameters or patients' outcome (Table 2). However, it added predictive power of outcome to pathologic stage: patients with pT3/pT4 tumours had a median OS time of 14.7 months (95% CI 6.9–22.6, $p = 0.004$), which was reduced to 9.2 months (95% CI 1.5–17.0, $p = 0.008$) if the tumours were CD147 positive.

All cases showed some degree of heparanase immunoexpression (Fig. 1), although 42 were graded as negative. Normal urothelium was not stained. Positive cases ($n = 35$) exhibited a heterogeneous pattern, with the invasive front being significantly more intensely stained than the tumour core. No association was found between heparanase immunoexpression and clinicopathological parameters or patients' outcome (Table 2).

Although the occurrence of embolic BVI (11 cases) did not correlate with the clinicopathological parameters, it affected significantly patients' prognosis ($p = 0.004$ for 5-year DFS and $p = 0.002$ for 5-year OS) (Table 2). Occurrence of LVI by isolated malignant cells (31 cases) was significantly correlated with pT3/pT4 stage ($p = 0.008$) and infiltrating type of lesion ($p = 0.007$), and had a significant impact in outcome ($p = 0.038$ for 5-year DFS and $p = 0.044$ for 5-year OS) (Table 2).

Development of a tumour aggressiveness scoring system

We developed a scoring system of tumour aggressiveness as a categorical variable using clinicopathological (stage and grade) and molecular (BVI and LVI) factors closely

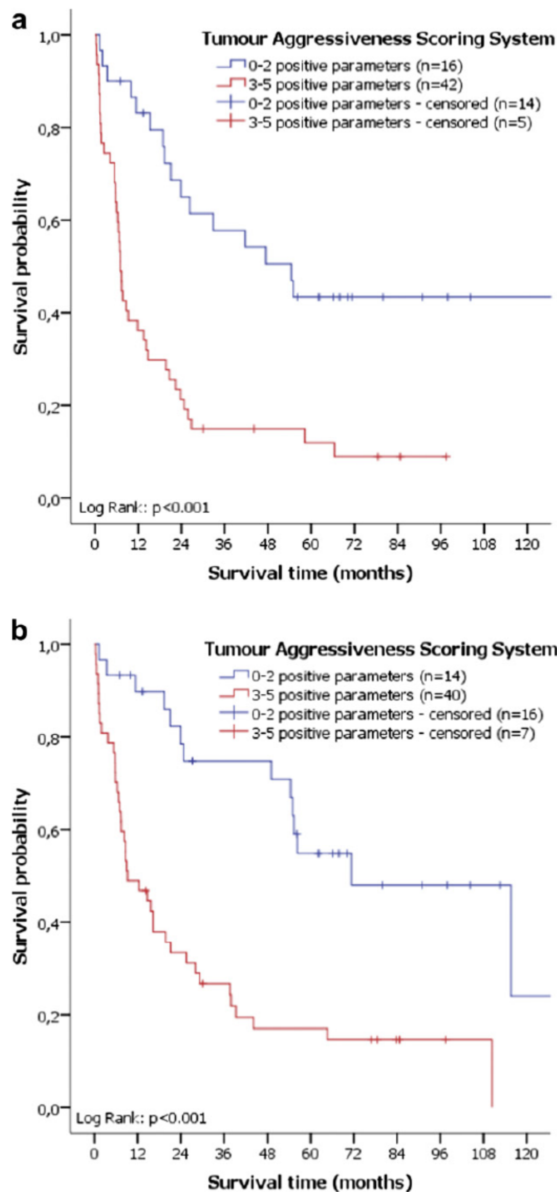

Figure 2. Kaplan–Meier curves demonstrating 5-year disease-free survival (a) and 5-year overall survival (b) based on the tumour aggressiveness scoring system (includes T3/T4 pathologic stage, grade III, occurrence of blood vessel invasion by malignant emboli, occurrence of lymphatic vessel invasion by isolated malignant cells and CD147 overexpression) ($n = 77$).

related with disease recurrence and tumour-specific death, as determined in the univariate analysis of 5-year DFS and OS. Additionally, we decided to include CD147 immunoexpression in the model, due to its known biological relevance as a prognostic factor probably associated with chemotherapy resistance,[12–14] and to the significant influence of the expression of this parameter in the OS of patients with pT3/pT4 tumours included in our series. For statistical analysis, we considered two groups: group 1 (low aggressiveness profile), in which cases with none, one or two of the above mentioned parameters were present; group 2 (high aggressiveness profile), which included cases with three to five positive parameters. The model revealed a significant association with 5-year DFS ($p < 0.001$) and OS rates ($p < 0.001$) (Table 2, Fig. 2). The rate of CD147 immunoexpression was significantly different between the low aggressiveness profile (60% of cases were CD147 positive) and the high aggressiveness profile (87.2% of cases were CD147 positive) ($p = 0.012$).

Multivariate analysis

In multivariate analysis, we included the parameters that significantly influenced the 5-year DFS and OS rates and that were entered in the tumour aggressiveness scoring system proposed above (tumour stage, grade and occurrence of BVI and/or LVI). The model of tumour aggressiveness also had a significant impact on survival rates. Multivariate analysis of these data revealed that the high aggressiveness profile remained as an independent prognostic factor of disease-free survival (HR 3.746; 95% CI 1.244–11.285; $p = 0.019$) and overall survival (HR 3.247; 95% CI 1.015–10.388, $p = 0.047$) (Table 3).

Discussion

In our study, we aimed to define urothelial bladder tumours with an aggressive phenotype by the combined analysis of clinicopathological and biological parameters. There were some limitations in the study. First, it included a population of bladder cancer patients that did not receive neoadjuvant or adjuvant treatments, and only some of the patients were treated with chemotherapy, in palliative setting, after progression (this may not be representative of all patients). Second, the study had a small sample size. However, despite these limitations, the classical prognostic factors, as stage pT3/pT4, grade III, infiltrating type of lesion, locoregional dissemination and lymphovascular invasion were related to a worse outcome, as previously reported.[21] Heparanase immunorreactivity did not reveal any relevant prognostic information in our series. In pT3/pT4 tumours, the median overall survival time was 14.7 months, which was reduced to 9.2 months if the tumours were CD147 positive. In accordance with our results, several authors have found that CD147 overexpression seems to be correlated with a worse outcome and a cisplatin-resistant profile.[12–14]

Using stage, grade and lymphovascular invasion as informative variables related to prognosis, and CD147 immunoexpression as an informative variable related to prognosis of patients with pT3/pT4 tumours, we attempted to organize a phenotype of UBC aggressiveness. Therefore, we developed a scoring system that classified patients with high grade superficial and invasive tumours in two aggressiveness profiles with different outcomes (low and high

Table 3
Multivariate survival-time regression for predictors of 5-year disease-free survival and overall survival after radical cystectomy for urothelial bladder cancer.

		5-year DFS rate			5-year OS rate		
		HR	95% CI	p	HR	95% CI	p
	Group 1	1.000	–	–	1.000	–	–
Stage	Group 2	0.741	0.251–2.186	0.587	0.694	0.211–2.288	0.549
	Group 3	0.672	0.230–1.961	0.467	0.783	0.243–2.524	0.682
Grade	II	1.000	–	–	1.000	–	–
	III	1.183	0.462–3.030	0.725	1.598	0.557–4.585	0.383
Blood vessel invasion (CD31 stain)	Non-occurrence	1.000	–	–	1.000	–	–
	Occurrence	1.653	0.801–3.413	0.174	1.728	0.835–3.578	0.141
Lymphatic vessel invasion (D2-40 stain)	Non-occurrence	1.000	–	–	1.000	–	–
	Occurrence	0.864	0.463–1.613	0.647	0.798	0.416–1.534	0.499
Tumour Aggressiveness Scoring System[a]	0–2 positive parameters	1.000	–	–	1.000	–	–
	3–5 positive parameters	3.746	1.244–11.285	0.019	3.247	1.015–10.388	0.047

DFS- disease-free survival, OS- overall survival, HR- hazard ratios, CI- confidence interval.
[a] Includes T3/T4 pathologic stage, grade III, occurrence of blood vessel invasion by malignant emboli, occurrence of lymphatic vessel invasion by isolated malignant cells and CD147 immunoexpression.

aggressiveness). This score proved to be an independent prognostic factor for 5-year disease-free survival and overall survival (95% CI 1.244–11.285, $p = 0.019$ for 5-year DFS; 95% CI 1.015–10.388, $p = 0.047$ for 5-year OS). In the group of highly aggressive tumours, the rate of CD147 immunopositive cases was 87.2%. However, one of the limitations of our study is the low number of cases involved, which is reflected in the wide confidence intervals. This score needs to be validated with a larger sample, preferentially in a multicentre study.

Nevertheless, our results suggest that CD147 overexpression may be a biological parameter related with worse prognosis. Xue et al. have recently reported that CD147 immunoexpression is as an independent prognostic factor for bladder cancer patients, playing an important role in tumour progression.[14] Takata et al. identified, in non-responder patients with invasive bladder cancer treated by M-VAC regimen, a gene (*SLC16A3*) that is closely associated with CD147.[9] Als et al. identified CD147 as a strong independent prognostic factor for response and survival after cisplatin-containing chemotherapy in patients with advanced bladder cancer.[12]Yang et al. found that CD147 is overexpressed in multidrug resistant (MDR) cancer cell lines, suggesting that during the development of a multidrug resistance phenotype, the expression of CD147 stimulates matrix metalloproteinases activity in MDR cells.[25] MDR1/P-glycoprotein or ABCB1 is one of the well-characterized members of the energy-dependent drug efflux pumps that reduce intracellular accumulation of anticancer drugs, leading to the MDR phenotype.[26] Recent studies have demonstrated the co-localization of CD147 with MDR1, highlighting the possible cooperative roles of these molecules in cancer drug resistance and progression.[27,28] In fact, increased expression of CD147 stimulates hyaluronan production, with MDR being induced in a hyaluronan-dependent manner.[29,30] The relationship between CD147 and MDR1 needs to be clearly elucidated. In our laboratory, studies are being conducted with bladder cancer cell lines that express CD147, in order to assess cisplatin resistance.

In conclusion, CD147 overexpression seems to be an important biomarker of prognosis that, when included in a scoring system of UBC aggressiveness, may help surgeons to identify patients who could benefit from a personalized therapeutic regimen. The definitive validation of this scoring system should be performed in a larger sample, in order to evaluate its internal consistency and its content, convergent-discriminant and construct validity.

Conflict of interest statement

There is no conflict of interest in this manuscript.

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CHAPTER 7 |

CD147 and MCT1 – Potential partners in bladder cancer aggressiveness and cisplatin resistance

The results presented in this chapter were:

(i) Submited as an original article in an international peer reviewed journal

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CD147 and MCT1:
Potential partners in bladder cancer aggressiveness and cisplatin resistance

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BACKGROUNG: The relapsing and progressive nature of bladder tumours, and the heterogeneity in the response to cisplatin-containing regimens, are the major concerns in the care of urothelial bladder cancer (UBC) patients. Biomarkers of tumour aggressiveness and response to treatment are urgently needed. The metabolic adaptations that alter the tumour microenvironment and thus contribute to chemoresistance have been poorly explored in UBC setting. We aimed to evaluate the clinical and prognostic significance of the microenvironment-related molecules CD147, monocarboxylate transporters (MCTs) 1 and 4, CD44 and CAIX expression in UBC patients, and to assess the therapeutic impact of CD147 downregulation *in vitro*.

METHODS: UBC sections from 114 patients were stained by immunohistochemistry for detection of the biomarkers. The immunohistochemical reactions were statistically correlated with the clinicopathological and the outcome parameters. Four UBC cell lines were assessed for cisplatin sensitivity. The RNA interference approach (siRNA) was used to silence CD147 expression in HT1376 cell line, in order to determine the effect of CD147 downregulation on MCTs expression and chemosensitivity to cisplatin.

RESULTS: Significant associations were found between the expressions of the biomarkers. CD44 expression was correlated with tumour progression. CAIX positivity was predominant in high grade papillary lesions. The presence of MCT1 and/or MCT4 expressions was significantly associated with unfavorable clinicopathological parameters. The incidence of CD147 positive staining significantly increased with advancing stage, grade and type of lesion, and occurrence of lymphovascular invasion. Similar associations were observed when considering the concurrent expression of CD147 and MCT1. This expression profile lowered significantly the 5-year DFS and OS rates. Moreover, when selecting patients who received platinum-based chemotherapy, the prognosis was significantly worse for those with MCT1 and CD147 positive tumours. On multivariate analysis, only stage remained as an independent prognostic factor. In the *in vitro* study, CD147 specific downregulation was accompanied by a decrease in MCT1 and MCT4 expressions and, importantly, an increase in chemosensitivity to cisplatin.

CONCLUSIONS: Our results provide novel insights for the involvement of CD147 and MCTs in bladder cancer progression and resistance to cisplatin-based chemotherapy. We consider that the possible cooperative role of CD147 and MCT1 in determining cisplatin resistance should be further explored as a potential theranostics biomarker.

KEYWORDS: CAIX, CD147, CD44, cisplatin, glycolytic metabolism, microenvironment, monocarboxylate transporters, urothelial bladder cancer, chemoresistance.

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INTRODUCTION

Urothelial bladder carcinoma (UBC), the most frequent type (90%) of bladder cancer and the second most common malignancy of the urogenital region, is a complex disease with variable natural history and clinical behaviour, representing an important cause of morbidity and mortality worldwide [1]. The natural history of UBC encompasses two main phenotypic variants: the majority of the tumours are non-muscle invasive (NMI) low-grade papillary lesions characterized by frequent recurrences; the remaining display a phenotype of muscle-invasive (MI) tumours. An intermediate sub-variant of high grade NMI

tumours harbours an enhanced risk of progression to MI disease [2-3]. Due to the high propensity of dissemination, MI tumours are generally treated by radical cystectomy (RC), pelvic lymphadenectomy and/or perioperative cisplatin-containing chemotherapy [4-5]. However, chemotherapy responses are very heterogeneous and frequently impaired by resistance [6]. To predict whose tumours will develop resistance remains a challenge that can only be overcome when biomarkers of tumour aggressiveness and response to chemotherapy are routinely evaluated in pathological specimens.

CD147 (or EMMPRIN, extracellular matrix metalloproteinase inducer) is a highly glycosylated transmembrane protein member of the immunoglobulin superfamily of receptors [7]. Originally identified as a matrix metalloproteinase (MMP) inducer [8], CD147 is also able to upregulate vascular endothelial growth factor (VEGF) [9], to associate with the laminin-interacting $\alpha3\beta1$ and $\alpha6\beta1$ integrins [10], and to stimulate hyaluronan production [11], co-localizing with the hyaluronan receptor CD44 [12]. Thus, this glycoprotein promotes extracellular matrix degradation, angiogenesis, migration and invasion, enhancing the metastatic potential of CD147-expressing tumour cells [7, 13]. Importantly, CD147, through hyaluronan-CD44 interaction, crosstalks with various multidrug transporters of the ABC (ATP-binding cassette) family classically associated with anti-apoptotic signalling and chemotherapy resistance [14]. Moreover, these constitutive interactions between hyaluronan, CD44, and CD147 contribute to the regulation of monocarboxylate transporter localization and function at the plasma membrane [12, 15].

Monocarboxylate transporters (MCTs) comprise fourteen members that share the same basic structure, although only the membrane-bound proton-coupled isoforms – MCT1, MCT2, MCT3 and MCT4 – transport monocarboxylates, namely lactate, through the plasma membrane [16]. The efflux of lactate from the malignant cells to the tumour microenvironment is crucial to maintain metabolic homeostasis. In fact, the malignant cells usually display high glycolytic rates even under aerobic conditions, a phenomenon known as the “Warburg effect” [17-18]. Hypoxia, a constitutive trait of tumours, is considered to be a trigger mechanism of the glycolytic phenotype [19]. Under hypoxic stress, hypoxia-inducible factor (HIF)-1α amplifies an adaptive response that promotes glycolysis and, importantly, induces the expression of pH regulators, such as carbonic anhydrase IX (CAIX) and MCTs, to assure intracellular pH balance. The high amounts of lactate extruded from the malignant cells, mainly through MCT1 and MCT4, contribute to acidification of the tumour microenvironment, which supports increased migration and invasion abilities of the primary tumour [20-21].

The preponderance of the tumour microenvironment in UBC setting has been poorly explored. A few studies have reported upregulation of microenvironment-related molecules, namely CD147 [22-25], CD44 [26-27], CAIX [28-29] and MCT4 [30], and their significant impact on the prognosis of the patients. In the study by Als et al. [24], CD147 positivity was able to predict response and survival following cisplatin-containing chemotherapy in patients with advanced UBC. Its downregulation signifycantly decreased proliferation, migration and invasion in UBC cell lines [23, 31]. However, the influence of CD147 downregulation on the response to cisplatin was not investigated. In other types of malignancies, increasing evidence suggest that upregulation of the aforementioned molecules strongly contributes to a hyper-glycolytic acid-resistant microenvironment that favours tumour growth, invasion and metastasis, suppresses host immune defenses, and impairs chemotherapy response [13, 32-36].

In order to elucidate the role of microenvironment-related molecules in UBC, namely their impact on chemoresistance, we aimed to assess, in 114 UBC patients, the clinical and prognostic significance of MCT1, MCT4, CD147, CD44 and CAIX expressions. Additionally, we intended to characterize the chemosensitivity of parental and CD147-silenced UBC cell lines to cisplatin.

MATERIALS AND METHODS

- Patients and Tissue Samples

Representative formalin-fixed paraffin-embedded surgical specimens were obtained from 114 patients with urothelial bladder carcinomas who underwent transurethral resection (TUR) and/or radical cystectomy (RC) at the Portuguese Institute of Oncology, Porto, from January 1996 to May 2006. In our cohort, we did not include patients diagnosed with urothelial carcinomas with variant histology, squamous cell or adenocarcinomas, patients who had an insufficient follow-up time and/or patients whose tumour samples were inadequate for further study. Prior approval was obtained from the Ethics Co-

mmittee of the Portuguese Institute of Oncology. The median age of the patients was 70 years (range 41-86); ninety-four (82.5%) were male and twenty (17.5%) were female. Additionally, tissue sections were obtained from normal-like areas of the urinary bladder of 6 autopsy patients without history of bladder cancer.
Each surgical product was examined according to the guidelines of the College of American Pathologists [37]. Hematoxylin-eosin (H&E)-stained sections were reviewed according to standard histopathological examination by two independent pathologists. Lesions were classified according to the American Joint Committee on Cancer [38] and to the World Health Organization 2004 [39] classification systems. For statistical analysis, tumours were divided into three groups based on T stage: group 1 (pTa, pT1 and pTis), group 2 (pT2 a and b) and group 3 (pT3 and pT4). Occurrence of lymphovascular invasion (LVI) was identified in 39 (34.2%) UBC samples (Table 1).
Forty-two (36.8%) patients underwent TUR with curative intention; 22 of these patients were treated by RC following disease recurrence and progression or when multiple CIS lesions were observed in the pathological specimen. Seventy-two (63.2%) patients had RC as their first treatment. Platinum-based chemotherapy regimens were administered to 31 (27.2%) patients (neoadjuvant: 6 patients, adjuvant: 9 patients, palliative: 16 patients). Twenty-seven (23.7%) patients presented loco-regional metastases at the time of RC. Mean and median follow-up were 38.2 and 37.0 months (range 1-132), respectively. Recurrence was defined as the reappearance of UBC (loco-regional dissemination or distant metastasis) more than 3 months after TUR/RC, occurring in seventy-four (64.9%) patients. Disease-free survival (DFS) was defined as the time from the TUR/RC until recurrence. Overall-survival (OS) was defined as the time from the TUR/RC until death by bladder cancer or the last clinical assessment.

- Cell Lines and General Cell Culture Procedures

In the present study, four urothelial bladder carcinoma cell lines were used: the 5637 NMI-UBC cell line and three MI-UBC cell lines (T24, MCR and HT1376). T24 was obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures; 5637, MCR and HT1376 were kindly provided by Professor Paula Videira, Universidade Nova de Lisboa, Lisboa, Portugal. The cell lines were cultured as a monolayer in RPMI Medium 1640 (Gibco®) supplemented with antibiotics (1% penicillin/streptomycin solution, Gibco®) and 10% fetal bovine serum (FBS, Gibco®). Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2, and were routinely subcultured by trypsinization.

- Immunohistochemistry and Immunocytochemistry

Representative 4µm-thick UBC sections were stained by immunohistochemistry, according to the streptavidin-biotin-peroxidase complex technique (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation) for MCT4, CD147, CD44 and CAIX detection, and to the avidin-biotin-peroxidase complex assay (VECTASTAIN Elite ABC Reagent, RTU, Vector Laboratories) for MCT1 detection, as previously described [25, 40-41]. The primary antibodies were obtained from Chemicon® (MCT1, AB3538P), Santa Cruz Biotechnology® (MCT4, H-90, sc-50329), Zymed® (CD147, 18-7344), AbD Serotec (CD44, MCA2726) and AbCam (CAIX, ab15086). These antibodies were used in 1:200 dilution (MCT1), 1:500 dilution (MCT4 and CD147), 1:1000 dilution (CD44) and 1:2000 dilution (CAIX), and incubated on the sections for 2 hours (MCT4, CD147, CD44, CAIX) or overnight (MCT1), at room temperature. Negative controls were carried out by replacing the primary antibodies with a universal negative control antibody (N1699, Dako). Colon carcinoma and gastric carcinoma sections were used as positive controls for MCT1, MCT4, CD147 and CD44 detection, and for CAIX detection, respectively.
The immunocytochemistry procedure for detecting MCT1, MCT4 and CD147 expression in the UBC cell lines was performed in 4µm-thick cytoblock sections, following the protocol mentioned for UBC sections, as described above. The paraffin cytoblocks were made from concentrated cell suspensions by centrifuging fresh cell suspensions at 1200 rpm for 5 minutes. Cell pellets were incubated overnight with formaldehyde 3.7%, re-centrifuged, processed in an automatic tissue processor (TP1020, Leica), and then included into paraffin (block-forming unit EG1140H, Leica).

- Evaluation of Immunohistochemistry and Immunocytochemistry Results

The immunostained tissue sections were evaluated by light microscopy for cytoplasmic and/or plasma membrane staining by two independent observers. Discordant cases were re-evaluated and classified by consensus. The grading system used was semi-

quantitative [25, 40-41], considering the sum of the percentage of immunoreactive cells (0, 0% of positive cells; 1, < 5% of positive cells; 2, 5-50% positive cells; score 3, >50% of positive cells) and the intensity of staining (0, negative; 1, weak; 2, intermediate; 3, strong); final scores ≥4 were considered positive for all of the biomarkers studied. Finally, the plasma membrane positive cases were analyzed separately.
The expression of the biomarkers on the cytoblocks sections was also assessed, distinguishing between cytoplasmic and plasma membrane staining.

- Downregulation of CD147 expression

Downregulation of CD147 expression in MCR and HT1376 cell lines was accomplished by reverse transfection of 50nM siRNA (siRNA for CD147, SASI_Hs01_ 00156882, Sigma-Aldrich®; control scramble siRNA, 4390843, Ambion®); lipofectamine (13778-075, InvitrogenTM) was used as permeabilization agent, following the manufacturer's instructions. Cells were transfected once and collected on days 5 and 8 after transfection. Specific silencing of the targeted gene was confirmed by Western blotting analysis.

- Western blotting

Parental UBC cell lines grown to 80% confluence, and siRNA cells grown until days 5 and 8 after transfection, were scraped in cold PBS and then homogenized in lysis buffer (supplemented with protease inhibitors) for 10 minutes. Cell lysates were collected after centrifugation at 13,000 rpm, 15 minutes at 4°C. The Bio-Rad Dc Protein Assay (500-0113, Bio-Rad) was used for protein quantification. Equal amounts (20 μg) of total protein were separated on 10% polyacrylamide gel by SDS-PAGE and transblotted onto nitrocellulose membranes (Amersham Biosciences) in 25 mM Tris-base/glycine buffer. MCT1, MCT4, CD147, CD44 and CAIX expressions were evaluated by incubating the membranes overnight at 4°C with specific primary polyclonal antibodies against MCT1 (1:200 dilution, H-1, sc-365501, Santa Cruz Biotechnology®), MCT4 (1:2000 dilution, H-90, sc-50329, Santa Cruz Biotechnology®), CD147 (1:200 dilution, sc-71038, Santa Cruz Biotechnology®), CD44 (1:1000 dilution, MCA2726, AbD Serotec) and CAIX (1:2000 dilution, ab15086, AbCam). β-Actin (1:300 dilution, I19, sc-1616, Santa Cruz Biotechnology®) was used as loading control. Blots were developed with enhanced chemiluminescence (Supersignal West Femto kit, 34096, Pierce) using anti-mouse or anti-goat Ig secondary antibodies coupled to horseradish peroxidase. Band densitometry analysis with the Image J software (version 1.41, National Institutes of Health) was performed for quantification of Western blot results.

- Cell Viability Assay

To assess the chemosensitivity of the UBC cell lines to cisplatin [CDDP, cis-diamminedichloroplatinum (II)], cells were seeded in triplicate into 48-well plates at different densities, based on the growth characteristics of each cell line ($1.2x10^4$ T24 and 5637 cells per well, $1.5x10^4$ HT1376 cells per well, $2x10^4$ MCR and siRNA-HT1376 cells per well and $3x10^4$ siRNA-MCR cells per well), and incubated for 2 (non-siRNA cell lines) or 5 (siRNA cell lines) days. The medium was then removed and replaced with fresh medium containing CDDP with varying concentrations (1-100 μg/ml). Stock solutions of 1 mg/ml CDDP in 10% NaCl were kindly provided by the Pharmaceutical Services of the Portuguese Institute of Oncology, Porto, Portugal, from which the working solutions were prepared. The effect of the treatment with CDDP on cell viability was determined at 72 hours by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium)] assay (G3580, Promega) according to the manufacturer's instructions. The IC_{50} values (CDDP concentration that corresponds to 50% of cell growth inhibition) were estimated from at least three independent experiments, using GraphPad Prism 5 Software.

- Cell Cycle Analysis

For cell cycle distribution analysis, cells were seeded in 6-well plates at different densities ($5x10^5$ T24 and 5637, $8x10^5$ HT1376 and $1x10^6$ MCR cells per well). After 42 hours of incubation, the cells were starved in FBS-free medium during 6 hours, and then treated with the specific CDDP IC_{50} dose during 72 hours. Cells were trypsinized and fixed in 70% ethanol (30 minutes at 4°C), followed by staining with propidium iodide (PI) solution [20 μg/ml of PI (81845, Sigma) + 250 μg/ml of RNAse (12091-021, Invitrogen™) diluted in 0.01% Triton X-100 in PBS] at 50°C during 50 minutes. PI stained cells were analyzed by flow cytometry (LSRII model, BD Biosciences), considering a total of 15.000 events, and the cell cycle distribution was determined with the FlowJo software (version 7.6, Tree Star, Inc). The assay was repeated at least three times.

- Cell Death Assay

Cell death rate was determined by the Annexin-V-FLOUS staining Kit (Roche Diagnostics), in order to assess apoptosis and/or necrosis occurrence induced by CDDP treatment in the parental UBC cell lines. Cells were seeded in 6-well plates at different densities ($5x10^5$ T24 and 5637, $8x10^5$ HT1376 and $1x10^6$ MCR cells per well). After 48 hours of incubation, cells were treated with the specific CDDP IC_{50} dose during 72 hours, followed by collection and staining with annexin V/PI, according to the manufacturer's instructions (15 minutes of incubition in the staining solutions, at room temperature). The percentage of cell death was assessed by flow cytometry (LSRII model, BD Biosciences), considering a total of 20,000 events, and the results were analyzed using the FlowJo software (version 7.6, Tree Star, Inc). The assay was repeated at least three times.

- Wound Healing Migration Assay

Cells were seeded in 6-well plates at different densities ($1x10^6$ T24 and 5637, $1.4x10^6$ HT1376 and $2x10^6$ MCR cells per well) and incubated for 24 hours. The medium was then replaced by fresh FBS-free medium containing the previously determined CDDP IC_{50} dose for each cell line, to assess the effect of the drug on the migration ability of the parental cell lines (CDDP-free control wells were also prepared); therefore, 48 hours after the beginning of the CDDP treatment, the cells were washed with PBS, and a scratch wound through the central axis of the wells was gently made using a plastic 200 μl pipette tip; the cells were then incubated with fresh CDDP-containing medium. The "wound" areas were monitored and photographed by phase contrast microscopy at 0 and 24 hours. The relative migration distances were quantified by the ratio of gap distance between 24 and 0 hours. The experiment was repeated at least three times.

- Invasion Assay

Invasion assays were performed with the parental cell lines treated with CDDP IC_{50} dose. Twenty-four-well BD Matrigel™ Invasion Chambers (354480, BD BioCoat™, BD Biosciences) were used, according to the manufacturer's instructions. After rehydrating the matrigel invasion chambers, cells were seeded at different densities ($2x10^4$ T24 and 5637, $3x10^4$ HT1376 and $4x10^4$ MCR cells per chamber) and incubated with the specific CDDP IC_{50} dose during 24 hours. Then, non-invading cells were swabbed and invading cells were fixed with methanol and stained with hematoxylin. Membranes were photographed at 16x magnification under an Olympus SZX16 stereomicroscope, and invading cells were counted using the Image J software (version 1.41, National Institutes of Health). Invasion was calculated as the percentage of cell invasion normalized for the control condition. Results were expressed as mean of triplicate experiments.

- Statistical analysis

The immunohistochemistry results were analyzed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 18.0. Associations between the immunoexpression of the biomarkers and the clinicopathological parameters were examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when n<5). Five-year DFS and OS rates were evaluated using Kaplan-Meier curves and differences were analyzed by Log-Rank or Breslow tests. p values lower than 0.05 were considered significant. Variables that achieved statistical significance in the univariate analysis were entered in a multivariate analysis using Cox proportional hazards analysis. The hazard ratios (HR) were estimated with their 95% confidence intervals (95% CI).

The results of the *in vitro* studies were analyzed using the GraphPad Prism 5 software, with the Student's t test, considering significant p values lower than 0.05.

RESULTS

Characterization of MCT1, MCT4, CD147, CD44 and CAIX Expressions in Urothelial Bladder Tumours

- Prognostic Significance of the Clinicopathological Parameters

The 5-year DFS and OS rates were significantly influenced by T3/T4 pathologic stage, infiltrating type of lesion, occurrence of lymphovascular invasion and presence of loco-regional metastases. (Table 1).

- Immunoexpression of the Biological Parameters

A total of 114 UBC samples and 6 non-neoplastic bladder samples were analyzed for MCT1, MCT4, CD147, CD44 and CAIX expressions. After testing different grading systems considering the semi-quantitative evaluation of extension and intensity of

			5-year DFS rate	p•	5-year OS rate	p•
Gender	Male	94	30.2%	0.939	44.1%	0.642
	Female	20	40.6 %		45.7%	
Age	≤ 70 years	61	35.1%	0.146	46.3%	0.089
	> 70 years	53	28.1%		42.3%	
TNM stage	pTa, pT1, pTis	46	44.0%	**<0.001**	55.0%	**<0.001**
	pT2	18	26.8%		47.0%	
	pT3, pT4	50	21.8%		27.5%	
WHO 2004 grade	NIP UC, low grade	10	55.6%	**<0.001**	85.7%	**<0.001**
	NIP UC, high grade	32	41.0%		56.3%	
	NI UC *in situ*	4	50.0%		50.0%	
	Infiltrating UC	68	23.1%		32.5%	
Lymphovascular invasion	Negative	75	37.9%	**0.002**	52.4%	**<0.001**
	Positive	39	20.2%		26.4%	
Loco-regional metastasis	Negative	87	38.1%	**0.007**	52.3%	**<0.001**
	Positive	27	12.1%		17.2%	

• Log-Rank or Breslow tests

DFS- disease-free survival, NI- non-invasive, NIP, non-invasive papillary, OS- overall survival, TNM- tumour, node, metastases, UC- urothelial carcinoma, WHO- World Health Organization

Table 1. Association between 5-year disease-free survival and overall survival rates, and clinicopathological parameters

staining (cytoplasmic expression, with or without plasma membrane staining), we adopted the final immunoreaction score ≥4 as the more suitable for explaining the results obtained with all of the studied biomarkers. Due to the membrane localization of the biomarkers, plasma membrane staining was additionally assessed separately.

Regarding MCT1 and MCT4 immunoexpressions (Figure 1A and 1B, respectively), 36 (31.6%) and 50 (43.9%) UBC cases were scored positive, respectively; plasma membrane staining was observed in 44 (38.6%) and 64 (56.1%) cases, respectively. Stromal and endothelial cells were negative for both biomarkers, and served as internal negative controls. None of the normal bladder samples expressed MCT1; two non-neoplastic sections showed cytoplasmic staining for MCT4, but the plasma membrane was negative in the six observed sections.

When considering the expression of the chaperones CD147 and CD44 (Figures 1C, 1D and 1E, respectively), the majority of the tumour tissues was positive both for global immunoreaction [CD147: 68 (59.6%); CD44: 57 (50.0%)] and plasma membrane staining [CD147: 70 (61.4%); CD44: 77 (67.5%)]. The stroma was negative for CD147 immunoreaction in all of the cases (Figure 1C); however, although CD44 positive tumours presented negative stromas (Figure 1D), CD44 negative tumours had their stromal cells stained (Figure 1E). Regarding the non-neoplastic bladder samples, the majority was negative for CD147 staining, while no difference was observed when evaluating CD44 expression.

CAIX positive immunoexpression (Figure 1F) was observed in the vast majority of the UBC samples [global immunoreaction: 72 (63.2%); plasma membrane staining: 92 (80.7%)]; plasma membrane positive cases exhibited a heterogeneous pattern, with the luminal face of NMI papillary lesions and the centre of MI lesions presenting a strong intensity of staining (Figure 1F). This pattern of expression was significantly different from the pattern observed in the non-neoplastic tissues – none of the normal bladders expressed CAIX.

- Associations among the Biological Parameters

Significant associations were found between the expression of MTCs and their chaperone CD147 (Tables 2 and 3) in the tumour samples. With regard to global immunoreaction, 91.7% MCT1 and 90.0% MCT4 immunoreactive cases were also CD147 positive ($p<0.001$ in both associations); when considering plasma membrane staining separately, 77.3% MCT1 and 71.9% MCT4 positive sections also expressed CD147 ($p=0.006$ and $p=0.012$, respectively). A similar pattern was observed when evaluating the correlation between MCTs and CD44 immunoreactions (Tables 2 and 3): 69.4% MCT1 and 66.0% MCT4 immunoreactive cases (global expression) were also positive for CD44 immunoexpression ($p=0.008$ and $p=0.004$, respectively). In accordance, and considering plasma membrane staining, 75.0% MCT1 and/or MCT4-expressing samples were also positive for CD44, although the differences were not significant due to the high number of cases that expressed CD44 but did not express MCTs.

CD147 and CD44 immunoreactions were also correlated: 70.2% (40/57) and 67.5% (52/77) of the positive sections for CD44 (global expression and plasma membrane staining, respectively) expressed CD147 ($p=0.035$ and $p=0.065$, respectively; data not shown). Additionally, significant associations

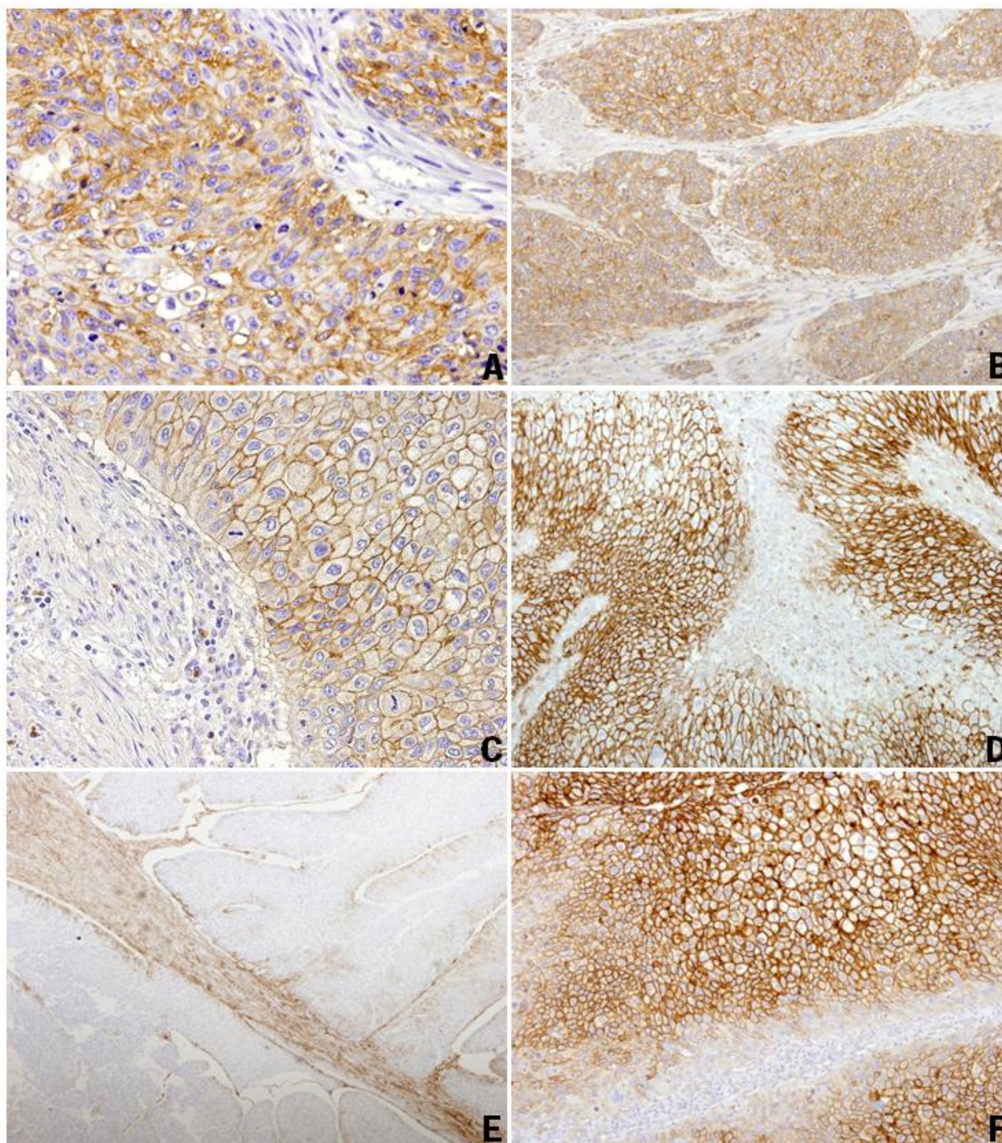


Figure 1. Immunohistochemical positive reactions for MCT1 (**A**, x200 amplification), MCT4 (**B**, x100 amplification), CD147 (**C**, x200 amplification), CD44 (**D**, x100 amplification; **E**, x40 amplification) and CAIX (**F**, x100 amplification) in urothelial bladder carcinoma cells. **A** to **D**, muscle-invasive tumours exhibiting cytoplasmic and membrane immunoexpression of the selected biomarkers in the malignant urothelium, with negative stromas. **E**, a non-muscle invasive tumour showing an inverted CD44 staining pattern, with negative malignant cells and positive stroma. **F**, a muscle-invasive tumour stained for CAIX in the plasma membrane of the malignant urothelial cells, where the tumour core is significantly more intensely stained than the invasive front.

were found when comparing immunoreactive samples for MCT4 (92%, 46/50, p=0.007), CD147 (86.9%, 59/68, p=0.046) and CD44 (89.5%, 51/57, p=0.018) with CAIX plasma membrane positive cases (data not shown).

- Clinical and Prognostic Significance of the Biological Parameters

The presence of MCT1 and/or MCT4 immunoexpression was significantly associated with unfavourable clinicopathological parameters, such as increasing stage (MCT1, p<0.001; MCT4, p=0.022), infiltrating morphological type of lesion (MCT1, p<0.001; MCT4, p=0.021) and occurrence of lymphovascular invasion (MCT1, p=0.002; MCT4, p=0.028) (Table 4). When considering plasma membrane staining separately (Table 5), this unfavourable phenotype was maintained for pT3/pT4 tumours (52.0%, p=0.003), for infiltrating tumours (51.5%, p=0.063) and for tumours with LVI occurrence (53.8%, p=0.025) that expressed MCT1. MCT1 expression (global immunoreaction) had a negative influence on 5-year DFS (p=0.053) and OS (p=0.065) rates (Table 6).

Regarding CD147 expression, 80.0% of pT3/pT4 tumours (p<0.001), 64.4% of high grade tumours

Table 2. Association between MCTs, and CD147 and CD44 global immunoreaction (cytoplasmic expression, with or without plasma membrane staining)

		n	CD147			CD44		
			Negative (%)		***p*•**	**Negative (%)**	**Positive (%)**	***p*•**
MCT1	**Negative (%)**	78	43 (55.1)	35 (44.9)	**<0.001**	46 (59.0)	32 (41.0)	**0.008**
	Positive (%)	36	3 (8.3)	33 (91.7)		11 (30.6)	25 (69.4)	
MCT4	**Negative (%)**	64	41 (64.1)	23 (35.9)	**<0.001**	40 (62.5)	24 (37.5)	**0.004**
	Positive (%)	50	5 (10.0)	45 (90.0)		17 (34.0)	33 (66.0)	

• χ^2 or Fisher's exact tests

Table 3. Association between MCTs, and CD147 and CD44 plasma membrane immunoexpression

		n	CD147			CD44		
			Negative (%)	**Positive (%)**	***p*•**	**Negative (%)**	**Positive (%)**	***p*•**
MCT1	**Negative (%)**	70	34 (48.6)	36 (51.4)	**0.006**	26 (37.1)	44 (62.9)	0.220
	Positive (%)	44	10 (22.7)	34 (77.3)		11 (25.0)	33 (75.0)	
MCT4	**Negative (%)**	50	26 (52.0)	24 (48.0)	**0.012**	21 (42.0)	29 (58.0)	0.070
	Positive (%)	64	18 (28.1)	46 (71.9)		16 (25.0)	48 (75.0)	

• χ^2 or Fisher's exact tests

(p=0.001), 75.0% of infiltrating tumours (p<0.001) and 84.6% of the tumours with LVI occurrence (p<0.001) were positive for CD147 cytoplasmic staining (with or without plasma membrane immunoreactivity) (Table 4). This expression profile lowered significantly the 5-year DFS (p=0.027) and OS (p=0.018) rates (Table 6).

In order to assess the clinical and prognostic significance of the combined analysis of MCT1 and CD147 immunoreaction, we considered two groups: group 1, including cases with 0 or 1 positive biomarkers, and group 2, including cases with two positive biomarkers. The concurrent immunoexpression of MCT1 and CD147 was associated with unfavourable clinicopathological parameters – 72.7% (24/33, p<0.001), 90.9% (30/33, p<0.001) and 60.6% (20/33, p<0.001) of the MCT1 and CD147 positive cases were pT3/pT4, infiltrating and with LVI occurrence tumours, respectively (data not shown) – and lowered significantly the 5-year DFS (p=0.033) and OS (p=0.037) rates (data not shown). Notably, when selecting patients who received platinum-based chemotherapy (n=31), the prognosis was significantly worse for those with MCT1 and CD147 positive tumours (n=11) – patients with 0 or 1 positive biomarkers had median DFS and OS times of 25.8 (95% CI 20.4-31.2) and 42.2 (95% CI 33.9-50.4) months, respectively, which were reduced to 11.7 (95% CI 6.7-16.2) and 12.4 (95% CI 1.0-32.5) months, respectively, if the tumours were MCT1 and CD147 positive (p=0.072 and p=0.026, respectively; data not shown).

CD44 plasma membrane positivity was predominant in pT3/pT4 (82.0%, p=0.013) and infiltrating (76.5%, p=0.032) UBC samples (Table 5). Conversely, the majority of the high grade papillary lesions (93.8%, p<0.001) were CAIX positive (Table 5). Regarding the global immunoreaction for CAIX, 87.5% of high grade papillary tumours (p=0.001), 69.3% of the tumours without LVI occurrence (p=0.068) and 67.8% of the cases without loco-regional metastasis (p=0.064) were scored CAIX positive (Table 4).

The aforementioned associations were found when analyzing a series of 114 UBC patients, which includes six patients that received neoadjuvant platinum-based chemotherapy regimens. Since this could introduce a bias variable, the statistical analysis was also performed without those six cases, however no differences were observed, and we decided to include the cases in the final results.

- Multivariate Analysis

The parameters that significantly influenced the 5-year DFS and OS rates, namely T3/T4 pathological stage, infiltrating type of lesion, occurrence of lymphovascular invasion and loco-regional dissemination, CD147 positive immunoreaction and the concomitant expression of MCT1 and CD147, were entered in the multivariate analysis model. None of the aforementioned variables was identified as an independent prognostic factor.

Immunoexpression of MCT1, MCT4 and CD147 in Urothelial Bladder Cancer Cell Lines

All UBC cell lines expressed MCT1, MCT4 and CD147, as detected by Western blot (Figure 2A) and

Table 4. Association between MCT1, MCT4, CD147, CD44 and CAIX global immunoreaction (cytoplasmic expression, with or without plasma membrane staining) and the clinicopathological parameters

Clinicopathological parameter		n	MCT1			MCT4			CD147			CD44			CAIX		
			Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*
TNM stage	pTa, pT1, pTis	46	42 (91.3)	4 (8.7)	**<0.001**	33 (71.7)	13 (28.3)	**0.022**	29 (63.0)	17 (37.0)	**<0.001**	27 (58.7)	19 (41.3)	0.076	13 (28.3)	33 (71.7)	0.187
	pT2	18	12 (66.7)	6 (33.3)		8 (44.4)	10 (55.6)		7 (38.9)	11 (61.1)		11 (61.1)	7 (38.9)		6 (33.3)	12 (66.7)	
	pT3, pT4	50	24 (48.0)	26 (52.0)		23 (46.0)	27 (54.0)		10 (20.0)	40 (80.0)		19 (38.0)	31 (62.0)		23 (46.0)	27 (54.0)	
WHO 2004 grade	NIP UC, low grade	10	9 (90.0)	1 (10.0)	**<0.001**	8 (80.0)	2 (20.0)	**0.021**	9 (90.0)	1 (10.0)	**<0.001**	6 (60.0)	4 (40.0)	0.417	5 (50.0)	5 (50.0)	**0.001**
	NIP UC, high grade	32	30 (93.8)	2 (6.2)		21 (65.6)	11 (34.4)		18 (56.2)	14 (43.8)		18 (56.2)	14 (43.8)		4 (12.5)	28 (87.5)	
	NI UC *in situ*	4	3 (75.0)	1 (25.0)		4 (100.0)	0 (0.0)		2 (50.0)	2 (50.0)		3 (75.0)	1 (25.0)		4 (100.0)	0 (0.0)	
	Infiltrating UC	68	36 (52.9)	32 (47.1)		31 (45.6)	37 (54.4)		17 (25.0)	51 (75.0)		30 (44.1)	38 (55.9)		29 (42.6)	39 (57.4)	
Lymphovascular invasion	Negative	75	59 (78.7)	16 (21.3)	**0.002**	48 (64.0)	27 (36.0)	**0.028**	40 (53.3)	35 (46.7)	**<0.001**	39 (52.0)	36 (48.0)	0.693	23 (30.7)	52 (69.3)	0.068
	Positive	39	19 (48.7)	20 (51.3)		16 (41.0)	23 (59.0)		6 (15.4)	33 (84.6)		18 (46.2)	21 (53.8)		19 (48.7)	20 (51.3)	
Loco-regional metastasis	Negative	87	60 (69.0)	27 (31.0)	0.817	49 (56.3)	38 (43.7)	1.000	38 (43.7)	49 (56.3)	0.262	44 (50.6)	43 (49.4)	1.000	28 (32.2)	59 (67.8)	0.064
	Positive	27	18 (66.7)	9 (33.3)		15 (55.6)	12 (44.4)		8 (29.6)	19 (70.4)		13 (48.1)	14 (51.9)		14 (51.9)	13 (48.1)	

* χ^2 or Fisher's exact tests

DFS- disease-free survival, NI- non-invasive, NIP, non-invasive papillary, OS- overall survival, TNM- tumour, node, metastases, UC- urothelial carcinoma , WHO- World Health Organization

Table 5. Association between MCT1, MCT4, CD147, CD44 and CAIX plasma membrane expression, and the clinicopathological parameters

Clinicopathological parameter		n	MCT1			MCT4			CD147			CD44			CAIX		
			Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*	Negative (%)	Positive (%)	p^*
TNM stage	pTa, pT1, pTis	46	37 (80.4)	9 (19.6)	**0.003**	23 (50.0)	23 (50.0)	0.453	19 (41.3)	27 (58.7)	0.654	21 (45.7)	25 (54.3)	**0.013**	8 (17.4)	38 (82.6)	0.609
	pT2	18	9 (50.0)	9 (50.0)		6 (33.3)	12 (66.7)		8 (44.4)	10 (55.6)		7 (38.9)	11 (61.1)		5 (27.8)	13 (72.2)	
	pT3, pT4	50	24 (48.0)	26 (52.0)		21 (42.0)	29 (58.0)		17 (34.0)	33 (66.0)		9 (18.0)	41 (82.0)		9 (18.0)	41 (82.0)	
WHO 2004 grade	NIP UC, low grade	10	9 (90.0)	1 (10.0)	**0.006**	5 (50.0)	5 (50.0)	0.758	5 (50.0)	5 (50.0)	**0.052**	3 (30.0)	7 (70.0)	**0.032**	2 (20.0)	8 (80.0)	**<0.001**
	NIP UC, high grade	32	25 (78.1)	7 (21.9)		16 (50.0)	16 (50.0)		10 (50.0)	22 (68.8)		15 (46.9)	17 (53.1)		2 (6.2)	30 (93.8)	
	NI UC *in situ*	4	3 (75.0)	1 (25.0)		2 (50.0)	2 (50.0)		4 (100.0)	0 (0.0)		3 (75.0)	1 (25.0)		4 (100.0)	0 (0.0)	
	Infiltrating UC	68	33 (48.5)	35 (51.5)		27 (39.7)	41 (60.3)		25 (36.8)	43 (63.2)		16 (23.5)	52 (76.5)		14 (20.6)	54 (79.4)	
Lymphovascular invasion	Negative	75	52 (69.3)	23 (30.7)	**0.025**	37 (49.3)	38 (50.7)	0.115	33 (44.0)	42 (56.0)	0.110	28 (37.3)	47 (62.7)	0.144	15 (20.0)	60 (80.0)	1.000
	Positive	39	18 (46.2)	21 (53.8)		13 (33.3)	26 (66.7)		11 (28.2)	28 (71.8)		9 (23.1)	30 (76.9)		7 (17.9)	32 (82.1)	
Loco-regional metastasis	Negative	87	52 (59.8)	35 (40.2)	0.652	37 (42.5)	50 (57.5)	0.661	36 (41.4)	51 (58.6)	0.366	30 (34.5)	57 (65.5)	0.485	15 (17.2)	72 (82.8)	0.402
	Positive	27	18 (66.7)	9 (33.3)		13 (48.1)	14 (51.9)		8 (29.6)	19 (70.4)		7 (25.9)	20 (74.1)		7 (25.9)	20 (74.1)	

* χ^2 or Fisher's exact tests

DFS- disease-free survival, NI- non-invasive, NIP, non-invasive papillary, OS- overall survival, TNM- tumour, node, metastases, UC- urothelial carcinoma, WHO- World Health Organization

		n	5-year DFS rate	p*	5-year OS rate	p*
MCT1	Negative	78	33.7%	**0.053**	45.8%	**0.065**
	Positive	36	28.1%		38.7%	
MCT4	Negative	64	28.5%	0.602	47.5%	0.071
	Positive	50	35.9%		39.1%	
CD147	Negative	46	40.4%	**0.027**	48.0%	**0.018**
	Positive	68	26.7%		39.7%	
CD44	Negative	57	27.6%	0.850	41.4%	0.869
	Positive	57	36.3%		46.7%	
CAIX	Negative	42	22.7%	0.270	35.6%	0.291
	Positive	72	38.3%		50.0%	

* Log-Rank or Breslow tests
DFS- disease-free survival, OS- overall survival

Table 6. Association between 5-year disease-free survival and overall survival rates, and MCT1, MCT4, CD147, CD44 and CAIX global immunoreaction (cytoplasmic expression, with or without plasma membrane staining)

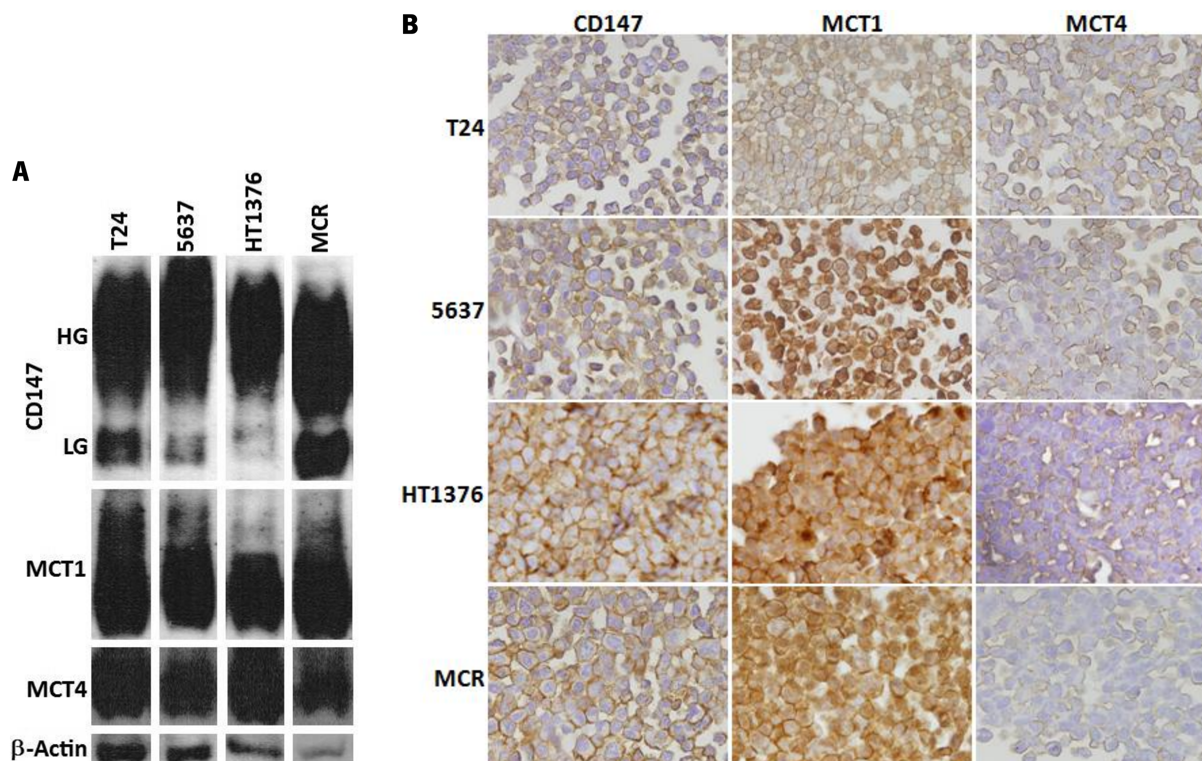


Figure 2. CD147 and monocarboxylate transporters (MCT1 and MCT4) expressions in bladder cancer cell lines, as detected by Western blot (**A**; molecular weights: 50-60 kDa for the highly glycosylated and 42 kDa for low glycosylated form of CD147, 50 kDa for MCT1, and 52 kDa for MCT4) and immunocytochemistry (**B**, x400 amplification). The biomarkers were expressed by the four UBC cell lines. The pattern of expression was predominantly membranous. 5637, HT1376 and MCR additionally exhibited a strong cytoplasmic immunoreaction for MCT1.

immunocytochemistry (Figure 2B). MCT4 and CD147 were expressed predominantly at the plasma membrane in the four cell lines. In T24 cell line, MCT1 expression was membranous, while in the remaining cell lines, both plasma membrane and cytoplasm were stained.

In Vitro Effect of CDDP in Urothelial Bladder Cancer Cell Lines

In order to characterize the response of four different parental UBC cell lines to CDDP, we started by measuring the effect of this drug on cell viability (Figure 3). For this, IC_{50} values were estimated after 72 hours of treatment. Ten different CDDP concentrations were used, ranging from 1 to 100 μg/ml. We observed that 5637 and T24 cell lines presented a gradual decrease in total biomass (MTS assay) in a CDDP dose-dependent manner; IC_{50} values were low: 3.1 μg/ml for 5637 and 3.5 μg/ml for T24 cells. HT1376 and MCR cell lines were less sensitive to CDDP effect: at the initial concentrations, only a slight decrease on cell viability was noted; IC_{50} values were 5.5 μg/ml for HT1376 and 8.8 μg/ml for MCR.

To further elucidate CDDP effect on cell cycle distribution (Figure 4A) and cell death (Figure 4B),

the UBC cell lines were treated with the CDDP IC_{50} predetermined doses. Comparing with the control condition, 5637, T24 and HT1376 cell lines presented a decrease in G0/G1 phase, an increase in S phase (the majority of HT1376 cells were arrested in S phase) and an increase in subG1 phase cell populations, although the differences were only statistically significant for T24 and HT1376. The drug induced cell death in 5637 and T24 cell lines: we observed a marked increase in late apoptotic/ necrotic cell populations (the differences were statistically significant for 5637); no effect was noted for HT1376. Regarding the cell cycle distribution of MCR cell line, we observed a significant decrease in G0/G1 and an arrest in S + G2 phase's cell populations, without observing any effect on the cell population of subG1 phase. We confirmed this cytostatic action of the drug in MCR cells through the cell death assay: no difference was found between control and treated cells.

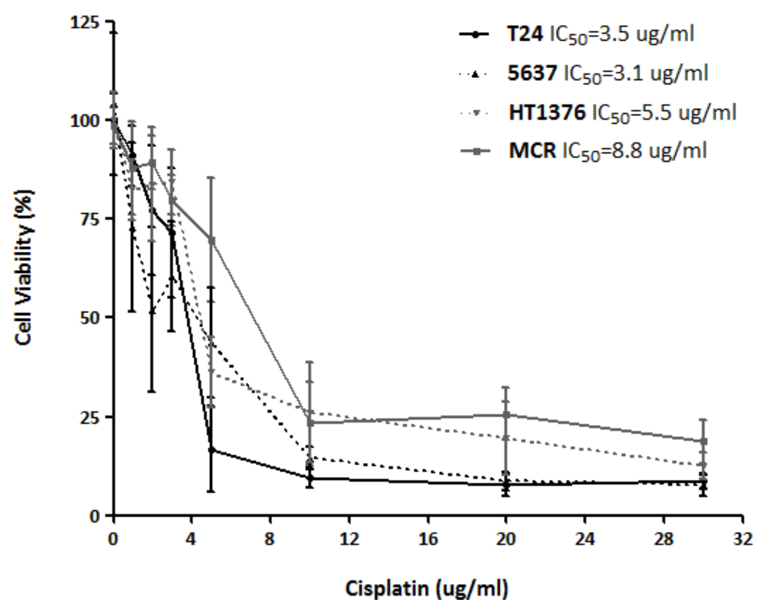


Figure 3. Effect of CDDP on the viability of bladder cancer cell lines, as detected by the MTS assay after 72 hours of treatment. Results are expressed as the mean±standard deviation of at least 3 independent experiments, each one in triplicate. T24 and 5637 viability was inhibited in a dose-dependent manner; HT1376 and MCR were less sensitive to CDDP effect at the initial concentrations.

The effect of CDDP treatment on UBC cells' migration and invasion capacities was studied by the wound healing migration (Figure 5A) assay and by the matrigel invasion assay (Figure 5B), respectively. The treatment significantly decreased T24 and HT1376 cells' migration ability; no effect was observed in 5637 cell line; conversely, MCR treated cells migrated significantly more than control cells. Regarding invasion assays, we observed that CDDP treatment induced a significant increase in T24 cell's invasion capacity, a decrease for 5637 and HT1376 cells, and no effect for MCR cells.

Effect of CD147 downregulation on Urothelial Bladder Cancer Cells' Biology and Response to CDDP Treatment

The characterization of the effect of CDDP treatment on cell viability, cell cycle distribution and cell death, as well as on the migration and invasion abilities of four parental UBC cell lines, allowed us to choose two of the cell lines for subsequent downregulation studies. HT1376 and MCR cells seemed to be less sensitive to CDDP effect. These cells showed the highest CDDP IC_{50} values, and the drug apparently exerted a cytostatic effect on them. Based on this, we used specific siRNA targeting CD147 mRNA to downregulate CD147. By Western blotting, we confirmed a marked decrease in CD147 expression in both cell lines, most notably following 6 and 10 days after reverse transfection; the transfection with scramble siRNA did not alter protein expression, as expected. Once the protocol has been optimized, we proceeded with CDDP treatment in siRNA-HT1376 and siRNA-MCR cells. Since we had previously determined CDDP IC_{50} values for the parental cell lines after 72h of exposure to the drug, we followed the same procedure with the siRNA cell lines, by treating the cells between days 5 and 8 after reverse transfection. Due to technical complications with MCR cell line (the cells did not tolerate CD147 downregulation and CDDP treatment, and became unviable at the end of repeated assays), we were only able to continue the experiment with HT1376 cell line. CD147 downregulation at days 5 and 8 after transfection was confirmed by Western blot; the decrease in CD147 expression was accompanied by a decrease in MCT1 and MCT4 expressions (Figure 6A). IC_{50} values were determined for siScramble-HT1376 and siCD147-HT1376 cells (Figure 6B), which allowed us to conclude that siCD147-HT1376 cells (CDDP IC_{50} = 7.4 μg/ml) were more sensitive to CDDP treatment than siScramble-HT1376 cells (CDDP IC_{50}= 24.1 μg/ml) (the disparity between IC_{50}

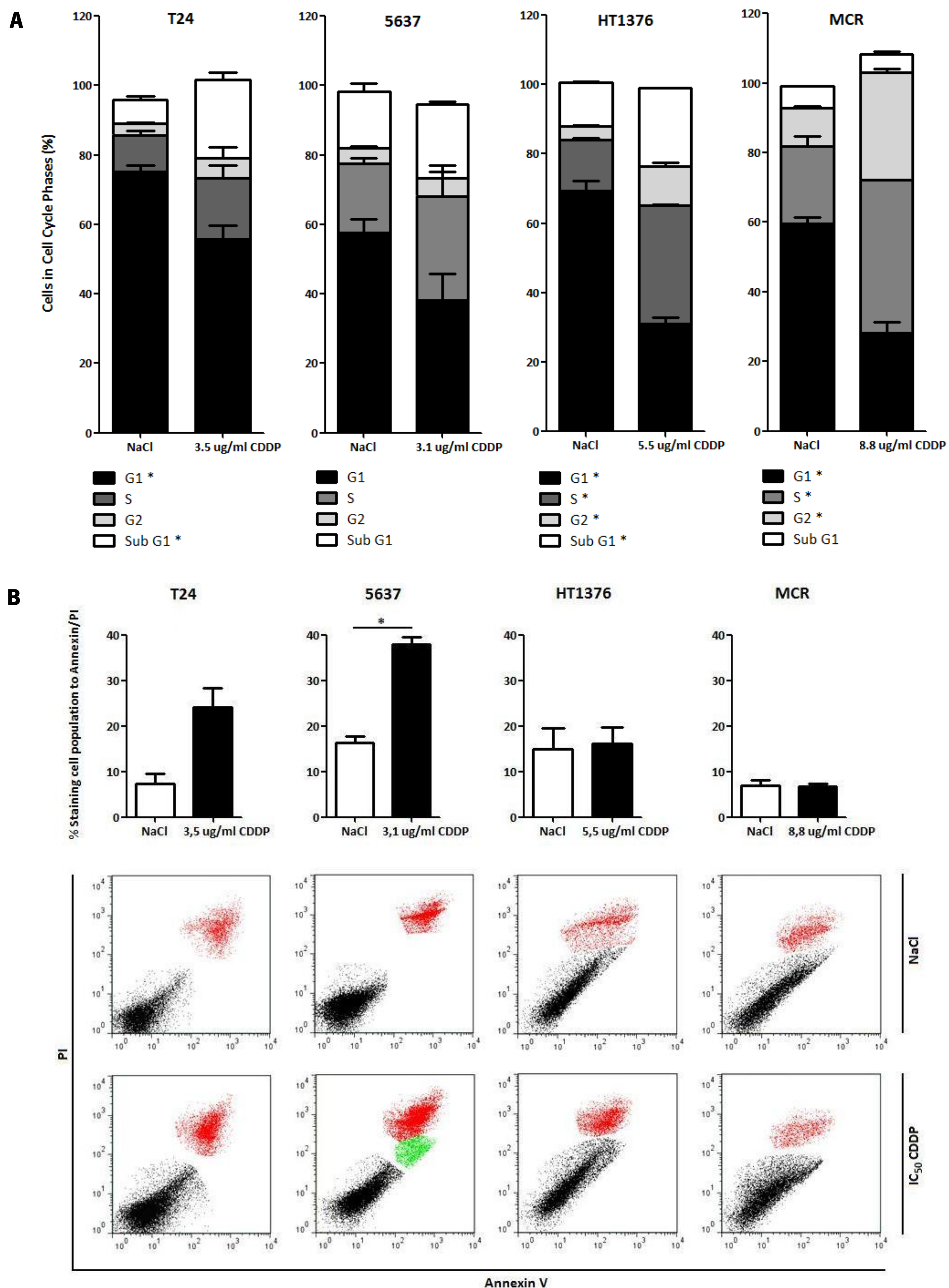
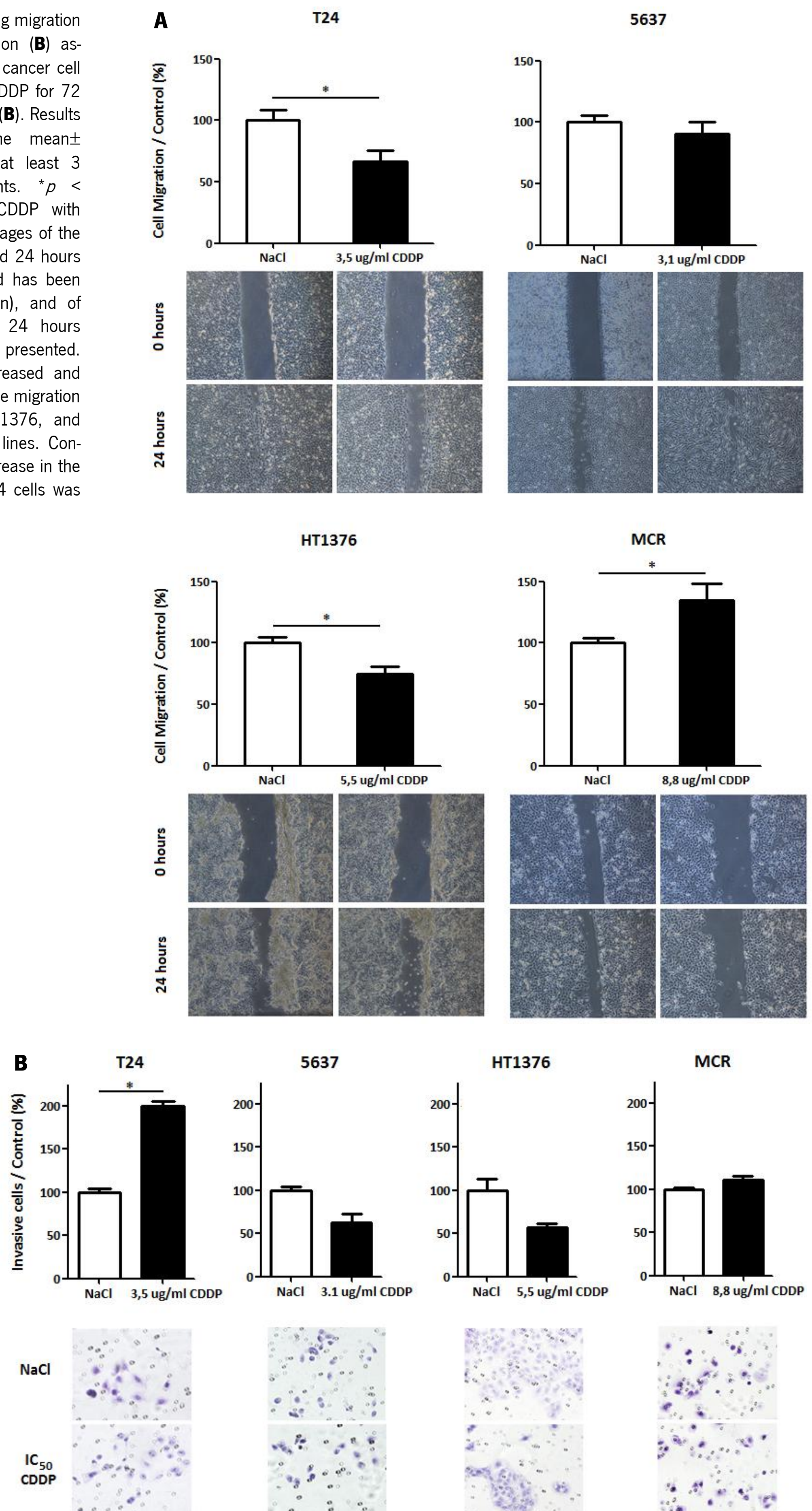


Figure 4. Cell cycle (**A**) and cell death (**B**) analysis of bladder cancer cell lines after 72 hours of treatment with IC_{50} values of CDDP, as detected by the propidium iodide (PI) and the Annexin V/PI assays, respectively (flow cytometry). Results are ex-pressed as the mean± standard deviation of at least 3 independent experiments. *$p < 0.05$, compared IC_{50} CDDP with NaCl. Representative dotplots of cell population distribution stained for Annexin V and PI are shown (cell population in bottom/left (black dots) = viable cells; cell population in upper/right = death cells (red dots, late apoptosis; green dots, necrosis)]. CDDP exerted a cytotoxic effect on T24 and 5637 cell lines, as confirmed by an increase in subG1 phase cell populations, in the cell cycle analysis, and an increase in late apoptotic/necrotic cell populations, in the cell death analysis. HT1376 and MCR cells were arrested in S phase (cell cycle analysis), and no difference was observed between control and treated conditions in the cell death analysis, which denotes a cytostatic action of CDDP.

Figure 5. Wound-healing migration (**A**) and matrigel invasion (**B**) assays results for bladder cancer cell lines treated with IC_{50} CDDP for 72 hours (**A**) and 24 hours (**B**). Results are expressed as the mean± standard deviation of at least 3 independent experiments. *p < 0.05, compared IC_{50} CDDP with NaCl. Representative images of the migration assay at 0 and 24 hours after the scratch wound has been made (x40 amplification), and of the invasion assay at 24 hours (x100 amplification), are presented. CDDP significantly decreased and significantly increased the migration ability of T24 and HT1376, and MCR (respectively) cell lines. Conversely, a significant increase in the invasive potential of T24 cells was observed.



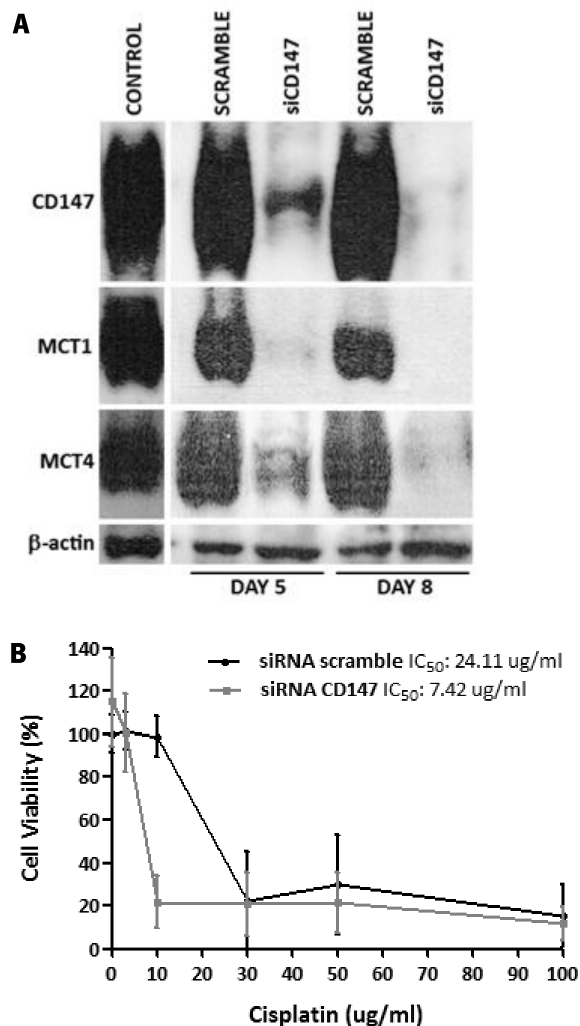


Figure 6. Effect of CD147 downregulation in HT1375 cell line on the expression of MCTs and on chemosensitivity to CDDP (treatment with CDDP between days 5 and 8 after reverse transfection). **A**, Western blot analysis of CD147, MCT1 and MCT4 expressions in control/scramble HT1376 cells and in siCD147 HT1376 cells showing that CD147 downregulation was accompanied by a decrease in MCT1 and MCT4 expressions (molecular weights: 50-60 kDa for the highly glycosylated and 42 kDa for low glycosylated form of CD147, 50 kDa for MCT1, and 52 kDa for MCT4). **B**, effect of CDDP on the viability of scramble and siCD147-HT1376 cells, as detected by the MTS assay after 72 hours of treatment, showing that siCD147 cells were more sensitive to CDDP. Results are expressed as the mean±standard deviation of at least 3 independent experiments, each one in triplicate.

values for parental-HT1376 and siScramble-HT1376 cells is due to the different number of cells plated per well).

DISCUSSION

Radical cystectomy with bilateral lymphadenectomy provides a cure for most of the UBC patients with muscle-invasive organ-confined lesions [6], but regional lymph node and visceral metastases are frequently found; in these cases, perioperative chemotherapy in fit patients is mandatory [5]. Multidrug platinum-based regimens provide the best response rates. Cisplatin is the main component of the MVAC (methotrexate, vinblastine, adriamycin and cisplatin) and GC (gemcitabine and cisplatin) combinations generally used to treat MI-UBC patients [42-44]. This alkylating agent has DNA as its primary cellular target. After entering the cell, cisplatin is activated by the replacement of its two chloride ligands with water molecules, being thereafter able to react with the N7-sites of purine bases in DNA, forming inter- and intra-strand crosslinks, and monofunctional adducts, which will eventually lead to apoptotic cell death [45-46]. Cisplatin exerts clinical activity against several solid malignancies, namely testicular, bladder, ovarian, colorectal, lung and head and neck cancers [47-49]. However, many patients are intrinsically resistant to cisplatin-based regimens, while others are initial responders but will eventually develop resistance [50-51]. Patient fragility is also an important limitation, due to the severe citotoxicity of cisplatin [52-54]. Still, intrinsic or acquired chemoresistance is the major drawback to its clinical usefulness, and UBC is not an exception [55-56].

Although poorly explored in UBC setting, the influence of the metabolic transformation events that alter the tumour microenvironment and thus mediate malignant progression and dissemination is gaining particular attention. In fact, solid malignancies are characterized by hypoxic regions and increased anaerobic and aerobic glycolysis, acidic-promoting conditions that facilitate metastasis and chemoresistance [18, 57-59]. In order to further unravel the role of microenvironment-related molecules in bladder cancer, we initiated our study by characterizing the clinicopathological and prognostic significance of MCT1, MCT4, CD147, CD44 and CAIX in a cohort of 114 UBC patients.

To our knowledge, this is the first study evaluating MCTs expression in bladder tumour tissue. We found a considerable percentage of tumour sections positive for MCT1 and MCT4. The malignant cells were stained in the cytoplasm and/or in the plasma membrane. The biomarkers were largely absent in the non-neoplastic sections. Plasma membrane expression was only relevant for MCT1, which probably indicates that this isoform is essential for the transport of lactate from the malignant glycolytic cells to the extracellular milieu. Additionally, the cytoplasmic expression found for both biomarkers

possible denotes their accessory role in the metabolism of UBC cells, by transporting monocarboxylates, namely lactate and pyruvate, across the membranes of cellular organelles. In fact, MCT1 and MCT4 have also been localized in the mitochondrial membrane [60-62]. UBC patients with positive tumours, particularly for MCT1, displayed unfavourable clinicopathological profiles. A near significant association was found between MCT1 expresion and poor prognosis. Therefore, it seems that MCT1 and MCT4 overexpression contributes to bladder cancer aggressiveness. In accordance, MCTs upregulation has also been observed in other malignant contexts, namely colorectal [40, 63-64], breast [40, 65], lung [40, 66] and prostate [67-68] carcinomas, glioblastomas [69-70] and ginecologic tract malignancies [40, 71-72].

In vivo and *in vitro* studies have described CD147 has a chaperone for MCT1 and MCT4 [15, 73-75], which was similarly supported by immunoexpression studies with human tissues [40, 65, 68-69, 76-77]. In our UBC cohort, MCT1 and MCT4 expressions were also significantly correlated with CD147 expression. Besides its function as a chaperone, CD147 directly promotes the malignant phenotype, being upregulated in several tumour types [40, 68-69, 77-79]. We have previously demonstrated that CD147 overexpression, included in a model of UBC aggressiveness, facilitates the discrimination of bladder cancer patients' prognosis [25]. In the current study, we evaluated CD147 expression in a larger and more comprehensive UBC series, which allowed us to further confirm our previous findings. In fact, CD147 was upregulated in bladder tumour tissue, significantly associating with tumour aggressiveness and lowering 5-year disease-free and overall survival rates. In accordance, a few studies have identified CD147 expression in UBC as an independent prognostic factor [22-24], being able to predict response to cisplatin-containing regimens [24]. In our cohort, the concurrent expression of MCT1 and CD147 significantly associated with unfavourable clinicopathological parameters and poor prognosis. Other studies with distinct malignancies have demonstrated that the prognostic value of CD147 is associated with its co-expression with MCT1 [65, 76]. MCTs seem to be necessary for proper membrane expression of CD147 [74, 80], and a cooperative role between the two types of biomarkers in determining chemotherapy resistance has been proposed [30, 72]. Importantly, the CD147/MCT1 double-positive profile discriminated, in our UBC cohort, a poor-prognosis group within patients who received platinum-based chemotherapy. Thus, besides acting as lactate transporters and pH regulators [16, 60], MCTs may also play indirect roles in angiogenesis, invasion, malignant dissemination and chemoresistance, by regulating and interacting with CD147 [7, 14]. It has been described that CD147 enhances tumour growth and chemoresistance via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in a hyaluronan-dependent manner [81]. In fact, CD147 stimulates hyaluronan production [11]. Besides its important structural function, this ubiquitous glycosaminoglycan plays also instructive roles in signalling via binding to specific cell-surface receptors, namely CD44 [82-83]. CD44 is a multifunctional transmembrane glycoprotein involved in cell adhesion and migration [84]. In our study, we observed that the majority of the UBC samples expressed CD44, mainly at the plasma membrane, which was significantly correlated with tumour progression. These results are in agreement with those obtained by other authors [26-27, 85]. Moreover, there was a substantial concordance between plasma membrane expression of MCTs and CD44, on one hand, and CD147 and CD44, on the other hand. It has been demonstrated that CD44 co-localizes with MCT1, MCT4 and CD147 at the plasma membrane of breast carcinoma cells, and that constitutive interactions among hyaluronan, CD44, and CD147 contribute to regulate MCTs localization and function. In fact, disruption of hyaluronan-CD44 signalling led to MCTs internalizetion and attenuation of their function [12]. Our results seem to support that theory. We may hypothesise that this interactive profile points out for a probable partnership between CD44, MCTs and CD147 in regulating the hyper-glycolytic and acid-resistant phenotype, and also chemotherapy resistance. CD147 stimulates hyaluronan production [11], but lactate – the end product of glycolysis extruded from the malignant cells through MCTs – also induces synthesis of hyaluronan and expression of CD44 variants in stromal [86] and tumour cells [87]. Moreover, hyaluronan-CD44 binding influences the activity of several downstream signalling pathways, namely the anti-apoptotic MAPK (mitogen-activated protein kinase) and PI3K-Akt pathways, consequently promoting tumour cell proliferation, survival, motility, invasiveness, and chemoresistance [88-89]. A few studies have shown that hyaluronan-CD44 signalling promotes cisplatin resistance in head and neck, and in lung cancers [90-93]. The aforementioned pathways seem to mediate the increased expression of multidrug membrane efflux

pumps of the ABC family, such as MDR1 (multidrug resistance protein 1) and MRP-1 (multidrug resistance-associated protein-1) [94-96]. However, MDR1 and MRP-1 do not seem to influence tumour response to cisplatin [97-98]. Other chemoresistance-mediating hyaluronan-dependent mechanisms have been described, namely EGFR (epidermal growth factor receptor)-mediated oncogenic signalling [90], or acquisition of cancer stem cell properties due to CD44 interaction with cancer stem cell markers and subsequent activation of microRNAs [93]. Additional studies are necessary to further clarify how cell surface interactions among hyaluronan, CD44, CD147 and MCTs contribute to initiate molecular responses that impair chemotherapy – namely cisplatin – effects.

In our immunohistochemistry study, we also evaluated CAIX expression. This catalyst mediates the reversible hydration of cell-generated carbon dioxide to bicarbonate and protons, activity that promotes intracellular pH regulation and extracellular trapping of acid. Thus, CAIX clearly contributes to the generation of the acid-resistant phenotype under hypoxic conditions [99]. We did not observe CAIX expression in the non-neoplastic tissues, but the vast majority of the UBC samples expressed this biomarker, and a heterogeneous pattern was noted, with the luminal face of NMI papillary tumours and the core of MI tumours being intensely stained. CAIX positivity was predominant in high grade papillary lesions, and seemed to associate with a low aggressiveness profile. Several authors have also reported a higher expression of CAIX in NMI than in MI tumours [29, 100-101], although their reports generally pointed out for an association between CAIX upregulation and occurrence of recurrence, progression and poor overall survival. In the study by Hussain et al. [100], there was a tendency towards longer survival for patients with tumours expressing CAIX strongly. Probably, in their study, as well as in our cohort, the high rate of CAIX expression in papillary lesions influenced the clinicopathological and survival data. Interestingly, significant associations were found when we compared immunoreactive samples for MCT4, CD147 and CD44 with CAIX plasma membrane positive cases. These results most likely reflect the adjustment to a hypoxia-mediated glycolytic metabolism that upregulates MCTs and their chaperones, and thus contributes to an acid-resistant microenvironment that favours tumour dissemination and impairs chemotherapy response.

Our important results on the prognostic and platinum-response discriminatory significance of CD147 in UBC patients led us to further explore its biological role in an *in vitro* assay. We started by confirming the expression of CD147, MCT1 and MCT4 in four parental UBC cell lines. We then characterized the effect of cisplatin treatment on cell viability, cell cycle distribution and cell death, as well as on the migration and invasion abilities of the cell lines. Different and controversial responses were obtained, mostly in the migration and invasion assays, which probably reflect the natural heterogeneity in UBC pathology, biology and response to treatment. Overall, the NMI 5637 cell line and the MI T24 cell line were the most sensitive to cisplatin treatment, as observed by the effective decrease in cell viability, the increase in S and subG1 phase cell populations, and the higher apoptotic rate. Similar results were obtained by Pinto-Leite et al. [102]. The MI HT1376 and MCR cell lines were less sensitive to cisplatin treatment, and the drug seemed to exert a cytostatic effect on these cells. Based on these observations, we downregulated CD147 expression on MCR and HT1376 cells using the RNA interference (siRNA) approach, although we were not able to conclude the assay with MCR cells, due to technical limitations. CD147 downregulation in HT1376 cells was accompanied by a marked decrease in MCT1 and MCT4 expres-sions, confirming that MCTs rely on CD147 for their proper expression and function. Moreover, CD147 downregulation clearly increased chemosensitivity to cisplatin, which supports the hypothesis that this multifunctional protein mediates chemoresistance in UBC. In accordance, Wang et al. [103] and Zhu et al. [104] used a similar RNA interference approach in gastric and laryngeal cell lines, and also demonstrated that suppression of CD147 expression sensitizes cells to cisplatin. These results indicate that CD147 may be a promising therapeutic target for malignancies frequently hampered by cisplatin resistance, and additional *in vitro* and *in vivo* studies are demanded to clarify the molecular mechanisms involved in this biological scenario, namely the cooperation with MCTs.

In summary, our findings indicate that microenvironment-related molecules, particularly CD147 and MCT1, are implicated in bladder cancer progression and resistance to cisplatin-based chemotherapy, unraveling new possibilities for target therapeutic intervention. CD147 and MCT1 should be further explored as potential theranostics biomarkers.

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CHAPTER 8 | General Discussion

8.1. Contribution to the State of the Art – An Overview

While, in the past, etiology of heterogeneous clinical behavior and response to treatment in cancer patients has eluded science, currently there is no doubt that prognostic and/or predictive biomarkers will eventually guide clinical-decision making. In fact, the extraordinary progresses achieved in cancer genetics and genomics are positively affecting the management of solid tumours. This important step towards personalized medicine has already allowed significant survival benefits and improvements in the quality of life of numerous patients, such as breast cancer patients with HER2 (human epidermal growth factor receptor 2)-positive tumours treated with trastuzumab, or advanced non-small-cell lung cancer patients harbouring specific EGFR (epidermal growth factor receptor) mutations and, thus, selected for gefitinib and erlotinib treatments [1-2]. Conversely, although urothelial bladder carcinoma (UBC) is relatively genetically well-characterized, it has largely been excluded from validation trials on potential biomarkers. Due to its unique divergent natural history among epithelial malignancies [3-4], UBC represents a major challenge in the oncology field, and this clearly reflects the delay in translating biology into the clinic [5-7]. However, areas in which biomarkers may prove valuable are evident, encompassing the three most important risk factors that threaten survival and life quality of bladder cancer patients [8]. First, the majority of UBCs emerge as non-muscle invasive (NMI), low grade, papillary lesions. Due to their high risk of recurrence, current guidelines recommend intense follow-up that classically relies in invasive techniques such as cystoscopy and biopsy, causing significant patient discomfort and implicating substantial costs. Thus, prediction of tumour recurrence through non-invasive methods would be of great value [9]. Second, an important proportion of NMI tumours, such as high grade or carcinoma *in situ* lesions, incur at an increased risk of progression to muscle-invasive (MI) disease. Timely prediction of progression would guide a vigilant surveillance, and would help clinicians to identify patients in need of early, aggressive management, while avoiding over-treatment in others [10]. Third, the risk of metastasis is the main pitfall for MI-UBC patients, and the majority of bladder cancer deaths occur as a consequence of metastatic disease [11]. In this scenario, robust biomarkers could help to identify circulating or lymph-node occult micrometastases, could represent potential therapeutic targets, and could forecast and stratify responses to conventional cytotoxic therapies or to emerging targeted therapies (the so called companion biomarkers) [7, 12-14]. Hence, UBC represents a considerable opportunity and challenge for biomarkers' research.

In the last years, efforts have been taken to uncover prognostic and/or predictive biomarkers that

might be useful in the clinical care of UBC patients. Traditional approaches of single-molecule or single-pathway profiling are being replaced by investigations on panels of biomarkers encompassing several hallmarks of cancer [6, 8, 15-17]. While the few biomarkers of potential clinical relevance that have been identified so far are mainly related to the key molecular pathways of bladder tumourigenesis [e.g. *FGFR3* (fibroblast growth factor receptor 3) and *TP53* (tumour protein p53) mutations] [8, 17-18], there is the need to expand the research into poorly explored scenarios of the malignant phenotype, in an attempt to unveil novel promising markers that can be integrated into a molecular signature with accurate prognosis and predictive power. A cancer-related biomarker must be a molecule produced by the tumour, detectable and measurable in patient specimens (tissue, blood or urine), representative of various tumour properties, and reproducible, specific and sensitive [8, 19]. Immunohistochemical approaches in tissue arrays are well suited for the detection task, by being practical methods that can easily allow the translation of new described biomarkers into clinical practice [17, 20]. In this line of investigation, we used immunohistochemistry to study, in a cohort of well-characterized UBC samples, the clinical and prognostic significance of several poorly studied putative biomarkers encompassing and overlapping three hallmarks of cancer: inducing tumour angiogenesis (and lymphangiogenesis), activating invasion and metastasis, and reprogramming cellular energetics and the tumour microenvironment. We additionally performed validation assays with bladder cancer cell lines. Our research efforts have resulted in important findings concerning some biological parameters that seem to influence bladder cancer aggressiveness and chemoresistance, and thus should be further explored as potential prognosis and predictive biomarkers, as well as new therapeutic targets.

8.1.1. Tumour Angiogenesis and Lymphangiogenesis

The role of angiogenesis in UBC is well established. Both VEGF (vascular endothelial growth factor) levels and high blood vessel density (BVD) counts independently predicted progression and lymph node metastasis, significantly lowering survival rates [21-25]. Large scale approaches have also confirmed VEGF as an independent prognosis factor [26]. Moreover, although studies on lymphangiogenesis occurrence and its usefulness in urothelial malignancies are fewer in number, the general tendency points out for an important task of lymphatic vessel formation in malignant dissemination [27-29]. VEGF-C levels were associated with high lymphatic vessel density (LVD) counts, predicting lymph node metastasis [29-32]. Both blood and lymphatic vessels participate in the metastatic cascade, and lymphovascular invasion (LI) has been identified as an independent prognostic factor for recurrence and

overall survival [33-35]. Importantly, it has been demonstrated that the LI status helps to stratify N0 UBC patients who are at increased risk of bladder cancer recurrence and death [35-37]. Despite these important associations, LI occurrence is not routinely described on the pathology reports, due to the lack of diagnosis reproducibility [38-39].

In our research, we assessed angiogenesis, lymphangiogenesis and lymphovascular invasion occurrence in a series of 83 UBC tissue sections from patients who underwent radical cystectomy (CHAPTER 3, [40]). An immunohistochemical method was used to differentiate between blood and lymphatic endothelial cells. Although we aimed to confirm previous findings on angiogenesis and lymphangiogenesis preponderance in UBC setting, our main goal was to investigate different ways of counting vessel invasion. Thus, we did observe that tumour neovascularization occurrence determines bladder cancer aggressiveness, although no significant association with outcome variables was found. While contradicting a few prior reports [22-25], others have also failed to demonstrate correlations among BVD and prognosis [41], and it has been advocated that, due to the inconsistency among various studies, BVD alone does not capture the real effect of angiogenesis occurrence on tumour progression and metastasis [18, 42]. On the other hand, in our study it was noted that intratumoural lymphatic vessels, described as collapsed and non-functional by some authors [43-46], had visible lumens in a significant proportion of cases, and no edema was observed, which supports an efficient lymphatic flow. Moreover, these intratumoural vessels, when functional, seem to actively cooperate in malignant dissemination, as observed by the presence of single malignant cells in the well-preserved intratumoural lymphatic vessels, which portended a low overall survival rate. Similar results have been obtained by others [28]. Regarding our major aim – to evaluate different methods of quantifying vessel invasion – we obtained interesting results. The specific staining of blood and lymphatic endothelium significantly contributed to an accurate evaluation of LI occurrence, and to a specific distinction between blood vessel invasion (BVI) and lymphatic vessel invasion (LVI). This was particularly important in the accurate detection of isolated malignant cells invading lymphatic capillaries, which have a higher propensity to survive in the lymphatic flow, when comparing with the rigors of the blood circulation. In fact, malignant emboli – easily detectable in hematoxylin and eosin (H&E) stained sections if no stromal retraction is observed – are more prone to invade the chaotic and hyperpermeable structure of the blood vasculature and to overcome the hostilities inherent to blood flow, such as serum toxicity, high shear stress and mechanical deformation [47-48]. Conversely, lymph flows slowly, and has a composition similar to interstitial fluid, being ideal for the survival and dissemination of single malignant cells [49-50]. These are more difficult to detect in H&E sections. Thus, and according to our results, the

specific staining of lymphatic endothelium contributes to accurately diagnose LVI occurrence, which significantly impairs overall survival, as well as BVI by malignant emboli. BVI was identified as an independent prognostic factor in our cohort. In another study (CHAPTER 6, [51]) where we developed a model of bladder cancer aggressiveness by the combined analysis of clinicopathological – stage and grade – and biological – specifically highlighted BVI and LVI, and CD147 expression – parameters in 77 UBC patients, we found that BVI and LVI clearly contributed to separate between low and high aggressiveness groups. BVI and LVI occurrence may, therefore, represent potential prognostic biomarkers that can guide personalized selection of patients likely to benefit from perioperative chemotherapy regimens and/or targeted therapies. In accordance, a recent review has emphasized that LI should be routinely reported in the pathological report, and that immunohistochemistry identification of blood and lymphatic vessels should be employed in histologically equivocal cases for confirmation [39].

In order to further elucidate the role of lymphangiogenesis in urothelial malignancy, we additionally assessed VEGF-C and VEGFR-3 (VEGF receptor 3) expression in our cohort of 83 UBC patients (CHAPTER 3, [40]). Although others have found significant associations between VEGFR-3 expression, poor clinicopathological parameters and short disease-free survival [52], in our series VEGFR-3 was monotonously expressed by all tumour cases. VEGF-C overexpression was well-defined in the group of poor prognosis patients; however, no significant association with survival rates was found. Some authors have also failed to demonstrate correlations among VEGF-C and poor prognosis [52-53]. We and others [29-30] observed significant correlations among LVD counts and VEGF-C levels, confirming its role as a lymphangiogenic factor. Moreover, intratumoural BVD was considerable enhanced by VEGF-C overexpression, supporting the expression of its fully processed form, which also activates VEGFR-2, and induces angiogenesis [30, 54-56]. Recent *in vitro* and *in vivo* assays demonstrated that VEGF-C depletion suppresses malignant progression and lymph node metastasis, and enhances chemosensitivity of urothelial malignant cells [57-58]; more studies are being developed to unveil the inherent biological mechanisms [58]. Although VEGF-C has been proposed as a potential prognostic biomarker for UBC patients [59], caution is recommended due to some controversial results, and additional studies with larger and more comprehensive series are demanded.

Angiogenesis and lymphangiogenesis represent potential targets for therapeutic intervention in UBC setting, and several compounds targeting the most relevant neovascularization signalling pathways are being tested in clinical trials [60-61]. However, caution is recommended, due to the risk of refractoriness to VEGFs/VEGFRs signalling blockade. In fact, compensation mechanisms to VEGF

abrogation in UBC cells lines have been described [62]. In alternative, mTOR pathway, besides transducing signals that activate the translational machinery and promote cell growth [63], is also an important signalling mediator in hypoxia-induced angiogenesis [64]. Some rapamycin analogues have demonstrated anti-angiogenic effects in UBC pre-clinical [65] and clinical trials [66]. Nevertheless, the levels of mTOR activation in UBC tissue sections have been poorly explored, and controversial results were found [67-72]. We assessed phospho-mTOR (p-mTOR) levels in a series of 76 UBC sections with representative tumour and non-tumour (normal-like or hyperplasic) areas, where blood and lymphatic vessels were also stained by immunohistochemistry, in order to correlate angiogenesis and lymphangiogenesis occurrence with p-mTOR expression (CHAPTER 4, submitted results). No significant associations were found between the clinicopathological parameters and vascular density, and p-mTOR expression. Even though, we observed that p-mTOR decreased with increasing stage, and was lost from non-tumour to tumour urothelium, particularly in MI lesions, where immunoexpression was observed in a few spots of cells. Angiogenesis occurrence was impaired in pT3/pT4 negative tumours; conversely, pT3/pT4 positive cases had worse survival rates, as reported by other authors [67, 69]. In NMI tumours, p-mTOR was evenly distributed within the malignant urothelium, although staining was stronger at the superficial layers of cells, resembling the pattern of expression that was observed in the non-tumour urothelium, where p-mTOR expression was restricted to umbrella cells and some superficial cells of the intermediate layer. This pattern of expression has been similarly described in other studies [69, 73]. We hypothesized that umbrella cells from non-tumour urothelium express p-mTOR constitutively, as part of their metabolic plasticity, and that NMI lesions with increasing malignant potential extend immunoexpression to the inner layers. The two patterns among MI tumours – absence of expression or expression in cell clusters – probably indicate divergent biological scenarios encompassing the mTOR pathway. Our preliminary results need to be further explored, and the next step will be to assess the immunoexpression of the remaining upstream and downstream actors of the mTOR pathway.

8.1.2. INVASION AND METASTASIS

High risk NMI and, more often, MI-UBC, carry a significant threat of invasion and metastasis despite radical surgical treatment [11]. Timely detection of biomarkers that enable malignant cells with invasive and metastatic properties would allow identifying patients that could benefit from early aggressive approaches such as radical cystectomy and perioperative chemotherapy, and would guide

the development of targeted therapies. In the pursuit of these objectives, we studied the immunoexpression of the endoglycosidase heparanase in a cohort of 77 UBC patients (CHAPTER 6, [51]). Heparanase cleaves heparan sulfate into smaller fragments, regulating the functions of this highly sulfated polysaccharide abundantly present in the extracellular matrix [74-75]. We observed that heparanase was upregulated in malignant urothelium, and exhibited a heterogeneous pattern, with the invasion front of the tumours being more intensely stained than the tumour core, which apparently supports its role in the disassembly of the extracellular matrix. However, heparanase immunorreactivity did not reveal any clinicopathological and prognostic information in our series. Conversely, other authors have demonstrated that heparanase overexpression associates with tumour progression, high BVD, invasion, metastasis, and poor prognosis [76-78], and its depletion in *in vitro* assays significantly inhibited those traits of malignancy [79-80]. Therefore, although our results do not support that hypothesis, heparanase may represent a new prognostic biomarker, and additional studies are necessary to validate such potential function.

While inhibiting biomarkers of invasion and metastasis emerges as an attractive therapeutic strategy, restoring the function of metastasis suppressor proteins is not less appealing. The preponderance of the metastasis suppressor RKIP (Raf kinase inhibitor protein) in UBC setting is largely unknown, although low mRNA levels have been reported in NMI tumours, when compared with normal urothelium [81]. We evaluated RKIP expression in a cohort of 81 tumour sections from UBC patients. Blood and lymphatic vessels were also immunostained, in order to correlate BVI and LVI occurrence with RKIP levels (CHAPTER 5, [82]). To the best of our knowledge, this is the first study evaluating RKIP immunoexpression in bladder cancer tissue samples. We observed a homogeneous expression of RKIP in normal urothelium and in tumour sections with a favourable clinicopathological profile, namely NMI tumours where LVI was absent. Conversely, a heterogeneous pattern of expression, with loss of RKIP expression intensity from the tumour centre to the invasion front, was associated with LVI occurrence. Moreover, low RKIP expression significantly lowered disease-free and overall survival, remaining as an independent prognostic factor for disease-free survival. RKIP loss or diminution had been previously reported in other types of aggressive cancers, significantly impairing prognosis. Clinically, RKIP expression is higher in benign tumors than in malignant tissues while its expression is completely absent in metastases [83]. Additional studies in bladder cancer setting need to be urgently developed, in order to confirm our promising results and to expand the research into therapeutic strategies that can potentially restore RKIP functionality. Besides acting as a prognostic biomarker, RKIP status may also

have a role as a predictive biomarker, once it has been demonstrated that its expression may potentiate apoptosis induced by chemotherapeutic agents, which might be useful in defining therapy response profiles [84-85].

8.1.3. ENERGY METABOLISM REPROGRAMMING AND THE TUMOUR MICROENVIRONMENT

Altered energy metabolism, although only recently emerged as a new hallmark of cancer [86], is proving to be as widespread in tumour cells as the classical traits of malignancy. In fact, cancer growth is characterized by deregulated cell proliferation and corresponding adjustments of energy metabolism, such as the adoption of the Warburg effect. This necessarily involves different inputs to the tumour microenvironment, namely the extrusion of high amounts of lactate from the malignant cells that will sculpt an acid-resistant phenotype, which supports increased migration and invasion, favouring metastasis [87-89]. Molecules and pathways involved in this intricate backstage of malignancy potentially represent new areas of therapeutic intervention.

The biological mechanisms that reprogram cellular energetics and model the tumour microenvironment are poorly characterized in bladder cancer. Thus, we elected a panel of five microenvironment-related molecules and investigated their expressions in a subset of tumour tissue sections from 114 UBC patients treated by transurethral resection and/or radical cystectomy (CHAPTER 7, submitted results). The central player was CD147, a tumor cell surface molecule implicated in extracellular matrix remodeling, angiogenesis and tumour growth, and related with chemoresistance-promoting events [90-91]. We had previously demonstrated the prognostic impact of CD147 overexpression in bladder cancer patients, when we developed a model of UBC aggressiveness that included clinicopathological and biological parameters (CHAPTER 6, [51]). In fact, CD147 expression was largely preponderant in the high aggressiveness group, and clearly added prognostic information to the model. For that reason, we decided to re-evaluate this glycoprotein in a larger series, together with other molecular companions. Thus, we observed that CD147 was upregulated in bladder tumour tissue, significantly associating with a dismal clinicopathological profile and poor prognosis. Other authors have identified CD147 expression in UBC as an independent prognostic biomarker [22-24], and have additionally proposed it as a predictive biomarker in the setting of cisplatin-containing regimens [24]. To confirm this hypothesis, we established four CD147-expressing UBC cell lines and studied the effect of cisplatin treatment on cell viability, cell cycle distribution and cell death, as well as

on the migration and invasion abilities of the cells. CD147 expression was then downregulated in a cisplatin less-sensitive cell line. Importantly, we found that CD147 downregulation clearly increased chemosensitivity to cisplatin. To the best of our knowledge, this was the first *in vitro* study demonstrating that CD147 depletion in UBC cells enhances the therapeutic action of cisplatin, highlighting this molecule as a potential prognostic and predictive biomarker.

In order to further elucidate CD147 interactions, we also analyzed monocarboxylate transporter (MCT) expressions in the cohort of 114 UBC patients (CHAPTER 7, submitted results). MCTs, particularly MCT1 and MCT4, play a key role in the promotion of the hyper-glycolytic acid-resistant phenotype, by exporting lactate from the glycolytic malignant cells to the tumour microenvironment [92]. CD147 has been described as a chaperone for the proper expression of MCTs at the plasma membrane [93-94], and our results support that function. In fact, we found significant associations among MCT1, MCT4 and CD147 expressions. MCT1 and MCT4 were upregulated in highly aggressive tumours, and MCT1 overexpression impaired overall survival. Although no studies with MCTs in bladder cancer have been reported so far (to the best of our knowledge), their upregulation has been observed in other malignancies [95-96]. Interestingly, a CD147 and MCT1 double-positive profile was significantly associated with unfavourable clinicopathological parameters and poor prognosis in our UBC series, and discriminated a poor prognosis group in cisplatin-treated patients. We hypothesized that MCT1 cooperates with CD147 in the promotion of a chemoresistance phenotype and, possibly, of other functions that are primarily attributed to CD147. In fact, it appears that CD147 maturation is affected by MCT expression [97-98]. In our *in vitro* study, CD147 depletion was accompanied by a marked decrease in the expression of MCT1 and MCT4, which suggests CD147 as an MCT1/4 chaperone. It would be interesting to silence MCT1 expression in the UBC cell line and to study CD147 expression levels, in order to confirm the opposite.

CD44 levels were also investigated in our UBC series (CHAPTER 7, submitted results), because this hyaluronan-receptor involved in cell adhesion and migration [99] also seems to cooperate with CD147 in the chemoresistance milieu. This is thought to occur through CD44-hyaluronan interaction, with multidrug resistance arising in CD147-overexpressing cells, in a hyaluronan-dependent manner [100]. In agreement with other authors [101-102], we observed that CD44 expression significantly correlated with UBC progression, and the concordance between expression of MCTs and CD44, and of CD147 and CD44, is allusive to a possible partnership among these biomarkers, which has also been suggested by others [103].

Finally, we studied the immunoexpression of the hypoxia marker CAIX (carbonic anhydrase 9)

(CHAPTER 7, submitted results). Hypoxia has been described as a trigger mechanism of the hyper-glycolytic phenotype [87], and CAIX promotes intracellular pH regulation and extracellular trapping of acid by mediating the reversible hydration of cell-generated carbon dioxide to bicarbonate and protons [104]. A few studies have demonstrated CAIX upregulation in bladder cancer, although expression levels are generally higher in NMI that in MI lesions [105-107]. We found similar results, and the pattern of expression – stronger at the core of infiltrative tumours – clearly suggests the occurrence of hypoxia in regions were the blood supply is limited. Moreover, significant associations were observed when comparing immunoreactive samples for MCT4, CD147 and CD44, with CAIX plasma membrane positive cases, which probably reflect the adjustment to a hypoxia-mediated glycolytic metabolism where MCTs and their chaperones support microenvironment tumour remodeling.

Overall, our results point out for an important role of CD147 and their companions in promoting a highly aggressive phenotype where glycolysis is upregulated, contributing to acidify the tumour microenvironment, enabling the malignant cells with growth, migration, invasion and chemoresistance abilities that can only be overcome if new approaches of target therapeutic intervention are investigated.

8.2. Combining Pathology and Biology – Is it useful for Urothelial Bladder Cancer Patients?

Currently, clinicians rely on the AJCC (American Joint Committee on Cancer) TNM (tumour-node-metastases) staging system [108] and on the WHO (World Health Organization) grading guidelines [109] to diagnose the disease and to predict outcomes. While representing irreplaceable diagnostic and prognostic tools, these staging and grading schemes fail to capture the real heterogeneous nature of bladder tumours. Risk stratification scores have been developed to predict recurrence and progression of NMI tumours after transurethral resection, namely the EORTC (European Organization for Research and Treatment of Cancer) [110] and the CUETO (Club Urológico Español de Tratamiento Oncológico) [111] tables. Additionally, nomograms that predict recurrence of MI tumours after cystectomy have also been tested in large UBC series, with significant improvements in the predictive accuracy over AJCC and WHO systems [112-114]. Artificial neural networks have also surpassed the classical clinical classifications in predicting outcomes [115-116]. However, the lack of information that reflects the individual tumour biology strongly limits the personalized management of patients with bladder cancer. Inclusion of prognostic and predictive biomarkers in the risk stratification tables, nomograms and artificial neural networks would certainly refine diagnosis, prognosis and therapeutic decisions [117-118].

In our research, we developed a model of tumour aggressiveness by the combined analysis of two clinicopathological parameters – stage and grade – with three biological parameters – BVI, LVI and CD147 overexpression (CHAPTER 6, [51]). The parameters included in the model had individual prognostic impact on the 77 UBC patients that were studied, as demonstrated in univariate analysis. However, the model was stronger in predicting prognosis, clearly separating a low aggressiveness from a high aggressiveness group, and remaining as an independent prognostic factor for disease-free and overall survival. Accordingly to our results, other authors have also demonstrated the potential impact of developing risk stratification tools that integrate clinicopathological and biological parameters. Moreover, it seems that combining biomarkers inherent to different cancer hallmarks improves predictive accuracy over one biomarker abnormality, as several biomarkers may help to elucidate individual biological features of the tumours [10, 15, 17, 20, 119-122]. In our scoring model, we included biomarkers that are manly associated with angiogenesis (BVI), lymphangiogenesis (LVI), energy metabolism reprogramming, invasion and chemoresistance (CD147). If an additional biomarker was included in the

model, namely immunoexpression of the metastasis suppressor RKIP, its accuracy would be further enhanced (data not shown). Therefore, combining pathology with biology will have undeniable impact for UBC patients, who may benefit, in the future, from accurate prediction of outcomes and response to therapy, and guided targeted therapy. There is the urgent need to transpose biomarker tests on small groups of patients to large-scale independent validation assays, encompassing multi-institutional collaborations, so that prospective validations and randomized trials based on the retrospective findings may then proceed [123].

8.3. LIMITATIONS OF THE RESEARCH

When interpreting the results of our research, it is important to acknowledge the limitations of each study. Thus, studies reported in chapters 3 to 6 had small sample sizes, and this necessarily reflects on the results. For instance, CD147 expression was first evaluated in a series of 77 patients, and its predictive power of outcome was restricted to pT3/pT4 tumours (CHAPTER 6, [51]). When we evaluated its expression in a larger series (114 UBC patients), CD147 arose as an important prognostic and predictive biomarker (CHAPTER 7, submitted results). Second, all of the patients included in the studies that have undergone radical cystectomy had limited pelvic lymphadenectomy, with only a few lymph nodes being removed, which compromised additional immunohistochemical evaluations that could further elucidate the functions of the studied biomarkers. It would be of great value to evaluate BVD and BVI, LVD and LVI, VEGF-C expression, or RKIP expression in the lymph nodes, due to their association with vascular invasion and metastasis. Third, the studies suffer from limitations inherent to analyses conducted via hospital patient medical record review, typical of retrospective approaches, with patients being lost to follow-up, which contributes to the heterogeneity in the follow-up periods. Nevertheless, we tried to standardize the definition of our UBC cohorts, eliminating confounding variables. In the *in vitro* study, technical complications with a cisplatin-resistant cell line limited the CD147 silencing assay to only one cell line.

8.4. Overall Conclusions and Future Perspectives

Despite the limitations inherent to our research, several important results were obtained that deserve to be distinguished. Thus, angiogenesis and lymphangiogenesis occur both in peritumoural and intratumoural regions, and clearly contribute to metastatic spread. Our immunohistochemical method of quantifying blood vessel and/or lymphatic vessel invasion, based on the specific staining of blood and lymphatic endothelium, allows an accurate discrimination between the two forms of LI and, more importantly, allows identifying LI images that could be missed during the classical evaluation on H&E stained tumour sections. We and others [39] stand up for the use of this method in histologically equivocal cases that require confirmation. VEGF-C expression may represent a potential prognostic biomarker for angiogenesis and lymphangiogenesis occurrence, although additional studies with larger and well-characterized series are necessary. A complete immunohistochemical and molecular approach to the PI3K/AKT/mTOR pathway should also be addressed, in an attempt to further clarify our results on p-mTOR loss of expression in MI tumours. Heparanase expression was upregulated in malignant urothelium, but the lack of other clinical and prognostic information advocates analyzing its expression in a more comprehensive series. RKIP emerged as an important prognostic biomarker in our UBC cohort. Based on those results, other directions on the assessment of RKIP function as a metastasis suppressor in bladder cancer need be taken. *In vitro* downregulation of RKIP expression should be the next step, in order to assess the impact of RKIP abrogation on parameters of aggressive behaviour, such as migration, invasion and colony formation abilities of the malignant cells, and also on the response to chemotherapy, in an attempt to unveil its predictive power in the setting of bladder cancer.

One of our stronger results was the identification of CD147 as a prognostic and predictive biomarker for UBC patients. Moreover, other microenvironment-related molecules, namely MCT1, MCT4, CD44 and CAIX, seem to contribute to the malignant phenotype, possibly cooperating among them and with CD147 in the implementation of a hyper-glycolytic, acid-resistant phenotype that promotes invasion, metastasis and chemoresistance. It is recommend improving the technical approach regarding the *in vitro* assay, namely by newly establishing cisplatin resistance in several UBC cell lines by culturing them in cisplatin-containing conditioned medium for, at least, six months. Downregulation studies with the RNA interference technique would be certainly facilitated under those conditions, and additional assays should be performed in order to evaluate the effect of CD147 depletion on cell viability, migration, invasion and colony formation abilities of the cells, and on the

response to cisplatin. Once MCT1 seems to be a potential partner of CD147 in determining cisplatin resistance, protein interaction strategies should be explored, as well as co-expression analyses, and MCT1 expression could be dowregulated in cisplatin-resistant cells, in order to determine the effect of MCT1 depletion on CD147 expression, and on the promotion of an aggressive malignant phenotype and drug resistance. It will also be important to mimic the microenvironmental conditions where these molecules act on, namely acidity and hypoxia. *In vivo* studies would be better suited to represent the real tumour conditions, including nutrient and oxygen availability. Importantly, alternative inhibition strategies that could be potentially applied in clinical setting must be searched and explored in pre-clinical trials, since MCTs and CD147 represent not only promising prognostic and predictive biomarkers, but also potential targets for therapeutic intervention in bladder cancer patients. Toxicological studies to determine side-effects of the inhibition treatments should also be developed.

In summary, the results presented in this thesis particularly highlight the roles of BVI and LVI occurrence, and RKIP, CD147 and MCT1 expressions, as relevant prognostic and/or predictive biomarkers, and as promising areas of therapeutic intervention, eliciting for the development of additional studies that can validate and further explore the potentialities of our research.

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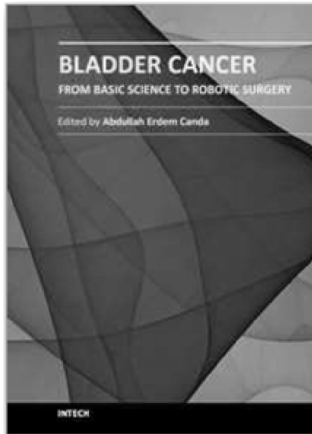
Appendix



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This book is an invaluable source of knowledge on bladder cancer biology, epidemiology, biomarkers, prognostic factors, and clinical presentation and diagnosis. It is also rich with plenty of up-to-date information, in a well-organized and easy to use format, focusing on the treatment of bladder cancer including surgery, chemotherapy, radiation therapy, immunotherapy, and vaccine therapy. These chapters, written by the experts in their fields, include many interesting, demonstrative and colorful pictures, figures, illustrations and tables. Due to its practicality, this book is recommended reading to anyone interested in bladder cancer.

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4

Angiogenesis, Lymphangiogenesis and Lymphovascular Invasion: Prognostic Impact for Bladder Cancer Patients

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1. Introduction

Bladder cancer is the second most common tumor of the urogenital tract. Urothelial carcinoma is the most frequent histologic type, being unique among epithelial carcinomas in its divergent pathways of tumorigenesis. Surgery continues to have a predominant role in the management of urothelial bladder cancer (Kaufman et al., 2009). However, the debate about the best treatment approach for T1G3 and muscle invasive tumors continually challenges all urologic surgeons and oncologists. This debate involves several aspects. First, a significant number of T1G3 tumors recurs and progresses rapidly after transurethral resection and BCG treatment (Wiesner et al., 2005). Second, half of patients with invasive tumors have a dismal outcome despite an effective treatment by radical cystectomy (Sternberg et al., 2007). Third, the extension of lymphadenectomy remains an issue of controversy, although clinical evidence suggests that an extended lymph node dissection may not only provide prognostic information, but also a significant therapeutic benefit for both lymph node-positive and lymph node-negative patients undergoing radical cystectomy (May et al., 2011). In muscle invasive bladder cancer, the presence of tumor foci in lymph nodes is an early event in progression, and the lymphatic vessels within or in the proximity to the primary tumor serve as the primary conduits for tumor dissemination (Youssef et al., 2011). Fourth, although urothelial bladder cancer is a chemo-sensitive tumor (Kaufman et al., 2000; von der Maase et al., 2000), adjuvant systemic chemotherapy does not reveal benefits (Walz et al., 2008), and neoadjuvant chemotherapy is not yet accepted as the best approach in invasive bladder cancer (Clark, 2009). Therefore, in order to solve the aforementioned problems, it is crucial to improve the knowledge about tumor microenvironment, regulation of cancer metabolism and neovascularization.
Blood and lymphatic neovascularization are essential for tumor progression and metastasis, by promoting oxygenation and fluid drainage, and establishing potential routes of dissemination (Adams and Alitalo, 2007). Therefore, the inhibition of tumor-induced neovascularization represents a powerful option for target therapy, in order to restrain the most efficient pathway of cancer spread.

2. Angiogenesis and Lymphangiogenesis: Molecular Regulation of Vasculature Development

During embryogenesis, the formation of the blood vascular system initiates by vasculogenesis: haemangioblasts proliferate, migrate and differentiate into endothelial cells, which in turn will organize a primitive vascular plexus. In parallel, angiogenesis promotes the remodeling and expansion of the primary capillary network, originating a hierarchical structure of different sized vessels that will mature into functional capillaries, veins and arteries (Risau, 1997). The lymphatic vascular system develops latter, when a group of blood endothelial cells differentiates into a lymphatic endothelium that subsequently sprouts to form the primary lymph sacs. By lymphangiogenesis, the lymphatic endothelial cells from the lymph sacs will further sprout, originating the peripheral lymphatic system (Sabin, 1902, as cited by Oliver & Detmar, 2002).
During postnatal life, blood and lymphatic vascular systems are, normally, in a quiescent state. Physiological angiogenesis and/or lymphangiogenesis occur to maintain or restore the integrity of tissues, namely during wound healing and the ovarian cycle. Conversely, the neovascularization machinery may be activated in pathological processes such as cancer and inflammatory diseases (reviewed in Lohela et al., 2009).
Similarly to physiological neovascularization, tumor-induced angiogenesis and/or lymphangiogenesis occur to satisfy the metabolic demands of a new tissue — the malignant tissue. Therefore, the molecular factors involved in the formation of the vascular systems during embryogenesis are newly recruited by the growing tumor (Papetti & Herman, 2002).

2.1. From Angiogenesis to Lymphangiogenesis in the Embryo

The proliferation, sprouting and migration of endothelial cells during vasculogenesis and angiogenesis is mainly guided by the vascular endothelial growth factor (VEGF) signaling through VEGF receptor-2 (VEGFR-2) (Risau, 1997).
VEGF (or VEGF-A), initially termed as vascular permeability factor (VPF) (Senger et al., 1983), is a specific mitogen and pro-survival factor for blood endothelial cells, also stimulating vascular permeability. It binds and activates two tyrosine kinase receptors primarily found on the blood endothelium: VEGFR-1 (or Flt-1, fms-like tyrosine kinase 1) and VEGFR-2 (or KDR/Flk-1, human kinase insert domain receptor/mouse foetal liver kinase 1) (reviewed in Carmeliet, 2005). Interaction of VEGF with VEGFR-1 negatively regulates vasculogenesis and angiogenesis during early embryogenesis (Fong et al., 1999). On the contrary, VEGFR-2 is the earliest marker for endothelial cell development: mouse embryos lacking VEGFR-2 die at embryonic day 8.5-9.5 due to no development of blood vessels as well as very low hematopoiesis (Shalaby et al., 1995). Regarding the ligand, even heterozygote mice for *Vegf* deficiency die at embryonic day 11-12: blood islands, endothelial cells and vessel-like tubes fail to develop (Carmeliet et al., 1996; Ferrara et al., 1996).
In humans, five weeks after fertilization, certain blood endothelial cells become responsive to lymphatic inducing-signals. The lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), a CD44 homologous transmembrane protein, is the first marker of lymphatic endothelial commitment. Initially, it is evenly expressed by the blood endothelium of the cardinal vein, which causes the blood endothelium to acquire the ability to differentiate in lymphatic endothelium (Banerji et al., 1999). The polarized expression of the prospero related homeobox gene-1 (Prox-1) transcription factor in a subpopulation of blood endothelial cells determines the establishment of the lymphatic identity and initiates the formation of the lymphatic vascular system. In mice, Prox-1 expressing cells are first observed at embryonic day 10 in the jugular vein (Wigle & Oliver, 1999). *Prox1* deletion leads to a complete absence of the lymphatic vasculature (Wigle et al., 2002). The expression of the transcription factor Sox18 [SRY (sex determining region Y) box 18] acts as a molecular switch to induce differentiation of lymphatic endothelial cells: it activates Prox-1 transcription by binding to its proximal promoter. Sox18-null embryos show a complete blockade of lymphatic endothelial cell differentiation (François et al., 2008).
Later, the sprouting, migration and survival of the newly formed lymphatic endothelial cells depends on the expression of VEGF-C by the mesenchymal cells surrounding the cardinal veins (Karkkainen et al., 2004) (Fig. 1).
VEGF-C, like VEGF, is a member of the VEGF family of growth factors and a mitogen for lymphatic endothelial cells. VEGF-D is also a pro-lymphangiogenic factor, although its deletion does not affect the development of the primitive lymphatic vessels (Baldwin et al. 2001). Conversely, in *Vegfc*$^{-/-}$ mice, Prox-1 positive cells appear in the cardinal veins, but fail to migrate and proliferate to form primary lymph sacs (Karkkainen et al., 2004). VEGF-C and VEGF-D interact with VEGFR-3 (of Flt-4, fms-like tyrosine kinase 4). Their affinity to VEGFR-3 is increased by proteolytic cleavage; the fully processed forms can also bind to VEGFR-2 (reviewed in Lohela et al., 2009).
VEGFR-3 is widely expressed at the early stages of embryonic blood vasculature, becoming virtually restricted to lymphatic endothelium in the later stages of embryonic development, (after the lymphatic commitment mediated by Prox-1 expression), and during adult life (Kaipainen et al., 1995). In mice, inhibition of VEGFR-3 expression at embryonic day 15 induces regression of the developing lymphatic vasculature by apoptosis of lymphatic endothelial cells (Makinen et al., 2001).
The subsequent development of the lymphatic vasculature involves the separation of the blood and lymphatic vascular systems, the maturation of lymphatic vessels and the formation of secondary lymphoid organs. The molecular regulation of these processes involves the coordinated expression of distinct genes from those involved

in the early events of lymphangiogenesis (reviewed in Alitalo et al., 2005) (Fig. 1). Moreover, several other growth factors, namely cyclooxygenase-2 (COX-2) fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs) and platelet-derived growth factor-B (PDGF-B) have been shown to induce lymphangiogenesis and/or angiogenesis in experimental models (reviewed in Cao, 2005). These are mainly protein tyrosine kinases, which play central roles in signal transduction networks and regulation of cell behavior. In the lymphatic endothelium, these tyrosine kinases are collectively involved in processes such as the maintenance of existing lymphatic vessels, growth and maturation of new vessels and modulation of their identity and function (Williams et al., 2010).

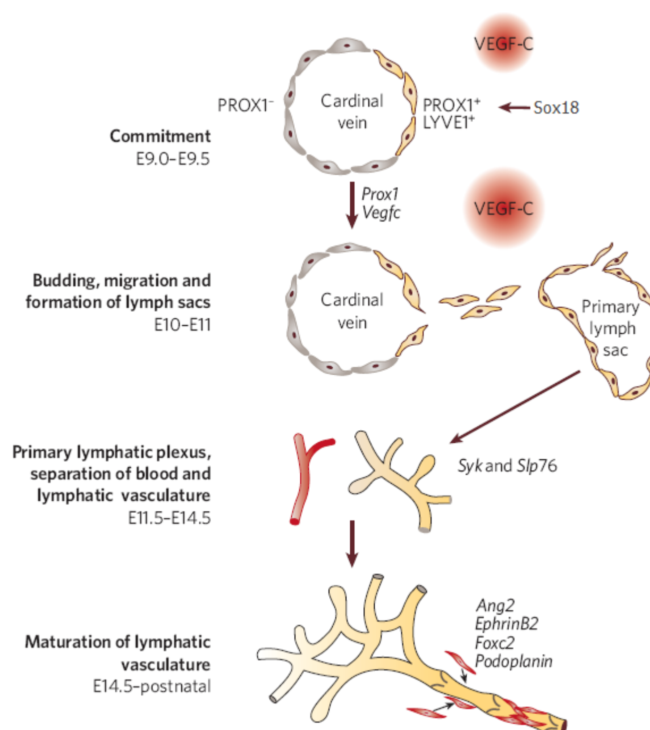

Fig. 1. Model for the development of mouse lymphatic vasculature (E- embryonic day; Syk- protein-tyrosine kinase SYK; Slp76- SH2 domain-containing leucocyte protein, 76-kDa; Ang2- angiopoietin 2; Foxc2- Forkhead Box C2) (adapted by permission from © 2005 Nature Publishing Group. Originally published in *Nature.* 438: 946-953)

2.2. Promotion of Angiogenesis and Lymphangiogenesis in the Malignancy Context

The major cause of cancer mortality is the metastatic spread of tumor cells that can occur via multiple routes, including blood and lymphatic vasculatures. For metastasis to occur, selected clones of malignant cells must be able to invade the newly formed vessels and disseminate. Induction of angiogenesis and/or lymphangiogenesis is, therefore, one of the first steps of the metastatic cascade (Alitalo & Carmeliet, 2002; Tobler & Detmar, 2006).

During the pre-vascular phase, the malignant tumor remains small (up to 1 or 2 mm^3); the preexistent surrounding blood vessels ensure the supply of oxygen and nutrients necessary for its survival. However, the expansion of the tumor mass is angiogenesis-dependent. As a compensatory response to hypoxia, proangiogenic factors such as VEGF are released by the malignant cells and infiltrating immune cells, namely monocytes. As a result, angiogenesis occurs and the tumor acquires its own blood supply. Neoplastic growth is thus promoted, as well as the potential for invasion and haematogenic metastasis (Kerbel, 2000).

Vegf is upregulated in hypoxia via the oxygen sensor hypoxia-inducible factor (HIF)-1α (Pugh & Ratcliffe, 2003). Another recently described VEGF activation mechanism is the induction of the transcriptional coactivator peroxisoma proliferator-activated receptor-gamma coactivator-1α (PGC-1α) in response to the lack of nutrients and oxygen (Arany et al., 2008). Additionally, VEGF gene expression can be upregulated by oncogene signaling, several growth factors, inflammatory cytokines and hormones (reviewed in Ferrara, 2004). Tumor cells secrete VEGF mainly in a paracrine manner, although it can also act in an autocrine manner to promote a protective/survival effect to endothelial cells, among other cell types (Brusselmans et al., 2005).

The mechanisms underlying tumor lymphangiogenesis are not clearly defined. Inflammation seems to promote lymphatic neovascularization: inflammatory cells that infiltrate in the growing tumor produce lymphangiogenic growth factors. Another lymphangiogenesis trigger mechanism may be the high interstitial pressure generated inside the tumors due to the excessive production of interstitial fluid (reviewed in Cao, 2005). On the other hand,

the extracellular matrix is of central importance for the generation of new lymphatic vessels as a response to the pathological stimulus. Integrins, a superfamily of cell adhesion molecules, are able to influence cell migration: integrin $\alpha 9\beta 1$ is a target gene for Prox1, and its direct binding to VEGF-C and VEGF-D stimulates cell migration (reviewed in Wiig, 2010).

VEGF-C and VEGF-D, via signaling through VEGFR-3, appear to be essential for tumor-associated lymphangiogenesis, leading to lymphatic vessel invasion, lymph node involvement and distant metastasis (reviewed in Achen & Stacker, 2008). Moreover, VEGF interaction with VEGFR-2 may also promote lymphatic neovascularization, namely inside the regional draining lymph nodes, even before lymph node metastasis occurrence. This probably corresponds to a pathophysiologic strategy of "soil" preparation by the primary tumor to ensure the success of its future dissemination (Hirakawa et al., 2005). In fact, sentinel lymph node metastasis is the first step in the spreading of many cancer types.

Preexisting blood and lymphatic vessels in the vicinity of the malignant mass may contribute to tumor spread. However, *de novo* formed vessels by tumor-induced angiogenesis and lymphangiogenesis seem to be the preferential routes for dissemination (reviewed in Cao, 2005). This is a consequence of the ultra-structure of the tumor-associated blood and lymphatic vessels.

2.3. Ultra-structure of Tumor-associated Blood and Lymphatic Vessels

Blood vessels present in malignant tissues show remarkable differences with vessels present in normal tissues. Tumor blood vessels are highly disorganized: they are tortuous, excessively branched and dilated. The basement membrane and the muscular coverage are incomplete or absent. The endothelial cells, abnormal in shape, overlap and are projected into the lumen rather than organizing a pavement layer below the basement membrane. Blood vessel invasion is facilitated by this aberrant structure, but the extravasation rate is high, and blood flow is variable. As a result, interstitial tumor hypertension occurs, and delivery of therapeutic agents into tumors is compromised (Jain & Carmeliet, 2001; reviewed in Cao, 2005). The intratumoral edema is pernicious to malignant cells; therefore, homeostasis needs to be re-established. The formation of a tumoral lymphatic vasculature could potentially resolve this problem.

The key function of lymphatic vessels is to collect the excessive amount of interstitial fluid back to the blood circulation for immune surveillance in lymph nodes. Unlike normal blood capillaries, lymphatic capillaries have a discontinuous or fenestrated basement membrane and are not ensheathed by pericytes or smooth muscle cells; the endothelial cells are arranged in a slightly overlapping pattern and lack tight interendothelial junctions. Specialized anchoring filaments of elastic fibers connect the endothelial cells to the extracellular matrix, which causes the vessels to dilate rather than to collapse when hydrostatic pressure rises (Alitalo et al., 2005; Tobler & Detmar, 2006). This structure facilitates the collection of interstitial fluid and is ideal for malignant cells' entry into the lymphatic flow.

A highly debated question is whether there are functional lymphatic vessels inside tumors (reviewed in Alitalo & Carmeliet, 2002; reviewed in Detmar & Hirakawa, 2002). On one hand, the elevated interstitial pressure generated by the proliferation of the malignant cells and by the high extravasion rate compromises the infiltration of new lymphatic vessels in the tumor stroma. Although intratumoral lymphangiogenesis may occur, the newly formed vessels are compressed and nonfunctional (Jain & Fenton, 2002). To compensate the lack of an intratumoral draining mechanism, the peritumoral lymphatic vessels enlarge due to an excess of pro-lymphangiogenic factors in that area. Therefore, in this model, the peritumoral lymphatic vessels passively collect interstitial fluid and, eventually, malignant cells (Carmeliet & Jain, 2000) (Fig. 2, A). However, some studies have demonstrated a relationship between the existence of functional intratumoral lymphatics, with cycling lymphatic endothelial cells and tumor emboli, and lymph node involvement (reviewed in Da et al., 2008). Additionally, peritumoral lymphangiogenesis occurs, and the new vessels actively contribute to metastatic spread (Padera et al., 2002) (Fig. 2, B). Probably, there are some organ-specific determinants that influence the occurrence of peritumoral and/or intratumoral lymphangiogenesis, as well as the function of the newly formed vessels.

2.4. Lymphovascular Invasion and Metastasis

Tumor metastasis involves a coordinated series of complex events that include promotion of angiogenesis and lymphangiogenesis, detachment of malignant cells from the primary tumor, microinvasion of the surrounding stroma, blood and/or lymphatic vessel invasion, survival of the malignant cells in the blood and/or lymphatic flow, and extravasion and growth in secondary sites. Because the large lymphatic vessels reenter the blood vascular system, malignant cells spread via the lymphatic system to the regional lymph nodes and, from this point, to distant organs (Alitalo & Carmeliet, 2002; Tobler & Detmar, 2006) (Fig. 3).

Follow-up data have shown that 80% of the tumors, mainly those of epithelial origin, disseminate through the lymphatic vasculature; the remaining 20% use the blood circulation to colonize secondary organs (reviewed in Saharinen et al., 2004; reviewed in Wilting et al., 2005).

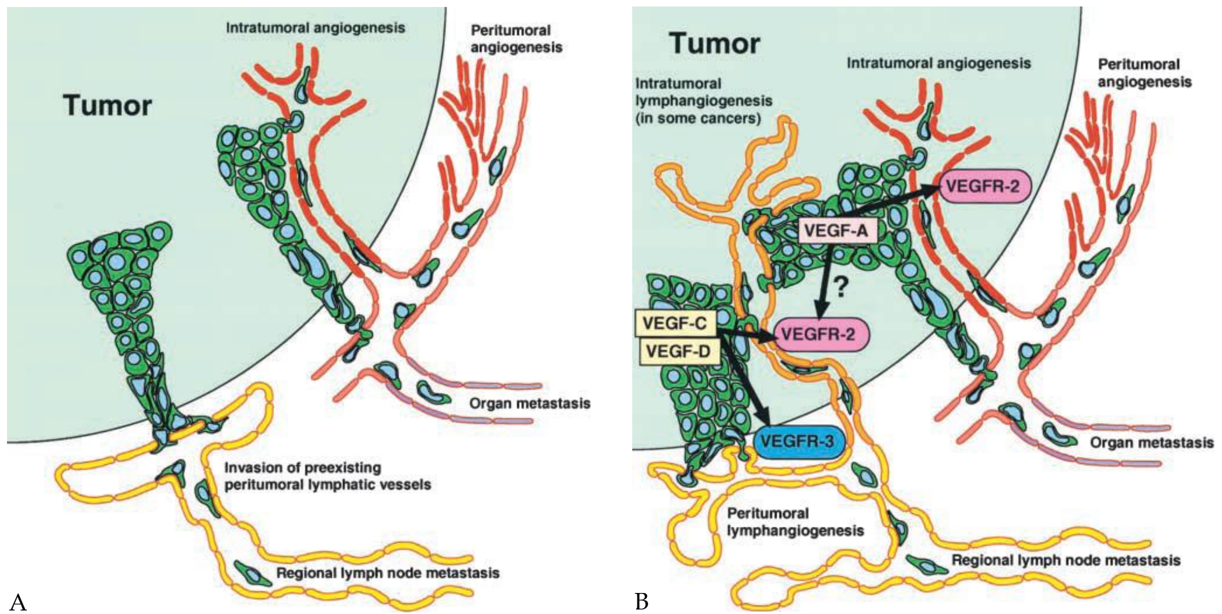

Fig. 2. (A) Traditional model of tumor metastasis via lymphatic and blood vessels. (B) Active lymphangiogenesis model of tumor metastasis (reprinted by permission from © 2002 Rockefeller University Press. Originally published in *J. Exp. Med.* 196: 713-718)

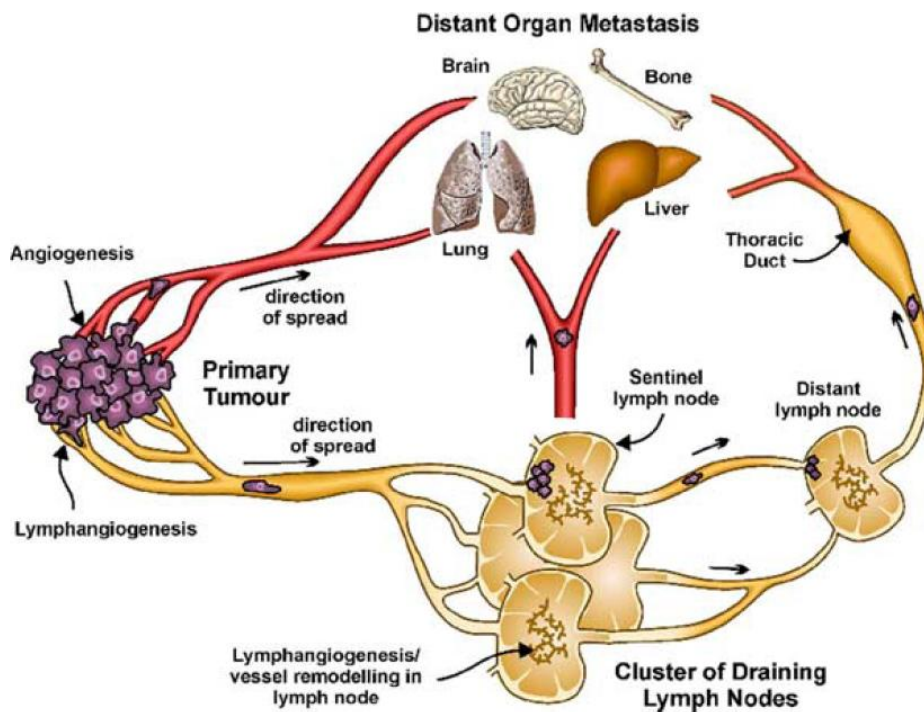

Fig. 3. Pathways of dissemination of malignant cells (reprinted by permission from © 2008 John Wiley & Sons, Inc. Originally published in *Ann. N. Y. Acad. Sci.* 1131: 225-234)

The blood vessels are not the best route for the success of malignant dissemination. Although their disorganized structure may contribute to the intravasion of malignant cells or emboli, in the bloodstream these cells experience serum toxicity, high shear stresses and mechanical deformation. Consequently, the viability of the tumor cells is seriously compromised (reviewed in Swartz, 2001). Conversely, the success rate of lymphogenous spread is high. As previously referred, the structure and function of the lymphatic capillaries facilitates intravasion of tumor cells or emboli. On the other hand, the composition of the lymph is similar to interstitial fluid, which provides an optimal medium for the survival of malignant cells. In collecting lymphatic vessels, muscle fibers assure lymph propulsion, that flows slowly, and valves prevent its backflow. Lymph nodes are areas of flow stagnation that represent ideal "incubators" for malignant cells' growth. Some cells exit the lymph node through the efferent

channels or high endothelial venules. Other cells may remain mechanically entrapped for long periods of time, originating micrometastases (Swartz, 2001; Van Trapen & Pepper, 2002). Martens and colleagues described the expression of a gene signature of scavenger and lectin-like receptors in the lymph node sinus, which are known mediators of tumour cell adhesion and, therefore, can contribute to selective metastasis in an organ-specific context (Martens et al., 2006). Probably, tumor-cell-specific characteristics, microenvironmental factors and crosstalk between tumor and host cells have a pivotal role in determining survival and growth of micrometastasis. Moreover, lymph node lymphangiogenesis may provide an additional mechanism to facilitate further metastatic spread throughout the lymphatic system (Ji, 2009). The occurrence of lymphangiogenesis prior to arrival of tumor cells indicates that signals derived from the primary tumor are transported to the draining lymph nodes (Hirakawa et al., 2005).

Different tumors metastasize preferentially to different organs, suggesting that tumor spread is a guided process. It has been reported that malignant cells may use chemokine receptor ligand interactions to guide the colonization of target organs (reviewed in Saharinen et al., 2004; reviewed in Achen & Stacker, 2008). Chemokines are a family of chemoattractant cytokines that bind to G protein-coupled receptors expressed on target cells, namely malignant cells (Laurence, 2006). For instance, breast cancer cells, that normally choose regional lymph nodes, bone marrow, lung and liver as their first sites of destination, overexpress CCR7 (chemokine, CC motif, receptor 7) and CXCR4 (chemokine, CXC motif, receptor 4). Their ligands, SLC/CCL2 (secondary lymphoid chemokine / CC-type chemokine ligand 21) and SDF-1 CXCL12/ (stromal cell-derived factor 1 / chemokine, CXC motif, ligand 12) are expressed at high levels by isolated lymphatic endothelial cells and lymphatic endothelium from vessels present in the preferred sites of metastasis (Muller et al., 2001). This guides chemoattraction and migration of tumor cells, and characterizes lymphatic vessel invasion as an active event.

3. Angiogenesis, Lymphangiogenesis and Lymphovascular Invasion in Urothelial Bladder Cancer

The metastatic profile of urothelial bladder carcinoma implies, as in most malignant tumors, the dissemination of tumor cells through the lymphatic vasculature, and the colonization of regional lymph nodes is an early event in progression. Smith & Whitmore reported the involvement of the internal iliac and obturator groups of lymph nodes in about 74% of patients who underwent radical cystectomy; the external iliac nodes were involved in 65% of the patients, and the common iliac nodes were involved in 20% of the cases (Smith & Whitmore, 1981). As already referred, controversy exists regarding the optimal extent of lymphadenectomy and the number of lymph nodes to be retrieved at radical cystectomy. An extended pelvic lymph node dissection (encompassing the external iliac vessels, the obturator fossa, the lateral and medial aspects of the internal iliac vessels, and at least the distal half of the common iliac vessels together with its bifurcation) has been suggested as potentially curative in patients with metastasis or micrometastasis to a few nodes (Karl et al., 2009; Abol-Enein et al., 2011). Wright and colleagues observed that an increased number of lymph nodes removed at the time of radical cystectomy associates with improved survival in patients with lymph node-positive bladder cancer (Wright et al., 2008). The recommendation from the Bladder Cancer Collaboration Group is that ten to fourteen lymph nodes should be removed at the time of radical cystectomy (Herr et al., 2004). The concept of lymph node density (the number of positive lymph nodes divided by the total number of lymph nodes) was introduced by Stein and colleagues and helps to select lymph node-positive patients after radical cystectomy for adjuvant treatment (Stein et al., 2003). However, the lymph node density threshold is a debatable question (Gilbert, 2008). In large series, the median number of total lymph nodes removed was nine, with high lymph node density (25%), which can lead to misleading N0 staging (Wright et al., 2008). Therefore, in this subgroup of patients (lymph nodes removed ≤ 9 and N0), another prognostic factor is needed to better select patients for adjuvant treatment. Moreover, according to Malmström, extending the boundaries of surgery will not drastically improve survival. The focus should be on exploring biomarkers that predict extravesical dissemination and improving on the systemic treatment concept (Malmström, 2011). In this line of investigation, angiogenesis, lymphangiogenesis and lymphovascular invasion occurrence have been implicated in bladder cancer progression, invasion and metastasis, and represent potential targets for guided therapy.

Several studies reported a significant association between VEGF overexpression — both in tumor tissue (Crew et al., 1997; O'Brien et al., 1995) and urine (Crew et al., 1999; Jeon et al., 2001) —, high blood vessel density (Goddard et al., 2003; Santos et al., 2003) and the occurrence of recurrence and progression in patients with non-muscle invasive bladder cancer. In this group of patients, it has been observed that angiotensin II type 1 receptor (AT1R) expression associates with high blood vessel density and is related to early intravesical recurrence (Shirotake et al., 2011). AT1R supports tumor-associated macrophage infiltration, which results in enhanced tissue VEGF protein levels (Egami et al., 2009). These results suggest that AT1R is involved in bladder tumor angiogenesis and may become a new molecular target and a prognostic factor for urothelial bladder cancer patients

In the subset of invasive urothelial bladder cancer, most studies also reported the association between angiogenesis occurrence and unfavorable prognosis. High blood vessel density was identified as an independent prognostic factor by several authors (Bochner et al., 1995; Chaudhary et al., 1999; Dickinson et al., 1994; Jaeger et al., 1995). Moreover, overexpression of VEGF associates with high blood vessel density (Sato et al., 1998; Yang et

al., 2004). Analysis of serum levels of VEGF has demonstrated its optimal sensitivity and specificity for predicting metastatic disease (Bernardini et al., 2001). Inoue and colleagues reported the importance of measuring blood vessel density and VEGF immunoexpression in identifying patients with invasive tumors who are at high risk of recurrence and development of metastasis after radical cystectomy and neoadjuvant systemic chemotherapy. The author highlighted the role of VEGF as a cell survival factor, not only by protecting the malignant cells in situations of hypoxia, but also during the occurrence of chemotherapy-induced apoptosis (Inoue et al., 2000).
Beyond VEGF signaling, other angiogenesis-related molecules have been implicated in bladder cancer recurrence, progression and metastasis, namely several proangiogenic factors — matrix metalloproteinases, fibroblast growth factors, platelet derived-growth factors, cyclooxygenases, integrins, angiopoietins, Notch signaling — and several antiangiogenic factors — thrombospondin-1, angiostatin-endostatin, platelet factor-4 (Chikazawa et al., 2008; Durkan et al., 2001; Grossfeld et al., 1997; Patel et al., 2006; reviewed in Pinto et al., 2010; Shariat et al., 2010).
The relevance of lymphangiogenesis in bladder cancer setting has gained recent attention. A few articles suggest that lymphangiogenesis occurrence, detected using specific lymphatic markers, is associated with poor prognosis (Fernández et al., 2008; Ma et al., 2010; Miyata et al., 2006; Zhou et al., 2011; Zu et al., 2006). VEGF-C, VEGF-D and VEGFR-3 are overexpressed in bladder cancer and promote tumor-induced lymphangiogenesis. This correlates with tumor upstaging and lymph node involvement, and results in a worse prognosis (Afonso et al., 2009; Miyata et al., 2006; Suzuki et al., 2005; Herrmann et al., 2007; Zhou et al., 2011; Zu et al., 2006). Interestingly, VEGF-C overexpression also associates with angiogenic events, probably by interaction of the fully processed form with VEGFR-2 (Afonso et al., 2009; Miyata et al., 2006). On the other hand, tumor associated macrophages play an important role in promoting lymphangiogenesis by producing VEGF-C and VEGF-D, mainly in peritumoral areas (Schoppmann et al., 2002). The blockade of VEGF-C/D with a soluble VEGF receptor-3 markedly inhibited lymphangiogenesis and lymphatic metastasis in an orthotopic urinary bladder cancer model. In addition, the depletion of tumor associated macrophages exerted similar effects (Yang et al. 2011).
Lymphovascular invasion has been identified as an independent prognostic factor for bladder cancer patients in several studies (Cho et al., 2009; Leissner et al., 2003; Lotan et al., 2005; Quek et al., 2005). In patients with newly diagnosed T1 urothelial bladder cancer, lymphovascular invasion in transurethral resection of bladder tumor specimens predicts disease progression and metastasis (Cho et al., 2009). Lotan and colleagues observed that blood and lymphatic vessel invasion (accessed by Haematoxylin-eosin stain) is an independent predictor of recurrence and low overall survival in patients who undergo radical cystectomy for invasive urothelial bladder cancer and are lymph node negative. They emphasized that these patients represent a high risk group that may benefit from neoadjuvant or adjuvant treatments. However, in this study, the mean number of lymph nodes removed per patient at the time of radical cystectomy was 20,1±10,2 (Lotan et al., 2005).
The prognostic impact of lymphovascular invasion in patients with lymph node-negative urothelial bladder cancer treated by radical cystectomy has been recently validated in large multicentre trials (Bolenz et al., 2010; Shariat et al, 2010). May and colleagues emphasized that, besides the importance of performing extended lymphadenectomies, the information resulting from an assessment of lymphovascular invasion is critical for stratification of risk groups and identification of patients who might benefit from adjuvant treatments (May, 2011). Algaba underlined that, in this field, it would be necessary to reach a consensus on strict diagnostic criteria as soon as possible, to be able to incorporate this prognostic factor in clinical practice (Algaba, 2006). Leissner and colleagues endorsed that blood and lymphatic vessel invasion should be commented on separately in the pathology report (Leissner et al., 2003).
Afonso and colleagues reported the prognostic contribution of molecular markers of blood vessels (like CD31) (Fig. 4, A) and lymphatic vessels (like D2-40) (Fig. 4, B) to accurately assess the occurrence of blood and/or lymphatic vessel invasion. The use of endothelial markers is encouraged because immunohistochemistry antibodies are significantly more sensitive in detecting invasive events than the standard Haematoxylin-eosin staining method and, additionally, facilitate the discrimination between blood and lymphatic vessel invasion. This is particularly important in identifying isolated malignant cells invading lymphatic vessels, because their viability is more probable in the lymphatic flow than in the blood circulation. Conversely, emboli of malignant cells are better suited to survive in the bloodstream, and are more easily identified, even by the traditional Haematoxylin-eosin staining method. This advocates the use of lymphatic markers for purposes of counting invaded lymphatic vessels. In this study, blood vessel invasion by malignant emboli assessed by CD31 staining (Fig. 5, A), and lymphatic vessel invasion by isolated malignant cells assessed by D2-40 staining (Fig. 5, B) significantly affected patients' prognosis; blood vessel invasion remained as an independent prognostic factor (Afonso et al., 2009). When included in a model of bladder cancer aggressiveness, these parameters contributed to a clear separation between low and high aggressiveness groups (Afonso et al., 2011).
Both peritumoral and intratumoral lymphatic vessels seem to be functional for urothelial cells' dissemination. Some articles reported the existence of intratumoral lymphatic vessels in bladder tumors, and their possible participation in metastatic events. No intratumoral edema has been observed, which is consistent with the occurrence of efficient lymphatic neovascularization (Afonso et al., 2009; Fernández et al., 2008; Ma et al., 2010; Miyata et al. 2006). Lymphatic vessel invasion occurrence correlates with high lymphatic vessel density values, mainly in the intratumoral areas. Although most of the invaded lymphatic vessels were distorted and collapsed, single malignant cells were significantly observed in the well-preserved intratumoral lymphatic vessels (Fig. 5, B).

Moreover, the absence of intratumoral edema is a surrogate marker of an efficient lymphatic flow (Afonso et al., 2009).

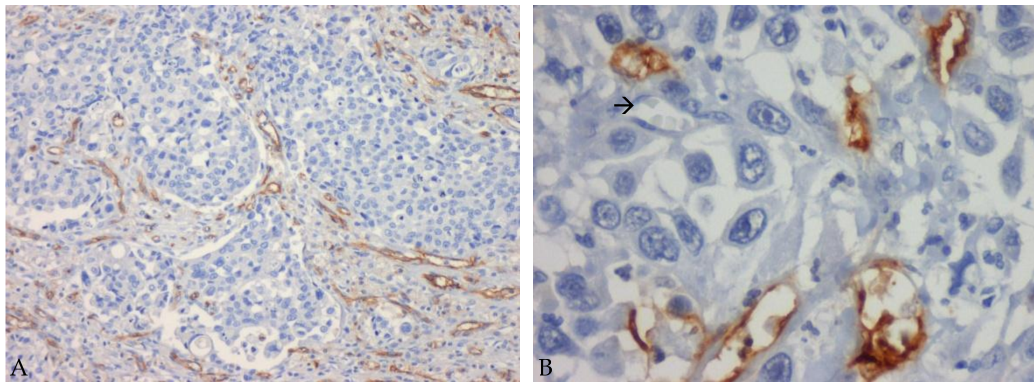

Fig. 4. Intratumoral blood vessels highlighted by CD31 (A), and intratumoral lymphatic vessels highlighted by D2-40 (B), in invasive urothelial bladder carcinoma. Evidence of internal negative control in A (D2-40 negative blood vessel →) (original magnification x100) (reprinted by permission from © 2009 John Wiley & Sons, Inc. Originally published in *Histopathol*. 55: 514-524)

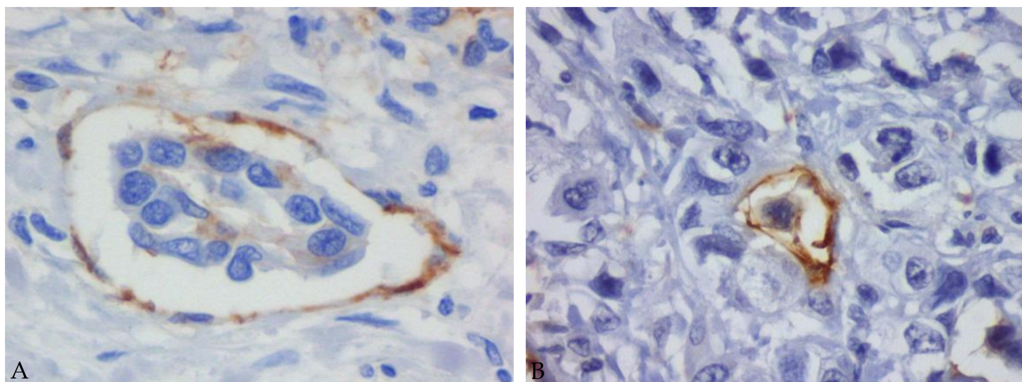

Fig. 5. Intratumoral blood vessel highlighted by CD31 invaded by a small malignant embolus (A), and intratumoral lymphatic vessel highlighted by D2-40 invaded by an isolated malignant cell (B), in invasive urothelial bladder carcinoma (original magnification x100) (reprinted by permission from © 2009 John Wiley & Sons, Inc. Originally published in *Histopathol*. 55: 514-524)

4. Angiogenesis and Lymphangiogenesis as Therapeutic Targets in Urothelial Bladder Cancer

Our current understanding of the importance of tumor-induced angiogenesis and lymphangiogenesis for the occurrence of haematogenous and lymphogenous metastasis suggests that, by blocking the activity of key molecules involved in these processes, it should be possible to suppress the onset of metastasis following diagnosis of cancer and its subsequent therapy. Moreover, prophylactic suppression of metastasis would be useful for patients who are at risk of recurrence (Thiele & Sleeman, 2006). Therefore, clinical trials evaluating novel agents and combinations including chemotherapeutic drugs, as well as targeted inhibitors, are desperately needed (Iyer et al., 2010).

Two types of neovascularization inhibitors have been described. The direct inhibitors refer to compounds that function directly on endothelial cells by blocking a common pathway of vessel growth. Indirect inhibitors are molecules that neutralize the functions of angiogenic and lymphangiogenic growth factors; due to their mode of action, these are preferred over the direct inhibitors (Cao, 2005; Folkman, 2003). The main strategies that have been tested focus on modulating the signaling of VEGF family of growth factors and receptors, and are based on the use of monoclonal antibodies or soluble versions of receptors to neutralize the ligand-receptor interaction, and the inhibition of the kinase activity of the receptors (Achen et al., 2006; Thiele & Sleeman, 2006).

In 2004, the U.S. Food and Drug Administration (FDA) has approved bevacizumab (Avastin®), a humanized monoclonal antibody that binds to VEGF-A, as the first drug developed solely for antiangiogenesis anticancer use in humans. Antiangiogenic drugs are presently approved in a wide number of tumor types, namely in breast, colorectal, lung, liver, glioblastoma and kidney cancer. Other compounds are currently in preclinical development, with many of them now entering the clinic and/or achieving approval (reviewed in Boere et al.,

2010; reviewed in Cook & Figg, 2010; reviewed in Pinto et al., 2010).
In anticancer therapy, an angiogenesis inhibitor may prevent the growth of new blood vessels. This should decrease the delivery of oxygen and nutrients – the "starving therapy" – which are indispensable elements for the support of uncontrolled cell division and tumor expansion. Angiogenesis inhibitors are predicted to be cytostatic, stabilizing tumors and perhaps preventing metastasis, rather than being curative (Zhi-chao & Jie, 2008). Therefore, there is the need to administrate this type of therapy for long periods of time. As a consequence, problems with bleeding, blood clotting, heart function and depletion of the immune system are common (Cohen et al., 2007). Nevertheless, inhibition of circulating VEGF reduces vascular permeability and thus tumoral interstitial pressure, permitting easier penetration of the tumor by conventional chemotherapeutic targets (Ferrara, 2005).
A second concern of anti-angiogenesis therapy is the approach to objectify the response to anti-angiogenic drugs. Chan and colleagues found that targeted contrast enhanced micro-ultrasound imaging enables investigators to detect and monitor vascular changes in orthotopic bladder tumors. Therefore, this technique may be useful for direct, noninvasive and in vivo evaluation of angiogenesis inhibitors (Chan et al., 2011). Lassau and colleagues demonstrated that dynamic ultrasound can be used to quantify dynamic changes in tumor vascularity as early as three days after the administration of the anti-angiogenic drug. These changes may be potential surrogate measures of the effectiveness of antiangiogenic therapy, namely by predicting progression-free survival and overall survival (Lassau et al., 2011).
Regarding antilymphangiogenic strategies, numerous compounds that could be used to block lymphangiogenesis already exist, although there is some delay in the translation to the clinic. These act mainly by targeting lymphangiogenic protein tyrosine kinases (Williams et al., 2010) (Table 1) or other indirect regulators of lymphangiogenic events. For instance, rapamycin (sirolimus), a classical immunosuppressant drug used to prevent rejection in organ transplantation, and a known inhibitor of the mTOR (mammalian target of rapamycin) signaling, has demonstrated potent antilymphangiogenic properties (Huber et al., 2007), and may suppress lymphatic metastasis (Kobayashi et al., 2007). mTOR is a member of the phosphoinositide-3-kinase-related kinase family, and is centrally involved in growth regulation, proliferation control and cancer cell metabolism (Rosner et al., 2008). Its inhibition impairs downstream signaling of VEGF-A as well as VEGF-C via mTOR to the ribosomal p70S6 kinase (a regulator of protein translation, and a major substrate of mTOR) in lymphatic endothelial cells (Huber et al., 2007). Other derivative compounds of rapamycin, like everolimus (RAD001) and temsirolimus (Torisel), have also demonstrated anti-tumor properties, namely by inhibiting tumor neovascularization (reviewed in Garcia & Danielpour, 2008). Recently, in patients with lymphangioleiomyomatosis (LAM, a progressive, cystic lung disease in women, which is associated with inappropriate activation of mTOR) sirolimus stabilized lung function, reduced serum VEGF-D levels, and was associated with a reduction in symptoms and improvement in the quality of life (McCormack et al., 2011).

Table 1. Protein tyrosine kinases involved in lymphatic biology, and available inhibitors (Tie- tyrosine kinase with immunoglobulin and EGF homology domain; EphB4- ephrin type-B receptor 4) (reprinted by permission from © 2010 BioMed Central Ltd. Originally published in *J. Ang. Res.* 2: 1-13)

Gene	Role in lymphatic vessels	Inhibitors available	Effect of pathway inhibition
VEGFR-2	Receptor for the VEGF family of ligands. Can also heterodimerize with VEGFR-3.	Yes	Secreted VEGFR-2 is a naturally occurring inhibitor of lymphatic vessel growth; however, Sorafenib† did not block VEGF-C/D induced tumor lymphangiogenesis.
VEGFR-3	Predominant receptor for VEGF-C and VEGF-D. Transduces survival, proliferation and migration signals.	Yes	Cediranib‡ blocks VEGFR-3 activity and inhibits lymphangiogenesis. Anti-VEGFR-3 antibody prevented tumor lymphangiogenesis with no effect on preexisting vessels.
Tie1	Not critical for lymphatic cell commitment during development, and no ligand has been shown.	None reported	Tie1 knockout mouse has lymphatic vascular abnormalities that precede the blood vessel phenotype.
Tie2	Receptor for Ang-1 and Ang-2. Appears to control vessel maturation.	Yes	Tie2-/- mice are embryonic lethal due to vascular defects. Inhibition of Ang-2 leads to tumor blood vessel normalization.
EphB4	Expressed on lymphatic capillary vessels. Involved in vascular patterning. Binds to the ephrinB2 ligand.	Yes	Mice expressing a mutant form of ephrinB2 lacking the PDZ binding domain show major lymphatic defects in capillary vessels and collecting vessel valve formation.
FGFR3	The ligands FGF-1 and FGF-2 promote proliferation, migration, and survival of cultured lymphatic endothelial cells. FGFR3 is a direct transcriptional target of Prox1.	Yes	Knockdown of FGFR3 reduced lymphatic endothelial cells' proliferation.
IGF1R	Both of the IGF1R ligands, IGF-1 and IGF-2, significantly stimulated proliferation and migration of primary lymphatic endothelial cells.	Yes	None reported.
PDGFRβ	The ligand PDGF-BB stimulated MAP kinase activity and cell motility of isolated lymphatic endothelial cells.	Yes	None reported.
MET	The ligand for c-Met, hepatocyte growth factor, has lymphangiogenic effect, but it is unclear if c-Met is expressed on lymphatic endothelial cells.	Yes	May be indirect effect.

†Sorafenib inhibits B-Raf, PDGFRb, VEGFR-2 and c-Kit. ‡Cediranib inhibits VEGFR-1, -2, -3, PDGFRb and c-Kit.

Inhibition of lymphangiogenesis has been shown to block lymphatic metastasis by 50-70% in preclinical animal models, with good safety profiles, which suggests that anti-lymphangiogenic therapy could possibly be used safely in cancer patients, without disrupting normal lymphatic function (reviewed in Holopainen et al., 2011). Optimally, the gold-standard strategy would be the one that could inhibit both angiogenic and lymphangiogenic cascades, in order to compromise the success of haematogenous and lymphogenous dissemination. Some potential compounds are being investigated (reviewed in Boere et al., 2010; reviewed in Cook & Figg, 2010; reviewed in Pinto et al., 2010; reviewed in Stacker & Achen, 2008).
Urothelial bladder carcinoma has experienced very few therapeutic successes, regarding antineovascularization therapy, in the last years. Compounds like bevacizumab (Avastin®), aflibercept (VEGF-Trap, AVE0005), sunitinib malate (Sutent, SU11248), sorafenib (BAY 43-9006), vandetanib (Zactima, ZD6474) and pazopanib (Votrient, GW786034) are being tested in preclinical and clinical trials (reviewed in Pinto et al., 2010) (Table 2).

Table 2. Selected ongoing or recently completed trials exploring antiangiogenic therapies in urothelial bladder carcinoma (reprinted by permission from © 2010 Elsevier. Originally published in *Commun. Oncol.* 7: 500-504)

Principal investigator / organization	Regimen	Patient population	Phase
Siefker-Radtke/MDACC	Methotrexate + vinblastine + doxorubicin+ cisplatin + bevacizumab	Neoadjuvant (muscle-invasive)	II
Kraft/MUSC	Gemcitabine + cisplatin + bevacizumab → cystectomy → paclitaxel + bevacizumab	Neoadjuvant/adjuvant (muscle-invasive)	II
Hahn/HOG	Gemcitabine + cisplatin + bevacizumab	First-line metastatic	II
Bajorin/MSKCC	Gemcitabine + carboplatin + bevacizumab	First-line metastatic (cisplatin-ineligible)	II
Rosenberg/CALGB	Gemcitabine + cisplatin ± bevacizumab	First-line metastatic	III
Garcia/Cleveland Clinic	Sunitinib	Neoadjuvant (muscle-invasive)	II
Sonpavde/HOG	Gemcitabine + cisplatin + sunitinib	Neoadjuvant (muscle-invasive)	II
Bellmunt	Sunitinib	First-line metastatic (cisplatin-ineligible)	II
Galsky/US Oncology	Gemcitabine + cisplatin + sunitinib	First-line metastatic	II
Hussain/University of Michigan	Sunitinib versus placebo	Maintenance after first-line chemotherapy	II
Gallagher/MSKCC	Sunitinib	Second-line metastatic	II
Milowsky/MSKCC	Gemcitabine + cisplatin + sorafenib	First-line metastatic	II
Kelly/Yale	Gemcitabine + carboplatin + sorafenib	First-line metastatic (cisplatin-ineligible)	II
Sternberg/EORTC	Gemcitabine + carboplatin ± sorafenib	First-line metastatic	II
Dreicer/ECOG	Sorafenib	Second-line metastatic	II
Choueiri/DFCI	Docetaxel ± vandetanib	Second-line metastatic	II
Vaishampayan/Mayo Clinic	Pazopanib	Second-line metastatic	II

MDACC = MD Anderson Cancer Center; MUSC = Medical University of South Carolina; HOG = Hoosier Oncology Group; MSKCC = Memorial Sloan-Kettering Cancer Center; CALGB = Cancer and Leukemia Group B; EORTC = European Organization for Research and Treatment of Cancer; ECOG = Eastern Cooperative Oncology Group; DFCI = Dana-Farber Cancer Institute

Bevacizumab, as has been already referred, is a monoclonal antibody that binds and neutralizes VEGF in the serum. Aflibercept is a soluble fusion protein of the human extracellular domains of VEGFR-1 and VEGFR-2, and the Fc portion of human immunoglobulin G. It binds, with a higher affinity than other monoclonal antibodies, to VEGF and additional VEGF-family members, namely VEGF-B and placental growth factor (PlGF). Sunitinib is an oral multi-targeted receptor tyrosine kinase inhibitor, with activity against VEGF receptors and PDGF receptors, among others. Sorafenib is a small, oral molecule that inhibits various targets along the EGFR/MAPK (epidermal growth factor receptor / mitogen-activated protein kinase) signal transduction pathway, and also through VEGFR and PDGFR families. Vandetanib is a tyrosine kinase inhibitor, antagonist of VEGFR and EGFR. Pazopanib is a multitargeted tyrosine kinase inhibitor against VEGF receptors, c-kit, and PDGF receptors (Cook & Figg, 2010).

4.1. Preclinical Studies

In the preclinical scenario, Videira and colleagues studied the effect of bevacizumab on autocrine VEGF stimulation in bladder cancer cell lines, and concluded that, at clinical bevacizumab concentrations, cancer cells compensate the VEGF blockade, by improving the expression of VEGF and related genes. This highlights the need to follow the patient's adaptation response to bevacizumab treatment (Videira et al., 2011). The antiangiogenic treatment of tumours may restore vascular communication and, thereby, normalize flow distribution in tumour vasculature. The use of antiangiogenic drugs leads to improved tumour oxygenation and chemotherapy drug delivery (Pries et al., 2010). However, these mechanisms may be also the cause of malignant dissemination, because tumours elicit evasive resistance. Caution is recommended, due to the divergent effects that VEGF inhibitors can induce on primary tumor growth and metastasis (Loges et al., 2009).
Yoon and colleagues, when exposing six human bladder cancer cell lines to an escalating dose of sunitinib alone or in combination with cisplatin/gemcitabine, demonstrated that sunitinib malate has a potent antitumor effect and may synergistically enhance the known antitumor effect of gemcitabine (Yoon et al, 2011).
The first study with vandetanib in bladder cancer cell lines demonstrated its potential to sensitize tumor cells to cisplatin. At vandetanib concentrations of ≤2microM, the combination with cisplatin was synergistic, especially when given sequentially after cisplatin , and additive with vandetanib followed by cisplatin (Flaig et al., 2009).

Li and colleagues studied the efficacy of pazopanib, both alone and in combination with docetaxel, in bladder cancer cell lines. They demonstrated that single-agent pazopanib has modest activity, but when given in combination with docetaxel, acted synergistically in docetaxel-resistant bladder cancer cells, with the potential of improved toxicity (Li et al., 2001).

Urothelial bladder carcinoma expresses mTOR signaling molecules, providing a rationale for clinical trials evaluating agents targeting this pathway (Tickoo et al., 2011). In fact, some studies using bladder cancer cell lines have demonstrated that sirolimus and related drugs inhibit the growth of cancer cells and decrease their viability (Fechner et al., 2009; Hansel et al., 2010; Pinto-Leite et al., 2009; Schedel et al., 2011). Similar results were obtained when treating bladder cancer animal models with sirolimus or everolimus (Chiong et al., 2011; Oliveira et al., 2011; Parada et al., 2011; Seager et al., 2009; Vasconcelos-Nóbrega et al., 2011).

4.2. Phase II Studies

The results of a phase II trial of cisplatin, gemcitabine, and bevacizumab (CGB) as first-line therapy for metastatic urothelial carcinoma revealed that CGB may improve overall survival — with a median follow-up of 27.2 months, overall survival time was 19.1 months. However, the rate of side effects was high, namely neutropenia, thrombocytopenia, anemia, and deep vein thrombosis/pulmonary embolism (Hahn et al., 2011).

In a phase II trial of gemcitabine, carboplatin, and bevacizumab in patients with advanced/metastatic urothelial carcinoma, Balar and colleagues concluded that addition of bevacizumab does not improve the response rate. However, bevacizumab can be safely added to gemcitabine and carboplatin, because the rate of venous thromboembolisms is similar to the one observed with gemcitabine and carboplatin alone (Balar et al., 2011). Moreover, in a pooled analysis of cancer patients in randomized phase II and III studies, the addition of bevacizumab to chemotherapy did not statistically significantly increase the risk of venous thromboembolisms *versus* chemotherapy alone. Probably, the risk for venous thromboembolisms is driven predominantly by tumor and host factors (Hurwitz et al., 2011). This type of side effect is primarily prevented by using anticoagulants simultaneously with cytotoxic chemotherapy (Riess et al., 2010). However, anticoagulant use during bevacizumab therapy may increase the risk of serious hemorrhage, although it is generally well tolerated (Bartolomeo et al., 2010). This controversial issue is still under scrutiny and more data are needed to clarify the optimal regime to reduce venous thromboembolisms in bladder cancer patients, particularly in those who are being treated with antiangiogenic drugs.

Patients with recurrent or metastatic urothelial carcinoma who had received a prior platinum-containing regimen were entered in a phase II trial with aflibercept as a second-line therapy. Aflibercept was well tolerated, but it had limited single agent activity in platinum-pretreated bladder cancer patients (Twardowski et al., 2009).

In a phase II study of sunitinib in patients with metastatic urothelial cancer designed to assess the efficacy and tolerability of this drug in patients with advanced, previously treated urothelial cancer, anti-tumour responses were observed. However, sunitinib did not achieve the predetermined threshold of ≥20% activity defined by the Response Evaluation Criteria in Solid Tumors, and side effects such as embolic events were reported (Gallagher et al., 2010).

In a multicenter phase II trial with sunitinib as first-line treatment in patients with metastatic urothelial cancer ineligible for cisplatin, on intention-to-treat analysis revealed that 38% of the patients showed partial responses (PRs), and 50% presented with stable disease (SD), the majority more than 3 months. Clinical benefit (PR + SD) was 58%. Median time to progression was 4.8 months and median overall survival 8.1 months (Bellmunt et al., 2011).

In a multicentre phase II trial of sorafenib as second-line therapy in patients with metastatic urothelial carcinoma, there were no objective responses to therapy. The 4-month progression-free survival rate was 9.5%, and the overall survival was 6.8 months (Dreicer et al., 2009).

Choueiri and colleagues conducted a double-blind randomized trial in which patients with metastatic bladder cancer and as many as three previous chemotherapy regimens received intravenous docetaxel with or without vandetanib. The results demonstrated that the addition of vandetanib to second-line docetaxel did not result in significant improvements in progression-free survival, overall survival or response rates (Choueiri et al., 2011).

The final results of a phase II study of everolimus in metastatic urothelial cell carcinoma have been presented at 2011 ASCO (American Society of Clinical Oncology) Annual Meeting. It was demonstrated that everolimus has clinical activity in patients with advanced urothelial bladder cancer. For the thirty-seven evaluable patients, the median progression-free survival was 3.3 months, and the median overall-survival was 10.5 months. Some side effects possibly related to everolimus were observed, namely anemia, infection, hyperglycemia, lymphopenia, hypophosphatemia and fatigue (Milowsky et al., 2011).

Dovitinib (TKI258) is an oral investigational drug that inhibits angiogenic factors, including FGFR and VEGFR. A multicenter, open-label phase II trial of dovitinib in advanced urothelial carcinoma patients with either mutated or wild-type FGFR3 is currently underway (Milowsky et al., 2011).

4.3. Phase III Studies

A randomized double-blinded phase III study comparing gemcitabine, cisplatin, and bevacizumab to gemcitabine, cisplatin, and placebo in patients with advanced urothelial carcinoma is open to enrollment. The primary end point is to compare the overall survival of patients with advanced urothelial carcinoma treated with gemcitabine hydrochloride, cisplatin, and bevacizumab *versus* gemcitabine hydrochloride, cisplatin, and placebo. The secondary end points are to compare the progression-free survival, the objective response rate and the grade 3 and greater toxicities of these regimens in the patients (Cancer and Leukemia Group B, 2011).

5. Conclusion

Bladder cancer represents a significant health problem, and the costliest type of cancer to treat. Although the majority of cases present as non-muscle invasive disease, the recurrence and progression rates are high, which demands for long-term follow-up and repeated interventions. Moreover, patients with advanced tumors treated by neoadjuvant or adjuvant regiments frequently progress and may develop chemotherapy resistance. Therefore, biomarkers of tumour aggressiveness and response to therapy are urgently needed, since the classical formulae based on stage and grade classification are insufficient to characterize bladder cancer. In this sense, angiogenesis, lymphangiogenesis and lymphovascular invasion have been described as surrogate markers of bladder cancer progression, invasion and metastasis, and represent potential fields of intervention. On one hand, the combined analysis of these biological parameters in tumor samples with the classical clinicopathological parameters may improve the individual characterization of bladder cancer, in what concerns to its clinical and prognostic course, and should allow therapeutic adequacy. On the other hand, the knowledge and modulating of biological phenomena related with bladder cancer progression may represent a significant improvement in the development of new drugs and in the pathological response to therapy, which ultimately will lead to an increase in disease-free survival and overall survival rates.

Targeted therapy has caused dramatic changes in the treatment of other types of tumors. However, in bladder cancer setting, clinical trials with molecularly targeted agents have been few in number and largely unsuccessful. Regarding antiangiogenic and antilymphangiogenic agents, these are still considered an investigational option for urothelial bladder cancer patients, and more results are needed to establish their roles in the treatment armamentarium. Research studies with anti-neovascularization drugs should not only provide effective agents to treat bladder cancer patients, but also predictive biomarkers for response to anti-neovascularization therapy, in order to implement the concept of personalized therapy.

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7. References

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