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The Association of p16^{INK4A} and Fragile Histidine Triad Gene Expression and Cervical Lesions

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■ **Abstract**

Objective. This cross-sectional study was intended to assess the association between immunohistochemical analysis of p16^{INK4A} and fragile histidine triad (FHIT) and the presence of precancerous cervical lesions.

Materials and Methods. Women seen at Pérola Byington Hospital, São Paulo, Brazil, with histologically confirmed cervicitis (n = 31), cervical intraepithelial neoplasia (CIN) 1 (n = 30), CIN 2,3 (n = 30), and cervical cancer (n = 7) had also cervical material collected for liquid-based cytology, human papillomavirus Hybrid Capture 2 (HC2) test, and p16 and FHIT immunohistochemical reactions.

Results. p16 and FHIT reactions were scored as the following: <1%, 1% to 5%, >5% to 25%, and >25%. Receiver operating curve analysis was used to select p16 and FHIT score cutoffs for further categorical analyses. All but one of the 37 CIN 2,3/cancer cases had a p16 score of greater than 1% to 5%. Among the 61 cervicitis/CIN 1 cases, 46 (75%) had a p16 score lower than 1% to 5%. In contrast, no association of FHIT expression and severity of cervical lesions could be demonstrated in this data set. Receiver operating curve analyses suggested the score of 1% to 5% for p16 as the cutoff that best discriminates CIN 2,3/cancer from cervicitis/CIN 1. No cutoff for FHIT scores could be suggested with data set.

Conclusions. p16, but not FHIT expression, has the potential to be used as complementary diagnostic tool to investigate human papillomavirus–induced cervical lesions, if these results are confirmed in larger studies. ■

Key Words: p16, FHIT, cervical cancer, HPV, liquid-based cytology

The ambiguity of morphological features to classify cervical lesions and its correct correlation with prognosis led many investigators to research new paradigms to assess this information [1, 2].

The major function of p16 protein, a product of *CDKN2A* gene, is to suppress the activity of cyclin-dependent kinase (CDK) 4 and CDK-6. This is an essential function to be considered in oncology because

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p16 is directly involved with the cell cycle regulation, because CDK-4 and CDK-6 cyclins regulate the G1 checkpoint [3]. In addition, p16 seems to hamper the transforming activity of the oncogenic human papillomavirus (HPV) gene E6; even so, E7 interaction with retinoblastoma protein can directly stimulate cyclin-inducing cell replication [4]. The effect of this pathophysiological phenomenon is p16 overexpression, which is presently accepted as an occurrence linked with the potential oncogenic activity of HPV infection in cervical and other genital lesions [3, 5]. Furthermore, p16 is deemed to be a powerful molecular biomarker for malignant and premalignant HPV-induced cervical lesions [6–8], and overexpression is recognized as a predictor of poor prognosis [9–12].

The fragile histidine triad (FHIT) gene encompasses the common chromosomal fragile site FRA3B. The HPV has been found to be able to integrate its genes into the chromosome 3 fragile site of cultured cells, deleting a piece of DNA that includes the FHIT gene [13]. The FHIT gene alteration is believed to occur fairly early in the development of some types of cancer. The FHIT inactivation seems to be a later event, probably related to evolution for a more aggressive neoplasia. Thus, FHIT immunohistochemical expression in premalignant lesions may give useful diagnostic and prognostic data [14, 15]. The FHIT gene loss of heterozygosity was found to be significantly associated with oncogenic HPV infection, suggesting a link between the integration of viral DNA and subsequent gene deletion in progression of cervical cancer. Recently, a microarray comparative genomic hybridization study has endorsed that FHIT deletion was the most common DNA losses present in 47% of the invasive carcinomas of the cervix [16].

The objective of our study was to investigate the association between HPV-induced lesions of the cervix and immunohistochemical analysis of $p16^{INK4A}$ and FHIT.

MATERIALS AND METHODS

This was a cross-sectional study performed at Pérola Byington Hospital, São Paulo, Brazil, from January through December 2002. Women with histologically confirmed cervicitis (n = 31), cervical intraepithelial neoplasia (CIN) 1 (n = 30), CIN 2,3 (n = 30), and cervical cancer (n = 7) had cervical material previously collected for liquid-based cytology (LBC), HPV Hybrid Capture 2 (HC2) test (Digene Co, Gaithersburg, MD) for high-risk HPV-DNA and p16 and FHIT immunohistochemical reactions (IHRs). p16 and FHIT IHRs were performed in all but 4 cases in whom FHIT could not be assessed for technical reasons. All laboratory tests were processed blindly at the Pathology Division of Adolfo Lutz Institute. The study protocol was approved by the institutional review boards of both institutions involved in the project.

Cytological and Histological Samples

Cervical samples were collected with a scored cervical brush included in the DNACitoliq LBC kit and stored in a universal collection medium (both from Digene Brasil, São Paulo, Brazil). Cytology results were reported in accordance to the Bethesda 2001 system [17]. Histological specimens were initially evaluated according to the World Health Organization [18], blinded to cytological results.

Immunohistochemistry for p16 and FHIT

The glass slides silane-treated with new 3-μm paraffin sections obtained for immunohistochemistry (IHC) analysis was maintained at 55°C for 6 hours. The IHC procedures were performed after removing paraffin in xylene and rehydrating baths in decreasing concentrations of ethyl alcohol and in distilled water. Antigen retrieval was performed using a 10-mmol/L concentration of citrate buffer (pH 6.0) in a pressure cooker for 10 minutes. The slides were allowed to cool down at room temperature and then subjected to immunostaining. The antibodies used in this study were $p16^{INK4A}$ (dilution, 1:500), obtained from MTM Laboratories AG (Heidelberg, Germany), and anti-FHIT (polyclonal rabbit; Zymed Laboratories, San Francisco, CA) (dilution, 1:2000), supplied by Dako AS (Glostrup, Denmark), both amplified by Envision peroxidase system (Dako Cytomation, Carpinteria, CA). The color of immunostaining was generated by chromogenic substrate diaminobenzidine (100 mg%, Sigma D5637), and hydrogen AQ2

Table 1. p16 IHR Scores According to Histopathological Results

	Histology		
p16	Cervicitis/CIN 1	CIN 2,3/cancer	Total
Negative (<1%)	36	0	36
1%–5%	10	1	11
>5%–25%	9	3	12
>25%	6	33	39
Total	61	37	98

IHR, immunohistochemical reaction; CIN, cervical intraepithelial neoplasia.
$\chi^2 < 0.0001$.

Table 2. Fragile Histidine Triad IHR Scores According to Histopathological Results[a]

	Histology		
FHIT	Cervicitis/CIN 1	CIN 2,3/cancer	Total
Negative (<1%)	3	6	9
1%–5%	10	6	16
>5%–25%	23	10	33
>25%	23	13	36
Total	59	35	94

IHR, immunohistochemical reaction; FHIT, fragile histidine triad; CIN, cervical intraepithelial neoplasia.
$\chi^2 = 0.37$.
[a]FHIT expression was not available in 4 patients.

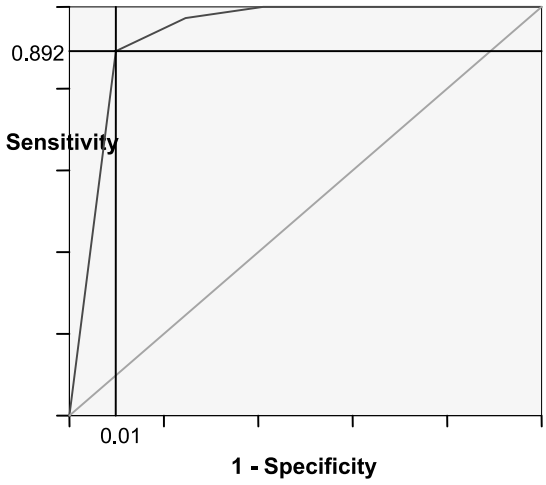
peroxide (0.1%). After light counterstaining in Harry hematoxylin, the slides were mounted with Entellan medium (Merck, Darmstadt, Germany) and analyzed using light microscopy.

Evaluation of the IHRs

Evaluation of $p16^{INK4A}$ IHR staining was scored, as published elsewhere [5]. Positive nuclear and cytoplasmic positive reactions were scored as follows: negative (no reaction or <1% of positive cells), sporadic (<5% isolated positive cells), focal (between 5% and 25% positive cells), and diffuse (>25% positive cells). A similar scoring system was applied to evaluate cytoplasmic FHIT IHR.

Hybrid Capture Test

The HC2 test was performed in accordance with the recommendations of the manufacturer (Digene Co) and reported in relative light units (RLU). Results were categorized as high (RLU, >20), intermediate (RLU, 5–19.9), and low (RLU, 1–4.99) [19]. Only high-risk HPV was tested.



Area Under the Curve

Test Result Variable(s): p16

Area	SE*	Asymptotic significance†	Asymptotic 95% CI	
			Lower bound	Upper bound
0.93	0.02	0.000	0.88	0.98

*Under the nonparametric assumption.

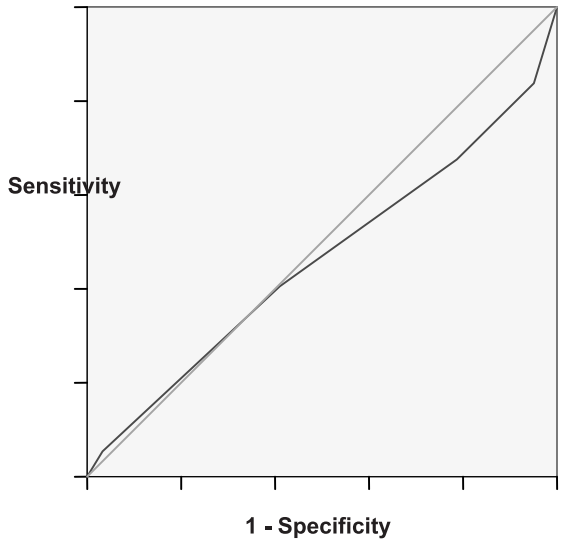
†Null hypothesis: true area = 0.5.

Coordinates of the Curve

Test Result Variable(s): p16

p16 score	Sensitivity	Specificity
<1%	1.00	0.59
1–5%	**0.97**	**0.75**
>5–25%	0.89	0.99

Figure 1. Receiver operating curve of the different p16 cutoffs to diagnose CIN 2,3/cancer lesions.



Area Under the Curve

Test Result Variable(s): FHIT

Area	SE*	Asymptotic significance†	Asymptotic 95% CI	
			Lower bound	Upper bound
0.46	0.06	0.51	0.34	0.58

*Under the nonparametric assumption.

†Null hypothesis: true area = 0.5.

Figure 2. Receiver operating curve of the different FHIT cutoffs to diagnose CIN 2,3/cancer lesions.

Statistical Analysis

The magnitude of p16 and FHIT association with histological results was compared by means of the Pearson χ^2 test. McNemar χ^2 test was used to compare p16 and FHIT scores with LBC results. For statistical analysis purposes, cytology results were lumped in 2 broad categories: cervicitis/CIN 1 and CIN 2,3/cancer. Similarly, histological examination results were also grouped as normal/CIN 1 or CIN 2,3/cancer categories. p16 and FHIT cutoffs that better discriminate CIN 2,3/cancer lesions were determined by the receiver operating characteristic curve (ROC) analyses. Cutoffs that maximized the areas under the curve were used to categorize p16 and FHIT scores in subsequent categorical analyses; *p* values of less than .05 were considered significant. Data were stored and analyzed using the SPSS statistical software, version 13.0 (SPSS Inc, Chicago, IL).

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RESULTS

Of the 37 histologically confirmed CIN 2,3/cancer cases included in the study, the result of LBC was abnormal in 34 cases (91.9%). The HC2 test turned out positive in all CIN 2,3/cancer cases. All but one of the 37 CIN 2,3/cancer cases had a p16 score of greater than 1% to 5% (Table 1). Among the 61 cervicitis/CIN 1 cases, 46 (75%) had a p16 score lower than 5%. In contrast, as it can be seen in Table 2, there was no significant association between FHIT scores and type of cervical lesion. Results of the ROC analyses shown in Figures 1 and 2 suggest the score of 1% to 5% for p16 as the cutoff that best discriminate CIN 2,3/cancer lesions from cervicitis/CIN 1 lesions. However, no cutoff for FHIT scores could be suggested with this data set. The 1% to 5% cutoff for p16 score (Table 3) has a sensitivity

T1 T2 F1 F2 AQ4 T3

Table 3. p16 IHR Scores Using the 1% to 5% Cutoff in Relation to Histological Examination Results AQ5

p16	Histology		Total
	CIN 2,3/cancer	Cervicitis/CIN 1	
>1%–5%	36	15	51
<1%	1	46	47
Total	37	61	98

IHR, immunohistochemical reaction; CIN, cervical intraepithelial neoplasia.
$\chi^2 < 0.00001$; odds ratio = 111.1; 95% CI = 14.2–1,000.

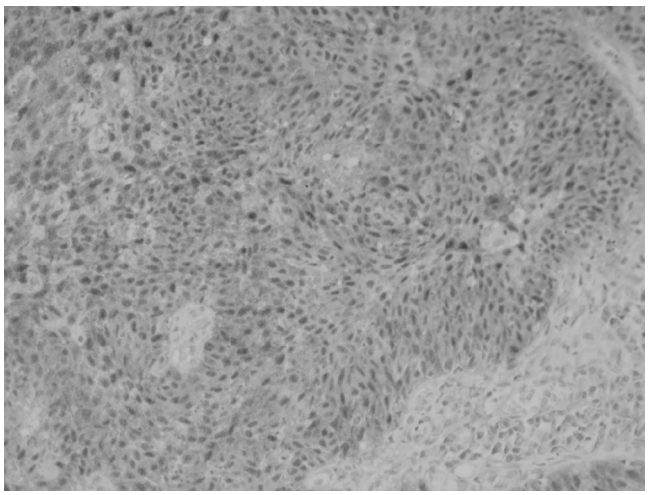

Figure 3. p16-positive reaction in CIN 2,3 cervical lesion (original magnification, ×20).

of 97.3%, specificity of 75.4%, positive predictive value of 70.6%, and negative predictive value of 97.9% for identifying CIN 2,3/cancer.

F3 F4 Figures 3 and 4 illustrate p16 and FHIT IHRs in CIN 2,3 cases.

DISCUSSION

The $p16^{INK4A}$, and FHIT immunohistochemical expressions were evaluated in a series of biopsy-proven cervical lesions. The results have shown that 97.3% of CIN 2,3/cancer cases had a p16 score of 1% to 5% or more, whereas LBC was reported as CIN 1 positive in 91.9%. The FHIT expression did not significantly correlate with high-grade lesions. The HC2 test for high-risk HPV turned out positive in 100% of CIN 2,3/cancer cases.

New molecular players have emerged in the cancer scenario; as a consequence, a number of interesting data are now available [20]. Among all recent feasible technical options, p16 IHC has been purposed as an alternative to optimize the recognition of HPV infection with potential of progression [20]. According to the data presently observed, $p16^{INK4A}$ expression in cervical high-grade lesions showed a sensitivity of 97.3% and a negative predictive value close to 100% because all but one of the 37 CIN 2,3/cancer cases had $p16^{INK4A}$ score of greater than 1% to 5%. In addition, 75% (46/61) of cervicitis/CIN 1 cases had a p16 score lower than 1% to 5%. These data strongly indicate that p16 expression increases with the severity of cervical lesions that corroborate, in part, the diagnostic potential of $p16^{INK4A}$ evaluation [21]. The optimism with this marker is justified based on the progressive intensity of p16 expression in minor lesions (cervicitis/CIN 1) to severe ones (CIN 2 and CIN 3), as herein demonstrated. However, the caveat is that the positive predictive value of p16 test for identifying CIN 2,3/cancer of 70.6% found in this study with 37.7% of diseased cases will be less impressive in populations with lower prevalence of cases. In addition, if $p16^{INK4A}$ has had an unambiguous performance in paraffin-embedded tissues, the same could not be observed in cytological samples. Indeed, the results are not so clear-cut when p16 expression is assessed in cytological samples. Actually, the contentious findings in cytological preparations strongly limit the use of $p16^{INK4A}$ under routine conditions [22]. Currently, when the combination of HPV HC2 test and LBC is the backbone of prevention of cervical high-grade lesions [23], the controversial results of $p16^{INK4A}$ should be judiciously ascertained in further studies with larger series to validate the data obtained with biopsy samples [22].

On the other hand, in this series, FHIT immunohistochemical score in CIN 2,3/cancer cases (65.7%) was unexpectedly greater than 1% to 5%. In contrast, other studies provided evidence indicating that FHIT expression seems to be a good prognostic marker [14–16]. The loss of FHIT gene in HPV-induced lesions is believed to represent a powerful option to predict cervical disease progression mainly in cigarette smoking–associated cervical carcinogenesis [24]. However, the mechanisms of FHIT inactivation and the real meaning of FHIT gene methylation in cervical cancer are not sufficiently understood. For this reason, caution is suggested in its use as a functionally relevant biomarker for cervical

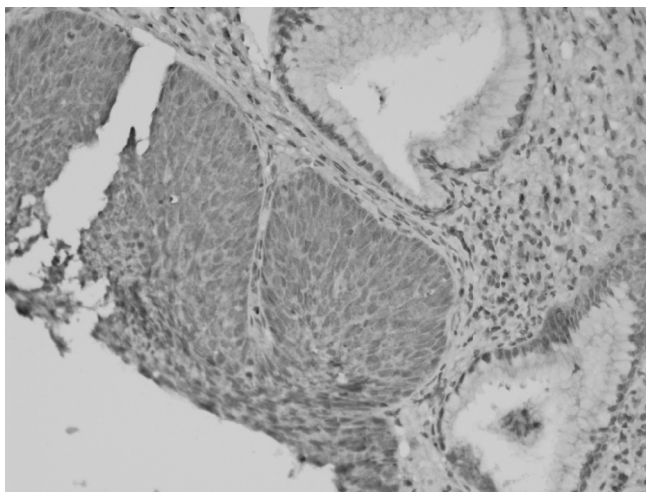

Figure 4. Fragile histidine triad positive reaction in CIN 2,3 cervical lesions (original magnification, ×20).

cancer [25]. The IHR performed with the commercially available antibody for FHIT is somewhat equivalent to FHIT protein expression, but its specificity should be confirmed by subsequent immunoblot analysis because of potential false-positive results [25]. This fact can explain, in part, the lack of specificity of FHIT immunoreaction in the present series. Certainly, it is supposed that best results have been reported with the use of the original anti–FHIT-glutathione *S*-transferase fusion antibody [26]. Even so, there are data obtained with this original antibody that clearly demonstrated ubiquitous distribution of aberrant FHIT expression in all types of cervical lesions, including cancer [27], similar to those reported in the present work with commercially FHIT antibody. Importantly, ROC analysis could not identify a cutoff of FHIT expression that could adequately discriminate CIN 2,3 from cervicitis/CIN 1 lesions in this study.

Finally, $p16^{INK4A}$ and FHIT markers have theoretical and interesting differences because of their apparently opposing expressions during cervical lesion development, which warrants additional investigation. Recently, cohypermethylation of p16 and FHIT genes was demonstrated to be a helpful biomarker for predicting the recurrence-associated prognosis of nonsmall lung cancer [28].

In a large study involving more than 200,000 women of the Kaiser Permanente Health Maintenance Organization [29], positive HPV HC2 test together with normal cytology was found in 3% of the women. Diagnostic accuracy in this clinical situation is likely to improve with the assessment of $p16^{INK4A}$, but not FHIT expression, if further well-controlled studies corroborate the results herein presented.

Acknowledgments

The authors thank Dr Ruediger Ridder from MTM Laboratories, Heidelberg, Germany, for providing the $p16^{INK4A}$ antibody, and Digene Brasil, São Paulo, Brazil, for providing the DCS system and Hybrid Capture 2 kits.

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