



*J Anal Res Clin Med*, 2018, 6(2), 66-71.  
doi: 10.15171/jarcm.2018.010, <http://journals.tbzmed.ac.ir/JARCM>



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Original Article

## Comparison of nuclear P16 immunostaining in atypical and normal endocervical glands: A descriptive analytical study

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### Article info

#### Article History:

Received: 27 Dec. 2017

Accepted: 01 Feb. 2018

ePublished: 10 June 2018

#### Keywords:

Cervical Cancer,  
P16 Genes,  
Gland

### Abstract

**Introduction:** Cervical cancer is one of the most common malignancies worldwide and a major problem in the healthcare system. Nowadays, the importance of biomarkers in the diagnosis of malignancies is proven. Some studies have pointed to the role of p16 in the diagnosis of cervical cancer. The purpose of this study was to compare the nucleic reactivity with p16 antibody in atypical versus normal endocervical glands.

**Methods:** In this descriptive-analytical study, we enrolled 60 patients who had undergone hysterectomy due to non-endocervical causes at Alzahra teaching hospital of Tabriz University of Medical Sciences, Tabriz, Iran. We selected 25 patients with atypical endocervical glands and 35 subjects with normal glands based on the pathologic examination using hematoxylin and eosin (H&E) staining. Then, we assessed the frequency of nucleic reactivity of the tissues with p16 antibody in both groups.

**Results:** No p16 expression was observed in any of the samples from normal subjects. However, only 2 (8.0%) out of 25 samples from the atypical group, were not reactive to the p16 antibody. In addition, 20 samples (80.0%) were diffusely stained continuous, whereas three samples (12.0%) were stained locally. Accordingly, in the group with atypical endocervical glands, the reaction with p16 antibody was significantly higher than that of normal endocervical glands ( $P = 0.001$ ).

**Conclusion:** P16 biomarker may play a role in the pathogenesis and progression of cervical cancer and can be used as a diagnostic marker for this purpose.

**Citation:** Esmaeili HA, Poulrak T, Ghamari B, Karkon-Shayan F, Sanaat Z, Gazi-Soltani G, et al. **Comparison of nuclear P16 immunostaining in atypical and normal endocervical glands: A descriptive analytical study.** *J Anal Res Clin Med* 2018; 6(2): 66-71. Doi: 10.15171/jarcm.2018.010

### Introduction

Cervical cancer is a common malignancy in the developing countries, accounting for approximately six percent of malignancies in women. The mortality rate for this cancer is high.<sup>1,2</sup> In the United States, cervical cancer is the sixth common solid malignancy after breast, lung, colorectal, endometrial, and ovarian carcinomas. The mean age of patients at the time of diagnosis is 52 years with a two-peaked distribution pattern, one at 35-39 years

and the other at 60-64 years of age. Cervical cancer is still one of the major causes of malignancy-related deaths among women.<sup>3-5</sup>

Squamous cell carcinoma (SCC) is the most common type of cervical cancer.<sup>6</sup> On the other hand, adenocarcinoma of cervix constitutes up to 15%-20% of the cervical malignancies in the developed countries. Recently, the prevalence of cervical adenocarcinoma has increased from 1.34% in the seventies to 1.73% in the nineties.

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Moreover, the ratio of adenocarcinoma to SCC of the cervix has increased due to the prevention of cervical SCC using screening programs and better identification of endocervical malignancies by pathologists.<sup>7,8</sup>

The main risk factors for cervical adenocarcinoma are African-American race, sexual and reproductive factors, smoking, oral contraceptives, and human papillomavirus (HPV).<sup>9,10</sup> The most common symptom of cervical adenocarcinoma is vaginal bleeding. Nevertheless, it has a nonspecific appearance in the colposcopic examination.<sup>11,12</sup> Diagnosis of the neoplastic epithelial lesions of the cervical glands is difficult because inflammation and hyperplastic changes of the epithelial glands can also mimic this condition. The precursors of cervical SCC have been well described, whereas the morphology of the precursors of adenocarcinoma in situ (AIS) including dysplasia or intraepithelial neoplasias of the cervical glands are still challenging among pathologists.<sup>13,14</sup>

Nowadays, studies have focused on the biomarkers that can be used to detect the transformation of HPV infection into dysplasia or cancer. P16 is a tumor suppressor protein used as a biomarker to identify cancerous changes in the cervix. This protein inhibits the cyclin-dependent kinase (CDK) 4/6. P16 is produced and accumulates in the nucleus of the cells infected with HPV, and it is detectable by immunostaining.<sup>15-17</sup> Although in all cases of high-grade cervical intraepithelial neoplasia (CIN 2 and 3), SCC and adenocarcinoma of cervix p16 is positive, it is rarely expressed in normal tissues or benign lesions of the epithelial glands or squamous tissues. Given that the p16 is considered to be positive in adenocarcinoma of the cervical gland and there is no information about the staining of p16 in the atypical endocervical glands,<sup>18,19</sup> in the present study we compared the nucleus reactivity with p16 antibody in atypical versus normal endocervical glands.

## Methods

In this descriptive-analytical study, we

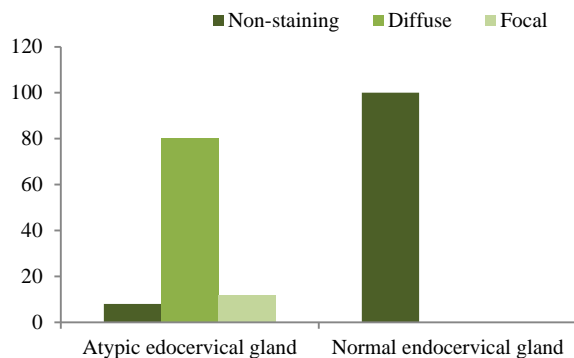
enrolled 60 patients who had undergone hysterectomy due to non-endocervical causes at Alzahra teaching hospital of Tabriz University of Medical Sciences, Tabriz, Iran, from February 2015 to February 2016.

We included every patient who underwent hysterectomy due to non-endocervical causes, had atypical or normal endocervical glands based on the pathologic reports and also informed consent to participate in the study. However, we excluded every subject with a history of any problem in the cervical area or uncertain outcome of the pathological examination. We also excluded patients who did not have informed consent to participate in the study.

We included 60 patients after confirming the study in the Ethics Committee of Tabriz University of Medical Sciences, taking into account the inclusion and exclusion criteria, explaining the purpose of the study to the patients, and obtaining the informed consent from the patients. This study was in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

We stained the specimens with hematoxylin and eosin (H&E), and a pathologist examined them afterwards. According to pathological reports, 25 patients had atypical endocervical glands and 35 patients had normal endocervical glands. We randomly selected these patients using convenient sampling and enrolled them to the study. We obtained the necessary sections from the hysterectomy specimens for evaluating p16. Then, we rinsed the paraffin blocks with xylol to deparaffinize them, and serially washed them with 70% alcohol and then with phosphate buffered saline (PBS) solution with pH = 7.2. Subsequently, we subjected all sections to citrate buffer with pH = 6, and then heated them in the microwave for 5 min. Finally, we incubated the specimens with anti-mouse p16 antibody for 60 min at 25 °C. A pathologist reviewed the immunohistochemical slides, and

considered positive cases with diffused reactivity of their nuclei and cytoplasm to p16 (Figure 1).



**Figure 1.** Percentage of different staining types of endocervical glands

We performed this study after explaining to all of the patients that their information would be kept confidential and their personal information would not be mentioned anywhere. We performed no additional diagnostic and therapeutic intervention during the whole study (except the evaluation of the reactivity of the nucleus with p16 antibodies in the endocervical glands of the hysterectomized individuals), and all patients received the necessary supportive and therapeutic measures. Moreover, we carried out the relevant tests with supports of the vice chancellor of Tabriz University of Medical Sciences. We received no additional costs from patients and their families.

We used Krishnappa et al.'s<sup>20</sup> study and G-power software to determine the sample size of the primary data. Based on the power of 80% and a type one error of a maximum of 5%, 25 patients were considered for each group. Finally, 25 patients with atypical endocervical glands and 35 patients with normal endocervical glands were decided to be included in the study. The data were expressed as frequency and percentage. We used chi-

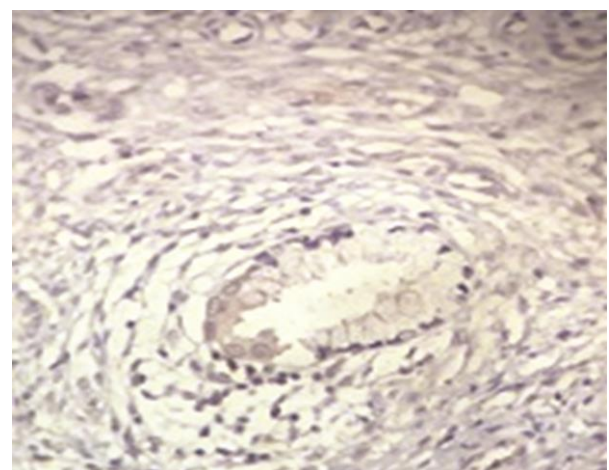
square test to compare the quantitative data between the two groups. We considered  $P \leq 0.05$  as statistically significant.

**Results**

In this study, a total of 60 immunohistochemistry (IHC) staining samples with p16 biomarker were assessed; of which 25 samples had atypical endocervical glands, and 35 samples had normal endocervical glands. Table 1 shows the comparison of the frequency of nuclei and cytoplasm reactivity with p16 antibody in two groups with atypical and normal endocervical glands.

Based on this table, in the group with normal glands, none of the samples reacted with p16 antibodies and did not have any staining. In contrast, in the group with atypical endocervical glands, 92.0% of the samples reacted with p16 antibodies. Accordingly, in the group with atypical endocervical glands, the reaction with p16 antibody was significantly higher than that of the other group ( $P = 0.001$ ).

Figure 2 shows the abundance of endocervical (normal and atypical) gland types.



**Figure 2.** Immunohistochemistry (IHC) staining with p16 marker

**Table 1.** The comparison of the frequency of nuclei and cytoplasm reactivity with p16 antibody in two groups with atypical and normal endocervical glands

Sample	Atypical endocervical glands (n = 25)	Normal endocervical glands (n = 35)
Non-stained [n (%)]	2 (8.0)	35 (100)
Diffusely stained [n (%)]	20 (80.0)	0 (0)
Locally stained [n (%)]	3 (12.0)	0 (0)

## Discussion

Cervical cancer is the second most common cancer in women worldwide, and is the fifth cause of cancer-related deaths.<sup>21</sup> Recent studies show that nearly 500000 new cases of cervical cancer are diagnosed annually worldwide. By introducing novel screening tests from the 1940s and performing Pap smear test, the prevalence and mortality of this cancer have reduced. Furthermore, the addition of cytological analyses and screening programs, resulted in early detection of premalignant lesions and appropriate treatment measures, leading to a reduction in the incidence of cancers.<sup>22</sup>

The most common risk factor for cervical cancer is HPV, which is involved in the progression of neoplasms through two oncoproteins. These oncoproteins react with the host regulatory proteins, and alter the function and expression of host cell genes, and thus destroy the cell cycle.<sup>23</sup> Previous studies have shown that E6 oncoprotein inhibits p53 tumor suppressor protein, which leads to the binding of E7 oncoprotein to the retinoblastoma protein.<sup>23</sup> As a result, the contrast between these two E2F transcription factors disturbs the progression of the cell cycle in the G1/S phase.<sup>24</sup> P16 protein inverts cell proliferation by preventing the retinoblastoma protein hyperphosphorylation through CDK 4/6 complex. Expression of this protein in cervical cancers is well documented. P16 is an indirect marker in cell cycle dysregulation, which is commonly found in cervical dysplasia and carcinomas associated with HPV.<sup>15</sup>

Regarding the high prevalence of cervical cancer and the importance of detecting pre-cancerous lesions in the prevention of these malignancies as well as improving the prognosis of patients, in the present study we compared the expression of p16 in atypical and normal endocervical glands. Based on the results of this study, 92% of the atypical endocervical glands reacted with the p16 antibody, but none of the normal gland specimens had this reaction; therefore, the response rate of p16 antibody in atypical

endocervical glands was significantly higher than that of normal glands ( $P = 0.001$ ).

Sharbatdaran et al. conducted a study to determine the role of p16 as a biomarker in the diagnosis of immature dysplasia and cervical metaplasia. The result of IHC staining showed that p16 biomarker was positive in 3 (10.0%) patients with the normal cervix, 9 (30.0%) patients with cervical metaplasia, 19 (63.3%) patients with cervical dysplasia, and 5 (83.3%) subjects with cervical cancer. In this study, p16 had 83.3% sensitivity for the prediction of cervical cancer. Moreover, its sensitivities for the prediction of dysplasia and immature metaplasia of cervix were 63.3% and 30.0%, respectively. P16 specificity for detecting dysplasia and SCC was found to be 80.0%.<sup>25</sup> In line with that in the present study, we found that p16 had 92.0% sensitivity in the detection of atypical endocervical glands.

Eleuterio et al. also conducted a study to investigate the relationship between expression of p16 and HPV. They showed that p16 expression was positive in 92.3% of cases with high-grade squamous intraepithelial lesions (HSILs) and 15.4% of low-grade SILs (LSILs), and was not seen in any of the normal tissues. Sensitivity, specificity, positive predictive value, and negative predictive value for high-grade lesions were 92.3%, 100%, 100%. 98.3%, respectively.<sup>26</sup> Similarly, we showed that expression of p16 in endocervical glands was higher than normal glands.

In another study, Meyer et al. showed a 9% expression of p16 protein in non-cancerous tissues, compared to an 81% expression in the HSIL tissues.<sup>27</sup> In the present study, we showed that the expression of p16 in the atypical glands (as the initial stage of malignancy) was significantly higher than that of the normal endocervical glands.

Furthermore, Tsoumpou et al. conducted a systematic review to investigate the expression of p16 in various histological samples of the cervix. The results of this study showed that this biomarker was expressed in only 12% of the normal smears.

However, its expression was 45% in atypical squamous cells of undetermined significance (ASCUS) as well as LSIL cases, and 89% in HSIL cases. The results of the study indicated that p16 expression increases as the lesion progresses to malignancy.<sup>16</sup>

### Conclusion

In conclusion, based on the results of this study and also on the basis of most studies in this field, it can be stated that the expression of p16 increases with the progression towards malignancy in cervical cancer and it can be used as a marker to determine the risk of malignancy.

### Acknowledgments

We are grateful to all our patients for their patience and contribution throughout this study. This study was supported by hematology and oncology research center of Tabriz University of Medical Sciences.

### Authors' Contribution

Study concept and design: Heydar Ali Esmaeili, Tala Poulrak

Acquisition of data: Behnaz Ghamari, Farid Karkon-Shayan, Farid Karkon-Shayan, Sepideh Fattahi

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Administrative, technical, and material support: Heydar Ali Esmaeili, Tala Poulrak

Study supervision: Heydar Ali Esmaeili, Tala Poulrak.

### Funding

This paper is based on Sepideh Fattahi's specialty dissertation (94/3-7/2) submitted to the Faculty of Medicine, Tabriz University of Medical Sciences.

### 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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