Universidade do Minho
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DNA repair genetic polymorphisms and breast cancer in the Portuguese population

Polimorfismos genéticos em genes de reparação de DNA e cancro da mama na população Portuguesa

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ABSTRACT/RESUMO

ABSTRACT

Breast cancer is the leading cause of death among women in developing countries. Approximately 10% of all cases of breast cancer are inherited, exhibiting a familial pattern of incidence, which have been attributable to mutations in high penetrance susceptibility genes, such as *BRCA1* and *BRCA2*. However, these mutations only account to approximately 25% of the families with inherited breast cancer; therefore, identification of genes that are associated with a small or modest cancer risk is an important step to define breast cancer risk. It has been determined that different genetic backgrounds due to the combination of subtle sequence variants or polymorphisms, within low-penetrance genes, can explain the remaining familial and sporadic breast cancer risks.

Many environmental factors have been associated with risk of breast cancer development, being sources of a wide range of DNA damage. The cellular response to DNA damage and its ability to maintain genomic integrity by DNA repair are crucial in preventing cancer initiation and progression. Previous studies have suggested an influence of gene variants in different DNA repair pathways regarding their capacity to repair. Therefore, polymorphisms in these genes may contribute to breast cancer susceptibility.

The general aim of this thesis was to understand the association of different polymorphisms (*XRCC1 Arg399Gln, XPD Lys751Gln, RAD51 G135C, XRCC3 Thr241Met, TP53 Arg72Pro* and *TP53 PIN3 Ins16bp*) belonging to the DNA damage signalling and repair mechanisms with breast cancer susceptibility, in familial and sporadic breast cancer, in the Portuguese population. Furthermore, we intended to characterize the protein expression profiles of the most relevant polymorphisms found in breast cancer patients and human breast cancer cell lines, correlating the protein expression profile with the polymorphic status.

Our findings identified *RAD51 G135C* polymorphism as a real risk modifier in familial breast cancer cases. Furthermore, we pointed out that *XRCC1 Arg399Gln* and *XRCC3 Thr241Met* polymorphisms as important biomarkers to sporadic breast cancer susceptibility. Moreover, our results also showed that *TP53 PIN3 A2* allele in a haplotype combination confer increased breast cancer susceptibility among women carriers of FH of the disease.

According to our findings from the association between the polymorphism and the clinical-pathological parameters from breast cancer patients, we clearly underlined the role of *XRCC1*

Arg399Gln and *RAD51 G135C* polymorphisms in the prediction of breast tumor aggressiveness and patients' survival. Furthermore, our results suggested *TP53 Arg72Pro* and *PIN3 Ins16bp* polymorphisms as predictive factors of presence of lymph node metastases. Additionally, we demonstrated that XRCC1, XRCC3 and P53 expressions did not correlate with the respective genetic polymorphisms analysed, in breast cancer patients and in human breast cancer cell lines.

RESUMO

O cancro da mama é a principal causa de morte por cancro em mulheres em todo o mundo. Aproximadamente 10% de todos os cancros da mama exibem um padrão de incidência familiar, tendo este sido atribuído a mutações em genes de susceptibilidade de elevada penetrância, tal como o *BRCA1* e *BRCA2*. No entanto, dados mais recentes têm demonstrado que mutações nestes genes contribuem apenas para cerca de 25% das doentes com história familiar de cancro da mama. Assim, a identificação de genes que possam estar associados a um risco pequeno ou moderado para cancro torna-se uma etapa importante na determinação de mais factores de risco para cancro da mama. Com o conhecimento de que dispomos, é perceptível que a existência de diferentes padrões genéticos, devido à combinação de pequenas alterações em determinados genes, designados de genes de susceptibilidade de baixa penetrância, possa explicar a diferente susceptibilidade para os restantes casos de cancro familiar da mama e para os casos da mama esporádicos. Além disso, muitos factores ambientais têm também sido associados com uma maior risco de desenvolvimento de cancro da mama, produzindo uma vasta gama de lesões no DNA. A resposta das células aos danos no DNA e a sua capacidade para manter a integridade genómica, através da reparação de DNA, é crucial para prevenir a iniciação e progressão do cancro. Trabalhos prévios têm sugerido uma forte associação de níveis elevados de danos no DNA e menor capacidade de reparação. Deste modo, polimorfismos nestes genes podem contribuir para a susceptibilidade para cancro da mama.

O objectivo geral desta tese foi compreender a associação de alguns polimorfismos genéticos em genes de reparação de DNA (*XRCC1 Arg399Gln, XPD Lys751Gln, RAD51 G135C, XRCC3 Thr241Met, TP53 Arg72Pro* e *TP53 PIN3 Ins16bp*) e a susceptibilidade para cancro da mama, familiar e esporádico, na população Portuguesa. Além disso, caracterizou-se o perfil de expressão proteica dos polimorfismos identificados como relevantes para risco de cancro da mama, numa série de pacientes com a doença, bem como em linhas celulares humanas de cancro da mama, correlacionando com o respectivo perfil polimórfico.

Os nossos resultados mostraram o polimorfismo *RAD51 G135C* como um importante modificador de risco para cancro da mama familiar. Adicionalmente, nós identificámos os polimorfismos *XRCC1 Arg399Gln* e *XRCC3 Thr241Met* como relevantes biomarcadores na susceptibilidade para cancro da mama esporádico. Este trabalho indicou também que uma

combinação haplotípica do alelo *TP53 PIN3 A2* conduz a um aumento do risco para cancro da mama familiar. Quando se relacionaram a presença destes polimorfismos com parâmetros clínico-patológicos numa série de carcinomas da mama, verificou-se que os polimorfismos *XRCC1 Arg399Gln* e *RAD51 G135C* claramente desempenham um papel na previsão da agressividade do tumor e sobrevivência dos doentes. Além disso, este estudo sugeriu os polimorfismos no gene *TP53, Arg72Pro* e *PIN3 Ins16bp*, como factores na previsão de metástases nos gânglios linfáticos. Por ultimo, demonstrou-se ainda que a expressão proteica da XRCC1, XRCC3 e P53 não se correlacionava com o respectivo polimorfismo estudado, nem em pacientes com cancro da mama nem em linhas celulares humanas de cancro da mama.

TABLE OF CONTENTS

TABLE OF CONTENTS

Abstract xiii

Resumo xv

Abbreviations xxiii

Aims xxix

Thesis Planning xxxiii

CHAPTER 1 – General Introduction 1

1.1. Breast Cancer 3

1.1.1. Breast normal development, anatomy and histology 3

1.1.2. Epidemiology and risk factors 5

1.1.3. Pathogenesis and etiology 10

1.1.4. Genetic polymorphisms as risk factors 15

1.2. DNA damage signalling and repair 17

1.2.1. Syndromes and cancers associated 17

1.2.2. Pathways 21

1.2.2.1. DNA damage signalling 21

1.2.2.2. Base excision repair 23

1.2.2.3. Nucleotide excision repair 24

1.2.3.4. Double strand break repair 25

1.2.3. Genetic polymorphisms, DNA repair capacity and breast cancer risk 27

1.2.4. XRCC1, XPD, RAD51, XRCC3 and TP53 polymorphic genes as good candidates to evaluate individual breast cancer susceptibility 31

1.3. References 34

CHAPTER 2 – DNA repair polymorphisms might contribute differentially on familial and sporadic breast cancer susceptibility: a study on a Portuguese population 53

2.1. Introduction 57

2.2. Materials and Methods 59

2.3. Results 61

2.4. Discussion 64

2.5. References 67

CHAPTER 3 – *XRCC1 Arg399Gln* and *RAD51 5'UTR G135C* polymorphisms and their outcome in tumor aggressiveness and survival of Portuguese breast cancer patients 77

3.1. Letter 81

3.2. References 83

CHAPTER 4 – Importance of *TP53 codon 72* and *intron 3 duplication 16bp* polymorphisms in prediction of susceptibility on breast cancer and presence of lymph node metastases 87

4.1. Introduction 91

4.2. Materials and Methods 92

4.3. Results 94

4.4. Discussion 98

4.5. References 101

CHAPTER 5 – Immunohistochemical expression profile of XRCC1, XRCC3 and P53 proteins in breast cancer: correlation with genetic polymorphic status 107

5.1. Introduction 111

5.2. Materials and Methods 114

5.3. Results 118

5.4. Discussion 123

5.5. References 126

CHAPTER 6 – General Discussion 135

6.1. References 143

ABBREVIATIONS

ABBREVIATIONS

HRT	Hormone replacement therapy
FH	Family history
BRCA	Breast cancer protein
TP53	Tumor protein 53
ATM	Ataxia telangiectasia mutated protein
CHEK or CHK	Checkpoint kinase
DCIS	Ductal carcinoma in situ
LCIS	Lobular carcinoma in situ
ER	Estrogen receptor
IR	Ionizing radiation
PRG	Progesterone receptor
PPARGC	Peroxisome proliferator-activated receptor-related estrogen receptor alpha coactivator
EP300	E1A-binding protein
COMT	Catechol-O-methyl transferase
CYP19	Aromatase (cytochrome P450 sub-family XIX)
HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2
EPHX	Epoxide hydrolase protein
UGT1A7	UDP glucuronosyltransferase 1 family, polypeptide A7
GSTP1	Glutathione S-transferase pi
Val	Valine
Leu	Leucine
His	Histidine
Gln	Glutamine
Gly	Glycine
Ser	Serine
Thr	Threonine
Met	Methionine
Ala	Alanine
Pro	Proline
Ile	Isoleucine
Arg	Arginine
Cys	Cysteine
C	Cytosine
T	Thymine
A	Adenine
G	Guanine
Tyr	Tyrosine
Lys	Lysine
UV	Ultraviolet
NER	Nucleotide excision repair
MMR	Mismatch repair
DSBR	Double strand break repair
HNPCC	Hereditary non-polyposis colorectal cancer

FA	Fanconi anemia
AT	Ataxia telangiectasia
LFS	Li-Fraumeni syndrome
BS	Bloom's syndrome
WS	Werner's syndrome
NBS	Nijmegen breakage syndrome
XP	Xeroderma pigmentosum syndrome
CS	Cockayne syndrome
TTD	Trichothiodystrophy
MLH1	Human MutL homolog protein
MSH	Human MutS homolog protein
PMS	Postmeiotic segregation increased protein
HRR	Homologous recombination repair
NHEJ	Non homologous end joining
GGR	Global genomic repair
XPA to XPG	Xeroderma pigmentosum group A to G proteins
ATR	ATM and Rad23-related protein
RAD51	Recombination protein 51
FANCA to M	Fanconi anemia A to M proteins
MRE11	Meiotic recombination 11
RAD50	Recombination protein 50
NBS1	Nijmegen breakage syndrome 1 protein
BLM	Bloom helicase protein
RPA or C	Replication protein A or C
WRN	Werner syndrome helicase protein
DSB	Double strand break
H2AX	Histone family member X protein
BER	Base excision repair
PCNA	Proliferating cell nuclear antigen
MDC1	DNA damage checkpoint 1
Cdc	Cell division cycle protein
AP	Abasic site
dRP	Abasic sugar phosphate
DNA Pol	DNA polymerase protein
FEN1	Flap endonuclease protein
XRCC	X-ray repair complementing protein
PARP-1	Poly ADP-ribose) polymerase-1
TCR	Transcription-coupled repair
hHR23B	Human Rad23B homolog
ERCC	Excision repair cross-complementing protein
LIG	Ligase protein
CSA or B	Cockayne syndrome A or B protein
Ku	Thyroid autoantigen subunit protein
DNA-PK	DNA protein kinase
FEN-1	Flap structure-specific endonuclease 1
OGG-1	8-oxoguanine DNA glycosylase
SSBR	Single strand break repair

BRCT	BRCA C-terminal domain
Trp	Tryptophan
Asp	Aspartic acid
Asn	Asparagine
UTR	Untranslated region
Glu	Glutamic acid
Thr	Threonine
IVS	Intron intervining sequence
bp	Base pair
IPO	Oncology Portuguese Institute
EDTA	Ethylene diamine tetracetic acid
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
OR	Odds ratio
CI	Confidence interval
SD	Standard deviation
OS	Overall survival
IHC	Immunohistochemistry
TMA	Tissue microarray
IPATIMUP	Institute of Molecular Pathology and Immunology of the University of Porto
PBS	Phosphate buffer solution
RT	Room temperature
WB	Western blot
IDV	Intensity density value

AIMS

A number of risk factors have been associated with susceptibility to breast cancer development, including endogenous and exogenous factors responsible for the production of a wide range of DNA damage, causing genome instability. Furthermore, some studies have been demonstrated a strong association of higher levels of DNA damage and lower DNA repair capacity in breast cancer patients. Several reports showed the presence of polymorphic alleles in DNA damage signalling/repair genes. Previous studies suggested an influence of gene variants in different DNA sigannling/repair mechanisms, as well as in its capacity to promote DNA repair and/or fidelity, maintaining the original sequence. Therefore, polymorphisms in these genes may contribute to breast cancer susceptibility.

GENERAL AIM

The general aim of this thesis was to understand the role of DNA damage signalling and repair genetic polymorphisms in breast cancer susceptibility, in familial and sporadic breast cancer, from a Portuguese population.

To achieve our research aim, the work was divided as follows:

1. COLLECTION OF PERIPHERIC BLOOD AND DNA EXTRACTION FROM SEVERAL GROUPS OF WOMEN

We collected peripheric blood from 3 different groups of women:

- 84 unrelated familial breast cancer cases were obtained from S. João Hospital at Porto and General Hospital at Vigo;

- 201 unrelated sporadic breast cancer cases were recruited from IPO-Porto (Oncology Portuguese Institute);

- 442 healthy women were randomly selected from blood banks during the same time period as the cases were collected.

DNA used for genotyping was extracted from peripheric blood lymphocytes.

2. GENOTYPING OF *XRCC1 ARG399GLN, XPD LYS751GLN, RAD51 G135C, XRCC3 THR241MET, TP53 ARG72PRO* AND *TP53 PIN3 INS16BP*

Polymorphisms were chosen based on theoretical effects on biological function of the protein, and an allelic frequency higher than 0.1. We selected the following polymorphisms: *XRCC1 Arg399Gln, XPD Lys751Gln, RAD51 G135C, XRCC3 Thr241Met, TP53 Arg72Pro and TP53 PIN3 Ins16bp,* and evaluated their genotypic frequencies in previous groups: familial and sporadic breast cancer patients and healthy women with no breast cancer family history, in a Portuguese population.

3. CORRELATION OF THE SELECTED POLYMORPHISMS WITH BREAST CANCER SUSCEPTIBILITY AND CLINICAL PATHOLOGICAL FEATURES OF PATIENTS

We applied appropriate statistical analysis to assess correlations between the genotyped polymorphisms with breast cancer susceptibility. Furthermore, we analysed associations of these genotypes and breast cancer features, as histological type and grade, axillary lymph node status, estrogen receptor status and survival and recurrence at last follow-up.

4. CHARACTERIZATION OF XRCC1, XRCC3 AND P53 PROTEIN EXPRESSIONS IN BREAST CANCER PATIENTS AND HUMAN BREAST CANCER CELL LINES

The characterization of XRCC1, XRCC3 and P53 immunohistochemical expressions were performed in paraffin embedded tissue microarrays from normal breast, benign breast lesions, *in situ* and invasive breast carcinomas.

5. CORRELATION BETWEEN XRCC1, XRCC3 AND P53 PROTEIN EXPRESSIONS IN BREAST CANCER PATIENTS AND HUMAN BREAST CANCER CELL LINES WITH THEIR POLYMORPHIC STATUS

We compared the expression profiles from breast cancer patients and from a series of human breast cancer cell lines with its respective genetic polymorphic status. We also evaluated the association of its expression with clinical-pathological factors, such as family history, histological grade, lymph node status and estrogen receptor status, and correlations between expressions of the different proteins.

THESIS PLANNING

THESIS PLANNING

The present thesis is organized in six different Chapters:

- In Chapter 1, a general introduction to the thesis theme is presented, including a review of the literature focused in the breast cancer epidemiology, risk factors and carcinogenesis, as well as the importance of DNA damage signalling/repair pathways and polymorphisms within these mechanisms in breast cancer.
- In Chapter 2, the work "DNA repair polymorphisms might contribute differentially on familial and sporadic breast cancer susceptibility: a study on a Portuguese population" is presented, showing the importance of *XRCC1 Arg399Gln* and *XRCC3 Thr241Met* DNA repair polymorphisms as biomarkers to sporadic breast cancer susceptibility, as well as, *RAD51 G135C* polymorphism in familial breast cancer cases.
- In Chapter 3, the study "*XRCC1 Arg399Gln* and *RAD51 5'UTR G135C* polymorphisms and their outcome in tumor aggressiveness and survival of Portuguese breast cancer patients" clearly underling the role of the above mentioned polymorphisms in the prediction of breast tumor aggressiveness and patients' survival.
- In Chapter 4, our findings regarding *TP53 Arg72Pro* and *PIN3 Ins16bp* polymorphisms and association with breast cancer susceptibility and clinical-pathological features are shown in the work "Importance of *TP53 codon 72* and *intron 3 duplication 16bp* polymorphisms in prediction of susceptibility on breast cancer and presence of lymph node metastases".
- In Chapter 5, a characterization of XRCC1, XRCC3 and P53 protein expression profiles is demonstrated in the study "Immunohistochemical expression profile of XRCC1, XRCC3 and P53 proteins in breast cancer: correlation with genetic polymorphic status".
- In last chapter, Chapter 6 is presented the discussion outline of the present thesis, as well as main conclusions of this work.

CHAPTER 1 – GENERAL INTRODUCTION

1.1. BREAST CANCER

Breast Cancer is an extraordinarily important disease all over the world. Over 1,2 million of women are diagnosed each year worldwide, and almost 411,000 of them will die of breast cancer (1). This disease affects not only the survival rate of these women but also their psychological and physical well-being, also influencing the people with close relation with them.

Over the last 30 to 40 years, significant progress has been made in the diagnosis and treatment of this disease. Breast cancer presents a multifactor etiology, leading to a variety of genetic changes that result in variable biological behaviours from one patient to the other. Remarkable efforts have been developed to define the risk factors that help to identify those women expected to develop breast cancer and the genetic factors that contribute for this risk.

1.1.1. BREAST NORMAL DEVELOPMENT, ANATOMY AND HISTOLOGY

In order to understand the changes that the human breast undergoes in normal and especially abnormal situations, it is important to know how its normal breast development occurs, its anatomy and histology.

Breasts begin developing in the embryo about 7 to 8 weeks after conception. They are unrecognizable at this stage consisting only of a thickening or ridge of tissue. From weeks 12 to 16, the various sub-components become more defined. Tiny groups of cells begin to branch out laying the foundation for future ducts and milk producing glands. Other tissues develop into muscle cells which will form the nipple (the protruding point of the breast) and areola (the darkened tissue surrounding the nipple). In the later stages of pregnancy, the mother's hormones, which cross the placenta into the fetus, cause breast cells to organize into branching tube-like structures, thus forming the ducts. In the last 8 weeks, lobules (milk producing glands) mature and actually begin to secrete a liquid substance called colostrum. In both female and male newborns, swellings underneath the nipples and areola can easily be felt and a clear liquid discharge, colostrum, can be seen. These represent the effect of the mother's hormones and collapse in the first few weeks of life (2,3).

Up until the onset of puberty, the breasts are much the same in males and females and their internal structure is similar – a collection of branching ducts ending in terminal ducts, with

minimal lobule formation. With the beginning of female menarche, the breast tissue responds to the release of female sex hormones, estrogen and progesterone. These stimulate the formation of lobules from the pre-existing terminal ducts, designed terminal duct-lobular unit (Figure 1B), leading to an increase in volume and elasticity of the connective tissue, the deposition of adipose tissue and the increase in vascularity (Figure 1A) (3-5).

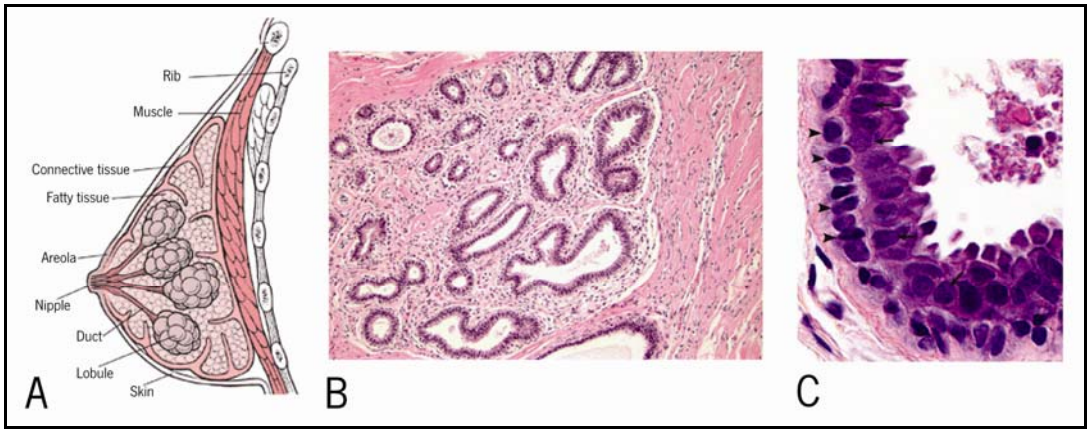

Figure 1 – Normal female breast. Macroscopic diagram (A), a microscopic low magnification of a terminal duct-lobular unit (B) and a microscopic high magnification of a intralobular terminal duct showing clear separation of epithelial (arrows) and myoepithelial cells (arrow heads).

Histologically, the epithelium of intralobular terminal ducts presents essentially 2 layers: an inner layer presenting a epithelium constituted by a continuous surface of epithelial cells with oval-like nuclei, and an outer discontinuous layer of prominent myoepithelial cells presenting a clear cytoplasm (Figure 1C) (5).

During each menstrual cycle there is a number of morphologic changes that happen in the breast. In the first half of the cycle, the lobules are relatively quiescent. After ovulation, under the enhancement of hormone levels, an increased cell proliferation and number of acini per lobule and vacuolization of epithelial cells take place. When menstruation occurs, falling hormone levels there are epithelial cells apoptosis, loss of the stromal edema and regression in lobules size (4).

Complete maturation of the breast tissue only occurs with lactation. The morphological alterations during pregnancy include increase of lobules number and size, so that in the end of the phase almost all the breast is composed of lobules with a slight amount of stroma, increase of melanin pigmentation in areola and vascularization of the nipple. After birth, the breast first produce the colostrum, and, with the decrease of progesterone levels, change to milk. After

ending lactation, the breast size diminished and the lobules regress and atrophy, but never to the appearance of the nulliparous breast (3,5).

As women age, especially with the loss of estrogen at menopause, the lobules involute and in some areas disappear, remaining only the ducts. The fibrous connective component of the stroma also diminishes, whereas stromal adipose tissue accumulation increases (2,3).

1.1.2. Epidemiology and Risk Factors

Breast cancer is the leading cause of death among women in developing countries. According to the World Health Organization, more than 1.2 million people worldwide will be diagnosed with breast cancer each year and nearly 320.000 cases in Europe (31% of all cancers in women) (1,6) (Figure 2).

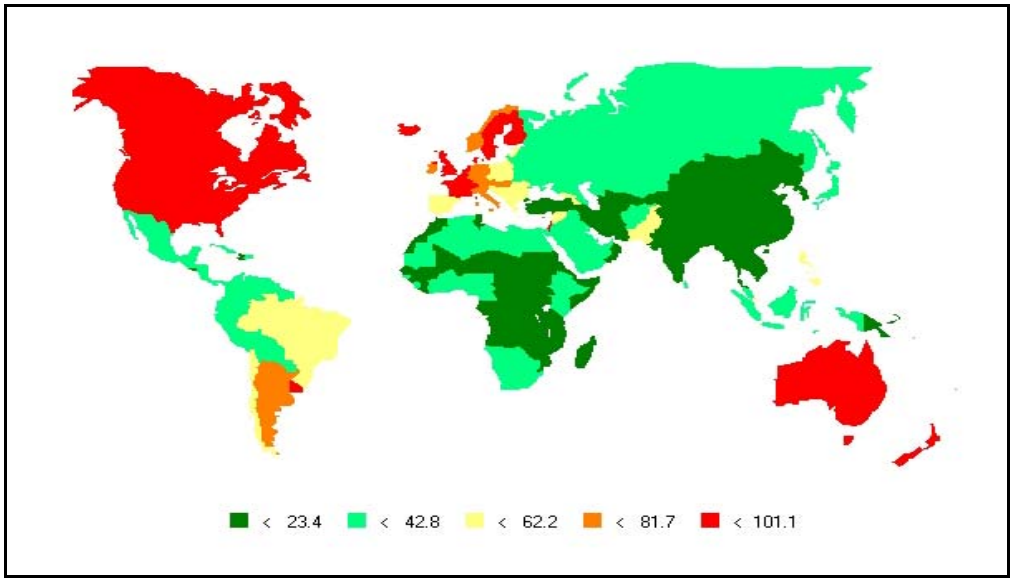

Figure 2 – Incidence rates of female breast cancer worldwide (per 100,000; all ages), according to GLOBOCAN 2002 (1).

In Portugal, it presents the highest incidence and mortality rates between the women diseases (1,7) (Figure 3). However, in the last decade, breast cancer mortality have been declined, in Portugal as well as in developing countries, due to multiple factors, including improvements in cancer screening and novel and more effective treatment regimens (3,8,9).

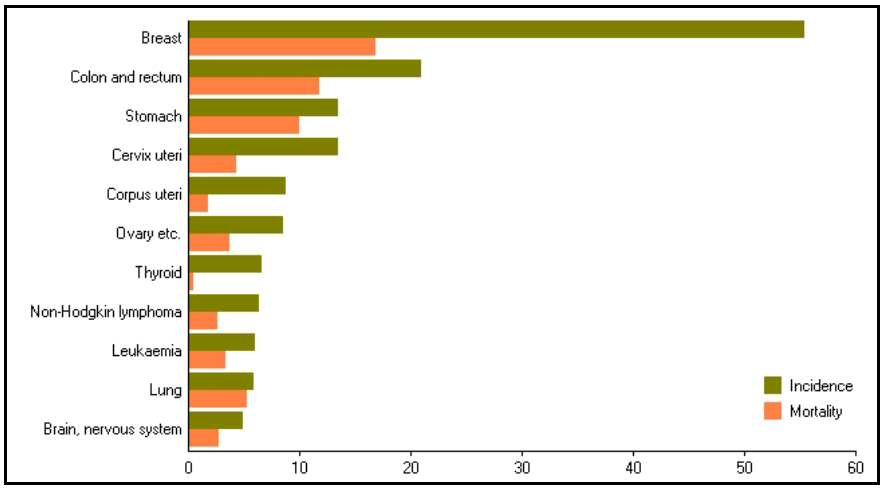

Figure 3 - Incidence and mortality rates of common cancer types in Portugal (per 100,000; all ages), according to GLOBOCAN 2002 (1).

As result of numerous epidemiological studies, several risk factors have been pointed out as well-established and probable in breast cancer, both as harmful or protective factors (Table 1).

Table 1 – Summary of breast cancer risk factors.

Risk Factor	Effect
Increasing Age	↑↑
Early menarche	↑
Late menopause	↑↑
Nulliparity	↑
Early age of first birth	↓↓
Breastfeeding	↓
High serum of sex hormones (oestradiol, prolatin and insulin-like growth factor-1)	↑↑
Prolonged HRT	↑
History of benign breast disease	↑↑
High breast density	↑↑
Obesity postmenopausal	↑
Obesity premenopausal	↓
Physical activity	↓
Fat intake and well-done food	↑
Vegetables and fruit intake	↓
Alcohol consumption	↑
Radiation exposure (child and young adulthood)	↑↑
Family history	↑↑
Mutations in high penetrance genes (*BRCA1, BRCA2, TP53, ATM, CHEK2)*	↑↑
Polymorphism in low penetrance genes	↑↓

↑ - low to moderate increased risk; ↑↑ - moderate to high increased risk; ↓ - low to moderate decreased risk; ↓↓ - moderate to high decreased risk

Breast cancer incidence is very low before age 25, except in certain familial cases, but increases with age, doubling every 10 years until the menopause, reaching its highest incidence rates in women over 50 years old (3,9). In menopause women the incidence rates slows dramatically (9), which suggests the important involvement of reproductive hormones in breast cancer etiology.

Mortality and, mainly, incidence rates of breast cancer shows significantly differences between more developed countries, which present high rates, and less developed countries and Japan, with low rates (9). Studies of migrants from low to high risk countries have shown that women assume the rate of the host country within one or two generations, indicating the relevance of the environmental and lifestyle factors as breast cancer risks (8,9).

The lifetime exposure to endogenous sex hormones has been described, for almost half a century, as the most well-established risk factor to breast cancer. This factor is determined by several variables, including age at menarche, age at first full-term pregnancy, breastfeeding and age at menopause. Women who start menstruating early in life (less than 12 years of age) or who have a late menopause (after the age of 55 years) present an increase risk to breast cancer (3,10). These findings could be explained by a prolonged exposure of mammary gland epithelium to estrogens and progesterone due to earlier or long-standing regular ovulatory menstrual cycles (11). The time and/or occurrence of pregnancy seem to have dual effect on breast cancer risk. On one hand, early age of first full-term pregnancy (less than 20 years versus more than 30 years) is a protective factor to breast cancer, independently of the number of pregnancies. In contrast, nulliparity or late age of first full-term birth represents increased risk factor (9,10). These findings are contradictory with the fact that high and continuous levels of estrogen are associated with increased risk to breast cancer, since during pregnancy the oestradiol reach high levels. However, this could be counteracted by the fact that other hormones are secreted during pregnancy, inhibiting the effect of estrogen, and the breasts at this time reach the differentiation maximum, reducing the probability of cancer development (12). Breastfeeding effect on breast cancer risk has been controversial. Recent studies have proved that it represents a protective factor only in women that experiment a prolonged lactation period time (more than 24 months), probably due to diminishing of ovulatory frequency (9,10).

During the last decade, several studies have looked to the effect of endogenous serum concentrations of hormones and breast cancer risk. It has been demonstrated that high serum oestradiol concentrations represent an increased risk to postmenopause women to develop

breast cancer. Furthermore, other sex hormones, such as insulin-like growth factor-1 and prolactin, have been suggest also to contribute to an increased breast cancer risk (9,11).

Recent studies aimed to elucidate the importance of hormone replacement therapy (HRT) in breast cancer risk. So far, it becomes clear that users of HRT (mainly, estrogen combined with progestin) have a higher increase risk of breast cancer after four to five years of therapy compared with women who never used HRT (9).

The presence of history of benign breast disease is also known to increase the risk of developing breast cancer. The diagnosis of atypical hyperplasia or atypia in epithelial mammary cells has been correlated with an increased risk of breast cancer (9,10).

Another consistent breast cancer risk factor is the breast density. Research studies have shown that higher breast density is linked with increased risk to breast cancer, both in pre- and postmenopausal women, especially nulliparious, where these two factors seem to act in synergy (10).

Obesity and the weight gain seem to have contradictories effects on breast cancer risk depending on the menopause status of the women. Obese postmenopausal women present higher risk to breast cancer, in contrast to obese premenopausal women that have a decreased risk (13).

Several studies have demonstrated that physical activity reduces breast cancer risk when performed during adolescence and young adulthood, since it could delay the age of menarche (10).

Numerous epidemiological studies have tried to clarify the role of diet and lifestyle (i.e. smoking, alcohol) as breast cancer risks. High intake of fat in the diet seems to be weakly associated with breast cancer risk (14,15). Moreover, consumption of well-done meat also has been correlated with increased risk, since in overcooked food there is production of extremely mutagenic compounds, like heterocyclic aromatic amines, which can lead to DNA damage, mutation accumulation and cancer initiation (15,16). In contrast, the fruits and vegetables intake, rich sources of natural antioxidants, seems to have a protective effect in breast cancer, mainly in postmenopausal women (14,15). Furthermore, intake of soya or some other foods that presents high levels of phytoestrogens seems to have some protective effect in breast cancer, given that these substances may block the effects of endogenous estrogens. However, contradictory results have been shown, whereas these substances seems to have also breast cancer promoting effects (14). Concerning alcoholic habits, several studies have demonstrated that daily alcohol uptake

increases breast cancer risk (14,15). No association of smoking habit and breast cancer risk was obtained by the majority of the reports (14,15).

Radiation exposed population has shown higher increase risk to breast cancer, mostly in women exposed before 40 years. However, low doses of radiation, including occupational and medical diagnostic exposures, remain uncertain in their effect to breast cancer risk (3).

Approximately 10% of all breast cancer is inherited, exhibiting a familial pattern of incidence. Women with at least one affected first-degree relative have an increased risk to breast cancer. This risk enhance significantly when: a) there are more than one affected relative; b) the relatives are close; c) early age of onset of the disease; c) there are cases of bilateral disease in the affected women or among relatives (17).

The family history (FH) as a risk factor to breast cancer indicates that genetic factors are important determinants of this disease risk. Mutations in *BRCA1* and *BRCA2* (*Breast Cancer 1 and 2*), the most commonly implicated high-penetrance genes in hereditary breast cancer, account for around 25% of families with this disease (18). In addition to these genes, mutations in three others were considered to establish them as also high penetrance genes associated with a moderate breast cancer risk (10,17). *TP53* mutations are rare events in breast cancer families; however, women carriers of them present significantly high risk of early onset breast cancer. Another gene that account to increased risk to hereditary breast cancer is *ATM* (*Ataxia-Telangiectasia*), especially under the age of 50. More recently, a gene that has been associated with hereditary breast cancer is *CHEK2*, presenting a frequency of 0.5-2.0% in the European population (19,20).

The high penetrance genes account for only 5-10% of all breast cancers. Therefore, the majority of breast cancer cases do not have any inherited or hereditary origin. In this way, identification of genes that are associated with a moderate or low cancer risk is an important step in defining breast cancer risk. It has been understandable that different genetic backgrounds due to the combination of subtle sequence variants or polymorphisms in the low-penetrance genes, in combination with endogenous and exogenous exposure, could explain the remaining familial and "sporadic" breast cancer risks. Numerous studies have been conducted to identify accurate low-penetrance susceptibility genes in breast cancer, and promising results have been obtained in genes encoded proteins implicated in the DNA repair and cell signalling pathways and in the metabolism of estrogen or various carcinogens (21-25).

1.1.3. PATHOGENESIS AND ETIOLOGY

Some years ago it was believed that breast cancer rose from mammary epithelium through a well-defined, but non-obligatory, sequence of histological changes, from normal epithelium through hyperplasia, atypical hyperplasia, *in situ* carcinoma and invasive malignant disease. However, new pathological findings and distinct genetic hereditary or somatic alterations have reflected a more complex, heterogenic and multi-step etiology for breast cancer.

Nowadays, breast tumors classification are made concerning not only to the morphology of the lesion, but also to their molecular profile (8). A vast variety of benign and malignant breast diseases has been identified, reflecting the high complexity and heterogeneity of the disease. The most common breast tumors have an epithelial origin (nearly 95% of all breast diseases) (3), and could be divided in three main groups: benign lesions, *in situ* and invasive carcinomas.

Benign tumors can be defined as well-differentiated tissue with similar origin morphology, usually with a slow growth rate and well demarcated areas of growth (3). The benign lesions may be divided in: a) nonproliferative lesions, including cysts, apocrine metaplasia and duct ectasia; b) proliferative lesions without atypia, such epithelial hyperplasia, sclerosing adenosis, complex sclerosing lesions, papillomas, and fibroadenoma; and c) proliferative lesions with atypia, as atypical lobular and ductal hyperplasia (3,8). Of the many types of benign lesions in human breast, only the atypical hyperplasias seem to present a pre-malignant phenotype, enclosing a relative loss of growth control, however, without the ability to invade and metastasize, features restricted to malignant lesions (3). Thus, malignant tumors are characterized by decreased levels of cellular differentiation presenting a loss of morphology regarding the origin tissue; with a disorganized growth and high proliferation rates, abnormal nuclei and several mitoses; with ability to invade adjacent tissues and metastasize to other organs (3).

The *in situ* carcinomas are divided mainly in two types: ductal (DCIS) and lobular (LCIS). Both present proliferation of the epithelium combined with cellular features of malignancy but without the capacity to invade the adjacent tissue. These two types of carcinomas present similar cellular origins, such as origin in terminal duct-lobular unit and undistinguishable genetic alteration at various loci (26). However, only a minority of LCIS cases seem to progress to invasive disease, in contrast to DCIS, which represents a high risk factor for progression to invasive carcinomas (8).

The majority of the invasive carcinomas are referred as ductal (85-95%), encompassing a heterogeneous group of tumors with particular names, as tubular, medullary, mucinous, papillary, metaplastic, apocrine, secretory and lipid-rich, and others classified as "not otherwise specified" (3,8). The abundance of invasive lobular carcinomas varies from 5-15% and is a much more homogenous group.

Recently, this morphological classification was totally remodelled using the expression profile analysis through cDNA microarrays. Perou et al (27) defined three groups of breast cancer that are related to different molecular features of mammary epithelial biology: the luminal (estrogen receptor (ER) positive), the epidermal growth factor-2 (HER-2) positive and the basal-like (ER and HER-2 negative).

In the classical model of general tumorigenesis, genetic variation is provided primarily by genetic mutations, and natural selection acts on this variation to provide a net survival advantage to the phenotypes (and genotypes) that are best adapted to the environment. Tumour growth, by comparison, is initiated by one or more mutations that a selective growth advantage to a cell. The clone derived from that cell then expands. Successive useful mutations or epigenetic changes occur, and it is thought that each is followed by waves of clonal expansion. This model has been confirmed by numerous molecular studies (28-30), and it has been established that cells have to acquire several genetic changes to allow tumour growth, invasion and metastasis. However, the normal mutation rate is insufficient to provide the genetic variation that is required for tumour growth. So, it is often proposed that mutations causing genomic instability occur as the initiating events and driving force to tumorigenesis. In general, these theories assume that genomic instability is derived from mutations in genes that are involved in processes such as DNA repair and chromosomal segregation, and mutations in these genes have no direct selective advantage or disadvantage, only an effect on the mutation rates of other genes. Genomic instability may arise as an additional effect of a mutation that has a larger direct selective advantage. Similarly, mutagenic environments might favour cells that bypass slow DNA repair mechanisms and therefore speed up progress through the cell cycle (31). Under these conditions, selection for rapid cellular proliferation might again indirectly raise the mutation rate. Finally, genomic instability mutations might 'hitch-hike' with new advantageous mutations in the same genome, as long as the overall selective advantage for the tumour cell is maintained.

The tumorigenesis model is also viable in breast organ. The transformation of normal breast epithelium into carcinoma is generally accepted as a multistep process, in which genetic changes

(either in tumour suppressor genes and oncogenes) (32), environmental factors, lifestyle and hormones exposure, may play a role (33), resulting in a very complicated picture (figure 4). However, most breast cancer encloses a complex, heterogenic and multifactorial origin. One consequence of this phenomenon implies that two or more groups of breast cancer cases may have been caused by different sets of events, or even different causes.

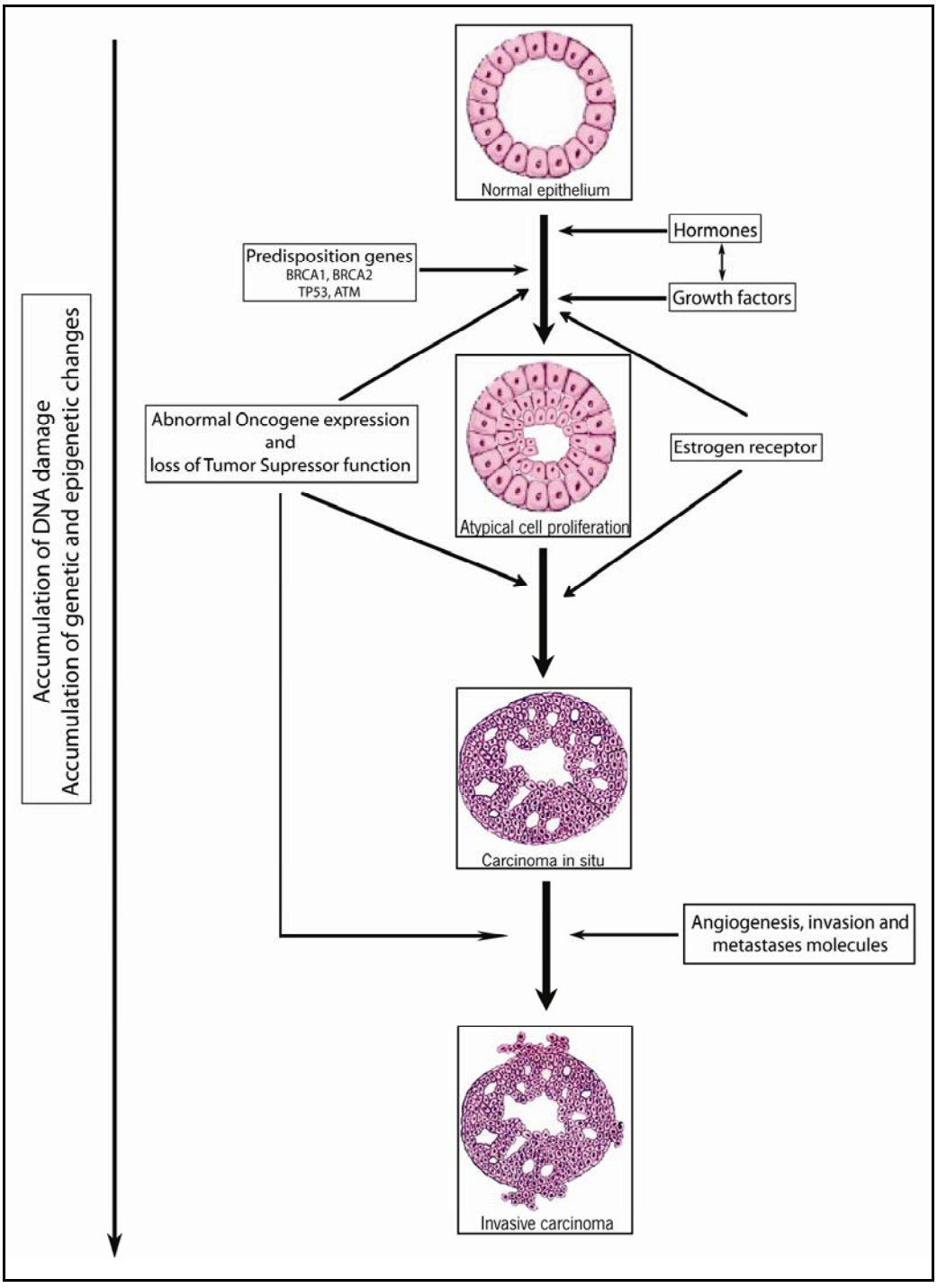

Figure 4 – Schematic model of hypothetical multistep carcinogenesis in breast cancer.

Regarding epidemiological findings, it is clear that one of the hypotheses to breast cancer etiology is due to excessive and cumulative exposure to endogenous hormones at different stages of breast cancer development. Several evidences suggest the involvement of hormones through two distinct ways: as genotoxic estrogen metabolites, which are able to induce DNA damage and thus cause initiation and progression of breast carcinogenesis; and as estrogen receptor (ER) mediated genomic and non-genomic signalling, affecting cell proliferation and apoptosis in breast tissue (34-37).

Importantly, estrogens by their mitotic effects on breast cells appear to control the growth of primary breast cancers by inducing estrogen-regulated proteins that function as autocrine and paracrine growth factors. Estrogens activate genes controlled by estrogen-responsive elements. In addition to these classical transcriptional effects, these ligands can also modulate other genes, not containing estrogen-responsive elements, via direct protein–protein interaction of ER with other transcription factors (35,37). The genes responsible for the mitogenic effect of estrogen probably include secreted growth factors, for example, epidermal growth factor and insulin-like growth factor-1 and their respective receptors (35,37). By contrast, the non-genomic effects of estrogens on signal transduction do not appear implicated in their mitogenic action, since all key events in cell cycle stimulation can occur in the presence of a mitogen-activated protein kinase-activating inhibitor (38).

Increasing evidences have suggested that estrogen metabolism produced mutagenic metabolites that may contribute to breast carcinogenesis. During the process of estrogen biotransformation and elimination several DNA damage, such as direct DNA adducts, lipids oxidative damage and production of reactive oxygen species, which can turn out multiple types of genetic insults contributing to the induction of genomic instability (39-41). In addition to the estrogen mutagenic potential, a number of environmental chemicals, resulting from lifestyle and environmental factors, are supposed to contribute to the initiation of breast cancer, through the accumulation of DNA mutations, arising presumably via DNA damage, in genes that normally function to guarantee genetic stability (15,42-44). Several studies have been performed to elucidate the role of DNA damage in breast cancer predisposition and initiation. Patel et al (45) showed that breast cancer patients and their first-degree relatives presented higher frequencies of chromatid breaks in peripheric blood lymphocytes compared with control women, following *in vitro* G_2 phase X-irradiation. Regarding ionizing radiation (IR), various reports have demonstrated higher levels of DNA damage in breast cancer patients (with or without FH of breast cancer)

compared to healthy women, after *in vitro* and *in vivo* exposure to IR (46-48). Some studies have also demonstrated that familial breast cancer patients, as well as theirs relatives and the sporadic breast cancer patients presented higher cromatid break frequencies when compared with controls, using as mutagens bleomycin, doxorubicin and N-methyl N-nitro N-nitrosoguanidine (49-51). Another common type of DNA damage is the bulky DNA adducts, produced by a wide range of chemical, such as polycylic aromatic amines, heterocyclic aromatic amines and benzo(a)pyrene, commonly found in well-cooked food and cigarette smoke. Rundle et al (52) design a case-control study to analyse polycylic aromatic amines-DNA adducts in tumors and nontumoral breast tissue from patient cases and benign tissue from controls. They observed a mean adduct levels significantly higher for the tumour tissue samples when compared with benign tissue samples. Furthermore, DNA adduct levels was significantly associated with breast cancer risk. In resume, all these findings corroborate the important involvement of DNA damage, and, in consequence, all the cellular mechanisms unchain by it (such as DNA damage recognition, signalling and repair pathways), in breast cancer etiology.

Some years ago, Adami et al (53) have proposed an etiological model with four key components. First, the probability of breast cancer occurrence depends on the number of proliferating cells, being supported by the evidence that breast density is a predictor of breast risk (10). Secondly, the number of target cells and their responsiveness to hormonal stimulation is determined early in life, since the prenatal period, the mammary gland is in an undifferentiated state that turns it to a "perfect place" for cancer initiation. Third, the occurrence of the first full-term pregnancy leads to the generation of terminally differentiated glandular tissue, which presents a lower rate of proliferation conferring long-term protection. Fourth, exposure to sex hormones, like estrogens, progesterone, prolactin and insulin-like growth factor 1, affect breast cancer risk by increasing the cellular proliferation, influencing clonal expansion and modulating growth enhancement of sub-clinical tumors (53).

A more recent theory to breast cancer etiology, gaining more and more recognition, is based on the stem cell concept (54). In summary, the epithelial stem cells are the primary targets of tumorigenesis in the adult mammary gland, are long-lived and have large replication potential, allowing them to accumulate the mutations required for malignant potential. According to Dontu et al (54), ER positive and negative stem cells or progenitor cells of normal breast epithelium are the founding cells of breast tumors. The transformed mutated cells become the cancer stem cells that maintain the functional properties of differentiation present in normal stem cell. This concept

would provide an explanation for the heterogeneity of breast cancer phenotypes. These last two theories could be consider to be linked, since the four components of the first one represent steps in a single biologic process that point the mammary gland stem cells as the core determinant of breast cancer risk (55).

1.1.4. Genetic Polymorphisms as Risk Factor

Polymorphisms have been historically classified as commonly occurring (>1%) genetic variations in the general population. Compared to mutations, polymorphisms have been perceived as functionally insignificant, however, current evidence emphasizes that a considerable fraction affects the intrinsic properties and proteins function to a variable degree (56,57).

Low-penetrance susceptibility alleles are defined as polymorphic genes with specific alleles that are associated with an altered risk for disease susceptibility. Usually, the variants in these genes are common within general population. Therefore, although each variant may be associated with a small increased risk for breast cancer in an individual, the attributable risk in the population as a whole and the genetic effect of combinations of relevant polymorphisms may additively or synergistically be higher than for rare, high-penetrance susceptibility genes.

Several reports have demonstrated the importance of polymorphisms in several cellular mechanisms, like estrogen and carcinogen detoxification metabolisms, cell cycle, apoptosis, cell signalling, growth factors and receptors molecules, cell adhesion, angiogenesis, DNA damage signalling and DNA repair, on breast cancer susceptibility (21,58-64). Polygenic models have also been proposed to explain the joint effect of many susceptibility alleles on breast cancer, but without considering specifically their possible interactions (65-68). Some examples of recent studies regarding this issue are shown in Table 2. Besides, these genetic variants might function through interactions with different genes and with behavioural, environmental and other external risk factors.

Table 2 – Studies of association of polymorphisms in different cellular mechanisms with breast cancer risk.

Gene Name	Polymorphism Name	Breast cancer risk (OR; 95% CI)#	Reference
ER α	*Xba*I	2.61 (0.65-10.49)	(69)
	*Pvu*II	0.95 (0.43-2.08)	
	*Xba*I	1.18 (0.73-1.89)	(70)
	*Pvu*II	1.30 (0.86-1.97)	
	*Xba*I	0.69 (0.46-1.03)	(71)
	*Pvu*II	0.92 (0.69-1.21)	
PRG	*Val660Leu*	1.69 (0.87-3.28)	(72)
	Val660Leu	1.47 (0.06-37.5)	(73)
	H770H	1.43 (0.06-35.3)	
	Q886Q	1.54 (0.20-11.8)	
	Val660Leu	1.40 (0.90–2.00)	(74)
PPARGC1A	*Gly482Ser*	0.90 (0.65-1.24)	(75)
	Thr612Met	3.02 (0.78-11.7)	
PPARGC1B	*Ala203Pro*	1.78 (0.61-5.15)	
	Pro388Pro	1.43 (0.76-2.68)	
EP300	*Ile997Val*	0.95 (0.65-1.38)	
COMT	*Val158Met*	3.72 (0.99-13.9)	(69)
		1.04 (0.76-1.44)	(76)
		1.20 (0.90-1.40)	(74)
		1.10 (0.93-1.30)	(66)
CYP19	*Arg264Cys*	2.07 (0.18-23.3)	(69)
	3'UTR C>T	0.90 (0.70-1.00)	(74)
HSD17B2	*Met226Val*	1.06 (0.49-2.29)	(77)
		1.14 (0.47-2.77) [a]	
		1.26 (0.47-3.41) [b]	
EPHX	*Tyr113His*	0.60 (0.43-0.84)	(78)
		1.42 (0.94-2.17)	(76)
SOD2	*Val16Ala*	1.20 (1.00-1.50)	(74)
		0.92 (0.66-1.27)	(76)
UGT1A7	*Lys131Arg*	1.00 (0.80-1.20)	(74)
GSTP1	*Ile105Val*	0.90 (0.66-1.25)	(78)

Homozygote variant genotype vs homozygote wild type genotype; * Study not realized; a) homozygote wild type vs heterozygote genotype between breast cancer patients with two close relatives with breast cancer vs healthy women; b) homozygote wild type vs heterozygote genotype between hereditary breast cancer patients vs healthy women.

1.2. DNA DAMAGE SIGNALLING AND REPAIR

"DNA is, in fact, so precious and so fragile that we now know that the cell has evolved a whole variety of repair mechanisms to protect its DNA from assaults by radiation, chemicals and other hazards. This is exactly the sort of thing that the process of evolution by natural selection would lead us to expect." (79).

As Crick postulated, multiple repair mechanisms have evolved in all organisms to minimize the consequences of cellular exposure to endogenous and exogenous agents that inflict deleterious alterations in DNA.

DNA damage occurs spontaneously and constantly throughout the life of an organism and can be further enhanced by exogenous DNA damaging factors. Therefore, an efficient response to DNA damage is essential for cellular life. DNA damage results from exogenous factors, including chemical carcinogens, such as some presented in diet and environmental pollution, IR, ultraviolet (UV) rays, and chemotherapeutic drugs (15). Spontaneous errors in fundamental cellular processes, such as DNA replication, and DNA damage produced by endogenous metabolic processes together with exogenous factors induce a wide range of DNA lesions such as reactive oxygen species, oxidized bases, bulky DNA adducts and DNA strand breaks (41,80).

The importance of DNA repair is underscored by DNA repair deficiency, that is associated with hypersensitivity to DNA-damaging agents leading to mutations accumulation in the genome (81), as well as with genomic instability syndromes, which dramatically increase cancer incidence (82). So far, several pathways that lead to genomic instability have been described, including the disruption of Nucleotide Excision Repair (NER), Double Strand Break Repair (DSBR), Mismatch Repair (MMR) and DNA damage signalling pathways.

1.2.1. SYNDROMES AND CANCERS ASSOCIATED

The acquisition of some form of inherent genomic instability is a hallmark to several diseases, most notably cancer. The failure in the maintenance of genomic integrity and DNA repair can predispose humans to some well described malignancies (83). Several DNA repair genes are linked to hereditary predisposition diseases, such as Hereditary Nonpolyposis Colon Cancer

(HNPCC), Hereditary Breast Cancer, Fanconi Anemia (FA), Ataxia Telangiectasia (AT), Li-Fraumeni syndrome (LFS), Bloom's Syndrome (BS), Werner's Syndrome (WS), Nijmegen Breakage Syndrome (NBS), Xeroderma Pigmentosum Syndrome (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD).

HNPCC syndrome, also termed as Lynch Syndrome, is the most common form of hereditary colorectal cancer counting for 1-3% of all colorectal cancer cases. Other cancers seen in HNPCC include small intestine, pancreas, brain, hepatobiliary tract, and urinary tract. This hereditary disease is characterised by germline mutations in any of five DNA MMR genes (*MLH1, MSH2, MSH6, PMS1* and *PMS2*) causing errors in DNA replication, known as microsatellite instability. These deficiencies in MMR instead of causing malignant transformation create the background that permits mutations to accumulate in other growth regulatory genes (84).

Hereditary breast cancer syndrome is the most common form of inherited breast cancer and is caused by germline mutations in *BRCA1* and *BRCA2* (85). Although a great proportion of the familial risk cases is not explained by germ line mutations in *BRCA1/2*, no other susceptibility genes exclusively associated with increased risk of breast cancer have been identified so far, except *CHEK2*, whose variant allele *1100delC* has been associated with a moderate increase in breast cancer risk in specific populations (86). BRCA1, BRCA2 and CHEK2 present important functions within the cellular network that responds to DNA damage and protects genomic integrity. BRCA1 protein seems to enclose multiple functions: acts directly in Homologous Recombination Repair (HRR), a sub-pathway of DSBR, through association and co-localization with RAD51 protein; promotes precise Non-Homologous End Joining (NHEJ) repair sub-pathway of DSBR, reducing the mutagenic potential of it; coordinates some roles of DNA replication; enhances Global Genome Repair (GGR) sub-pathway of NER by inducing genes of these machinery (*Xeroderma Pigmentosum C (XPC)* and others); is a phosphorylated downstream protein by protein kinases ATR (ATM- and Rad3-related) and ATM, having important roles in the various cell cycle checkpoints (87). The functions of BRCA2 protein are not well known, but it has an unquestionably role in HRR, since it interacts directly with the DNA recombination protein A (RAD51), being responsible for the transport of it to the nucleus and sites of DNA damage (87). Concerning CHEK2 protein, it has a role in DNA damage signalling, with direct impact on downstream effectors within cell cycle checkpoints, DNA repair and apoptosis machineries (86).

Fanconi Anemia is a rare recessive disorder characterized clinically by congenital defects, bone marrow failure, and cancer predisposition. Patients are diagnosed early in life by certain

clinical hallmarks, particularly hematological abnormalities such as aplastic anemia, myelodysplastic syndrome, and acute myeloid leukaemia (88). Abnormalities may also be present in many other organ systems, and the most notable features include radial and thumb hypoplasia, abnormal skin pigmentation, short stature, and infertility (89). Patients with FA are also susceptible to solid tumors, such as head and neck squamous cell carcinoma, gynaecologic squamous cell carcinoma, esophageal carcinoma, liver, brain, skin, and renal tumors (89). At the cellular level, a distint and diagnostic feature of FA is chromosomal instability and cellular sensitivity resulting from exposure to DNA interstrand crosslinkers, such as mitomycin C. Upon exposure to these genotoxins, cells from FA patient's exhibit increased chromosomal aberrations, including chromosomal breaks and tri-radial formations (88,89). So far, twelve complementation groups and responsible genes, named *FANCA-FANCM,* have been identified. A general model has become known for the FA pathway as an arm of the DNA-damage response following interstrand crosslinkers. Eight FA proteins (FANCA, B, C, E, F, G, L, and M) form a nuclear core complex with a putative DNA helicase (FANCM) and an E3 ubiquitin ligase (FANCL) subunit. Following DNA damage, the core complex is required for mono-ubiquitination of FANCD2, a downstream FA protein. Following modification, FANCD2 co-localizes to DNA damage foci, presumably DNA-repair complexes, with BRCA1, BRCA2 and the MRE11-RAD50-NBS1 complex (88,89).

Ataxia Telangiectasia is characterized by cerebellar degeneration, immunodeficiency, cancer predisposition, and acute sensitivity to IR. The affected individual has been found to be prone to develop T cell prolymphocytic leukemia, B cell chronic lymphocytic leukemia, as well as sporadic colon cancer with microsatellite instability (90). The ATM protein enlarges a central role in the cellular response to DNA damage. ATM is a nuclear serine/threonine protein kinase involved in activation of several cell cycle checkpoints (G1–S, S and G2–M by autophosphorylation after exposure of cells to DNA damage, as IR, and through phosphorylation of several substrates, such as BRCA1, NBS1 and P53. ATM also has a role in phosphorylating proteins that are recruited to the sites of DNA double strand breaks and this results in enhanced cell survival (90).

Li-Fraumeni syndrome originally was described as a familial cancer syndrome with an autosomal-dominant pattern of inheritance of early onset sarcomas of the soft tissues and bone, carcinomas of the breast and adrenal cortex, brain tumors, and leukemias. The underlying genetic defect in the majority of LFS families was identified as a germline mutation in the p53 tumor-suppressor gene (91).

Bloom's Syndrome is an autosomal recessive disorder characterized by growth retardation, sunlight sensitivity and a predisposition to the development of cancer. At the cellular level, BS is associated with inherent genomic instability. In comparison with cells isolated from unaffected individuals, BS cells show an increased frequency of several types of chromosomal aberrations, including breaks and translocations. BS is a result of mutations in a RecQ helicase gene, *BLM*. BLM seems to develop important roles to maintain the genomic stability. This protein interacts physically and functionally with a number of other nuclear factors in human cells. Most notably, BLM binds directly to topoisomerase IIIα, to RAD51 and to RPA (replication protein A), the major ssDNA (single-stranded DNA)-binding protein in human cells (92).

Werner's syndrome is an autosomal recessive disorder manifested by premature onset of age-related phenotypes (such as short stature, premature greying of the hair, progressive hair loss, mild diabetes and cataract formation), including cancer. In WS cells, the genomic instability appears as spontaneous chromosomal abnormalities: chromosome breaks, complex rearrangements and deletions. A striking characteristic of WS cells is the expansion of different structural chromosome rearrangements in different clones from the same cell line. Another sign of genomic instability is abnormal fluctuation of telomere length. Along with spontaneous genomic instability, cells from WS individuals show a delayed S phase and a hypersensitivity to agents that interfere with DNA replication. The mutated gene responsible for WS is *WRN,* a RecQ helicase family member. WRN protein has been found to physically interact and/or co-localize with several proteins involved in DNA replication or control of genetic stability during S phase, enclosing an important role in recombinatorial repair and replication fork (93).

NBS1 protein was the first component of the complex NBS1/MRE11/RAD50 to be associated with a genetic disease, the Nijmegen Breakage Syndrome. NBS is characterized by developmental defects, immune deficiency and a high incidence of cancer. NBS cells present genomic instability in the form of chromosome breaks and fail to arrest DNA synthesis following DNA damage. Furthermore, NBS cells are also sensitive to DNA-damaging agents causing replication fork stall. NBS1 protein forms a complex with MRE11/RAD50 and recruits them to sites of double strand breaks (DSBs). Recent knowledge showed that H2AX is phosphorylated by ATM in response to radiation and hence, ATM regulates this recruitment of the NBS1/MRE11/RAD50 complex through interaction of NBS1 with H2AX phosphorilated. Moreover, NBS1 is involved in signal transduction for cell-cycle checkpoints as a substrate of

ATM kinase and, when this mechanism is defective, induces impaired G2 checkpoint control and also allows continued DNA synthesis in the presence of DSBs (94).

The presence of defects in some NER proteins is responsible for three rare recessive syndromes: Xeroderma Pigmentosum Syndrome, Cockayne Syndrome and Trichothiodystrophy. The more common clinical manifestations of XP are freckling in sun-exposed areas, followed by other pigmentation changes, loss of elasticity and multiple skin cancers including basal and squamous cell carcinomas and malignant melanomas. XP can result from defects in any of eight genes (*XPA-XPG*) (82). The clinical features of CS have little in common with XP. However, they share some mutated genes responsible for the diseases, which in the case of CS are *XPB*, *XPD* and *XPG*. CS patients have many developmental defects including severe physical and mental retardation, microcephaly, long limbs, bird-like face, pigmented retinopathy, gait defects and sun sensitivity (82). The defining feature of TTD is sulphur-deficient brittle hair caused by a reduced level of cysteine-rich matrix proteins. Associated features include small stature, mental retardation, ichthyotic skin, β-thalassaemia trait, unusual facial features, and in many cases photosensitivity. Most photosensitive patients have mutations in the *XPD* gene, but there are patients mutated in *XPB* and others in another group, TTD, which gene has not yet been identified (82).

1.2.2. PATHWAYS

Approximately 150 human DNA repair genes were cloned and sequenced. DNA repair genes can be divided into 2 sub-groups: genes associated with signalling and regulation of DNA repair, and the genes associated with distinct repair mechanisms, such as Base Excision Repair (BER), NER, DSBR and MMR (95-97).

Next we will focus on the DNA repair pathways more related with breast cancer initiation and progression, namely BER, NER and DSBR and DNA damage signalling and regulation.

1.2.2.1. DNA DAMAGE SIGNALLING

The fidelity of eukaryotic genome is maintained by coordinated actions of cellular pathways, including DNA repair, chromatin remodelling, apoptosis, and cell cycle check-

points. The checkpoint pathways are signal-transduction pathways, responsible mainly for the control of cell cycle arrest, control of the activation of DNA repair mechanisms, movement of DNA repair proteins to sites of DNA damage, activation of transcriptional programmes and induction of cell death by apoptosis (98). These DNA damage control mechanisms minimize the risk of DNA to be converted to inheritable mutations, and are believed to be of critical importance in the prevention of carcinogenesis.

As in all signal-transduction processes, the DNA checkpoint pathways involve sensors, responsible for DNA damage recognition and signal initiation, transducers, being in charge of transmitting and amplifying the signal, and effectors molecules, that control the biological consequences of triggering the pathway.

In Figure 5, we visualized a schematic diagram of DNA damage signalling. In mammalian systems, the proteins responsible for the sensing and initiation of DNA damage responses, caused by various genotoxic agents, are two protein kinases of the PI-3-kinase-like kinase family: ATM and ATR. The kinase activity of ATM is activated when DNA DSBs occur (99,100). A crucial sensor for the ATM pathway seems to be the MRE11-NBS1-RAD50 complex. This complex is required for the damage-induced chromatin association of ATM and for efficient ATM autophosphorilation after damage (100). In contrast to ATM, the ATR responds to damage rather than DSB, such that caused by hydroxyurea and UV-light (98). Activation of the ATR kinase requires its associated protein ATR-interacting protein and two protein complexes, that seem to be the trimeric proliferating cell nuclear antigen (PCNA) and the replication factor C (RFC) (100).

Some proteins, the transducers, are crucial to the activation of specific subsets of ATM or ATR substrates. ATR-dependent pathway requires the function of several proteins including BRCA1, Claspin and mediator of DNA damage checkpoint 1 (MDC1). In the case of ATM, P53 binding protein-1 and MDC1 also appear to be critical for the phosphorilation of many ATM substrates (100).

With the help of mediators, checkpoint signals are transmitted, in the form of protein phosphorylation, to two major signal-transducing checkpoints kinases—CHK1 and CHK2. These two kinases in their turn regulate downstream targets, such as the phosphatases (Cdc25A, Cdc25C), and P53, controlling cell cycle progression and DNA synthesis. CHK2 is the kinase target of ATM, and seems to phosphorilate P53 and BRCA1 (98,101). On the

other side, CHK1 is the target of ATR-dependent pathway and responsible for Cdc25 phosphorilation (100,101).

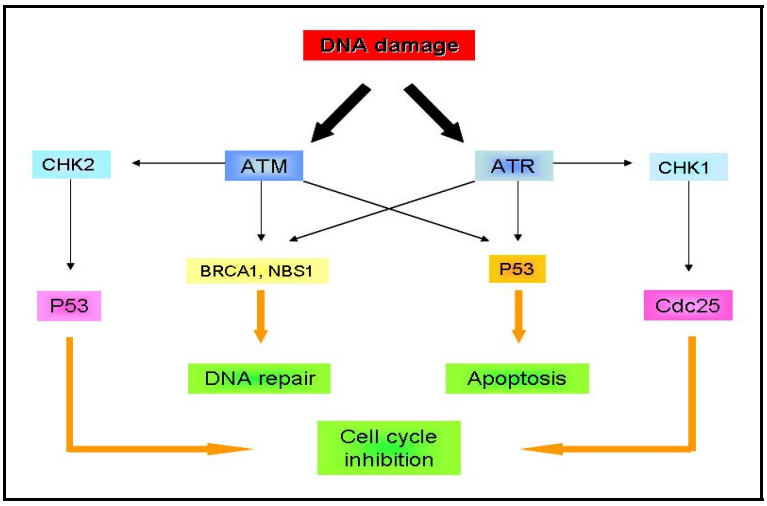

Figure 5 - Simplified scheme of DNA damage signalling.

1.2.2.2. Base Excision Repair

Base excision repair pathway is the main mechanism to repair DNA damage with an endogenous origin, mainly DNA oxidation by reactive oxygen species which are generated by a wide range of normal metabolism and spontaneous deamination of DNA bases, and with an exogenous origin, including ionising radiation and long-wave UV light, as already mentioned (102).

Briefly, BER is initiated by a DNA glycosylase that releases the target base to form an abasic site (AP) in the DNA (Figure 6). AP endonuclease (APE1) is the second enzyme in the pathway and hydrolyses the phosphosdiester bond 5' to the abasic sugar phosphate (dRP) site to generate a nick. The insertion of the first nucleotide is performed by DNA polymerase β (DNA Polβ) (103). The removal of 5'dRP upon the insertion of the first nucleotide is the critical step in the decision between the two sub-pathways in BER: short-patch and long-patch. Besides polymerisation activity, DNA Polβ also exerts lyase activity in the hemiacetal form of 5'-dRP residues from incised AP sites. In contrast, oxidised or reduced AP sites are resistant to β elimination by DNA Polβ. Upon dissociation of DNA Polβ from damaged DNA, strand displacement and DNA synthesis is accomplished by DNA Polε and DNA Polδ together with PCNA and RFC, resulting in longer repair patches of up to 10 nucleotides. The removal of deoxyribosephosphate flap

structure is executed by flap endonuclease (FEN1) stimulated by PCNA. The ligation is performed by ligase I, in interaction with PCNA and Polβ, in long-patch BER, and by ligase III, that interact with X-ray repair complementing 1 (XRCC1), Polβ and PARP-1 [poly(ADP-ribose) polymerase-1], in short-patch BER (97,103).

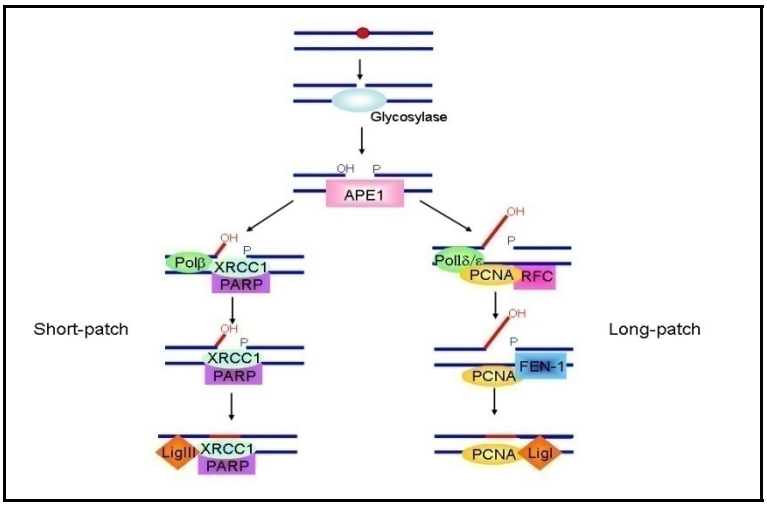

Figure 6 - Simplify diagram of BER pathway.

1.2.2.3. Nucleotide Excision Repair

The NER is the most versatile and flexible DNA repair pathway and is the major repair system for removing bulky DNA lesions, such as UV-light-induced photolesions and cyclobutane pyrimidine dimers, intrastrand cross-links, large chemical adducts, bulky adducts, generated from exposure to genotoxic agents and oxidative damage (104,105).

This pathway consists of 2 distinct sub-pathways designated GGR and transcription-coupled repair (TCR) (Figure 7). GGR seems to be responsible for the repair of the non-transcribed domains of the genome. In contrast, TCR removes lesions from the transcribed strand of active genes. The first step involved in NER is the recognition of damaged residues and bubble formation, performed by XPC-hHR23B (human Rad23B homolog) and the nine subunits of transcription factor-IIH (TFIIH), XPA and RPA, respectively. The dual incision of the damaged DNA strands 5' and 3' to the lesion is executed by 2 endonucleases, XPG and ERCC1(excision repair cross-complementing)-XPF. DNA Polδ and Polε jointly with the sliding clamp PCNA, the pentameric clamp loader RFC and DNA ligase I (LIG I), are responsible for the release of an oligonucleotide containing the damage, synthesis and ligation of the resulting gap. With the

exception of XPC-hHR23B, all the genes involved in GGR are also required for TCR. In addition, TCR requires other genes, including CSA and CSB genes, also responsible for the Cockayne Syndrome (97,103).

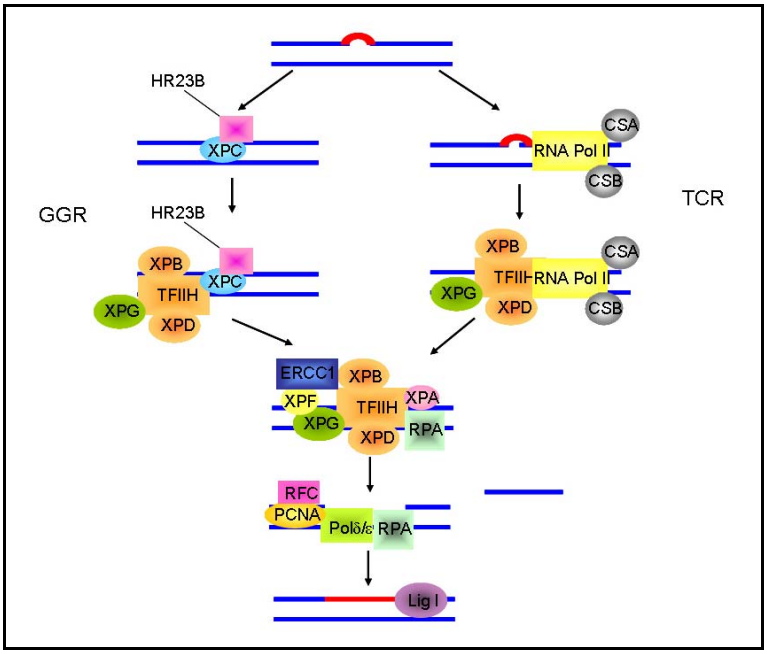

Figure 7 - Elementary illustration of NER pathway.

1.2.2.4. Double Strand Break Repair

Double-strand break is the most dangerous type of DNA damage in the cells. DSB could result from exogenous agents, such as IR and certain chemotherapeutic agents, from endogenous origin, for instance reactive oxygen species, mechanical stress on chromosomes and replication errors (99). Several genes described as breast cancer susceptibility genes are important DSBR genes and for DSBR pathway, such as *BRCA1, BRCA2, ATM, P53* and *CHEK2* (99).

The repair of DSB involves 2 types of pathways (Figure 8): HRR and NHEJ mechanisms, which are error-free and error-prone, respectively. The occurrence of HRR or NHEJ depends on the cell cycle phase. HRR occurs during the late S and G2 phases, whereas NHEJ occurs mainly in G0/G1 phases (97).

HRR pathway uses extensive regions of DNA homology as coding information. The homologous DNA is usually the sister cromatid and may also be the homologous chromosome. The first step in HRR is the nucleolytic resection of the DSB in the 5'-3' direction by the MRE11-

Rad50-NBS1 complex. The resulting 3' single-stranded DNA is bound by a heptameric ring complex formed by Rad52 proteins. The search for a homologous template and the formation of the joint molecules are performed by Rad51 nucleoprotein filament, which reunion is facilitated by five different paralogues of Rad51 (Rad51B, C and D, XRCC2 and XRCC3). The BRCA2 interacts directly with RAD51, through its BRC repeats and through a domain in this carboxyl terminus (85,106). The interaction between these molecules is essential to RAD51 nucleoprotein filament formation (107). Furthermore, the BRCA1, having important functions in DNA damage checkpoints, seems to be important to RAD51 functions, but the nature of this interaction is still unknown. After strand exchange, the resulting structures are resolved according to the classical model of Holliday (108).

In contrast to HRR, NHEJ is a conceptually simple pathway that involves the religation of broken ends and does not require a homologous template (109). NHEJ is initiated by the binding of a heterodimer complex consisting of the Ku (thyroid autoantigen) 70 and Ku80 proteins to the damaged DNA, protecting DNA from exonucleases digestion. The Ku heterodimer associates with the catalytic subunit of DNA protein kinase (DNA-PK). One of the targets of DNA-PKs is XRCC4 which forms a stable complex with DNA ligase IV (LIG IV), which binds to the ends of DNA molecules and links duplex DNA molecules with complementary but non-ligatable ends. The XRCC4-LIG IV complex cannot directly re-ligate most DSB, being these processed first. The processing of DSB is mainly performed by MRE11-Rad50-NBS1 complex. Two other proteins that seem to be involved in the removal of 5' and 3' overhang are FEN1 and Artemis (109).

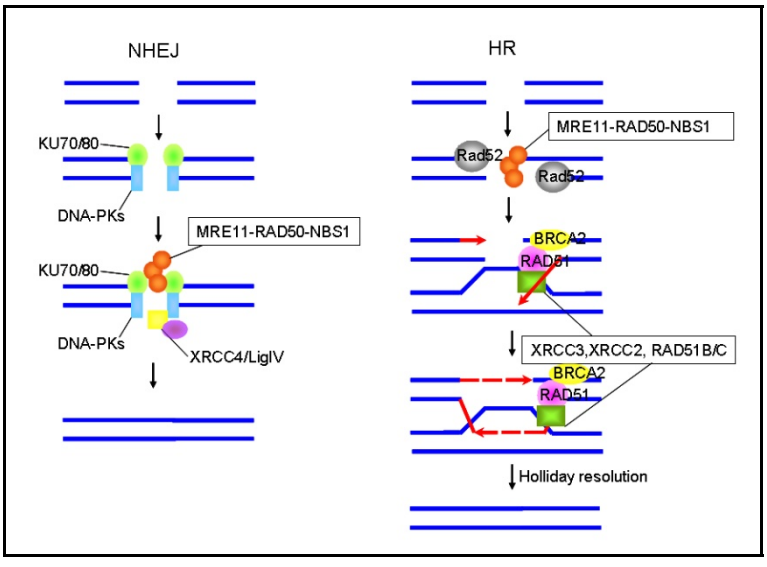

Figure 8 - Simplified diagram of DSBR pathway.

1.2.3. GENETIC POLYMORPHISMS, DNA REPAIR CAPACITY AND BREAST CANCER RISK

The DNA damage signalling and repair pathways are essential mechanism to the genome viability. The important role of DNA repair in the maintenance of a normal cellular genotype and a cancer-free state is obvious in cancer with family history, in which the presence of rare variant alleles but highly penetrance at a number of loci is associated with a high risk for cancer.

A huge number of common polymorphisms have been described in DNA damage signalling and repair genes (110-112). Observations of inter-individual differences in measurements of DNA damage suggest that these polymorphisms may alter the functional properties of DNA repair enzymes. For all the evidences, it seems of great importance to define the meaning of DNA damage signalling and repair polymorphisms in the context of protein and pathway functions and their contribution to breast cancer risk.

Several *BER* genes as been described to present polymorphic regions, namely, *APE1, XRCC1,* glycosylases (8-oxoguanine DNA glycosylase - *OGG1), LIG I* and *LIG III* (112). Several studies have been conducted with the aim of determine the influence of BER polymorphisms in breast cancer risk (Table 3). Until today, more than 200 polymorphisms have been identified in the NER pathway (111,112) and several studies have been performed to associate them with breast cancer risk. In Table 4, we show reports associating breast cancer susceptibility and NER polymorphisms. Cells evolved two sub-pathways to repair DSB lesions: NHEJ and HRR. Some polymorphisms have been demonstrated in some NHEJ and HRR genes (110), several of them have been examined in case-control studies for breast cancer risk (Table 5). An overview of the different studies associating breast cancer and DNA signalling polymorphic genes is presented in Table 6. From the analysis of the tables above, we can conclude that there are a lack of consistent findings between polymorphisms and breast cancer risk. Several points could be responsible for these differing reports. First of all, breast cancer is a heterogeneous disease, but the majority of the analysis treats it as one, using in the same analysis different types of tumors. Secondly, risk factors of breast cancer behind the genetic factors, such as environmental, lifestyle, endogenous metabolism, are very difficult to identify, and therefore so complicated to be included in case-control studies. Third, although it has been shown that >95% of genetic variation is shared across populations and that <10% is specific to a single population (113), it is also well known that prevalence of the allele variants differs across racial/ethnic groups (114). This is

phenomenon which adds considerable importance to studies that comprise a diverse group of racial/ethnic groups or populations with different ancestry.

Table 3 – Studies of association of BER genetic polymorphisms with breast cancer risk and functional phenotype.

Gene Name	Polymorphism Name	Breast cancer risk (OR; 95% CI)#	DNA repair capacity/environment interaction	Reference
OGG1	*Ser326Cys*	0.98 (0.52-1.86)	*	Vogel et al, 2003 (115)
		1.3 (0.92-1.93)	*	Choi et al, 2003 (116)
XRCC1	*Arg194Trp*	1.61 (0.10-26.1)	No	Moullan et al, 2003 (117)
	Arg399Gln	1.00 (0.57-1.76)	No	
		2.24 (0.91-5.53)[a]		
	Arg194Trp	2.46 (0.98-6.17)[b]	*	Smith et al, 2003 (118)
		1.58 (0.39-6.39)[c]		
		0.92 (0.53-1.58)[a]		
	Arg399Gln	0.57 (0.32-1.02)[b]	*	
		0.74 (0.31-1.72)[c]		
		1.05 (0.59-1.87)	*	Smith et al, 2003 (119)
	Arg399Gln	1.20 (0.85-1.69)	Yes	Shu et al, 2003 (120)
		1.03 (0.77-1.37)	No	Han et al, 2003 (121)
	Gln632Gln	0.90 (0.69-1.16)	No	
		0.89 (0.46-1.72)	*	Forsti et al, 2004 (122)
	Arg399Gln	0.88 (0.57-1.37)	Yes	Figueiredo et al, 2004 (123)
		1.27 (0.62-2.61)	*	Deligezer et al, 2004 (124)
	Arg194Trp	2.78 (0.82-9.40)	*	Chacko et al, 2005 (125)
	Arg280His	1.69 (0.29-9.63)	*	
	Arg399Gln	2.69 (1.10-6.57)	*	
		0.97 (0.73-1.29)	Yes	Shen et al, 2005 (126)
DNA Pol β	*Lys289Met*	3.76 (1.87-7.56)	*	Sliwinski e tal, 2007 (127)
	Pro242Arg	1.96 (1.15-3.34) [d]	*	

Homozygote variant genotype vs homozygote wild type genotype; * study not realized; ** visualized in the test; a) variant genotypes vs wild type genotype between healthy women with FH vs healthy women without FH; b) variant genotypes vs wild type genotype between breast cancer patients without FH vs healthy women without FH; c) variant genotypes vs wild type genotype between breast cancer patients with FH vs healthy women without FH; d) homozygote wild type genotype vs heterozygote genotype.

Table 4 – Studies of association of NER genetic polymorphisms with breast cancer risk and functional phenotype.

Gene Name	Polymorphism Name	Breast cancer risk (OR; 95% CI)#	DNA repair capacity/environment interaction	Reference
XPC	*Lys939Gln*	0.91 (0.47-1.73)	*	Försti et al, 2004 (122)
		0.91 (0.47-1.73)	*	Zhang et al, 2005 (128)
XPG	*Asp1104His*	1.19 (0.57-2.50)	No	Kumar et al, 2003 (129)
XPD	*Asp312Asn*	0.51 (0.27-0.94)	*	Försti et al, 2004 (122)
	Lys751Gln	1.00 (0.57-1.77)	*	
	Asp312Asn	2.06 (0.63-6.69)[a]	Yes	Shi et al, 2004 (130)
	Lys751Gln	1.49 (0.46-4.86)[a]	Yes	
	Asp312Asn	2.06 (1.39-3.07)[b]	*	Justenhoven et al, 2004 (131)
	Lys751Gln	1.32 (0.94-1.86)[b]	*	
		1.18 (0.91-1.53)[a]	Yes	Terry et al, 2004 (132)
	Asp312Asn	0.80 (0.12-3.23)	*	Lee et al, 2005 (133)
		1.38 (1.11-1.73)[b]	*	
	Lys751Gln	1.01 (0.82-1.25)[b]	*	Kuschel et al, 2005 (134)
	Asp312Asn	10.2 (0.77-1.36)[b]	*	
		0.77 (0.54-1.10)[b]	*	
ERCC1	*3'UTR C8092A*	0.58 (0.38-0.89)[c]	No	Lee et al, 2005 (135)
	C354T	1.08 (0.84-1.39)[a]	Yes	
XPF	*T835C*	1.30 (0.57-2.74)	*	Lee et al, 2005 (133)

Homozygote variant genotype vs homozygote wild type genotype; * study not realized; a) Variant genotypes vs wild type genotype between breast cancer patients vs healthy women; b) homozygotes variant genotype vs wild type genotype between breast cancer patients vs healthy; c) homozygote variant genotype vs wild type and heterozygote genotypes between breast cancer patients vs healthy women.

Table 5 – Studies of association of DSBR genetic polymorphisms with breast cancer risk.

Gene Name	Polymorphism Name	Breast cancer risk (OR; 95% CI)#	Reference
NBS1	*Glu185Gln*	0.70 (0.39-1.28)	Försti et al, 2004 (122)
		0.70 (0.39-1.28)	Zhang et al, 2005 (128)
RAD51	*5'UTR G135C*	0.69 (0.27-1.80)	Webb et al, 2005 (136)
		0.89 (0.67-1.17)[a]	
	5'UTR G172T	0.84 (0.56-1.26)[a]	Lee et al, 2005 (135)
RAD52	*3'UTR C2259T*	1.33 (1.02-1.75)[a]	
XRCC2	*Arg188His*	1.12 (0.88-1.44)[a]	Han et al, 2004 (23)
		1.01 (0.77-1.33)[a]	Webb et al, 2005 (136)
XRCC3	*Thr241Met*	1.48 (0.64-3.43)	Smith et al, 2003 (118)
		0.98 (0.67-1.41)[a]	Smith et al, 2003 (119)
		1.72 (0.94-3.15)[b]	Försti et al, 2004 (122)
		0.74 (0.34-1.60)[c]	
	5'UTR A4541G	1.10 (1.00-1.20)[a]	
	IVS5-14 A>G	1.04 (0.87-1.25)[a]	Han et al, 2004 (23)
		0.92 (0.76-1.11)[a]	
	Thr241Met	1.47 (1.00-2.15)[a]	Figueiredo et al, 2004 (123)
		0.84 (0.64-1.09)[a]	Webb et al, 2005 (136)
		1.79 (0.98-3.26)	Zhang et al, 2005 (128)
BRCA2	*Asn372His*	1.65 (0.36-7.58)	Ishitobi et al, 2003 (137)
	Met784Val	2.03 (1.07-3.87)[a]	
	Thr1915Met	2.20 (0.20-23.7)[d]	Górski et al, 2005 (138)
		5.40 (1.20-24.6)[e]	

Homozygote variant genotype vs homozygote wild type genotype; a) Variant genotypes vs wild type genotype; b) Finish population; c) Polish population; d) under age 40 years; e) above age 41 years.

Table 6 – Studies of association between DNA damage signalling genetic polymorphisms with breast cancer risk.

Gene Name	Polymorphism Name	Breast cancer risk (OR; 95% CI)	Reference
ATM	*IVS22-77 T>C*	1.67 (1.00-2.81)	Angéle et al, 2003 (139)
	IVS48+238 C>G	1.66 (1.00-2.76)	
	G5557A	1.07 (0.35-3.24)	
	-5144 A>T	1.13 (0.92-1.39)[a)]	Lee et al, 2005 (140)
	IVS21+1049 T>C	1.39 (1.09-1.77)[a)]	
	IVS33-55 T>C	1.19 (0.96-1.47)[a)]	
	IVS34+60 G>A	1.29 (1.04-1.60)[a)]	
	3393 T>G	1.24 (1.00-1.54)[a)]	
TP53	*PIN3 Ins16bp*	5.30 (1.10-25.6)[a)b)]	Wang-Gohrke et al, 2002 (141)
		1.20 (0.90-1.60)[a)]	
	Arg72Pro	2.30 (0.70-7.60)[a)b)]	
		1.10 (0.80-1.40)[a)]	
	MspI Intron6	2.80 (0.80-19.3)[a)b)]	
		1.20 (0.90-1.50)[a)]	
	Arg72Pro	2.14 (1.21-3.79)	Huang et al, 2003 (142)
		1.26 (0.96–1.67)	Cox et al, 2007 (143)

´# Homozygote variant genotype vs homozygote wild type genotype; a) Variant genotypes vs wild type genotype between breast cancer patients vs healthy women; b) breast cancer patients with FH.

1.2.4. *XRCC1, XPD, RAD51, XRCC3* AND *TP53* POLYMORPHIC GENES AS GOOD CANDIDATES TO EVALUATE INDIVIDUAL BREAST CANCER SUSCEPTIBILITY

XRCC1 protein is thought to act as scaffold protein for both single-strand break repair (SSBR) and BER activities (144). Three domains have been identified within its 633 amino acid protein: the NH2-terminal domain (residues 1–183), which interacts with DNA Pol-β and DNA containing a single-strand break; a central BRCA C-terminal (BRCT)-I domain (residues 315–403), which interacts with PARP-1; and the COOH-terminal BRCT-II (residues 538–633), which interacts with the COOH-terminal domain of DNA LIG III. XRCC1 is responsible for the assembly of these proteins in lesion area (144). Several polymorphisms have been found in *XRCC1* gene and some have been linked with a variety of cancers (145). A common *XRCC1* polymorphism, *Arg399Gln*, located in exon 10, results in an amino acid substitution from arginine to glutamine (Arg→Gln), within the BRCT-I domain, where PARP-1 binds, therefore affecting complex assembly and the repair efficiency of BER (146).

XPD protein is a DNA helicase, being a subunit of the TFIIH complex and presenting important roles both in transcription and NER pathway. XPD participates in the locally unwind of DNA helix

allowing RNA transcription machinery to access the promoter and to permit the NER machinery access to the lesion (104). *XPD* is a highly polymorphic gene and correlation of its polymorphisms and cancer risk have been intensively studied (147,148). One of the most common *XPD* polymorphism, *Lys751Gln*, has been associated with a differential DNA repair capacity (149-151). This polymorphism, located in exon 23, is characterized by an A to T substitution causing a lysine to glutamine amino acid exchange (Lys→Gln) at codon 751. *Lys751Gln* is situated in the important domain of interaction between XPD protein and its helicase activator, inside the TFIIH complex, suggesting a biological importance in XPD function (152).

In the HRR pathway, RAD51 is an important protein in DSBR pathway promoting DNA homologous pairing and strand exchange, in association with other proteins of the heterodimer complex with several other genes (such as *XRCC2*, *XRCC3*, *BRCA2* and others) (153). In cultured mammalian cells, RAD51 is involved in spontaneous gene conversion as well as in HRR induced by radiation, alkylating agents and replication elongation inhibitors. More precisely, RAD51 controls DSB repair via gene conversion associated or not with crossing over (154). RAD51 also partly participates in induced sister chromatid exchange in mammalian cells. *RAD51* gene presents a low rate of polymorphisms and the majority of them are described in UTRs, such as *5'UTR G135C* polymorphism, occurring in the 5'UTR region at 135 position by a substitution of a guanine by a cytosine. The biological effect of this polymorphism is currently unknown. However, in two studies, an elevated breast cancer risk associated with *RAD51 135C* allele was reported in BRCA2 mutation carriers, but not in BRCA1 mutation carriers (155,156).

Another important HRR protein is XRCC3, that interacts directly with RAD51, helping in the assembly of the nucleofilament protein and in the selection and interaction with appropriate recombination substrates (157), being required for genomic stability. The *XRCC3 Thr241Met* polymorphism, located in exon 7, is a very common variant in the general population, and results from the substitution of a C to a T, which induces in an amino acid substitution of a threonine by a methionine (Thr→Met) at codon 241. Some findings have demonstrated that this polymorphism may affect the enzyme's function and/or its interaction with other proteins involved in DNA damage and repair (158). Some functional studies have shown that *XRCC3 241Met* variant present a decreased DNA repair function and some deficiencies in mitotic events (158,159). Molecular epidemiological studies have linked this *XRCC3* polymorphism to increased risk of some types of cancer, among them breast cancer (123,160).

The *TP53* tumour suppressor gene, also designated the "guardian of the genome", is essential in the preservation of genome integrity. From the numerous biological functions of p53 protein, inhibition of cell cycle progression, DNA repair and apoptosis are the major cellular pathways where it is involved (161). *TP53* gene mutations are widely detected in breast cancer, being correlated with specific clinical phenotypes (162,163). Furthermore, several polymorphisms have been identified within *TP53* gene, both in non-coding and coding regions (164). One of the most well studied *TP53* gene polymorphism is *Arg72Pro*, located in codon 72 on exon 4, leading to arginine-proline substitution (Arg→Pro), which in its turn results in a structural alteration of the protein giving rise to variants with distinct electrophoretic mobility (165). This polymorphism occurs in a proline-rich region of p53, which is known to be important for the growth suppression and apoptotic functions of this protein (165). Another common polymorphism is 16 base pair (bp) duplication in intron 3 of the *TP53* gene (*PIN3 Ins16bp*). However, until now only a single report has shown an altered mRNA expression linked to the presence of this polymorphism (166). Consistent with this altered functional activity, several studies have correlated the intron 3 duplication with an increased risk of various cancers, including breast cancer (167-169).

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CHAPTER 2 – DNA REPAIR POLYMORPHISMS MIGHT CONTRIBUTE DIFFERENTIALLY ON FAMILIAL AND SPORADIC BREAST CANCER SUSCEPTIBILITY: A STUDY ON A PORTUGUESE POPULATION

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ABSTRACT

The purpose of this study was to evaluate the role of polymorphisms in DNA repair genes as genetic indicators of susceptibility to familial and sporadic breast cancer. We analysed DNA samples from 285 breast cancer patients and 442 control subjects, for *XRCC1 Arg399Gln*, *XPD Lys751Gln*, *RAD51 G135C* and *XRCC3 Thr241Met* polymorphisms using PCR-RFLP. We observed that women carriers of *XRCC1 399Gln* genotypes and without FH of breast cancer have a protective effect concerning this disease (OR= 0.54 95% CI 0.35-0.84; p=0.006). Furthermore, we found that carriers of *XRCC3 241Met* genotypes without FH have an increased susceptibility of breast cancer (OR= 2.21 95% CI 1.42-3.44; p<0.001). Additionally, we verified an increased risk of breast cancer in women with FH and carrying *RAD51 135C* genotypes (OR= 2.17 95% CI 1.19-3.98; p=0.012). Our results suggest *XRCC1 Arg399Gln* and *XRCC3 Thr241Met* DNA repair polymorphisms as important biomarkers to sporadic breast cancer susceptibility, as well as, *RAD51 G135C* polymorphism as a real risk modifier in familial breast cancer cases.

2.1. INTRODUCTION

Breast cancer is the leading cause of death among women in developing countries. In Portugal, it presents the highest incidence and mortality rates among women diseases (1). It is a disease caused by a complex combination of genetic and environmental factors. Well-established risk factors have been described to breast cancer, such as early menarche, late menopause, age of first child's birth, nulliparity and family history (2). Family history of breast cancer is a particularly important high risk factor for this disease. Two genes were identified as the major susceptibility genes in high risk families, namely *BRCA1* and *BRCA2*. However, these genes account for only a minority of the overall family risk of breast cancer (3). Furthermore, approximately only 10% of all breast cancer cases exhibit a familial pattern of incidence (4). In this way, the identification of genetic susceptibility factors that account from low to moderate breast cancer risk is an important step in the definition of individual risk to this malignancy.

Many environmental factors have been associated with risk of breast cancer development, such as ionized radiation and chemical carcinogens, such as some presented in diet and environment (5). These mutagens sources, together with endogenous and exogenous estrogens,

produce a wide range of DNA lesions such as reactive oxygen species, oxidized bases, bulky DNA adducts and DNA strand breaks (6,7). Mammalian cells have evolved distinct pathways to repair different types of DNA damage in order to maintain genome integrity. Therefore, DNA repair capacity determines cellular susceptibility to endogenous and exogenous substances. Some studies have demonstrated a strong association of higher levels of DNA damage and lower DNA repair capacity in breast cancer patients and in healthy women with a positive family history of breast cancer (8,9). Genetic polymorphisms in DNA repair genes are very common events (10-12), and some studies have shown a significant effect of some of these polymorphisms in DNA repair capacity (13-15).

XRCC1 protein is thought to act as scaffold protein for both single-strand break repair and BER activities (16). It has been shown that XRCC1 interacts with DNA Polβ, DNA LigIII and APE1, through a BRCT-I domain at the C-terminus (16). Several polymorphisms have been found in *XRCC1* gene and some have been linked with a variety of cancers (17). One common *XRCC1* polymorphism, *Arg399Gln*, located in exon 10, lies within the BRCT-1 domain (18).

Another important DNA repair protein is XPD protein is a subunit of the TFIIH complex and has important roles in transcription and NER pathway. XPD participates in the locally unwind of DNA helix to allow RNA transcription machinery to access the promoter and to permit the NER machinery to access the lesion (19). *XPD* is a highly polymorphic gene and correlation of its polymorphisms and cancer risk have been intensively studied (14,20). One common *XPD* polymorphism, *Lys751Gln*, has been associated with a differential DNA repair capacity (21-23). This polymorphism, located in exon 23, is situated in the important domain of interaction between XPD protein and its helicase activator, inside the TFIIH complex (24).

Double strand break damage is the most injurious lesions observed because it causes cell death or loss of genetic material. HRR and NHEJ are two distinct mechanisms in the repair of DSB in mammalian cells. In the HRR pathway, RAD51 is an important protein in DSBR pathway that forms a heterodimer with several genes (such as *XRCC2*, *XRCC3*, *BRCA2*) playing central role in strand exchange (25). The majority of *RAD51* polymorphisms described are in UTRs, such as *5'UTR G135C*. Another HRR protein is XRCC3, that interact directly with RAD51, helping in the assembly of the nucleofilament protein and in the selection and interaction with appropriate recombination substrate (26). The *XRCC3 Thr241Met* polymorphism, located in exon 7, could affect the enzyme function and/or its interaction with other proteins involved in DNA damage and repair (15).

We hypothesize that those common polymorphisms of DNA repair genes could modify either DNA repair capacity or fidelity, which may contribute to familial and sporadic breast cancer susceptibility. In a case-control study, we analysed polymorphisms of genes involved in different DNA repair pathways, since the DNA damage produced by breast mutagens are repaired by different pathways (6,7): *XRCC1 Arg399Gln* in BER, *XPD Lys751Gln* in NER and *RAD51 5'UTR G135C* and *XRCC3 Thr241Met* in HRR.

2.2. Materials and Methods

Study Population

We analysed a total of 727 DNA samples. From 285 breast cancer cases, 84 unrelated familial breast cancer cases were selected from S. João Hospital at Porto and General Hospital at Vigo, and 201 unrelated sporadic breast cancer cases were recruited from IPO-Porto (Oncology Portuguese Institute), during 1998-2003, form patients that were receiving treatment. All cases were histological confirmed at the Departments of Pathology. Familial case group presented a mean age of 41.05, with an age range of 21-77 years. The high-risk family history of breast cancer group included women with the follow features, based on the Breast Cancer Linkage Consortium criteria (27): early onset (≤40 years) and/or bilaterality; or more than three cases of breast cancer in the family; or more than one case of ovarian cancer in the family; or more than two first-degree relatives involved; or male breast cancer. Sporadic cases group presented a mean age of 53.56, with an age range of 41-88 years. Control women were randomly selected from blood banks in the same region during the same time period as the cases were collected. The selection criteria include no prior history of cancer, and controls were frequency matched to the cases by age (±5 years). 442 healthy women, presenting a median age of 42.29 and an age range of 21-85 years, were used as control group of familial breast cancer cases. 226 healthy women, with a median age of 53.05 and an age range of 41-85, were selected according to age of diagnosis higher than 40 years, and it was used as control group of sporadic breast cancer cases. All participants provided informed consent.

Genotyping

Blood samples from all study participants were collected in tubes with EDTA as an anticoagulant. Genomic DNA was isolated from buffy coat using Puregene® DNA Purification Kit by Gentra Systems. All the polymorphisms were assessed by PCR-RFLP technique. The PCR and RFLP conditions to perform genotyping are presented in Table 1.

Table 1 – Primers, amplification parameters and PCR product fragments weight used as PCR conditions, and specific restriction enzymes and corresponding digestion product fragments weight used as RFLP conditions, for the polymorphisms studied.

PCR Conditions	**Polymorphism**			
	XRCC1 Arg399Gln	*XPD Lys751Gln*	*RAD51 G135C*	*XRCC3 Thr241Met*
Primers				
Forward	5'-CAA GTA CAG CCA GGT CCT AG-3'	5'-CTG CTC AGC CTG GAG CAG CTA GAA TCA GAG GAG ACG CTG-3'	5'-TGG GAA CTG CAA CTC ATC TGG-3'	5'-GCC TGG TGG TCA TCG ACT C-3'
Reverse	5'-CCT TCC CTC ATC TGG AGT AC-3'	5'-AAG ACC TTC TAG CAC CAC CG-3'	5'-GCG CTC CTC TCT CCA GCA G-3'	5'-ACA GGG CTC TGG AAG GCA CTG CTC AGC TCA CGC ACC-3'
Annealing temperature	58°C/30 seconds	60°C/30 seconds	53°C/30 seconds	60°C/30 seconds
Number of cycles	32	32	32	32
PCR product (bp)	268	161	159	136
RFLP Conditions				
Restriction enzyme	*Bcn I* (Fermentas)	*Pst I* (Fermentas)	*Mva III* (Fermentas)	*Nla III* (New England Biolabs)
Digestion products (bp)				
W	91 and 177	161	71 and 88	136
M	268	41 and 120	159	35 and 101

Statistical Analysis

Analysis of data was performed using the computer software SPSS version 14.0. Chi-square (χ^2 test) analysis was used to compare categorical variables. A 5% level of significance was used in the analysis. The Odds Ratio (OR) and its 95% confidence interval (CI) were calculated to measure the association between polymorphic variants and breast cancer risk. Logistic regression analysis was used to calculate the adjusted OR and 95% CI for the influence of different genotypes in the risk of breast cancer, adjusted for age. Whenever appropriate, the observed number of each genotype in control groups were compared with that expected for a population in the Hardy-Weinberg Equilibrium by using a goodness of fit χ^2 test. We hypothesized that DNA repair genotypes may alter the onset of disease in these cases, as performed before by

Medeiros et al (28). We therefore considered the waiting time for the onset of disease as the interval between the time of initial exposure to the risk factor (DNA repair genotypes) and the time of onset of disease. We estimated the cumulative probabilities for having disease (cumulative hazard function plots) by the Kaplan–Meier methodology. The primary analysis of time-to-event end points for waiting time for the onset of disease was performed with the use of a two-sided log-rank test at the 5% level of significance.

2.3. RESULTS

The genotypic and allelic frequencies of DNA repair polymorphisms studied for cases, familial and sporadic breast cancer, and controls are presented in Table 2.

Table 2 – Allelic and genotype frequencies of polymorphisms in *XRCC1, XPD, RAD51* and *XRCC3* genes and familial and sporadic breast cancer cases and controls.

Polymorphisms	Genotype or Allele	Familial Risk Cases n (%)	Controls n (%)	Sporadic Cases n (%)	Controls n (%)
XRCC1 Arg399Gln					
P_{HWE}=0.781	*Arg/Arg*	28 (34.6)	153 (34.7)	84 (48.0)	75 (34.1)
	Arg/Gln	42 (51.9)	222 (50.3)	67 (38.3)	116 (52.7)
	Gln/Gln	11 (13.6)	66 (15.0)	24 (13.7)	29 (13.2)
	Arg	98 (60.5)	528 (59.9)	235 (67.1)	266 (60.5)
	Gln	64 (39.5)	354 (40.1)	115 (32.9)	174 (39.5)
XPD Lys751Gln					
P_{HWE}=0.415	*Lys/Lys*	34 (42.0)	216 (48.9)	93 (46.3)	115 (52.8)
	Lys/Gln	41 (50.6)	179 (40.5)	84 (41.8)	81 (37.2)
	Gln/Gln	6 (7.4)	47 (10.6)	24 (11.9)	22 (10.1)
	Lys	109 (67.2)	611 (69.1)	270 (67.2)	311 (71.3)
	Gln	53 (32.7)	273 (30.9)	132 (32.8)	125 (28.7)
RAD51 5'UTR G135C					
P_{HWE}=0.842	*GG*	64 (78.0)	381 (87.6)	152 (83.1)	177 (83.9)
	GC	18 (22.0)	53 (12.2)	27 (14.8)	33 (15.6)
	CC	0 (0.0)	1 (0.2)	4 (2.2)	1 (0.5)
	G	146 (89.0)	815 (93.7)	331 (90.4)	387 (91.7)
	C	18 (11.0)	55 (6.3)	35 (9.6)	35 (8.3)
XRCC3 Thr241Met					
P_{HWE}=0.002	*Thr/Thr*	40 (49.4)	225 (52.2)	68 (38.6)	121 (57.3)
	Thr/Met	29 (35.8)	140 (32.5)	77 (43.8)	61 (28.9)
	Met/Met	12 (14.8)	66 (15.3)	31 (17.6)	29 (13.7)
	Thr	109 (67.3)	590 (68.4)	213 (60.5)	303 (71.8)
	Met	53 (32.7)	272 (31.6)	139 (39.5)	119 (28.2)

We tested each polymorphism for association with breast cancer risk in two breast cancer subgroups: negative presence of FH of breast cancer, designated as sporadic cancer, and positive FH of breast cancer, family risk cases, and the results are presented in Table 3.

Concerning *XRCC1 Arg399Gln* polymorphism, *399Gln* genotypes were observed in lower frequency in all breast cancer cases (56.3%) and sporadic breast cancer cases (52.0%) compared with the respective control group (65.3% and 65.9%, respectively). The *399Gln* genotypes showed a protective effect to sporadic breast cancer risk (p=0.006; OR= 0.54, 95% CI 0.35-0.84). However, no statistically significant difference was observed regarding familial breast cancer risk (p=0.932; OR= 1.02, 95% CI 0.61-1.71).

No statistically significant associations were found between the *XPD Lys751Gln* polymorphism and risk of familial and sporadic breast cancer risk (table 3).

Regarding *RAD51 G135C* polymorphism, our results showed that carriers of variant *135C* present an increased risk of familial breast cancer (p=0.012; OR=2.17, 95% CI 1.19-3.98), while no statistically significant correlation was observed with sporadic breast cancer risk (p= 0.477; OR= 1.22, 95% CI 0.70-2.14).

The *XRCC3 241Met* genotypes carriers with negative FH were at a 2.21-fold (95% CI = 1.42–3.44; p<0.001) increased risk of breast cancer compared with the respective control group. No statistically significant differences were observed in genotypes frequencies comparing positive FH breast cancer cases and respective control group.

Table 3 – Polymorphisms in DNA repair genes as biomarkers of breast cancer susceptibility in presence or absence of family risk.

Polymorphism	Familial Risk Cases		Sporadic Cases	
	OR (95% CI)*	P value	OR (95% CI)*	P value
XRCC1 Arg399Gln *Gln* carriers	1.02 (0.61-1.71)	0.932	0.54 (0.35-0.84)	0.006
XPD Lys751Gln *Gln* carriers	1.31 (0.80-2.15)	0.278	1.45 (0.95-2.19)	0.082
RAD51 G135C *C* carriers	2.17 (1.19-3.98)	0.012	1.22 (0.70-2.14)	0.477
XRCC3 Thr241Met *Met* carriers	1.16 (0.71-1.89)	0.558	2.21 (1.42-3.44)	<0.001

* OR adjusted for age among variant carriers vs wild type carriers (logistic regression analysis)

Regarding the waiting time-to-onset of disease, the cumulative probabilities of having familial and sporadic breast cancer according to the presence or absence of the variants genotypes are shown in Figure 1 and 2. We observed statistically significant differences in the mean waiting time-to-onset of sporadic breast cancer for carriers of the *399Gln* genotypes (Figure 2 (a)) and for carriers of the *241Met* genotypes (Figure 2 (d)) in comparison for non-carriers (log-rank test: p=0.028 and p<0.001, respectively).

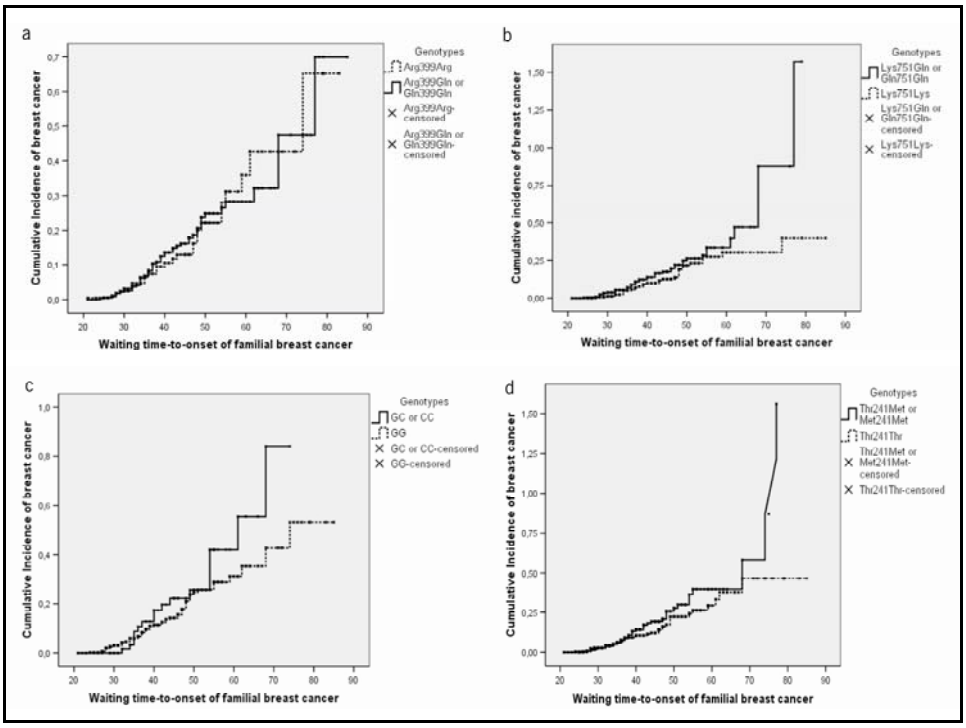

Figure 1 - Association between *XRCC1 Arg399Gln* (a), *XPD Lys751Gln* (b), *RAD51 G135C* (c) and *XRCC3 Thr241Met* (d) polymorphisms and the waiting time-to-onset on familial breast cancer cases. Cumulative hazard function plots by the Kaplan–Meier methodology and Log-rank test.

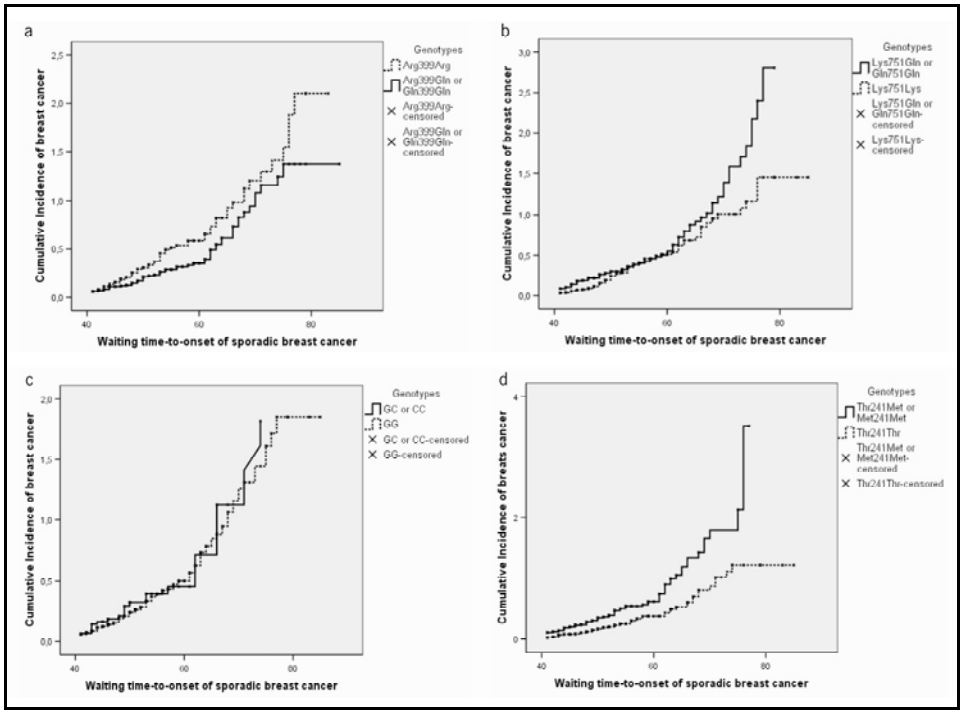

Figure 2 - Association between *XRCC1 Arg399Gln* (a), *XPD Lys751Gln* (b), *RAD51 G135C* (c) and *XRCC3 Thr241Met* (d) polymorphisms and the waiting time-to-onset on sporadic breast cancer cases. Cumulative hazard function plots by the Kaplan–Meier methodology and Log-rank test.

2.4. DISCUSSION

The individual susceptibility to the development of cancer may be influenced by several factors. Increasing evidences suggest that genetic polymorphisms may have an important contribution on cancer susceptibility and tumour behaviour, particularly in hormone related cancers (28-31).

Maintenance of the genomic integrity by DNA repair genes is an essential step in normal cellular growth and differentiation (32). There is increasing data supporting the hypothesis that genetic polymorphisms in various DNA repair genes result in reduced DNA repair capacity, in this way, being associated with increased susceptibility to various human solid tumours (13-15,17,20-23).

In our study, the allelic frequencies for the different polymorphisms were in accordance with earlier reports from European populations (33,34). The genotype frequencies in the controls are in agreement with those expected under Hardy-Weinberg equilibrium, except for *XRCC3 Thr241Met* polymorphism genotypes. Hardy-Weinberg equilibrium depends on a series of features about the tested population, including, for example, the sample population size, random mating, no migration, no genetic drift and no selection taking place (35). As the discrimination of the other genotypes for this assay was good, this deviation could be due to chance or violation of these assumptions, rather than to genotyping errors.

In this case-control study we investigated the role of polymorphisms of DNA repair genes involved in BER, NER and HRR. Our findings suggest a protective effect of the *399Gln* genotypes of *XRCC1* polymorphism to development of breast cancer in women with no FH. These results are consistent with those from previous studies of *XRCC1* polymorphisms and cancer risk, that reported a protective association between the *399Gln* carriers and cancer (34,36,37). However, previous studies, performed in breast cancer, observed a positive, specially when gene-exposure interactions were considered (38-40), or no association (41,42) between the *399Gln* carriers and breast cancer risk, also concerning in some studies the presence of FH of breast cancer (43-46). Functional studies of *XRCC1* suggest that the *399Gln* allele may be associated with higher levels of different types of DNA damage in some cancers (15,47,48). These contradictory effects of *399Gln* genotypes could be explained by two points. First, the gene variants could independently confer particular function to XRCC1 protein, since the effects of *XRCC1* alleles could potentially depend upon competing biochemical pathways operating in the tissue being analyzed (49), as

well as the effects of any given genetic variant will depend upon other genetic and environmental factors that interact with that variant (50). Secondly, these variants could diminish the efficiency of the protein but still provide decreased cancer risk, since, in the presence of excessive damage, cells carriers of these variants would have decreased ability to repair and might be more likely to undergo apoptosis.

In this study, we did not find an association between the *XPD Lys751Gln* polymorphism and breast cancer. To date, the results evaluating *XPD Lys751Gln* polymorphism are controversial. Our results are in agreement with other studies where no correlation was found between this polymorphism and breast cancer risk, among women without FH (38,51,52) and with FH of breast cancer (43,44). In contrast, significant association between *XPD 751Gln* allele and breast cancer risk was seen in recent studies (53). Furthermore, the functional significance of *XPD* polymorphism seems to be dependent from environmental factors that interact with that variant. Some studies showed that *751Gln* allele was associated with reduced DNA repair efficiency of UV-light DNA damage, while others showed a suboptimal repair of X-ray-induced DNA damage related with *751Lys* allele (47,54).

Regarding *RAD51 5'UTR G135C* polymorphism, our results showed an association of *RAD51 135C* genotypes and increased breast cancer risk only among women with FH of breast cancer, suggesting that this polymorphism contributed to the familial breast cancer in the Portuguese population, in opposition to reported results in the Brazilian population (44). Other studies have reported an association of *RAD51* genotypes with familial breast cancer risk, only in women carriers of *BRCA2* mutations (55-57). Since the number of *BRCA1* and/or *BRCA2* mutations detected in our familial breast cancer cases were too small (58), it was not possible to perform a separate analysis. The biological effect of the *RAD51 G135C* polymorphism is currently unknown. This polymorphism could affect mRNA splicing, regulation of transcription, translation or mRNA stability by association of *5'UTR* region with regulatory elements (59). Furthermore, linkage disequilibrium could occur between *RAD51* gene with another sequence change in a regulatory region of the gene or with another nearby gene affecting the incidence of breast cancer in familial breast cancer. Concerning sporadic breast cancer risk, similar results to our findings were obtained by others studies in the Australian women (60) and in the Anglo-Saxon population(10), where no association was obtained.

Our results demonstrate a strong association of increased breast cancer susceptibility in women carriers of *XRCC3 241Met* genotypes and negative FH of breast cancer. This is

consistent with an earlier report in the United Kingdom (10). Functional data supporting the hypothesis of damaging consequences due to *XRCC3 Thr241Met* polymorphism supports our results. The *241Met* variant has been associated with higher levels of bulky DNA adducts, mitotic defects and lower DNA repair capacities of X-ray-induced DNA damage (15,61). However, other studies report no risk association (44,45,60,62-64).

The variance in results of association in different case-control studies on *XRCC1*, *XPD*, *RAD51* and *XRCC3* polymorphisms may be connected to variation in genetic/ethnic origin and different carcinogenic exposures of the studied populations. Too small sample size and/or the inadequate controlling for certain confounders such as age and family history of breast cancer may also contribute to differing results.

The natural history of breast cancer can be influenced by several factors. We hypothesize that under the influence of genetic polymorphisms, chronic exposure to higher levels of several endogenous (e.g. estrogens) and exogenous breast carcinogens resulting in consequent higher accumulation of DNA damage during an individual's lifetime, may alter the waiting-time-to onset of disease. Moreover, it has been suggested that DNA repair genes are associated to age related disease (65). Our results are consistent with this hypothesis that *XRCC1 Arg399Gln* and *XRCC3 Thr241Met* polymorphisms seem to influence directly the waiting time-to-onset of sporadic breast cancer.

In conclusion, our findings suggest the *RAD51 G135C* polymorphism as a real risk modifier in familial breast cancer cases. Furthermore, we point out that *XRCC1 Arg399Gln* and *XRCC3 Thr241Met* polymorphisms as important biomarkers to sporadic breast cancer susceptibility. A possible interpretation for different associations depending on presence of FH may be that FH broadly represents shared genes and environmental factors, and the presence of a single polymorphism, with likely weak effects on the individual phenotype, may not be measurable except in the context of these supporting factors. Among individuals without a familial predisposition, the effect may be hidden by sum effects of other unidentified genetic and environmental factors (45). Further evidence from other studies and functional data is required to confirm the real significance of the results observed by us in breast cancer risk.

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CHAPTER 3 - *XRCC1 ARG399GLN* AND *RAD51 5'UTR G135C* POLYMORPHISMS AND THEIR OUTCOME IN TUMOR AGGRESSIVENESS AND SURVIVAL OF PORTUGUESE BREAST CANCER PATIENTS

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3. LETTER TO THE EDITOR

Breast cancer is the most common type of cancer in female, including Portugal, where this disease presents the highest incidence and mortality rates (1). Breast cancer risk factors, like prolonged exposure to estrogen and/or IR, *BRCA1, BRCA2, TP53, ATM* and *CHEK2* mutations (2,3), are related with an increased chance of DNA damage, acting as initiators of cellular alterations. DNA repair pathways are critical processes in order to maintain genome integrity. Therefore, genetic polymorphisms in DNA repair genes are common events (4). We previously showed correlations of some of these genetic variations, as *XRCC1 Arg399Gln, RAD51 5'UTR G135C* and *XRCC3 Thr241Met*, with changeable breast cancer susceptibility (5).

In the present study, we aimed to investigate the possible correlations between DNA repair polymorphisms with breast cancer clinical-pathological phenotypes, identifying subgroups of patients according to their genetic background.

We analysed DNA from 165 breast cancer patients, including 33 unrelated FH and 132 sporadic breast cancer cases from Surgical Departments of S. João Hospital and the IPO, at Porto. All participants provided informed consent. Patients presented a mean age of 51.01 years (standard deviation (SD) ±12.68).

We determined *XRCC1 Arg399Gln, RAD51 5'UTR G135C* and *XRCC3 Thr241Met* genotypes by PCR-RFLP technique, as previously described (6).

Chi-square ($\chi 2$ test) analysis was used to compare different variables. Logistic regression analysis was applied to calculate the adjusted p value for age and FH in identification of subgroups of patients according to genotypes. The Kaplan-Meier method was used to estimate overall survival (OS). OS was defined as minimal 60 months follow-up, from clinical diagnosis until death or censorship (were alive at the end of the follow-up time period). Differences on OS were obtained by Log Rank test.

The correlation of the analysed DNA repair polymorphisms with some clinical-pathological features is presented in Table 1. According to our results, *XRCC1 Gln/Gln* genotype seems to be associated with less aggressive tumors, since this genotype was correlated with well differentiated tumors (p=0.022 adjusted for age and breast cancer FH, using logistic regression analysis). Deficient efficiency of the XRCC1 protein has been described in *XRCC1 Gln* variant (7,8). Furthermore, repair of more complex base lesions (9-11) by BER pathway can potentially

convert non-lethal lesion into lethal DSBs (12,13). Thus, deficiency in BER, by low efficiency of XRCC1, may actually reflect a well differentiated nature of the tumor cells in less aggressive tumors, since less lethal lesions are produced.

We also observed that *RAD51 C135* genotypes show a relationship with more aggressive tumors and also with a poorer OS, since we found a significant association of *RAD51 C135* genotypes with moderate to poor differentiated grade (p=0.011, adjusted for age and breast cancer FH, using logistic regression analysis).

Table 1 – Correlation between DNA repair polymorphisms and clinical-pathological parameters in Portuguese breast cancer patients.

Parameters	*XRCC1 Arg399Gln*		*RAD51 G135C*		*XRCC3 Thr241Met*	
	Gln/Gln	Others	*GC* or *CC*	*GG*	*Met/Met*	*Others*
Histological Type						
Invasive ductal	23 (92.0)	115 (86.5)	32 (91.4)	113 (86.9)	23 (88.5)	120 (88.2)
Invasive lobular	0 (0.0)	3 (2.3)	0 (0.0)	3 (2.3)	1 (3.8)	2 (1.5)
Others	2 (8.0)	15 (11.3)	3 (8.6)	14 (2.3)	2 (7.7)	14 (10.3)
P value	0.654		0.606		0.665	
Histological Grade						
I	5 (23.8)	8 (7.1)	6 (18.2)	9 (8.3)	1 (4.0)	14 (12.4)
II	6 (28.6)	61 (54.0)	21 (63.6)	51 (47.2)	11 (44.0)	57 (50.4)
III	10 (47.6)	44 (38.9)	6 (18.2)	48 (44.4)	13 (52.0)	42 (37.2)
P value	0.021*		0.017**		0.269	
Axillary lymph node status						
Negative	10 (41.7)	52 (40.0)	12 (40.0)	54 (41.5)	8 (32.0)	57 (43.5)
Positive	14 (58.3)	78 (60.0)	18 (60.0)	76 (58.5)	17 (68.0)	74 (56.5)
P value	0.878		0.877		0.285	
Estrogen receptor status						
Negative	1 (33.3)	9 (31.0)	2 (28.6)	7 (25.9)	1 (33.3)	8 (27.6)
Positive	2 (66.7)	20 (69.0)	5 (71.4)	20 (74.1)	2 (66.7)	21 (72.4)
P value	0.935		0.888		0.833	
Survival status at last follow-up						
Death	3 (15.0)	13 (12.5)	2 (8.3)	15 (14.0)	1 (4.0)	16 (15.4)
Alive	17 (85.0)	91 (87.5)	22 (91.7)	92 (86.0)	24 (96.0)	88 (84.6)
P value	0.760		0.454		0.131	
Recurrence at last follow-up						
No	14 (77.8)	74 (82.2)	19 (86.4)	74 (80.4)	20 (87.0)	72 (80.0)
Yes	4 (22.2)	16 (17.8)	3 (13.6)	18 (19.6)	3 (13.0)	18 (20.0)
P value	0.658		0.519		0.444	

* p value= 0.022, adjusted for age and history family of breast cancer to compare the influence of different genotypes in histological grade (I vs II/III grade), using logistic regression analysis. ** p value= 0.011, adjusted for age and history family of breast cancer to compare the influence of different genotypes in histological grade (I/II vs III grade), using logistic regression analysis.

Additionally, assessment of OS demonstrated that patients with *RAD51 C135* genotypes (102.87 months mean OS) presented a poorer survival compared with others genotypes (136.36 months mean OS) (Figure 1). These results can be explained by the location of this polymorphism in the UTRs, may be affecting mRNA stability and/or translation efficiency, leading to altered RAD51 protein levels (14). Thus, RAD51, the key factor of homologous recombination process, can disturb the activity of the multi protein DNA repair complex, including BRCA1, BRCA2 and XRCC3, contributing to high levels of genetic instability (15), and as a result, being correlated with more aggressive breast tumors and poor survival.

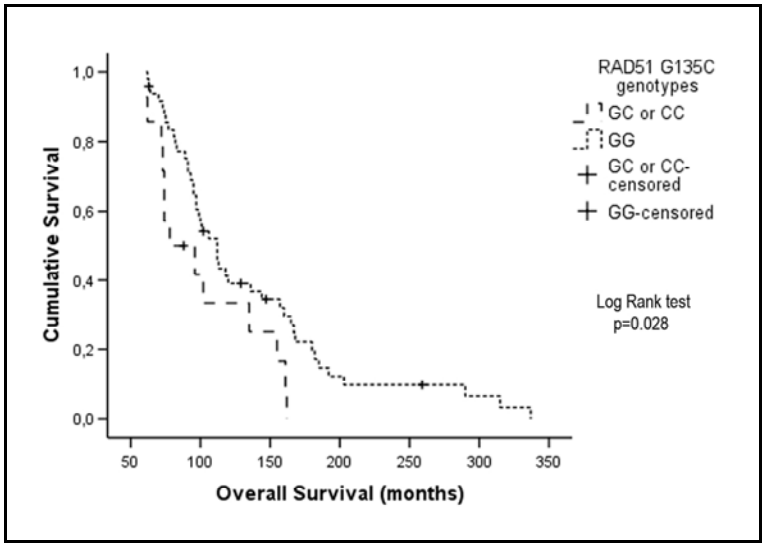

Figure 1 – Kaplan-Meier overall survival curve in breast cancer patients relating with *RAD51 G135C* polymorphism. Log-rank test for statistical analysis.

We had previously showed *XRCC1 Arg399Gln* and *RAD51 5'UTR G135C* as important polymorphism to predict breast cancer risk (16). According to the present results, we clearly underlie the role of these same polymorphisms in the prediction of breast tumor aggressiveness and patients' survival.

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CHAPTER 4 - IMPORTANCE OF *TP53 CODON 72* AND *INTRON 3 DUPLICATION 16BP* POLYMORPHISMS IN PREDICTION OF SUSCEPTIBILITY ON BREAST CANCER AND PRESENCE OF LYMPH NODE METASTASES

The results presented on this chapter were submitted for publication as:

Costa, S., Pinto, D., Pereira, D., Rodrigues, H., Cameselle-Tejeiro, J., Medeiros, R. and Schmitt, F. Importance of *TP53 codon 72* and *intron 3 duplication 16bp* polymorphisms in prediction of susceptibility on breast cancer and presence of lymph node metastases. (2007)

ABSTRACT

TP53 is one of major tumour suppressor genes, also known as the guardian of the genome, being essential in preservation of genome integrity. Two very common polymorphisms have been demonstrated to contribute to cancer susceptibility and tumour behaviour. The purpose of this study was to evaluate the role of *Arg72Pro* and *PIN3 Ins16bp* polymorphisms in *TP53* gene as genetic susceptibility and predictive markers to breast cancer. We analysed DNA samples from 264 breast cancer patients and 440 controls. We observed that women with *A2A2* genotype have increased risk for developing breast cancer, either in women with or without FH of the disease (OR=4.40, 95% CI 1.60-12.0; p=0.004; OR=3.88, 95% CI 1.18-12.8; p=0.026, respectively). In haplotype analysis, statistically significant differences were found between *TP53 Arg-A2* haplotype frequencies and familial breast cancer cases and the respective control group (OR=2.10, 95% CI 1.08-4.06; p=0.028). Furthermore, both *TP53* polymorphisms are associated with higher incidence of lymph node metastases. Our findings suggest *PIN3 Ins16bp* polymorphism as a risk modifier in breast cancer disease, with evidence for relative differential effect by family history. Moreover, *Arg72Pro* and *PIN3 Ins16bp* polymorphisms are suggested as predictive factors of lymph node metastases.

4.1. INTRODUCTION

Breast cancer disease have been associated with well-established risk factors, such as high estrogen exposure, many environmental factors (e. g. diet and ionizing radiation) and family history (1,2). Family history of breast cancer is a particularly important high risk factor for this disease. Two genes were identified as the major susceptibility genes in high risk families, namely *BRCA1* and *BRCA2*. However, these genes account for only a minority of the overall family risk of breast cancer (3). Furthermore, approximately only 10% of all breast cancer cases exhibit a familial pattern of incidence (4,5). In this way, the remaining familial and sporadic risk may be due to common low to moderate penetrance genetic variants, which are also referred as genetic polymorphisms. One strong candidate for genetic susceptibility factor to familial and/or sporadic breast cancer is the *TP53* gene. This gene is frequently somatically mutated in breast cancer (6,7) and *TP53* germline mutations are associated with increased risk for developing diverse

malignancies, including 25–30% of hereditary breast cancer cases (8). Furthermore, based on its pivotal role in DNA damage repair and its physical and functional interactions with BRCA1 and BRCA2 proteins (9,10), *TP53* seems to be a strong candidate breast cancer predisposition.

The *TP53* tumour suppressor gene, also designated the guardian of the genome, is essential in preservation of genome integrity. From the numerous biological functions of p53 protein, inhibition of cell cycle progression, DNA repair and apoptosis are the major cellular pathways where it is involved (6).

TP53 gene mutations are widely detected in breast cancer, being correlated with specific clinical phenotypes (11,12).

Predisposition to several human cancers has been associated with genetic polymorphisms, which may represent an important contribution to cancer susceptibility and tumour behaviour (13-16). Several polymorphisms have been identified within *TP53* gene, both in non-coding and coding regions (17). One of the most well studied *TP53* gene polymorphism is *Arg72Pro*, located in codon 72 on exon 4, leading to arginine-proline substitution, which in its turn results in a structural alteration of the protein (18). Another common polymorphism is 16 base pair (bp) duplication in intron 3 of the *TP53* gene (*PIN3 Ins16bp*).

In this case-control study, we hypothesize that the two common polymorphisms of *TP53* gene play a role either apoptosis, cell cycle control efficiency, as well as DNA repair capacity, which ultimately may contribute to an increase of breast cancer susceptibility within familial and/or sporadic cases, as well as represent an additional tool for prognosis prediction.

4.2. Materials and Methods

Study Population

We analysed a total of 264 DNA breast cancer cases: 73 unrelated familial breast cancer cases were selected from the Oncology and Surgical Departments from S. João Hospital at Porto and Vigo Hospital, and 191 unrelated sporadic breast cancer cases were recruited from IPO-Porto, during 1998-2003, from patients that were receiving treatment. All cases were histological confirmed at the Department of Pathology. Clinical-pathological parameters were obtained when possible from hospital clinical records. Familial case group presented a mean age of 42.07 years, with an age range of 24-77 years. The high-risk familial breast cancer group, also

designated by us as family history (FH) breast cancer cases, included women with the follow features, based on the Breast Cancer Linkage Consortium criteria (19): early onset (≤40 years) and/or bilaterality; or more than three cases of breast cancer in the family; or more than one case of ovarian cancer in the family; or more than two first-degree relatives involved; or male breast cancer. These high-risk breast cancer cases are *BRCA1/BRCA2* mutations non-carriers (20). Sporadic cases group (with no presence of FH) presented a mean age of 53.41 years, with an age range of 41-88 years. Control women were randomly selected from blood banks in the same region during the same time period as the cases were collected. The selection criteria include no prior history of cancer, and controls were frequency matched to the cases by age (±5 years). A total of 440 healthy women presenting a median age of 42.29 years and an age range of 21-85 years, were used as control group of familial breast cancer cases. From the above control group 216 healthy women were selected according to age of diagnosis higher than 40 years, with a median age of 53.05 and an age range of 41-85, being used as control group of sporadic breast cancer cases. All participants provided informed consent.

Laboratory Methods

Genomic DNA was isolated from lymphocytes of peripherical blood using Puregene® DNA Purification Kit (Gentra Systems, Minneapolis, USA). All the polymorphisms were assessed by PCR-RFLP technique. *TP53 Arg72Pro* polymorphism (rs1042522) was detected by amplifying genomic DNA with the forward primer 5'-GAA GAC CCA GGT CCA GAT GA-3' and the reverse primer 5'-CTG CCC TGG TAG GTT TTC TG-3'. The PCR amplification parameters were 32 cycles each of 30 sec at 94°C, 30 sec at 54°C, and 30 sec at 72°C. The 152bp PCR product was digested with *Bsh1236I* (Fermentas, Ontario, Canada) at 37°C overnight. Digested products were separated by electrophoresis in a 3% agarose gel (Seakem® LE Agarose, Rockland, USA) and visualized by ethidium bromide staining. Wild type alleles resulted in 50 and 102bp fragments and the variant alleles resulted in 152bp fragment following restriction enzyme digestion. *TP53 PIN3 Ins16bp* polymorphism (rs17878362) was detected by amplifying genomic DNA with the forward primer 5'-CTG AAA ACA ACG TTC TGG TA-3' and the reverse primer 5'-AAG GGG GAC TGT AGA TGG GTG-3'. The PCR amplification parameters were 32 cycles each of 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C. The PCR product was separated by electrophoresis in a 4% agarose gel (Seakem® LE Agarose, Rockland, USA) and visualized by ethidium bromide staining. Wild type alleles, designated *A1* allele (no duplication) resulted in

119bp fragment and the variant alleles, designated *A2* allele (with 16bp duplication) resulted in 135bp fragment.

To ensure quality control of all genotyping results, one percent of the samples was randomly selected and sequenced using an ABI automated sequencer.

Statistical Analysis

Analysis of data was performed using the computer software SPSS version 14.0 (SPSS Inc., Chicago, USA). Chi-square ($\chi 2$ test) analysis was used to compare categorical variables. Whenever necessary, the Fisher test was used when number of samples was equal or inferior to 5. A 5% level of significance was used in the analysis. The OR and its 95% CI were calculated to measure the association between *TP53* polymorphic genotypes and breast cancer risk. Logistic regression analysis was used to calculate the adjusted OR and 95% CI for the influence of *TP53* genotypes in the risk of breast cancer, adjusted for age and/or FH. Whenever appropriate, the observed number of each genotype in control groups were compared with that expected for a population in the Hardy-Weinberg Equilibrium by using a goodness of fit $\chi 2$ test. The frequencies of expected haplotypes were estimated by using the statistical methodologies implemented by HPlus software (21). The nonparametric test, Kruskal-Wallis test, was used to compare mean age of diagnostic between the different genotype polymorphisms. The Kaplan-Meier method was also used to estimate OS. OS was defined as time (months) from clinical registration until death or censorship (were alive at the end of the follow-up time period). A follow-up period of at least 60 months was considered. The Log rank test was applied to evaluate effect of the *TP53* polymorphisms on OS. The OS analysis was conducted only in a group of sporadic breast cancer patients were it was possible to get these data.

4.3. RESULTS

The distribution of the genotype frequencies in both *Arg72Pro* and *PIN3 Ins16bp* polymorphisms among control group and subgroup (Table 1) is in agreement with those expected under Hardy-Weinberg equilibrium, excepted for *Arg72Pro* in the control group.

Table 1 – *TP53 Arg72Pro* and *PIN3 Ins16bp* genotypic and allelic frequencies. Association with familial and sporadic breast cancer risk.

TP53 Polymorphism	Genotype	Positive FH Cases	Controls	OR* (95% CI)	Negative FH Cases	Controls	OR* (95% CI)
Arg72Pro							
P_{HWE}=0.223	*Arg/Arg*	39 (53.4)	256 (59.0)	Reference	98 (56.0)	124 (58.5)	Reference
	Arg/Pro	25 (34.2)	142 (32.7)	1.19 (0.68-2.08)	61 (34.9)	70 (33.0)	1.26 (0.79-2.02)
	Pro/Pro	9 (12.3)	36 (8.3)	1.58 (0.68-3.67)	16 (9.1)	18 (8.5)	1.35 (0.63-2.88)
Alleles							
	Arg	103 (70.5)	654 (75.3)	Reference	257 (73.4)	318 (75.0)	Reference
	Pro	43 (29.5)	214 (24.7)	1.28 (0.85-1.91)	93 (26.6)	106 (25.0)	1.09 (0.78-1.52)
PIN3 Ins16bp							
P_{HWE}=0.889	*A1A1*	46 (65.7)	299 (68.0)	Reference	122 (63.9)	147 (68.1)	Reference
	A1A2	15 (21.4)	130 (29.5)	0.80 (0.43-1.49)	56 (29.3)	65 (30.1)	1.07 (0.67-1.70)
	A2A2	9 (12.9)	11 (2.5)	4.40 (1.60-12.0)	13 (6.8)	4 (1.9)	3.88 (1.18-12.8)
Alleles							
	A1	107 (76.4)	728 (82.7)	Reference	300 (78.5)	359 (83.1)	Reference
	A2	33 (23.6)	152 (17.3)	1.48 (0.94-2.31)	82 (21.5)	73 (16.9)	1.34 (0.93-1.94)

* OR adjusted for age (logistic regression analysis); FH – family history; OR – odds ratio; CI-confidence interval.

Concerning *TP53 Arg72Pro* polymorphism in the familial breast cancer cases, frequencies of *Arg72Arg*, *Arg72Pro* and *Pro72Pro* were 53.4%, 34.2% and 12.3%, respectively. In sporadic breast cancer, 56.0%, 34.9% and 9.1% were homozygous to *72Arg* allele, heterozygous and homozygous to *72Pro* allele, respectively. No statistically significant associations were found between the *TP53 Arg72Pro* polymorphism and risk of familial and sporadic breast cancer risk (Table 1).

Frequencies of *TP53 PIN3 Ins16bp* polymorphism genotypes were 65.7% to *A1A1,* 21.4% to *A1A2* and 12.9% to *A2A2*, in familial breast cancer cases. Regarding sporadic breast cancer group, we observed 63.9%, 29.3% and 6.8% frequencies for homozygous to *A1* allele, heterozygous and homozygous to *A2* allele, respectively. We observed that *A2A2* genotype carriers with positive FH were at a 4.40-fold (95% CI = 1.60-12.0; p=0.004) increased risk of breast cancer compared with the respective control group. Moreover, statistically significant differences were observed in *A2A2* genotype frequencies comparing negative FH breast cancer cases and respective control group (p=0.026). Our results showed that carriers of *A2A2* genotype with no FH present an increased risk of breast cancer (OR=3.88, 95% CI 1.18-12.8).

We investigated haplotype effects of the two polymorphisms studied in breast cancer risk (Table 2). Compared the common *TP53 Arg-A1* haplotype with the other expected haplotypes; we only observed statistically significant differences regarding *TP53 Arg-A2* haplotype between the familial breast cancer cases and respective control group (p=0.028). Carriers of *TP53 Arg-A2* haplotype and presence of FH of breast cancer presented an increased risk of develop breast cancer (OR= 2.10; 95% CI 1.08-4.06).

Table 2 – Expected haplotype frequencies between *Arg72Pro* and *PIN3 Ins16bp* polymorphisms. Association with familial and sporadic breast cancer risk.

Haplotypes	Positive FH Cases	Controls	OR (95% CI)	Negative FH Cases	Controls	OR (95% CI)
Arg-A1	0.607	0.711	Reference	0.695	0.705	Reference
Arg-A2	0.091	0.041	2.10 (1.08-4.06)	0.048	0.045	1.06 (0.53-2.12)
Pro-A1	0.150	0.111	1.49 (0.86-2.58)	0.098	0.119	0.80 (0.49-1.32)
Pro-A2	0.151	0.137	1.27 (0.72-2.24)	0.160	0.131	1.27 (0.83-1.95)

FH – family history; OR – odds ratio; CI- confidence interval

We examined the relationship between age at onset and genotypes and found a positive correlation in the FH group. The mean age of FH patients group with *A2A2* genotype was 33.43 (±8.08) years, whereas the mean age of patients with *A1A1* and *A1A2* genotypes was 42.44 (±12.14) and 44.80 (±10.85) years, respectively (Kruskal Wallis test p=0.056; Figure 1 b). Therefore, the carrier's status of *A2A2* genotype was associated with an earlier age at onset cancer with respect to the patients with *A1* genotypes. However, this difference was in the frontier of statistically significant, possibly because of the smaller size of the group (7 patients to *Pro/Pro* genotype). No association was observed relating age at onset and *Arg72Pro* polymorphism (Kruskal Wallis test p=0.747; Figure 1 a).

The analysis of the *TP53* polymorphisms with respect to some clinical-pathological factors is presented in Table 3. No significant statistically association was found concerning histological type and grade and hormone receptor status. However, we found a significant association of *Pro* or *A2* genotypes with the presence of lymph node metastases (p=0.009 and p<0.001, respectively, adjusted for age and breast cancer family history, using logistic regression analysis.). Patient carriers of *Pro* or *A2* genotypes (69.7% and 78.6%, respectively) shown higher incidence of lymph node metastases than carriers of *Arg/Arg* or *A1A1* genotypes (50.6% and 50.5%, respectively).

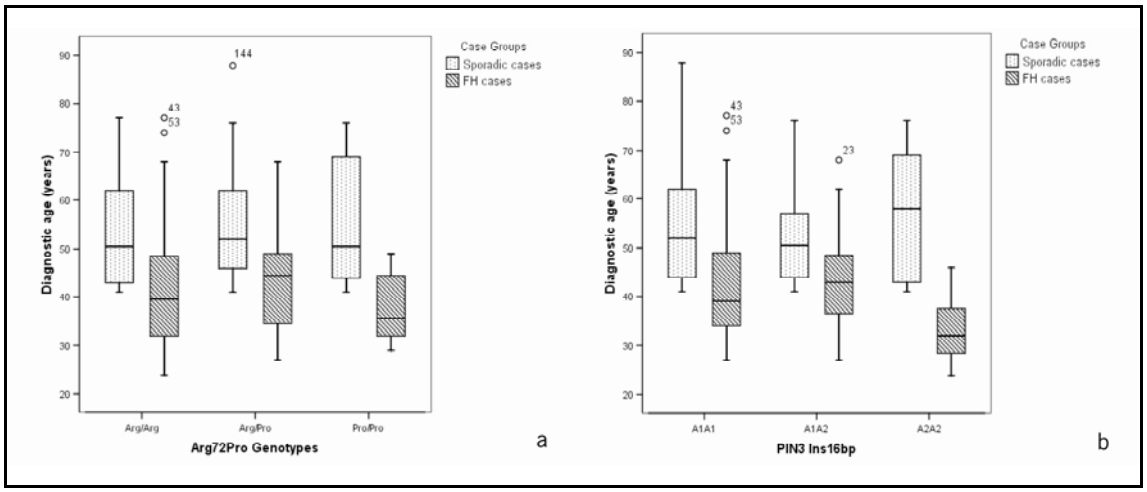

Figure 1 - Association of age of familial and sporadic breast cancer age at onset with *TP53 Arg72Pro* (a) and *PIN3 Ins16bp* (b) polymorphisms.

Table 3 – Correlation between *TP53 Arg72Pro* and *PIN3 Ins16bp* polymorphisms and clinical-pathological parameters in breast cancer patients.

Parameters	*TP53 Arg72Pro*		*TP53 PIN3 Ins16bp*	
	Arg/Arg	*Arg/Pro* or *Pro/Pro*	*A1A1*	*A1A2* or *A2A2*
Histological Type				
Invasive ductal carcinoma	68 (85.0%)	70 (92.1%)	88 (86.3%)	58 (90.6%)
Invasive lobular carcinoma	3 (3.8%)	2 (2.6%)	5 (4.9%)	1 (1.6%)
Others	9 (11.3%)	4 (5.3%)	9 (8.8%)	5 (7.8%)
P value	0.359		0.510	
Histological Grade				
I	8 (12.1%)	6 (9.1%)	11 (12.6%)	4 (7.4%)
II	33 (50.0%)	34 (51.5%)	45 (51.7%)	27 (50.0%)
III	25 (37.9%)	26 (39.4%)	31 (35.6%)	23 (42.6%)
P value	0.852		0.522	
Axillary lymph node status				
Negative	43 (49.4%)	20 (30.3%)	53 (49.5%)	12 (21.4%)
Positive	44 (50.6%)	46 (69.7%)	54 (50.5%)	44 (78.6%)
P value	0.017*		0.001**	
Oestrogen receptor status				
Negative	14 (18.7%)	14 (21.9%)	20 (21.3%)	9 (16.1%)
Positive	61 (81.3%)	50 (78.1%)	74 (78.7%)	47 (83.9%)
P value	0.638		0.435	
ırvival status at last follow-up				
Alive	59 (86.8%)	52 (88.1%)	8 (88.9%)	114 (87.0%)
Death	9 (13.2%)	7 (11.9%)	1 (11.1%)	17 (13.0%)
P value	0.816		0.871	
ːcurrence at last follow-up				
Yes	8 (13.3%)	12 (23.5%)	3 (37.5%)	21 (18.4%)
No	52 (86.7%)	39 (76.5%)	5 (62.5%)	93 (81.6%)
P value	0.164		0.189	

* p value=0.009, adjusted for age and breast cancer family history, using logistic regression analysis; ** p value<0.001, adjusted for age and breast cancer family history, using logistic regression analysis.

The overall survival analysis was performed in the patients where was possible to get follow-up of at least 60 months (Figure 2). The comparison of survival in patients with *Pro/Pro vs Arg/Pro*

vs Arg/Arg genotypes and *A2A2 vs A1A2 vs A1A1* genotypes suggested a poorer survival in the first ones. However, these differences were not statistically significant, possibly because of the smaller size of the groups (7 patients to *Pro/Pro* and 5 patients to *A2A2*).

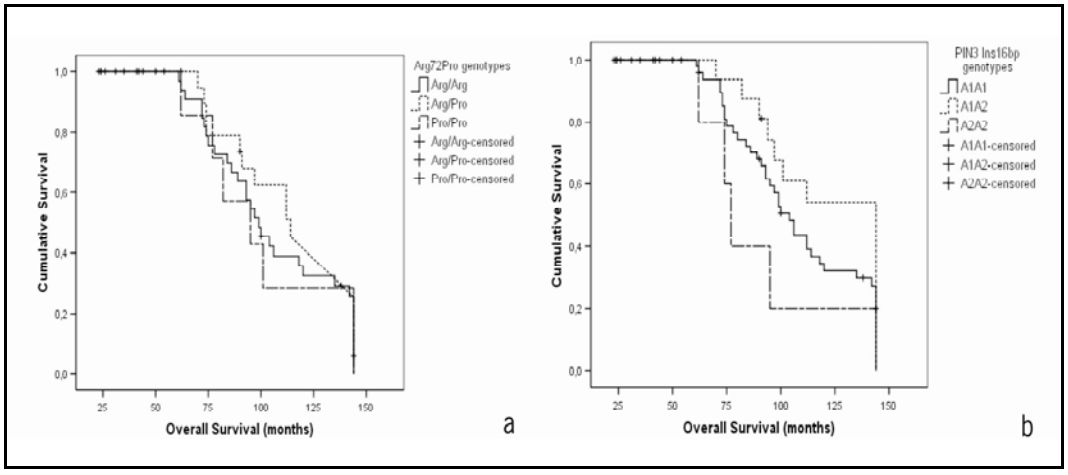

Figure 2 – Kaplan-Meier overall survival curves in breast cancer patients relating with *TP53 Arg72Pro* (a) and *PIN3 Ins16bp* (b) polymorphisms. Log-rank test for statistical analysis.

4.4. DISCUSSION

Breast cancer is an heterogeneous disease, as sustained by wide variable morphological appearance, many risks factors and distinct gene expression profile (2,22). Common genetic alterations (e.g. polymorphisms), with possible effects on function and/or protein expression, within genes involved in essential cellular pathways, such as carcinogen metabolism, DNA repair, cell cycle control and cell proliferation, could predispose individuals to cancer (15,23-25), including breast cancer (15,26-29).

The *TP53* is one of the major tumour suppressor genes which carry out essential functions in preservation of genome integrity. Thus, when the cell is under stress, particularly stress which will involve DNA damage, p53 promotes growth arrest, allowing the cell to repair the DNA lesions. If the damage is excessively hazardous, then p53 will lead to cell apoptosis. Several genetic polymorphisms have been described in *TP53* gene (18) and some of these variants seem to confer different functions among the p53 (30-32).

In the present study, we evaluated two separate *TP53* polymorphisms, *Arg72Pro* and *PIN3 Ins16bp*, in two groups of breast cancer, familial and sporadic cases, as well as in matching

control groups. The allelic frequencies of our control group for the different polymorphisms are in accordance with earlier reports from European populations (16,33).

Concerning the *codon 72 TP53* polymorphism (*Arg72Pro),* we did not find any association between this polymorphism and breast cancer. Our results are in agreement with other studies (33-35), however, the literature remains highly controversial regarding the role of this polymorphism in breast cancer risk (36-41). One study showed that *TP53 72Pro* variant induces transcription activation more efficiently than *TP53 72Arg* variant (39). On the other hand, other authors revealed that *TP53 72Pro* variant induce cell cycle arrest better than *72Arg* (31). Other studies have showed that *TP53 72Arg* variant is more efficient in inducing apoptosis (32,41). Beside apoptosis and cell cycle control, p53 protein seems to be crucial in the regulation of the different DNA repair pathways (42). A recent study demonstrated the influence of *TP53 Arg72Pro* in DNA repair capacity, showing that *TP53 72Pro* variant activates several *TP53* dependent target genes involved in DNA repair and DNA damage repair much more efficiently than the *72Arg* variant expressing cells (30). These contradictory results could be explained by the differential effects of this alteration in p53 function. Several *in vitro* evidences have demonstrated that both *TP53 Arg72Pro* variants may selectively regulate specific cellular functions.

In *TP53 PIN3* polymorphism, our findings suggest an association of *A2A2* genotype and increased breast cancer risk among women with FH and sporadic breast cancer, suggesting that this polymorphism contributed to enhance susceptibility for breast cancer among Portuguese population, regardless of the presence of FH. Our results are supported by previously reported studies suggesting an association of *PIN3 A2* genotypes with breast cancer risk (43). Although, the biological effect of the *TP53 PIN3 Ins16bp* polymorphism is currently unclear, theoretically, this polymorphism could affect mRNA splicing, altering the coding regions and therefore being implicated in regulation of gene expression and DNA-protein interactions, resulting in a defective protein (44,45). Until now, just a single study had show *PIN3 A2* allele presents reduced mRNA stability (46).

The linkage disequilibrium between *TP53* polymorphisms region could be an important factor affecting the incidence of cancer in general (47,48), and breast cancer, in particular (41,43,49). Thus, haplotype analysis would be important to confirm the significance of this variant on breast cancer susceptibility. A statistical significant association was found between *Arg-A2* haplotype and breast cancer susceptibility among women with FH of breast cancer. On the other hand, a recent study has found that *Pro-A1* haplotype individuals present increased breast cancer risk, however,

in women *BRCA2* mutation carriers (41). Nevertheless, other reports have also demonstrated a positive association of *Arg-A2* haplotype with cancer (43,47). Moreover, functional studies have shown that, in a specific haplotype combination, *A2* allele is associated with decreased apoptotic and DNA repair capacity (33,47).

Our findings suggest the *Pro/Pro* and *A2A2 TP53* genotypes as predictor factors for the presence of lymph node metastases, being in agreement with previously functional studies in the biological consequences of these variations in P53 protein functions (39,46)

The natural history of breast cancer can be influenced by several factors. We hypothesize that under the influence of *TP53* genetic polymorphisms, chronic exposure to higher levels of several endogenous (e.g. estrogens) and exogenous breast carcinogens resulting in consequent higher accumulation of DNA damage during an individual's lifetime, may alter the age at onset of disease. Moreover, it has been suggested that *TP53* polymorphisms are associated to familial breast cancer by the age of 50 years (33). Our results are consistent with this hypothesis, since *TP53 PIN3 Ins16bp* polymorphism seems to influence directly the age to onset of familial breast cancer.

In conclusion, our findings suggest *TP53 PIN3 Ins16bp* polymorphism as a real risk modifier in breast cancer disease, with evidence for relative differential effect by family history of breast cancer. Moreover, our results also suggest that *PIN3 A2* allele in a haplotype combination confer increased breast cancer susceptibility among women carriers of FH of the disease. Subsequently, these results will be crucial in the characterization of the genetic breast cancer susceptibility profile, within familial breast cancer cases non-carriers of *BRCA1/BRCA2* mutations. Furthermore, our findings suggest *TP53 Arg72Pro* and *PIN3 Ins16bp* polymorphisms as predictive factors of presence of lymph node metastases.

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CHAPTER 5 – IMMUNOHISTOCHEMICAL EXPRESSION PROFILE OF XRCC1, XRCC3 AND P53 PROTEINS IN BREAST CANCER: CORRELATION WITH GENETIC POLYMORPHIC STATUS

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NOTE

In our previous results, we showed that *XRCC1 399Gln* genotypes conferred a protective effect to the development of sporadic breast cancer. Furthermore, we also showed a strong association between increased breast cancer susceptibility in women carriers of *XRCC3 241Met* genotypes and sporadic breast cancer. Additionally, we demonstrated an association of *RAD51 135C* genotypes and increased breast cancer risk among women with FH of breast cancer, suggesting that this polymorphism contributes to familial breast cancer in the Portuguese population. Moreover, our findings suggested that *PIN3 A2* allele in a haplotype combination confer increased breast cancer susceptibility among women carriers of FH of the disease. In order to understand the role of these polymorphisms, we decided to evaluate XRCC1, XRCC3, RAD51 and P53 protein expression profiles in a series of invasive ductal breast carcinoma and in a panel of human breast cancer cell lines, assessing the possible correlations between the different expression profiles and the genetic polymorphic status.

The expression profiles were evaluated by immunohistochemical and western blot techniques and were studied to XRCC1, XRCC3 and P53 proteins. We excluded from this analysis the RAD51 protein screening due to technical reasons. After several attempts, using different antibodies and technical conditions, we did not find a RAD51 antibody that gives feasible results.

ABSTRACT

The important role of DNA repair in the maintenance of a normal cellular genotype and a cancer-free state is obvious in familial hereditary breast cancer, demonstrated by increase risk related to important genes involved in DNA repair pathways, such as *BRCA1*, *BRCA2*, *TP53*, *ATM*, and *NBS1* genes. Although, several DNA repair polymorphisms have been associated with differential DNA repair capacity and risk to breast cancer, such as *XRCC1 Arg399Gln, XRCC3 Thr241Met* and *TP53 PIN3 Ins16bp*, nothing is known concerning the correlation between protein expression in breast cancer and their polymorphic status. Our aim was to evaluate XRCC1, XRCC3 and P53 protein expression profiles in a series of invasive ductal breast carcinoma and in a panel of human breast cancer cell lines and to assess the possible correlations between their respective genetic polymorphic status and clinical-pathological features. Our results showed that XRCC1 nuclear expression is a common event in human breast tissues, either in normal-like, benign lesions, DCIS and invasive carcinomas. Moreover, we showed that XRCC3 expression may be associated with the regulation of P53 and XRCC1 expression in breast tumors and possibly an important factor in prediction of less aggressive breast tumors. Furthermore, we demonstrated that XRCC1, XRCC3 and P53 protein expressions do not correlate with their genetic polymorphisms status either in breast cancer patients or human breast cancer cell lines.

5.1. INTRODUCTION

Breast cancer is the leading cause of death among women in developing countries. In Portugal, it presents the highest incidence and mortality rates in women diseases (1). Well-established risk factors have been described to breast cancer, such as early menarche, late menopause, age of first child's birth and nulliparity (2), most of them reflecting a prolonged exposure to estrogen (3). However, these factors account for only half of the breast cancer cases. Several environmental factors have been associated with risk for breast cancer development, such as ionized radiation and chemical carcinogens (diet and environment) (4-6). These mutagens sources, together with endogenous and/or exogenous estrogens, produce a wide range of DNA lesions, such as reactive oxygen species, abasic sites, oxidized bases, bulky DNA

adducts and DNA strand breaks (7-9). However, during evolution, mammalian cells have developed distinct pathways to repair different types of DNA damage, in order to maintain the genome integrity. Therefore, DNA repair capacity determines cellular susceptibility to endogenous and exogenous substances and factors. The crucial role of DNA repair in the maintenance of a normal cellular genotype and a "cancer-free" state is obvious in familiar hereditary breast cancer, demonstrated by the increased risk related to mutations in important genes involved in DNA repair pathways, such as *BRCA1*, *BRCA2*, *TP53*, *ATM*, and *NBS1* genes (10). Interestingly, several DNA repair polymorphisms have been associated with differential risk to breast cancer and with differential DNA repair capacity (11,12).

The XRCC1 and XRCC3 carry important functions in DNA repair; however, they work through distinct pathways. XRCC1 plays a central role in the BER mechanism, which is responsible for lesions repair from distinct origin, like oxidative and IR (13). On the other hand, XRCC3, another member of this family, is a HRR crucial player repairing DSBs produced by endogenous sources, like oxidative stress, mechanical stress on chromosomes and replication errors, and exogenous sources, such as IR and some chemicals. It has been shown that XRCC1 interacts with DNA Polβ, DNA Lig III and APE1, through a BRCT-I at the C-terminus (14). Several polymorphisms have been found in *XRCC1* gene and some have been linked with a variety of cancers (15). Interestingly the most common *XRCC1* polymorphism, *Arg399Gln*, located in exon 10, is known to lay within the BRCT-I domain (16). XRCC3 interacts directly with RAD51, helping in the assembly of the nucleofilament protein, as well as in the selection and interaction with appropriate recombination substrate (17). The *XRCC3 Thr241Met* polymorphism, located in exon 7, is very common and seems to be responsible for a lower DNA repair capacity in healthy individuals, probably affecting the enzyme function and/or its interaction with other proteins involved in DNA damage and repair (18,19).

An important protein interacting with DNA repair processes, such as BER and HRR, is P53 (20-22). P53 is activated in response to a large range of stress, such as genotoxic damages (UV and IR radiation, chemical carcinogenesis and oxidative stress) and non-genotoxic damage, including hypoxia and oncogene activation (23). Its activation abolishes a wide variety of biological activities, including cell-cycle checkpoints, induction of apoptosis, DNA recombination, chromosomal segregation, cellular senescence and enhancement of DNA repair (20). Several polymorphisms have been identified within *TP53* gene, both in non-coding and coding regions

(24), as the common polymorphism resulted from a 16 bp duplication in intron 3 (*PIN3 Ins16bp*). However, information about its biological consequences is still scarce.

Our previous results showed the importance of some DNA repair polymorphisms in breast cancer susceptibility (25). Although it is well established the crucial role of XRCC1 and XRCC3 proteins in the maintenance of genomic integrity, there is no studies revealing their expression profile in normal and tumor tissues and its correlation with other DNA repair proteins, such as P53.

Thus, the aim of the present work was to determine the XRCC1, XRCC3 and P53 expression profile in breast tissues, analysing normal, benign and neoplastic lesions (*in situ* and invasive ductal carcinomas) by immunohistochemistry (IHC) in tissue microarrays (TMA). We evaluated the correlation of their expression profiles and the respective genotypes and clinical-pathological features, such as family history, histological grade, nodal and ER status, as well as correlations between their expressions. We further performed a XRCC1, XRCC3 and P53 expression screening in a set of human breast cancer cell lines correlating it with the respective genetic polymorphic status.

5.2. Materials and Methods

Study Population

One hundred and eighty one breast tissue samples were obtained from the Hospital São João and Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal. Eight out of these were normal-like parenchima samples adjacent to the tumor, seven samples were classified as benign breast disease (such as fibroadenoma, adenosis, and typical ductal hyperplasia); thirteen samples were identified as DCIS and one hundred and fifty three samples were invasive ductal carcinomas. Patients with invasive breast carcinoma presented a mean age of 51.79 years (SD ± 14.08), ranging from 19 to 83 years. Clinical-pathological features were obtained from hospital records when it was available. From the invasive ductal carcinomas, thirty nine presented clinical features of familial breast cancer based on the Breast Cancer Linkage Consortium criteria (26): early onset (≤40 years) and/or bilaterality; or more than three cases of breast cancer in the family; or more than one case of

ovarian cancer in the family; or more than two first-degree relatives involved; or male breast cancer. The remaining cases were considered sporadic breast cancer.

From all invasive breast carcinoma, it was possible to obtain DNA from peripheric blood and tumor tissue samples for 49 patients, who were genotyped for the different polymorphisms in study.

Cell lines and paraffin cell blocks

The breast carcinoma cell lines, MDA-MB-231, MDA-MB-468, SKBr-3, BT-474, BT549, T47D, MCF-7, ZR-75-1, Hs578-T and MDA-MB-435 were kindly supplied by Elena P. Moiseeva (Cancer Biomarkers and Prevention Group, Leicester University, Leicester, United Kingdom); Eric W.-F. Lam (Cancer Research-United Kingdom Laboratories, Imperial College London, London, United Kingdom); and Mark Mareel (Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear Medicine, VIB-Ghent University, Ghent, Belgium). All cell lines were cultured in Dulbecco's Modified Eagles' Medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in humidified 5% CO_2. To produce paraffin cell blocks, cell pellets from all cell lines were washed in phosphate buffer solution (PBS) 1x, fixed with 10% formalin during 20 min and then treated with 22% bovine serum albumin (BSA) solution and then incubated with ethanol 95%. The final pellets were then fixed overnight in 10% formalin, and then histologically processed.

Genotyping

Blood samples from all the study participants were collected in tubes with EDTA as an anticoagulant. Genomic DNA was isolated from buffy coat using Puregene® DNA Purification Kit from Gentra Systems. *XRCC1 Arg399Gln*, *XRCC3 Thr241Met* and *TP53 PIN3 Ins16bp* polymorphisms were assessed by PCR-RFLP technique, as already described (25). Briefly, fragments containing the specific polymorphisms were amplified by PCR, which were then digested with specific restriction endonuclease enzymes. The results were obtained comparing different pattern fragments corresponding to specific genotypes, after fragment separation by electrophoresis in a 3% agarose gel.

Tissue Microarrays construction

The TMA construction was conducted as previously described (27). Briefly, representative areas of different lesions/normal breast tissue were carefully selected on hematoxylin and eosin (H&E)-stained sections. Tissue cores (2 mm in diameter) were obtained from each selected area in the corresponding paraffin block and deposited into a recipient paraffin block using a TMA workstation (TMA builder ab1802, Abcam, Cambridge, United Kingdom). The H&E-stained slide from each block was performed to confirm the presence of the morphological representative area.

Immunohistochemical analysis

Immunostaining was carried out using the streptavidin-biotin-peroxidase technique in each set of glass slides containing the TMAs. Briefly, slides were deparaffinised and rehydrated and antigen retrieval was done incubating TMA slides in boiling (98°C) 10 mM citrate buffer, pH 6.0, for 20 min followed by cooling at room temperature (RT) for 20 min. After washes in PBS, endogenous peroxidase activity was inactivated by incubation for 10 min in 3% (v/v) H_2O_2/methanol. Slides were then incubated with a blocking serum (LabVision Corporation, Fremont, USA) for 10 min, for blockage of non-specific protein binding, and then incubated with the primary antibody: XRCC1 monoclonal mouse antibody (clone 144, LabVision) diluted 1:150 for 2 hours at RT; XRCC3 epitope specific rabbit antibody (LabVision) diluted 1:100 for 2 hours at RT; and P53 monoclonal mouse antibody (clone DO-7, Novocastra) diluted 1:100 for 2 hours at RT. After washes, the slides were incubated with biotinylated secondary antibody, following by streptavidin-conjugated peroxidase (LabVision Corporation, Fremont, USA). Diaminobenzidine was used as chromogen. Tissues were then counterstained with hematoxylin and covered with a mounting solution.

The IHC staining was assessed independently by at least two of the authors, including one pathologist. In each run, a positive control was included (normal testis for XRCC1 and XRCC3, and breast carcinoma for P53). A negative control for each antibody was also included by replacing the primary antibody by PBS.

The expression of XRCC1 and XRCC3 was classified by the absence (negative, 0) or presence of nuclear staining (positive, 1). XRCC3 was also evaluated by the intensity of its cytoplasmic stain, being graded as negative (0), low (1+), moderate (2+) or strong (3+). The cases 0/1+ were considered as negative and the 2+/3+ as positive. P53 nuclear expression was classified

according to a score already described (28): moderate or strong diffuse staining in more than 50% of the neoplastic cells was considered positive.

The same protocol was used to perform and evaluate IHC expression in paraffin cell block slides.

Western-blot (WB) analysis

All lysates were made from cell monolayers at 90% of confluence, which were washed twice with PBS. Cells were lysed with PBS containing 1% Triton X-100 (Sigma-Aldrich, St. Louis, USA), 1% Nonidet P-40 (Sigma-Aldrich, St. Louis, USA), and 1:7 protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). After clearing the lysates, protein concentration was determined using the Rc Dc protein assay (BioRad, Richmond, USA). Total protein samples (75 µg) were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Hybond C) (Amersham Pharmacia Biotech, Piscataway, USA) and immunoblotted for 2 hours at RT using anti-XRCC1 monoclonal antibody (1:1000) (clone 144, Labvision, Fremont, USA), XRCC3 epitope specific rabbit antibody (1:250) (Labvision, Fremont, USA), P53 monoclonal antibody (1:10000) (clone DO-1, Novocastra, Newcastle, England), and goat anti-rabbit or anti-mouse antibodies (1:10000) (Santa Cruz Biotechnology, Santa Cruz, USA). Immunoreactive bands were then visualized by chemiluminescence (SuperSignal West Femto Maximun Sensitivity Substrate®, Pierce, Rockford, USA), following manufacturer's instructions. The membrane was then stripped and re-probed with monoclonal mouse α-tubulin (1:10000) (Sigma-Aldrich, St. Louis, USA). Each immunoblot was done at least three times, and the selected are representative experiments. Protein expression was quantified using the AlphaImager analysing software (Alpha Innotech, San Leandro, USA. The final Intensity Density Value (IDV) for each cell line was normalised to the α-tubulin levels. Protein expressions were classified based on IDV score established, being graded as negative (0) with an IDV range of 0-1000, low (1+) with an IDV range of 1001-5000, moderate (2+) with an IDV range of 5001-10000 or strong (3+) with an IDV range of >10000. The cases 0/1+ were considered as negative and the cases 2+/3+ as positive.

Statistical analysis

Data analysis was performed using the computer software SPSS version 14.0. Pearson's Chi-square test ($\chi 2$) test or Fisher's exact test (when n<5) was used to analyse the relationship of protein expressions with genetic polymorphic status and with clinical-pathological parameters. A 5% level of significance was used in the analysis. Logistic regression analysis was applied to calculate the adjusted p value for mean age in the identification of subgroups of disease patients according to clinical-pathological features. The Pearson Correlation test was used to analyse expression correlations between the different proteins.

5.3. RESULTS

5.3.1. XRCC1, XRCC3 and P53 protein expressions in normal, benign and malignant breast tissues samples and in human breast cancer cell lines

The predominant pattern for XRCC1, XRCC3 and P53 protein expression observed in all breast tissue samples was nuclear; although, XRCC3 expression was also detected in the cytoplasm. XRCC1 nuclear staining was observed in 66.7% epithelial cells from normal-like tissues, in 85.7% of BBD lesions, 66.7% of DCIS and 61.3% of invasive carcinomas (Figure 1 A-G). Regarding XRCC3 nuclear expression, we detected a positive staining in 25.0% of normal-like breast tissues, 28.6% of benign lesions, 30.8% of DCIS and 31.9% of invasive breast carcinomas. Cytoplasmic expression of XRCC3 was found in 75.0% of breast normal-like tissues, 42.9% of BBD, 53.8% of DCIS and 61.3% of invasive breast carcinomas (Figure 1 H-Q). About P53 nuclear expression, all normal-like breast tissues and BBD lesions were negative (Figure 1 R-S). In contrast, DCIS cases showed 27.3% of P53 positivity, whereas the invasive carcinomas showed 12.4% (Figure 1 T-Y).

We also assessed the correlation between XRCC1, XRCC3 and P53 IHC protein expression, where we found a statistically significant correlation between XRCC1 (Pearson p=0.023) and P53 (Pearson p=0.022) with XRCC3 cytoplasmic expression.

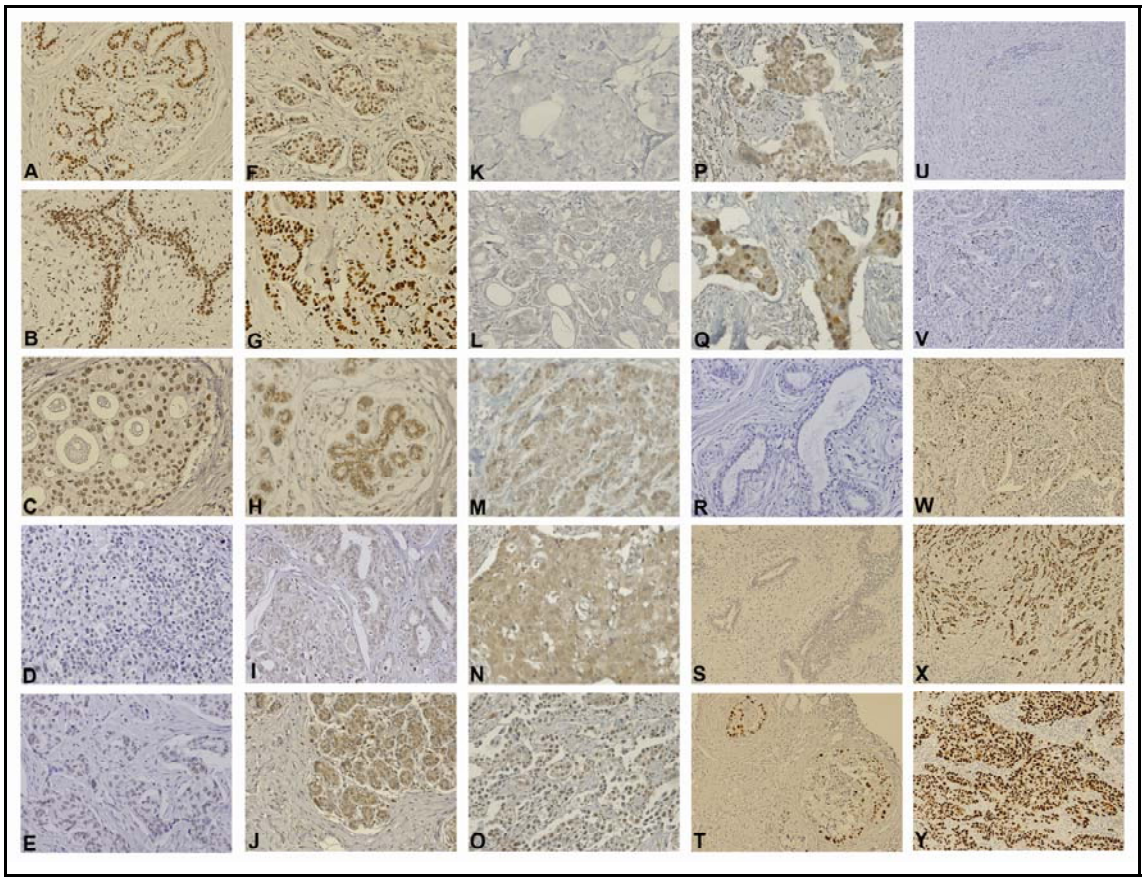

Figure 1 – Immunohistochemistry expression of XRCC1, XRCC3 and P53 protein in breast tissue samples. XRCC1 expression (A-G): (A) normal tissue (original magnification 200x); (B) – benign lesion (original magnification 200x); (C) – DCIS (original magnification 200x); (D-E) – negative staining of invasive ductal carcinomas (original magnification 200x); (F-G) - positive stain of invasive ductal carcinomas (original magnification 200x). XRCC3 expression (H-Q): (H) normal tissue (original magnification 200x); (I) – benign lesion (original magnification 200x); (J) – DCIS (original magnification 200x); (K-N) negative nuclear stain and low (L), moderate (M) and high (N) cytoplasmic intensity of invasive ductal carcinomas (original magnification 200x); (O-Q) – positive nuclear stain and low (O), moderate (P) and high (Q) cytoplasmic intensity of invasive ductal carcinomas (original magnification 200x). P53 expression (R-Y): (R) - normal tissue (original magnification 200x); (S) – benign lesion (original magnification 100x); (T) – DCIS (original magnification 100x); (U-W) – negative stain of invasive ductal carcinomas (original magnification 100x); (X-Y) - positive stain of invasive ductal carcinomas (original magnification 100x).

The XRCC1, XRCC3 and P53 expression levels in the 10 different breast cancer cell lines studied were determined by IHC and WB. A representative positive and negative nuclear IHC staining of the three proteins studied are revealed in Figure 2 and all the results are presented in Table1. Almost all cell lines presented a positive XRCC1 expression, except the BT-549 and MDA-MB-231 cell lines. Regarding XRCC3 expression, only MDA-MB-231 and MDA-MB-468 cell lines

showed a negative pattern to this protein. MCF7, BT-549, ZR-75-1 and MDA-MB-435 cell lines were negative for P53 expression.

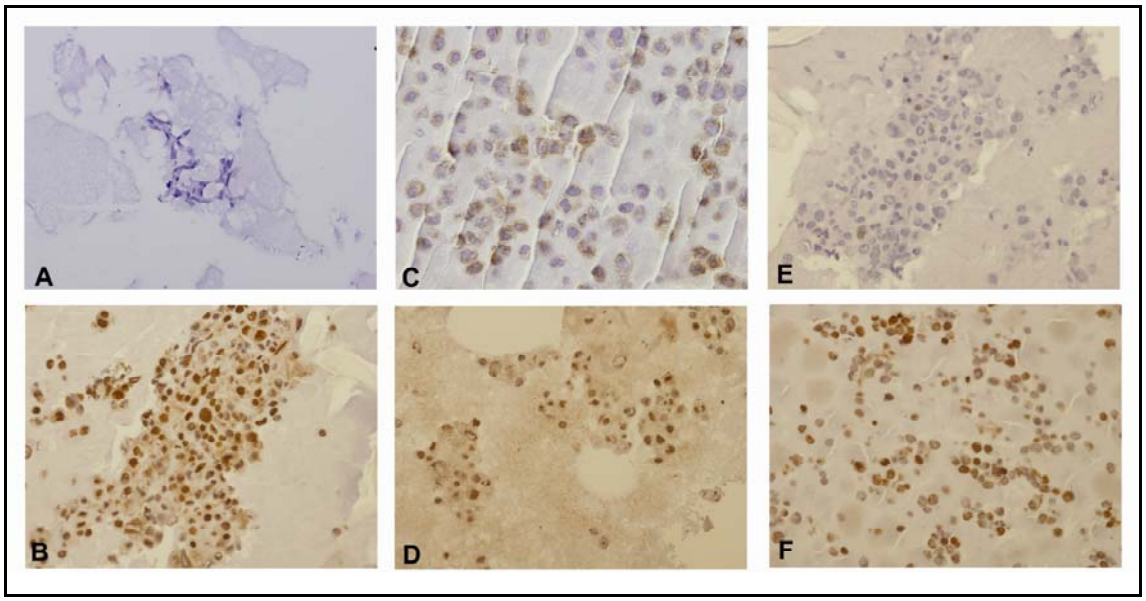

Figure 2 – Representative IHC expression of XRCC1, XRCC3 and P53 proteins in human breast cancer cell lines. XRCC1 negative (A) and nuclear staining expression (B) (original magnification 200x). XRCC3 negative nuclear and positive cytoplasmic expression (C) (original magnification 400x) and nuclear expression (with lack of cytoplasmatic expression) (D) (original magnification 200x). P53 negative (E) and positive (F) nuclear expression (original magnification 200x).

Table 1 – XRCC1, XRCC3 and P53 protein expression analysed by IHC and WB in a panel of 10 human breast cancer cell lines.

Human Breast	XRCC1		XRCC3		P53	
Cancer Cell Line	IHC	WB	IHC	WB	IHC	WB
MCF7	1	2+	1	2+	0	1+
ZR-75-1	1	3+	1	3+	0	0
SkBr3	1	3+	1	2+	1	2+
T47D	1	3+	ND	2+	1	3+
BT-474	1	0	1	2+	1	1+
BT-549	0	2+	1	2+	0	2+
Hs598T	1	2+	1	3+	1	2+
MDA-MB-231	1	2+	1	3+	1	3+
MDA-MB-435	1	3+	1	3+	0	3+
MDA-MB-468	1	2+	1	3+	1	3+

1- positive expression; 0 – negative expression; ND – not possible to determine.

The immunoblot expression pattern observed was presented in Figure 3. All the human breast cancer cell lines demonstrated moderate to strong expression levels of XRCC1 protein, except, BT-474 that expressed negative levels (Figure 3 – upper panel and Table 1). In relation to P53

expression, we observed moderate to strong levels in SkBr-3, T47D, BT-549, Hs578T, MDA-MB-231, MDA-MB-435 and MDA-MB-468 cell lines. In the other hand, MCF7, ZR-75-1 and BT-474 cell lines showed negative to low P53 expression (Figure 3 – middle panel and Table 1). XRCC3 protein was expressed in moderate to a strong manner in all the cell lines analysed (Figure 3 – lower panel and Table 1).

IHC analysis demonstrated expression patterns similar to WB analysis in almost all human breast cancer cell lines studied (correlation in 70 to 90%).

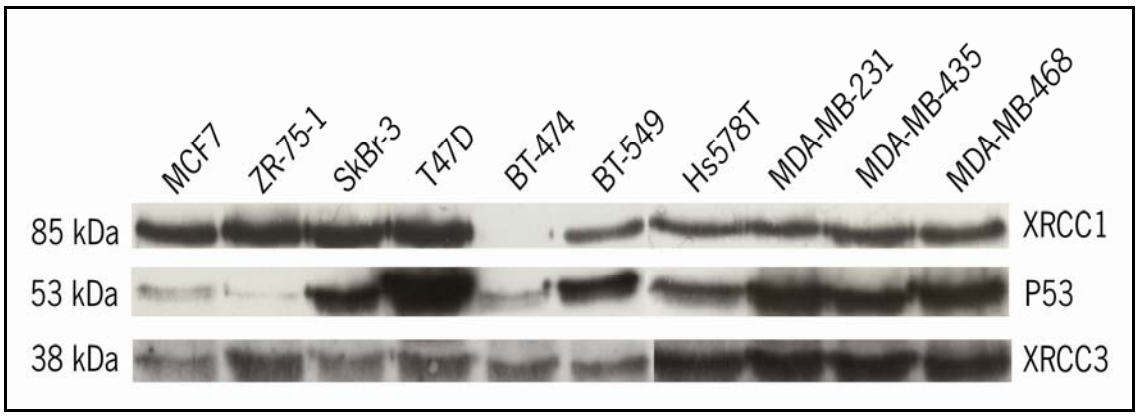

Figure 3 – Western blotting for XRCC1, P53 and XRCC3 expression protein analysis, from cell lysates derived from 10 human breast cancer cell lines used in this study. Protein expression levels were determined, intensity of the bands was measured, and normalised to an internal control.

5.3.2. Correlation of XRCC1, XRCC3 and P53 protein expressions in breast cancer patients and human breast cancer cell lines with genetic polymorphic status

The correlation analysis of XRCC1, XRCC3 and P53 IHC expression with their respective polymorphisms were performed in patients where we had both information. No correlation was found considering XRCC1, XRCC3 or P53 nuclear staining or XRCC3 cytoplasmic staining with respective genotypes (Table 2).

Next, we decided to use the WB results to see whether there was any correlation between the protein levels, expressed in the different cell lines, and their respective polymorphic status (Table 3). No correlation was found between XRCC1, XRCC3 and P53 protein levels and the respective polymorphic status.

Table 2 – Correlation between XRCC1, XRCC3 and P53 proteins IHC expression and *XRCC1 Arg399Gln, XRCC3 Thr241Met* and *TP53 PIN3 Ins16bp* polymorphisms.

Genotypes	Positive Nuclear staining (%)		P value	Positive Cytoplasmic staining (%)		P Value
	Negative	Positive		Negative	Positive	
XRCC1 Arg399Gln						
Arg/Arg	10 (58.8)	15 (53.6)				
Arg/Gln	5 (29.9)	11 (39.3)	0.742	NA		
Gln/Gln	2 (11.8)	2 (7.1)				
XRCC3 Thr241Met						
Thr/Thr	22 (55.0)	6 (66.7)		9 (56.3)	19 (57.6)	
Thr/Met	12 (30.0)	2 (22.2)	0.815	4 (25.0)	10 (30.3)	0.802
Met/Met	6 (15.0)	1 (11.1)		3 (18.8)	4 (12.1)	
P53 PIN3 Ins16bp						
A1A1	13 (48.1)	4 (80.0)				
A1A2	11 (40.7)	0 (0.0)	0.211	NA		
A2A2	3 (11.1)	1 (20.0)				

NA: not applicable.

Table 3 – *XRCC1 Arg399Gln, XRCC3 Thr241Met* and *TP53 PIN3 Ins16bp* polymorphisms genotypes and protein expression in human breast cancer cell lines.

Human Breast Cancer Cell Line	*XRCC1 Arg399Gln*		P value	*XRCC3 Thr241Met*		P value	*TP53 PIN3 Ins16bp*		P value
	Genotypes	WB		Genotypes	WB		Genotypes	WB	
MCF7	*Arg/Arg*	1		*Thr/Thr*	1		*A1A1*	0	
ZR-75-1	*Arg/Arg*	1		*Met/Met*	1		*A1A1*	0	
SkBr3	*Arg/Arg*	1		*Met/Met*	1		*A1A1*	1	
T47D	*Arg/Arg*	1		*Thr/Met*	ND		*A2A2*	1	
BT-474	*Gln/Gln*	0	0.200	*Met/Met*	1	ND	*A1A1*	0	0.301
BT-549	*Gln/Gln*	1		*Thr/Thr*	1		*A2A2*	1	
Hs598T	*Arg/Arg*	1		*Thr/Met*	1		*A1A1*	1	
MDA-MB-231	*Arg/Arg*	1		*Thr/Thr*	1		*A1A1*	1	
MDA-MB-435	*Arg/Arg*	1		*Met/Met*	1		*A1A1*	1	
MDA-MB-468	*Arg/Arg*	1		*Thr/Thr*	1		*A1A1*	1	

1- positive expression; 0 – negative expression; ND – not possible to determine.

5.3.3. Correlation of XRCC1, XRCC3 and P53 protein expressions in breast cancer patients and clinical-pathological features

The correlation between protein expression and genetic polymorphisms was performed by logistic regression analysis, adjusted for age. As shown in Table 4, there is no statistical significant relationship between XRCC1 nuclear staining and any of the clinical-pathological parameters considered. Likewise, no association was obtained between P53 nuclear staining or XRCC3 cytoplasmic expression with clinical-pathological features (Table 4). On the other hand,

positive XRCC3 nuclear staining showed correlation with lower tumor grade (grade I and II) (p=0.070), as well as estrogen receptor (ER) positivity status in the tumor (p=0.060) (Table 4), with relative statistically significant differences. No correlation was found between XRCC3 nuclear expression and any other of the clinical-pathological parameters analysed.

Table 4 – Correlation between XRCC1, XRCC3 and P53 proteins IHC expression and clinical-pathological features of invasive breast carcinomas.

Features	XRCC1 Nuclear Staining		P value	XRCC3 Nuclear Staining		P value	XRCC3 Cytoplasmic Staining		P value	P53 Nuclear Staining		P value
	Negative	Positive		Negative	Positive		Negative	Positive		Negative	Positive	
Family History												
Negative	27 (71.1)	75 (75.0)	0.920	71 (68.9)	45 (86.5)	0.141	43 (78.2)	73 (73.0)	0.371	91 (79.1)	18 (85.7)	0.797
Positive	11 (28.9)	26 (25.0)		32 (31.1)	7 (13.5)		12 (21.8)	27 (27.0)		24 (20.9)	3 (14.3)	
Histological Grade												
I	5 (20.0)	10 (16.7)		11 (17.2)	6 (23.1)		7 (21.9)	10 (17.2)		15 (20.8)	1 (11.1)	
II	14 (56.0)	28 (46.7)	0.426	26 (40.6)	15 (57.7)	0.156	16 (50.0)	25 (43.1)	0.239	37 (51.4)	4 (44.4)	0.358
III	6 (24.0)	22 (36.7)		27 (42.2)	5 (19.2)[a]		9 (28.1)	23 (39.6)		20 (27.8)	4 (44.4)	
Lymph Nodes												
Negative	8 (61.5)	17 (48.6)	0.436	23 (63.9)	7 (41.2)	0.221	15 (71.4)	15 (46.9)	0.080	25 (53.2)	1 (25.0)	0.407
Positive	5 (38.5)	18 (51.4)		13 (36.1)	10 (58.8)		6 (28.6)	17 (53.1)		22 (46.8)	3 (75.0)	
ER status												
Negative	4 (20.0)	10 (19.6)	0.892	15 (27.3)	1 (4.5)	0.060	5 (17.9)	11 (22.4)	0.574	11 (18.0)	2 (33.3)	0.417
Positive	16 (80.0)	41 (80.4)		40 (72.7)	21 (95.5)		23 (82.1)	38 (77.6)		50 (82.0)	4 (66.7)	

a – comparison grade I/II vs III, p=0.007, p value adjusted for mean age, using logistic regression analysis.

5.4. DISCUSSION

Breast cancer cells, as most of the cancer cells, have high genomic instability, representing a critical feature to enable tumor initiation and progression (29). In this way, it is of extremely importance to maintain a DNA damage/repair balance, through the perfect function of DNA damage signalling pathways and cell cycle checkpoints, such as those regulated by P53, BER, NER and HRR DNA repair pathways. In breast cancer, the importance of these systems is evident, since *TP53*, *ATM*, *BRCA1* and *BRCA2* genes, with a direct or indirect role in DNA repair processes, present mutations highly correlated with breast cancer development (30,31). Furthermore, several molecular epidemiological studies had shown that polymorphisms in genes involved in these pathways result in differential susceptibility to breast cancer (25,32-35). In previous studies (25), we showed that *XRCC1 399Gln* genotypes conferred a protective effect to development of sporadic breast cancer. Furthermore, we demonstrated a strong association of increased breast cancer susceptibility in women carriers of *XRCC3 241Met* genotypes and

negative FH of breast cancer. In addition, other study performed by us suggested that *PIN3 A2* allele in a haplotype combination confer increased breast cancer susceptibility among women carriers of FH of the disease. Therefore, we decided to evaluate the XRCC1, XRCC3 and P53 proteins expression profiles in a series of invasive ductal breast carcinoma and in a panel of human breast cancer cell lines assessing the possible correlations between their respective genetic polymorphic status.

To the best of our knowledge, this study represents the first report where XRCC1 expression profile in breast tissues was evaluated. Our results showed that XRCC1 nuclear expression is a common event in human breast tissues, either in normal-like, benign lesions, DCIS and invasive carcinomas. Similar results where obtained in other types of human cancer. Crnogorac-Jurcevic et al (36) described positive nuclear XRCC1 IHC expression in 100% of normal pancreatic tissues evaluated, whereas just 77% of the neoplastic pancreatic samples presented moderate/strong expression. In invasive bladder carcinoma, a mean percentage of positive nuclear staining of near 95% was observed (37). No correlation of XRCC1 expression was found with clinical-pathological features.

Regarding XRCC3 expression, the frequency of cytoplasmic positive breast cancer cases is comparable to what has been previously reported (38). However, XRCC3 nuclear expression frequency differs from that obtained by Honrado et al (38), mainly in the sporadic breast cancer patients. These differences could be explained by clinical features heterogeneity in sporadic cases used in both studies. Moreover, our study demonstrated that XRCC3 nuclear expression correlates with well/moderate differentiated tumors and with positivity to estrogen receptors. XRCC3 expression reveals an operational protein, working in order to achieve a state of relative genomic stability. Thus, cancer cells with active XRCC3 can exist in less aggressive tumors, traduced in more differentiated tumors and positive estrogens receptors.

The frequency of P53 positive nuclear expression in invasive breast carcinoma samples (12.4%) was similar to other studies (39-41). Additionally, we did not obtain any association between P53 IHC expression and clinical-pathological characteristics, which is in agreement with other studies, demonstrating the weak value of P53 IHC in the prognostic and prediction of breast cancer (42).

XRCC3 gene is required for several functions, like repair of double strand breaks through the HRR pathway (43), DNA cross-linking repair (44), and chromosomal segregation (45). During HRR, the XRCC3 protein interacts with the RAD51 protein, enabling RAD51 protein multimers to

assemble at the site of damage (44,46). Furthermore, RAD51 has also been found to colocalize with the XRCC1 protein after base damage, suggesting coordination between XRCC1-dependent single strand break repair and recombination events during DNA replication (47). This is in agreement to our results, since we found a great correlation between XRCC1 positive and XRCC3 cytoplasmic positive expression in breast cancer tissues. Furthermore, we also observed a correlation between P53 positive expression and XRCC3 cytoplasmic positive expression. As already mentioned, P53 takes part directly or indirectly in several DNA repair pathways, such as HRR. Several studies demonstrated that P53 wild-type downregulates HRR through interaction with RAD51 or it paralogs, like XRCC3 (22). Since P53 expression suggests a deregulation of P53 function, thus upregulation of HRR players, like XRCC3, can occur. The biologic meaning of cytoplasmic XRCC3 protein localization is unknown. However, evidences from *in vitro* studies have shown XRCC3 staining increased in the cytoplasm region after DNA damage (48,49), suggesting a biological role of this protein in the cytoplasmatic compartment.

No association was found concerning the XRCC1, XRCC3 and P53 protein expression in breast cancer and their polymorphic status, either in case group and cell lines. The reason for it can be due to the high genetic heterogeneity within tumors, so that polymorphisms in these genes produce only subtle alterations in protein activity and effectiveness, which were not perceptible in IHC analysis. Furthermore, interpretation of IHC is a subjective issue, depending on the experience of the interpreter.

In summary, our study was the first to describe XRCC1 IHC expression profile in breast tissues. We showed that XRCC3 expression may be associated with P53 regulation and XRCC1 expression in breast tumors and XRCC3 positivity expression may be an important factor in prediction of less aggressive breast tumor. Moreover, we demonstrated that XRCC1, XRCC3 and P53 expressions do not correlate with the respective genetic polymorphisms analysed, in breast cancer patients and human breast cancer cell lines.

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CHAPTER 6 – GENERAL DISCUSSION

6. GENERAL DISCUSSION

Breast cancer is a heterogeneous disease, sustained by a wide variable morphological appearance, many risks factors and distinct gene expression profiles (1,2). Common genetic alterations (e.g. polymorphisms), with possible effects on function and/or protein expression, within genes involved in essential cellular pathways, such as carcinogen metabolism, DNA repair, cell cycle control and cell proliferation, could predispose individuals to cancer (3-6), including breast cancer (4,7-10).

Breast cancer cells, like all cancer cells, exhibit high genomic instability, representing a critical feature to enable tumor initiation and progression (11). In this way, it is of extremely important the maintenance of DNA damage/repair balance, through the perfect functions of DNA damage signalling/repair pathways and cell cycle checkpoints, such as those regulated by P53, and the BER, NER and HRR DNA repair pathways. In breast cancer, the importance of these systems is evident, since *TP53*, *ATM*, *BRCA1* and *BRCA2* genes, with a direct or indirect role in DNA repair processes, present mutations highly correlated with breast cancer development (12,13). Moreover, there is increasing data supporting the hypothesis that genetic polymorphisms in various DNA repair genes result in reduced DNA repair capacity (14-21). Furthermore, several molecular epidemiological studies have shown that polymorphisms in genes involved in these pathways result in differential susceptibility to breast cancer (22-26).

In our Portuguese case-control study, we investigated the role of some of these DNA repair genes polymorphisms involved in BER, NER and HRR pathways (*XRCC1 Arg399Gln, XPD Lys751Gln, RAD51 G135C* and *XRCC3 Thr241Met*) and two separate *TP53* polymorphisms (*Arg72Pro* and *PIN3 Ins16bp*), in two groups of breast cancer, familial and sporadic cases, as well as in matching control groups. The allelic frequencies of our control group for the different polymorphisms are in accordance with earlier reports from European populations (27-30).

XRCC1 protein is thought to act as a scaffold protein for both single-strand break repair and base excision repair activities (31). It has been shown that XRCC1 interacts with DNA Polβ, DNA LigIII and APE1, through a BRCT domain at the C-terminus (32). XRCC1 seems to be essential to mammalian viability, since its disruption in mice leads to embryonic lethality (33). Several polymorphisms have been found in *XRCC1* gene and some have been linked to several types of cancer (17). Our findings suggest a protective effect of the *399Gln* genotypes of *XRCC1*

polymorphism to the development of breast cancer in women with no FH. These results are consistent with those from previous studies of *XRCC1* polymorphisms and cancer risk, which reported a protective association between the *399Gln* carriers and cancer (28,34,35). However, other studies in breast cancer showed contradictory results. Some observed a positive correlation of *399Gln* genotypes with breast cancer risk, specially when gene-exposure interactions were considered (36-38); others demonstrated no association (39,40) having also in account the presence of FH (9,41-43). Functional studies about XRCC1 suggest that the *399Gln* allele may be associated with higher levels of different types of DNA damage in some cancers (16,44,45). These contradictory effects of *399Gln* genotypes can be explained by two points. First, the gene variants can independently confer particular function to XRCC1 protein, since their effects can potentially depend from other biochemical pathways operating in the tissue being analyzed (46). The effects of any given genetic variant can also depend from other genetic or environmental factors that interact with it (47). Secondly, these variants can diminish the protein efficiency but still provide decreased cancer risk, since, in the presence of excessive damage, cells carriers of these variants will have decreased ability to repair and may be more likely to undergo apoptosis.

XPD is a highly polymorphic gene and the correlation of its polymorphisms and cancer risk have been studied extensively (15,18,48). XPD protein is a subunit of the TFIIH complex and has important roles in transcription and NER pathway. It participates in the locally unwind of DNA helix to permit RNA transcription machinery to access the promoter and to permit the NER machinery to access the lesion (49). Some common *XPD* polymorphisms have been associated with a differential DNA repair capacity (19-21,50,51). In this study, we did not find an association between the *XPD Lys751Gln* polymorphism and breast cancer risk. To date, the results evaluating *XPD Lys751Gln* polymorphism are controversial. Our results are in agreement with other studies where no correlation was found between this polymorphism and breast cancer risk, among women without (36,52,53) and with breast cancer FH (9,41). In contrast, significant association between *XPD 751Gln* allele and breast cancer risk was seen in other recent studies (54). Furthermore, the functional significance of *XPD* polymorphism seems to be dependent from environmental factors which interact with that variant. Some studies showed that *751Gln* allele was associated with reduced DNA repair efficiency of UV-light DNA damage, while others showed a suboptimal repair of X-ray-induced DNA damage related with *751Lys* allele (44,48).

RAD51 is a protein participating in DSBR pathway that forms a heterodimer with several genes (such as XRCC2, XRCC3, BRCA2) playing an important role in HRR (55). The majority of

RAD51 polymorphisms described are in UTRs. Regarding *RAD51 5'UTR G135C* polymorphism, our results showed an association of *RAD51 135C* genotype and increased breast cancer risk, only among women with FH of breast cancer. This suggests that this polymorphism contributes to the familial breast cancer in the Portuguese population, in opposition to reported results in the Brazilian population (9). Other studies have demonstrated an association of *RAD51* genotypes with familial breast cancer risk, only in women carriers of *BRCA2* mutations (56-58). Since the number of *BRCA1* and/or *BRCA2* mutations detected in our familial breast cancer cases was too small (59), it was not possible to perform a separate analysis. The biological effect of the *RAD51 G135C* polymorphism is currently unknown. This polymorphism can affect mRNA splicing, regulation of transcription, translation or mRNA stability by association of the *5'UTR* region with regulatory elements (60). Furthermore, linkage disequilibrium can occur between *RAD51* gene and another sequence in a regulatory region of the gene or with another nearby gene, affecting the incidence of breast cancer in familial breast cancer. Concerning sporadic breast cancer risk, others studies had obtained similar results in the Australian women (61) and in the Anglo-Saxon population (62), where no association was obtained.

Another important protein in HRR is XRCC3, interacting directly with RAD51, helping the assembly of the nucleofilament protein and the selection and interaction with appropriate recombination substrates (63). Some common polymorphisms were described in *XRCC3* gene (62), such as *Thr241Met*. Our results demonstrate a strong association of increased breast cancer susceptibility in women carriers of *XRCC3 241Met* genotypes and sporadic breast cancer. This is consistent with an earlier report in the United Kingdom population (62). Functional data supporting the hypothesis of damaging consequences due to *XRCC3 Thr241Met* polymorphism supports our results. The *241Met* variant has been associated with higher levels of bulky DNA adducts, mitotic defects and lower DNA repair capacities of X-ray-induced DNA damage (16,64). However, other studies report no risk association (9,42,61,65-67).

The *TP53* is one of the major tumour suppressor genes which carry out essential functions in preservation of genome integrity. Thus, when the cell is under stress, which involves DNA damage, p53 promotes growth arrest, allowing the cell to repair the DNA lesions. If the damage is excessively hazardous, then p53 will lead to cell apoptosis. Several genetic polymorphisms have been described in *TP53* gene (68). Concerning the *Arg72Pro* polymorphism, we did not find any association with breast cancer risk. Our results are in agreement with other studies (30,69,70), however, the literature remains highly controversial regarding the role of this

polymorphism in breast cancer risk (71-76). One study showed that *TP53 72Pro* variant induces transcription activation more efficiently than *TP53 72Arg* variant (74). On the other hand, other authors revealed that *TP53 72Pro* variant induces cell cycle arrest better than *72Arg* (77). Other reports have showed that *TP53 72Arg* variant is more efficient inducing apoptosis (76,78). Beside apoptosis and cell cycle control, p53 protein seems to be crucial in the regulation of the different DNA repair pathways (79). A recent study demonstrated the influence of *TP53 Arg72Pro* in DNA repair capacity, showing that *TP53 72Pro* variant activates several *TP53* dependent target genes involved in DNA repair much more efficiently than the *72Arg* variant expressing cells (80). These contradictory results in molecular epidemiologic studies can be explained by the differential effects of this alteration in p53 function.

Another common *TP53* polymorphism is the insertion of 16bp in intron 3 of the gene. Our findings suggest an association of *A2A2* genotype and increased breast cancer risk among women with familial and sporadic breast cancer, suggesting that this polymorphism contributed to enhance susceptibility for breast cancer among Portuguese population, regardless of the presence of FH. Our results are supported by previously reported studies which suggested the same association (81). Although the biological effect of the *TP53 PIN3 Ins16bp* polymorphism is currently unclear, theoretically, this polymorphism can affect mRNA splicing, altering the coding regions and therefore being implicated in regulation of gene expression and DNA-protein interactions, resulting in a defective protein (82,83). Until now, just a single study had showed that *PIN3 A2* allele presents reduced mRNA stability (84). The linkage disequilibrium between *TP53* polymorphisms region can be an important factor affecting the incidence of cancer in general (85,86), and breast cancer, in particular (76,81,87). Thus, haplotype analysis would be important to confirm the significance of *TP53* variants on breast cancer susceptibility. A statistical significant association was found between *Arg-A2* haplotype and breast cancer susceptibility among women with familial breast cancer. On the other hand, a recent study has found that *Pro-A1* haplotype individuals present increased breast cancer risk, however, only in *BRCA2* mutation carriers (76). Nevertheless, other reports have also demonstrated a positive association of *Arg-A2* haplotype with cancer (81,85). Moreover, functional studies have shown that, in a specific haplotype combination, *A2* allele is associated with decreased apoptotic and DNA repair capacity (30,85).

The variance in results of association in different case-control studies on *XRCC1*, *XPD*, *RAD51*, *XRCC3* and *TP53* polymorphisms may be connected to variation in genetic/ethnic origin

and different carcinogenic exposures of the studied populations. Too small sample size and/or the inadequate controlling for certain confounders such as age and breast cancer FH may also contribute to differing results.

The natural history of breast cancer can be influenced by several factors. We hypothesize that under the influence of genetic polymorphisms, chronic exposure to higher levels of several endogenous (e.g. estrogens) and exogenous breast carcinogens, resulting in a higher accumulation of DNA damage during an individual's lifetime, may alter the waiting-time-to onset of disease. Moreover, it has been suggested that DNA repair genes are associated to age related disease (88), and *TP53* polymorphisms are associated to familial breast cancer by the age of 50 years (30). Our results are consistent with this hypothesis, in which *XRCC1 Arg399Gln*, *XRCC3 Thr241Met* and *TP53 PIN3 Ins16bp* polymorphisms seem to influence directly the age to onset sporadic breast cancer and familial breast cancer, respectively.

In this work, we performed the analysis of possible associations between *XRCC1 Arg399Gln, XPD Lys751Gln, RAD51 G135C, XRCC3 Thr241Met* and *TP53 Arg72Pro* and *PIN3 Ins16bp* polymorphisms and breast cancer clinical-pathological features. According to our results, *XRCC1 Gln/Gln* genotype seems to be associated with less aggressive tumors, since this genotype was correlated with well differentiated tumors. Deficient efficiency of the XRCC1 protein has been described in *XRCC1 Gln* variant (44,45). Furthermore, repair of more complex base lesions (89-91) by BER pathway can potentially convert non-lethal DNA lesion into lethal DSB (92,93). Thus, deficiency in BER, by low efficiency of XRCC1, may actually reflect a well differentiated nature of the cancer cells in less aggressive tumors, since less lethal DNA lesions are produced.

We also observed that *RAD51 C135* genotypes show a correlation with more aggressive tumors, since we found a significant association of them with moderate to poor differentiated grade. Additionally, assessment of the OS demonstrated that patients with *RAD51 C135* genotypes presented a poorer survival compared with others genotypes. These results can be explained by the location of this polymorphism in the 5' UTR, affecting mRNA stability and/or translation efficiency, leading to altered RAD51 protein levels (60). Thus, RAD51, the key factor of homologous recombination process, can disturb the activity of the multiprotein DNA repair complex, including BRCA1, BRCA2 and XRCC3 proteins, contributing to high levels of genetic instability (94), and as a result, being correlated with more aggressive tumors as described for breast carcinomas associated with *BRCA1* mutations (95).

No correlation was found between *XPD Lys751Gln* and *XRCC3 Thr241Met* polymorphisms and clinical-pathological features, such as histological grade, axillary lymph node metastases, estrogen receptor, survival and recurrence at last follow-up.

Additionally, our findings suggest the *Pro/Pro* and *A2A2 TP53* genotypes as predictor factors for the presence of lymph node metastases, being in agreement with previously functional studies regarding the biological consequences of these variants in P53 protein function, such as, on one hand, transcription activation and apoptosis inhibition, and, on the other hand, cell cycle arrest induction and DNA repair activation, by the *72Pro* allele (74,77,78,80,84).

In summary, we showed that *XRCC1 399Gln* genotypes confer a protective effect to the development of sporadic breast cancer. Furthermore, we also demonstrated a strong association of increased breast cancer susceptibility in women carriers of *XRCC3 241Met* genotypes and negative FH. In addition, our results suggested that *PIN3 A2* allele in a haplotype combination confer increased breast cancer susceptibility among women FH carriers. Therefore and based on the above mentioned results, we decided to evaluate the XRCC1, XRCC3 and P53 protein expression profiles in a series of invasive ductal breast carcinomas and in a panel of human breast cancer cell lines, assessing the possible correlations between their expression and with their respective genetic polymorphic status.

Importantly, this work is the first report regarding XRCC1 protein expression in breast tissues. Our results show that XRCC1 nuclear expression is a common event in human breast tissues, either in normal-like, benign lesions, DCIS and invasive carcinomas. Similar results where obtained in other types of human cancers. Crnogorac-Jurcevic et al (96) described positive nuclear XRCC1 IHC expression in 100% of normal pancreatic tissues evaluated, whereas just 77% of the neoplastic pancreatic samples presented moderate/strong expression. In invasive bladder carcinoma, a mean percentage of positive nuclear staining of near 95% was observed (97). No correlation of XRCC1 expression was found with clinical-pathological features within our series.

Regarding XRCC3 expression, our percentage of cytoplasmatic positive breast cancer cases is comparable with the only other existent report (98). However, our frequency of XRCC3 nuclear expression differs from that obtained by Honrado et al (98), mainly in the sporadic breast cancer patients. These differences can be explained by clinical features heterogeneity in sporadic cases used in both studies. Moreover, our study demonstrated that XRCC3 nuclear expression correlates with well/moderate differentiated tumors and with positivity to estrogen receptors.

XRCC3 expression reveals an operational protein, working in order to achieve a state of relative genomic stability. Thus, cancer cells with active XRCC3 can exist in less aggressive tumors, translated in more differentiated tumors and estrogens receptors positive, corroborating our findings.

Our frequency of P53 positive nuclear expression in invasive breast carcinoma samples (12.4%) were similar to other studies (99-101). Additionally, the lack of association between P53 IHC expression and clinical-pathological characteristics was also in agreement with previous reports, which showed that prognostic and predictive value of P53 positive expression in breast cancer was found to be weak (102).

XRCC3 gene is required for several functions, like repair of double strand breaks through the HRR pathway (103), for repair of DNA cross-linking (104), and chromosomal segregation (105). During HRR, the XRCC3 protein interacts with the RAD51 protein, enabling RAD51 protein multimers to assemble at the site of damage (104,106). Furthermore, RAD51 has also been found to colocalize with the XRCC1 protein after base damage, suggesting coordination between XRCC1-dependent single strand break repair and recombination events during DNA replication (107). This comes in direction to our finding, where we got a good correlation between XRCC1 positive and XRCC3 cytoplasmatic positive expression in breast cancer tissues. Furthermore, we observed a correlation between P53 positive expression and XRCC3 cytoplasmatic expression. As already mentioned P53 takes part directly or indirectly in several DNA repair pathways, such as HRR. Several studies demonstrated that P53 wild-type downregulates HRR through interaction with RAD51 or it paralogs, like XRCC3 (108). Since P53 expression suggests a deregulation of P53 function, so an upregulation of HRR players, like XRCC3, can occur. The biological meaning of cytoplasmatic XRCC3 protein localization is still unknown. However, evidences of *in vitro* studies have shown increased XRCC3 staining in the cytoplasm region after DNA damage (109,110), suggesting a function of this expression in the biological role of the protein.

Concerning the XRCC1, XRCC3 and P53 protein expression in breast cancer and no correlation was found with their polymorphic status, either in case group and human cell lines. The reasons for the lack of associations can be explain by the high genetic heterogeneity of the tumors, so that these polymorphisms in these genes produce only subtle alterations in protein activity and effectiveness, which are not perceptible in IHC analysis. Furthermore, interpretation of IHC is a subjective issue, depending on the experience of the interpreter.

In conclusion, our findings imply the *RAD51 G135C* polymorphism as a real risk modifier in familial breast cancer cases. Furthermore, we point out that *XRCC1 Arg399Gln* and *XRCC3 Thr241Met* polymorphisms are important biomarkers to sporadic breast cancer susceptibility. Moreover, our results also propose that *PIN3 A2* allele in a haplotype combination confer increased breast cancer susceptibility among women carriers of FH of breast cancer. A possible interpretation for different associations depending on the presence of FH may be due to this factor broadly represents shared genes and environmental factors. The presence of a single polymorphism has most likely weak effects on the individual phenotype, not being measurable except in the context of these additional supporting factors, such as the family history. Among individuals without a familial predisposition, the effect may be hidden by sum effects of other unidentified genetic and environmental factors (42). According to our findings with clinical-pathological parameters, we clearly underlie the role of *XRCC1 Arg399Gln* and *RAD51 G135C* polymorphisms in the prediction of breast tumor aggressiveness and patients' survival. Furthermore, our results suggest *TP53 Arg72Pro* and *PIN3 Ins16bp* polymorphisms as predictive factors of presence of lymph node metastases. Additionally, we demonstrated that XRCC1, XRCC3 and P53 expressions do not correlate with the respective genetic polymorphisms analysed, in breast cancer patients and human breast cancer cell lines.

This work brings a significantly contribution to the characterization of the genetic breast cancer susceptibility profile, within familial breast cancer cases non-carriers of *BRCA1/BRCA2* mutations and sporadic breast cancer patients, in the Portuguese population. These findings can be helpful for an early and more efficient screening of breast cancer disease.

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