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Original Article

Survey of p16INK4a immunohistochemistry in diagnosis of dysplastic changes in cervix

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Abstract

Introduction: Cervical cancer is the third most common cancer and the second most frequent cause of mortality from malignancies of genital organs in women, which can be prevented by diagnosis of pre-neoplastic changes in cervix. This study aimed to evaluate the $p16^{INK4a}$ biomarker in different grades of cervical intraepithelial neoplasia (CIN) using immunohistochemistry (IHC) method.

Methods: The present cross-sectional study was carried out on the paraffin-embedded blocks of cervical tissue of 100 women with previous histopathological diagnosis of CIN referred to Al-Zahra Hospital, Tabriz, Iran, during 2015-2016. The samples were divided into 4 groups, 31 with normal cervical finding, 30 with low-grade CIN (CIN I), and 39 with high-grade CIN (16 CIN II and 23 CIN III). $p16^{INK4a}$ was investigated on the samples using IHC technique. Data was analyzed by SPSS using chi-square and Mann-Whitney U tests.

Results: Thirteen out of 30 (43%), 12 out of 16 (75%) and 23 out of 23 (100%) of the CIN I, CIN II, CIN III were positive for $p16^{INK4a}$, respectively. None of the normal samples were positive for $p16^{INK4a}$. Sensitivity of $p16^{INK4a}$ for detection of CIN I, CIN II, CIN III was calculated as 63%, 80% and 100%, respectively. The overall sensitivity of the biomarker for detection of CIN lesions was 76.6% and the specificity was 100% for all CIN grades.

Conclusion: The $p16^{INK4A}$ biomarker is a suitable diagnostic tool for high-grade CIN, yet for low-grade ones it has lower sensitivity. $p16^{INK4a}$ can be a helpful tool beside histopathology for diagnosis of CIN lesions.

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Introduction

Cervical cancer ranks as the fourth most common malignancy of females worldwide, affecting 527,624 women every year and 265,672 annual deaths from the disease. Underdeveloped countries account for 84% of cervical cancer cases. Almost all cervical cancers are resulted from infection with human papillomavirus (HPV), which is the most common sexually transmitted infection (STD) all around the globe.[1] HPV is reported to be present in 99.7% of cervical cancers in the United States.[2] The incidence of cervical cancer can be reduced in two ways: preventing the pre-cancerous lesions in the first place, and detecting them before they turn into cancer.[1]

Cervical intraepithelial neoplasia (CIN) is defined as intraepithelial squamous abnormalities which exhibit nuclear atypia in epithelial layers and have the potential of becoming invasive carcinoma if not diagnosed and removed well. CIN is subdivided into categories of high- and low-risk of cancer, which is based on the association between CIN III and subsequent invasive carcinoma. However, in practice, a small proportion may become invasive carcinoma. The trend among pathologists is to classify CIN I, as a process either identical

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to or closely resembling low-grade, and CIN II and CIN III as lesions within the spectrum of high-grade CIN.[3]

Cytological screening of cervical abnormalities has been shown to reduce mortality and morbidity of cervical cancer, yet cytology relies on some diagnostic parameters with variable sensitivity and specificity.[4,5] In order to improve the detection of abnormalities on Pap smears and histopathology, additional biomarkers may need to be employed for better identifying the high-grade abnormalities. Detection of high-risk HPV DNA has also been reported to be highly sensitive, yet less specific than the cytological and histopathological examination.[1,5] Women with abnormal screening test results for cervical cancer are commonly referred to colposcopy after some triage test.[6]

Cyclin dependent kinase (CDK) inhibitor 2A also known as $p16^{INK4a}$ limits cell cycle through negative feedback control to check the cell proliferation by regulation of CDK4 and CDK6. Thus, in the cervical neoplasia and dysplasia, the overexpression of $p16^{INK4a}$ happens.[7,8] Identification of $p16^{INK4a}$ as a biomarker for neoplastic transformation of squamous epithelial cells of cervix allows the identification of transformed cells in the specimens for cytopathology or histopathology. Reports have demonstrated that $p16^{INK4a}$ immunohistochemistry (IHC) significantly improves the diagnostic accuracy of histopathology diagnoses. Moreover, it is been demonstrated that $p16^{INK4a}$ cytology shows higher sensitivity for detection of cervical pre-cancer abnormalities compared to conventional Pap tests.[6]

The present study aimed to evaluate $p16^{INK4a}$ immunohistochemical staining for diagnosis of CIN in patients who had definite histopathological diagnosis with low and high-grad cervical lesions.

Methods

In the present cross-sectional study, 100 paraffin-embedded tissues from cervical samples were used with previous histopathological diagnosis of CIN I, CIN II and CIN III. The sample was derived from women who referred to Al-Zahra Hospital, Tabriz, Iran, during 2015-2016. Of total 100 women who enrolled in the study, 31 had normal findings in cervix epithelium, 30 had low-grade CIN (CIN I), and 39 had high-grade CIN (16 CIN II and 23 CIN III) with mean age of 40.6 years.

Hematoxylin and eosin staining (H&E): The paraffin-embedded tissues were subjected to H&E in order to confirm the histopathological findings. Tissue sections were prepared with 3 µm thickness using microtome device and the staining procedure was performed as described before.[9]

IHC: IHC for $p16^{INK4a}$ was performed on all of the samples (100 samples). The procedure of IHC has performed according to the manuals of antibody detection systems. Tissue sections with 4 µm thickness were prepared on the positive charged adhesive slides (Leica Biosystems, USA) and incubated overnight at 37 °C for the tissues to be adhered on the slide. The tissue sections underwent deparaffinization with xylene and hydration with ethanol 100%, 96% and 7%. The antigen retrieval process was done using antigen retrieval solution citrate buffer (AR Citra Plus, pH:6.2, BioGenex®, USA) in 95 °C for 25 minute, cooled in room temperature (RT) and endogenous peroxidase was blocked using 30% H_2O_2 for 10 min. Anti-$p16^{INK4a}$ (BioGenex®, USA) was added to the slides as the first antibody and incubated for 20 min at RT, washed twice in IHC wash buffer (Tris-buffered saline Tween-20) each 7 min and once in phosphate buffered saline (PBS) for 5 min. For visualization, the slides were incubated with anti-mouse/rabbit EnVision® Duo FLEX double stain system, horseradish peroxidase (HRP)/3,3'-diaminobenzidine (DAB) (Dako, Denmark) for 30 min. The washing procedure was repeated again as the previous washing step. DAB was diluted (50 µl in 1000 µl) and added to the slides and incubated for 7 min for enzymatic reaction to develop. The slides were washed in distilled water, dehydrated and mounted.

Table 1. p16^{INK4a} immunohistochemistry (IHC) among low-grade and high-grade CIN lesions and its sensitivity and specificity to distinguish the high-grade lesions from low-grade ones

Grade	p16^{INK4a}		Total	P	Sensitivity (%)	Specificity (%)
	Positive	Negative				
Low-grade	13	17	30	< 0.001	-	-
High-grade	35	4	39		90.64	69.76
Total	48	21	69		-	-

IHC staining for p16^{INK4a} was interpreted as positive when a strong and diffuse nuclear and/or cytoplasmic staining in cells was observable with the cytomorphologic features of CIN (Figure 1).

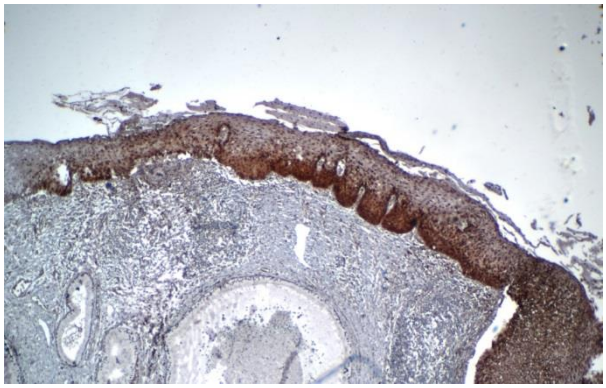

Figure 1. Positive immunohistochemistry (IHC) staining of P16^{INK4a} basal and parabasal cells in cervix epithelium with cervical intraepithelial neoplasia (CIN) II lesion (100X magnification)

Positive and negative control: Cervical carcinoma was used as positive control. The same slide without adding anti-p16^{INK4a} antibody was also used as negative control.

Sensitivity and specificity of p16^{INK4a} IHC: Sensitivity and specificity of p16^{INK4a} IHC was calculated using the following equation:

$$\text{Sensitivity} = \frac{TP}{TP+FN}, \text{Specificity} = \frac{TN}{TN+FP}$$

TP: True positive; FN: False negative; TN: True negative; FP: False positive

Data were analyzed with SPSS software (version 20, IBM Corporation, Armonk, NY, USA) using the Kolmogorov-Smirnov, chi-square, and Mann-Whitney tests.

Results

Among the studied women, 13 out of 30 (43.0%) low-grade and 35 of 39 (89.7%) high-grade CIN lesions were positive for p16^{INK4A}. Considering the high-grade CIN lesions, 12 out of 16 (75.0%) and 23 out of 23 (100) CIN II and CIN III were positive for p16^{INK4A}, respectively (Table 1). None of the normal samples were positive for p16^{INK4A}. The difference between the expression of p16^{INK4A} among different grades of CIN lesions were statistically significant (P = 0.001) (Table 2).

Sensitivity of p16INK4A IHC for detection of CIN I, CIN II, CIN III was calculated as 63%, 80% and 100%, respectively. The overall sensitivity of the biomarker for detection of CIN lesions was 76.6% and the specificity was 100% for all CIN grades (Table 2). The sensitivity and specificity of p16INK4A for distinguishing the high-grade lesions from low-grade ones was 94.64% and 69.76%, respectively (Table 1).

Using one-sample Kolmogorov-Smirnov test showed that the data for the women's age was not normally distributed (P = 0.037). Thus the non-parametric Mann-Whitney test was used for estimating the P-value for mean ages among positive and negative P16^{INK4a} IHC. The results showed a significant difference (P = 0.003) which is available in table 3.

Table 2. p16^{INK4a} immunohistochemistry (IHC) for detection of different cervical intraepithelial neoplasia (CIN) lesions and their sensitivity and specificity

CIN	p16INK4a		P	Total	Sensitivity (%)	Specificity (%)
	Positive [n (%)]	Negative [n (%)]				
CIN I	13 (43.9)	17 (56.1)	< 0.001	30	63	100
CIN II	12 (75.0)	4 (25.0)		16	80	100
CIN III	23 (100)	0 (0)		23	100	100
Normal	0 (0)	31 (100)				
Total	48 (48.0)	52 (52.0)		100	76.6	100

CIN: Cervical intraepithelial neoplasia

Table 3. Estimated P-value for mean ages among positive and negative $p16^{INK4a}$ immunohistochemistry (IHC)

	Age		Mann-Whitney U	Z	P
$p16^{INK4a}$	Mean rank	Sum of ranks	Age		
Positive	39.36	1889.50	713.5	-2.969	0.003
Negative	55.99	2575.50			

Discussion

Cervical cancer is the third most common cancer among women and the second most frequent cause of mortality from malignancies of genital organs in women, which can be prevented by diagnosis of pre-neoplastic changes in cervix.[10] The present study aimed to evaluate the diagnostic value of $p16^{INK4a}$ biomarker in different grades of CIN using IHC method. Results of the preset study showed that 13 out of 30 (43.0%) low-grade and 35 out of 39 (89.7%) high-grade CIN lesions were positive for $p16^{INK4A}$. Sensitivity of $p16^{INK4A}$ for detection of CIN I, CIN II, CIN III was calculated as 63%, 80% and 100%, respectively. The overall sensitivity of the biomarker for detection of CIN lesions was 76.6% and the specificity was 100% for all CIN grades. The sensitivity and specificity of $p16^{INK4A}$ for distinguishing the high-grade lesions from low-grade ones was 94.64% and 69.76%, respectively (Table 1).

We found that $p16^{INK4a}$ IHC is a great tool for detection of high-grade CIN lesions (CIN III), yet it is not absolutely sensitive for diagnosis of low-grade ones (CIN I, CIN II). Moreover, the specificity of $p16^{INK4A}$ IHC was 100%, which means there were no false positives in all grades of CIN lesions. The difference between $p16^{INK4A}$ positivity among different CIN lesions was statistically significant, which shows that the detection of the biomarker is far different among different lesions (CIN I < CIN II < CIN II). $p16^{INK4a}$ IHC can be recommended for the diagnosis of high-grade lesions; however, it is not recommended to be the only diagnostic method for low-grade CIN lesions. Yet, it can be used as a helpful diagnostic assistant beside histopathological findings.

There are several studies that evaluated $p16^{INK4a}$ as a diagnostic marker for cervical abnormalities and some are mentioned below. Tabrizi et al.[5] evaluated $p16^{INK4a}$ IHC for detection of high-grade lesions of cervix in 454 women and $p16^{INK4a}$ was positive in 321 out of 454 (71%) women. Similar to the findings of the present study, $p16^{INK4a}$ was reported as a good marker for high-grade lesions, yet not for low-grade ones. $p16^{INK4a}$ was able to detect 54%, 78% and 90% of CIN I, CIN II and CIN III lesions, which are close to the findings of the present study, except for CIN III that was completely detected by $p16^{INK4a}$ in our study.

Forouhesh Tehrani et al. studied the expression of $p16^{INK4a}$ in the normal and tumoral tissues of myometrium of 136 women.[11] Similar to the findings of the present study, all of the normal myometrium were negative for $p16^{INK4a}$. According to their report, 4 out of 62 (6.5%) leiomyoma and 12 out of 12 (100%) leiomyosarcoma (LMS) samples were positive for $p16^{INK4a}$. They concluded that $p16^{INK4a}$ is a good biomarker for differentiation of normal from tumoral tissue. In another study, Kava et al. evaluated $p16^{INK4a}$ IHC for detection of cervical intraepithelial neoplasm. Based on their report, $p16^{INK4a}$ was positive in 8 out of 15 (53.3%) samples of cervical intraepithelial neoplasms.[12]

In 2017, da Costa et al.[13] discussed $p16^{INK4a}$, Ki-67 and cytokeratin 7 (CK7) as potential markers for low-grade CIN progression. The highest frequency of positivity of each marker was associated with progression to high-grade squamous intraepithelial lesion (HSIL). The percentage of positivity of each marker was reported higher in the low-grade squamous intraepithelial lesion (LSIL) group who showed progression to HSIL ($p16^{INK4a}$ 45.4%, Ki-67 54.5%, and CK7 63.7%), in comparison with women who kept the primary LSIL diagnosis (32%, 40%, and 56%, respectively) and those in whom the lesion regressed (15.8%, 42.1%, and 42.1%, respectively). They

concluded that p16^{INK4a}, CK7, and Ki-67 may be useful biomarkers to identify LSIL lesions that require special attention.[13]

p16^{INK4a} is reported as a potential diagnostic complementary for prediction of high-grade cervical lesions in cytology (liquid-based) with HPV testing and histopathological correlation. Wong et al.[14] reported that, 36 out of 57 (63.2%) liquid-based cervical cytology samples showed immunoreactivity for p16^{INK4a} and 43 out of 57 (75.4%) were infected by high-risk HPV. For prediction of CIN grade II and higher, p16^{INK4a} showed a sensitivity and specificity of 93.5% and 60.0%, respectively. They concluded that p16^{INK4a} is useful for prediction of severity of cytological abnormalities. However, they mentioned that p16^{INK4a} is more specific, yet less sensitive than high-risk HPV in detecting high-grade cervical lesions. Furthermore, combination of tests, high-risk HPV and p16^{INK4a}, failed to show significant improvement in diagnostic values, such as sensitivity, specificity and predictive value.[14] In the present study, the sensitivity and specificity of p16^{INK4a} IHC for detection of CIN II was 80% and 100%, respectively, and for CIN III, both were 100%, which was higher that the report form Wong et al.[14]

Conclusion

The p16^{INK4A} biomarker is a suitable diagnostic tool for high-grade CIN lesions, yet for low-grade ones it has lower sensitivity. p16^{INK4A} can be a helpful tool beside histopathology for diagnosis of CIN lesions.

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Authors' Contribution

Heidar Ali Esmaieli designed the study, analyzed the data and prepared the manuscript. Siamak Berenjian carried out the laboratory work and cooperated in manuscript preparation.

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Conflict of Interest

Authors have no conflict of interest.

Ethical Approval

This study was approved by the Medical Ethics Committee of Tabriz University of Medical Sciences.

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