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P16 staining patterns and their relationships with clinical parameters in cases of cervical intraepithelial neoplasia

Muruvvet Akcay Celik[1], Havva Erdem[1], Soner Cankaya[2], Yeliz Kasko Arici[3]

[1]Ordu University, Faculty of Medicine, Department of Pathology, Ordu, Turkey
[2]Ondokuz Mayis University, Faculty of Sport Sciences, Department of Sports Management, Samsun, Turkey
[3]Ordu University, Faculty of Medicine, Department of Biostatistics and Medical Informatics, Ordu, Turkey

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Abstract

The aim of this study is to investigate the contribution of the p16 immunohistochemical marker in the cervical intraepithelial neoplasia (CIN) cases in the distinction between CIN1, CIN2, CIN3 and the relationship of CINs with age, localization. CIN1, CIN2, CIN3 preparations of 2015 in the pathology department were retrospectively analyzed. New sections of 3 µm thickness were obtained on polylysine slides from paraffin blocks of these tissues. Samples with immunohistochemical staining with p16 were evaluated under a light microscope.p16 expression was semiquantitatively assessed according to staining intensity with 4 staining levels as score 0,1,2,3. As a result of the Kruskal-Wallis test, a significant difference was found between the scores in the CIN groups (p <0,001). As a result of the multiple comparison test, p16 scores of patients in the CIN2 and CIN3 group were found to be significantly higher than in the CIN1 group (p <0.001). While the mean age of patients in the CIN3 group was significantly higher than those in the CIN2 group (p <0.05), it was not different from the patients in the CIN1 group (p> 0.05). The variations between CIN groups and anatomical locations on the cervix were examined with Chi-square test. There was no change in the frequency distribution of CIN groups in the anatomical locations on the cervix (p> 0.05). In cervical intraepithelial neoplasms, p16 was found to show increased staining with the severity of the lesion. It was observed that p16 expression is important in CIN evaluation and reduces subjective evaluation.

Keywords: Cervical intraepithelial neoplasia, p16, cervical precancerous lesion

Introduction

Preinvasive cervical intraepithelial neoplasm (CIN) is the precursor of invasive cervical cancer [1]. World Health Organization (WHO) guidelines have adopted the use of the term squamous intraepithelial lesion (SIL) of the Bethesda System, used for screening and treatment of precancerous lesions [2]. In the latest classification system of the World Health Organization (WHO); SIL is divided into low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL) [1]. While a low grade squamous intraepithelial lesion (LSIL) is an infectious lesion created by HPV, a high squamous intraepithelial lesion (HSIL) is a neoplasia that may contain CIN2 and CIN3 [3]. However, according to the degree of epithelial involvement, CINs are classified as CIN1 (mild), CIN2 (moderate), and CIN3 (severe) [4].

**Corresponding Author: Muruvvet Akcay Celik, Ordu University, Faculty of Medicine, Department of Pathology, Ordu, Turkey
E-mail: drmakcaycelik@gmail.com

In CIN1, undifferentiated cells are limited to the lower layer of the epithelium [4]. Maturation loss, cytological atypia, and mitosis are observed in the lower two-thirds of the epithelium in CIN2 and in the full layer of the epithelium in CIN3. CIN's progress to cancer is a dynamic process and represents a morphological continuity. CIN3 is the most advanced precancerous lesion [5]. CIN1 patients should be followed without treatment because most cases of CIN1 regress spontaneously and progress at a low rate [6,7].Excisional treatment or close observation approaches differ between clinics for CIN2 due to the high regression rate of CIN2 [8].CIN3 lesions are associated with a high risk of cervical cancer and these are typically treated with cervical excision or ablation. Excisional procedures can lead to negative obstetric outcomes for women who plan future childbearing [9,10]. In particular, the differential diagnosis of CIN1, CIN2 with immature squamous metaplasia, as well as the differential diagnosis of low grade lesions (CIN1) and high grade lesions (CIN2 and CIN3) can be difficult [4].

Despite well-defined criteria, histopathological diagnoses may show variations among pathologists [11].Cervical screening programs can also be used to detect cervical precancerous

lesions [12]. Cytology, histology, and colposcopy are important in detecting preinvasive cervical intraepithelial neoplasm lesions [13,14]. Early detection and treatment of cervical intraepithelial neoplasia (CIN) are beneficial in preventing cervical cancer [15,16].

Related research shows that the human papilloma virus (HPV) can greatly increase the incidence of cervical cancer [17]. When HPV continues to infect the cervical region and invade mucosal squamous epithelial cells, CIN1, CIN2, and CIN3 lesions gradually develop. When the basement membrane is invaded,it is transformed into invasive cervical cancer [18].

p16, a cyclin-dependent kinase inhibitor, is a cell cycle regulating protein. p16 is also known as p16INK4a [19,20]. Its function is to regulate cell proliferation in the G1-S phase and p16 negatively affects cell proliferation in a mutual relationship with another tumor suppressor protein, pRb. Over-expression of p16 can be found in cells with inactive pRb commonly found in HPV infection [21].

There is no expression of p16 in the normal cervical squamous epithelium [22] or it is rarely observed as focal positive [23]. Overexpression of protein p16INK4a encoded by the tumor suppressor gene INK4a is a feature of dysplastic and neoplastic changes of the cervical epithelium. The degree of staining of P16INK4a-positive samples increases according to the order of CIN1- CIN2- CIN3- invasive carcinoma [1,24].p16-positive immunohistochemical reactivity is widely used as a biomarker to identify HPV in cervical squamous neoplasms [25].However, CIN1 and CIN2/3 sometimes show similar p16 expression, and p16 can also be found in inflammatory cervical lesions [19,20].

Personal experience is very important in the diagnosis of CIN. Histomorphological examination of Hematoxylin&Eosin (H&E) stained preparations or evaluation with immunohistochemical findings are in question [3,12].

Grading of cervical intraepithelial neoplasia (CIN) with p16 immunoscore system is so useful in reducing variations among pathologists for CIN1,2,3 differentiation in current CIN grading. Accurate colposcopy and histological evaluation of cervical precursor lesions are important to determine clinical management [5].

In our study, it was aimed to emphasize the importance of p16 in the classification of CIN, to reveal its usefulness in planning treatment, and also to examine the relationship between age and localization in CIN.

Materials and Methods

Before beginning the study, permission was granted by the Clinical Research Ethics Committee dated 23/05/2016 and numbered 2016/48.

This study used 80 preparations with CIN diagnosis in 2015 in the Training and Research Hospital Pathology Department and 41 CIN1, 16 CIN2, 23 CIN3 cases were detected.

Preparations were removed from the archive and reevaluated in line with the study aims. New sections with 3µm thickness were obtained on polylysine slides from paraffin blocks of the collected CIN cases.

Immunohistochemical staining was performed with Leica Bond automatic tissue staining device with p16 antibody (Anti-p16-rabbit clonal antibody, R19-D, (1: 100) DB BIOTECH). Stained samples were assessed with a light microscope (BX51, Olympus, Tokyo, Japan).

H&E stained preparations were examined by light microscopy and the areas with the most dysplastic features of the epithelium were selected and CIN areas were evaluated with p16 especially in those regions.

p16 immunoreactivity was evaluated positively when both nuclear and cytoplasmic diffuse staining were seen in basal or parabasal cells. [26].

p16 staining was rated as 4 scores; score 0 (no staining), score 1 (seldom staining of singly scattered cells), score 2 (patchy but strong staining, usually not continuous from basement membrane), and score 3 (strong and diffuse staining, often continuous staining from the basement membrane and extending upward in proportion to lesion grade) [27].

In our study, sites, where lesions were detected on the cervix of each patient after conization or biopsy were determined and then the cervix was divided into anatomical location

The cervix was grouped as upper quadrant (from 10 to 3 o'clock), lower quadrant (from 4 to 9 o'clock), upper + lower quadrant, unknown localization, ECC (endocervical curettage).

Statistical Analysis

Normal distribution control of data with Kolmogorov-Smirnov test, homogeneity control of group variances with Levene's test were done. Groups for the variables providing assumptions were compared with the one-way ANOVA following Tukey's test. The groups that did not provide assumptions were compared with the Kruskal-Wallis test following Dunn-Bonferroni test.

The relationship between categorical variables was examined with Chi-square test.All calculations were made with SPSS v26 statistical software.

Results

A total of 80 cases were included in the study. The average age of patients is 45.24 ± 9.61 and their ages vary between 29-66. According to One-way ANOVA, the mean age of the patients varied according to the CIN groups (Table 1).While the average age of patients in the CIN3 group was significantly higher than those in the CIN2 group ($p < 0.05$), it was not different from patients in the CIN 1 group ($p > 0.05$).At the same time, there was no difference in the mean age of patients in the CIN1 group and in the CIN2 group ($p > 0.05$) (Figure1-3).

Frequency distributions of CIN groups according to scoring are given in Table 2.Chi-square test scores were observed to vary according to CIN groups ($p < 0.001$).While the percentage of CIN1 group with p16 scores of 0 was higher (41.5%), CIN2 (43.8%) and CIN3 (39.1%) groups had a higher percentage of p16 scores of 3(Figure 4-6).

Table 1. Age distribution and comparison results of CIN groups

	n	Mean	SD	Min.	Max.	P
CIN1	41	44.927[ab]	9.501	31	66	
CIN2	16	40.533[b]	9.92	28	61	0.029*
CIN3	23	48.870[a]	8.46	32	63	

*:<0.05, Means that do not share a letter are significantly different (p<0.05)
CIN: cervical intraepithelial neoplasia

Table 2. Comparison results of CIN groups with p16 staining scores

		CIN1	CIN2	CIN3	Total	P
p16 scoring	0	17 (41.5%)	1 (6.3%)	3 (13.0%)	21 (26.3%)	<0.001
	1	13 (31.7%)	3 (18.8%)	3 (13.0%)	19 (23.8%)	
	2	10 (24.4%)	5 (31.3%)	8 (34.8%)	23 (28.7%)	
	3	1 (2.4%)	7 (43.8%)	9 (39.1%)	17 (21.3%)	
Total		41 (100.0%)	16 (100.0%)	23 (100.0%)	80 (100.0%)	

***:<0.001
CIN: cervical intraepithelial neoplasia

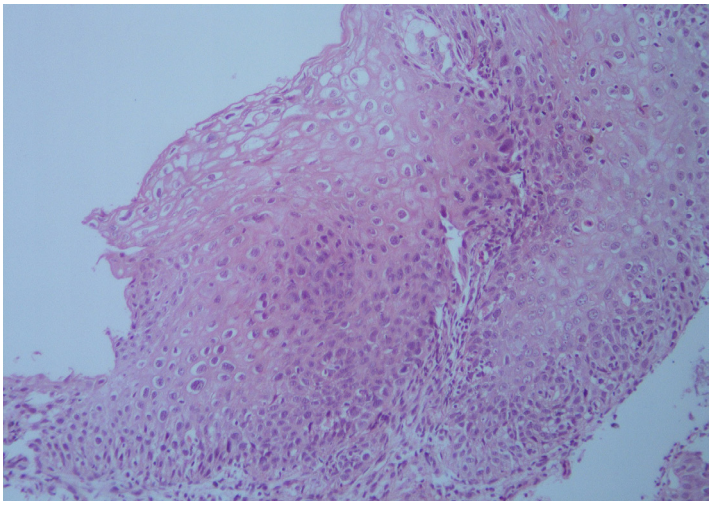

Figure 1. CIN1 lesion (H&Ex200)

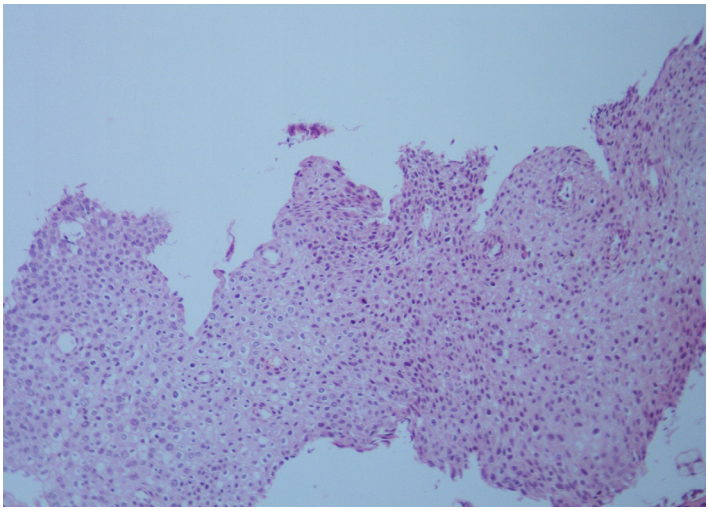

Figure 2. CIN2 lesion (H&Ex200)

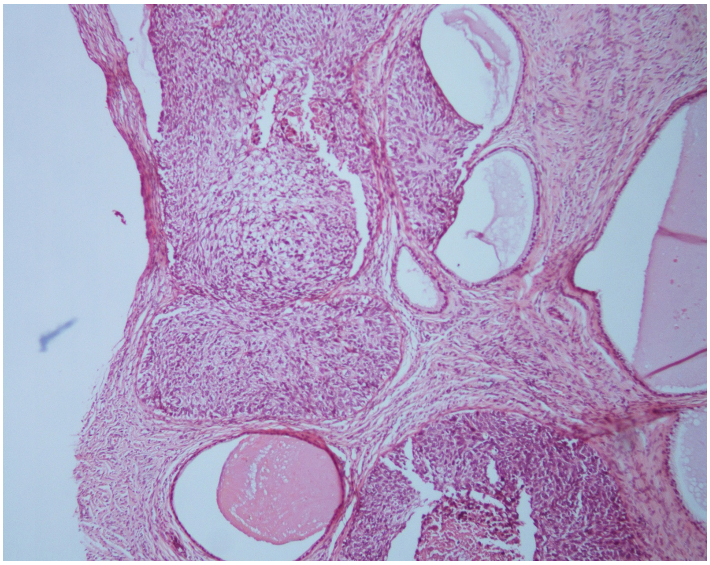

Figure 3. CIN3 lesion (H&Ex200)

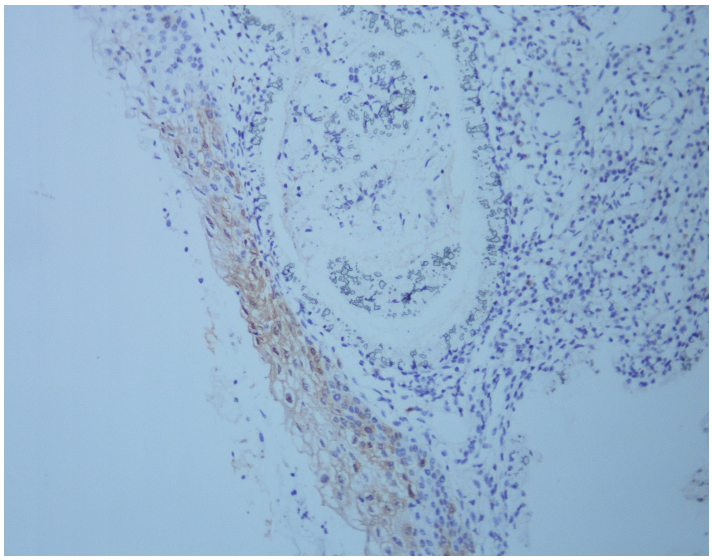

Figure 4. Immunohistochemical staining with p16 in CIN1lesion, mild cytoplasmic staining is seen (p16x100)

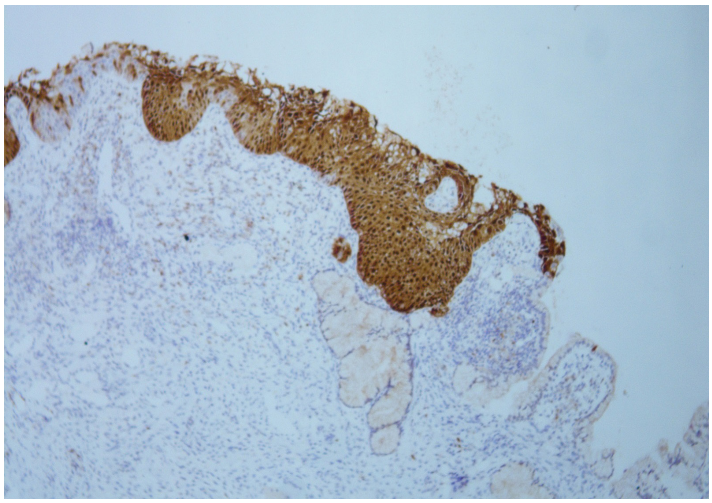

Figure 5. Immunohistochemical staining with p16 in CIN2 lesion, moderate cytoplasmic and nuclear stainings are seen (p16x100)

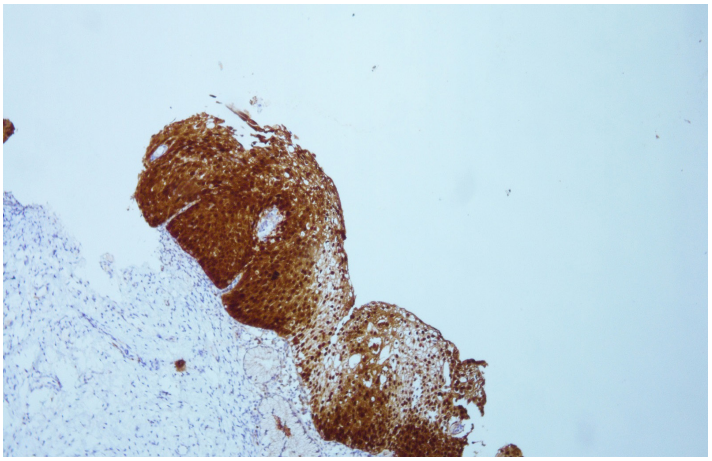

Figure 6. Immunohistochemical staining with p16 in CIN3 lesion, severe cytoplasmic and nuclear stainings are seen (p16x100)

As a result of the Kruskal-Wallis test, a significant difference was found between the scores in the CIN groups (p <0.001, Table 3). As a result of the multiple comparison test, the scores of patients in the CIN2 and CIN3 group were significantly higher than those in the CIN1 group (p <0.001).

The variation between CIN groups and anatomical locations on the cervix were examined with Chi-square test. There was no change in the frequency distribution of CIN groups in the anatomical locations on the cervix (p> 0.05, Table 4).

Table 3. Comparison results of CIN groups with p16 staining scores

		n	Mean	SD	Min.	Max.	Mean (Rank)	p
p16 scoring	CIN1	41	1.878	0.872	1	4	28.83[b]	<0.001
	CIN2	16	3.125	0.957	1	4	54.25[a]	
	CIN3	23	3	1.044	1	4	51.74[a]	

Means that do not share a letter are significantly different (p<0.001)
CIN: cervical intraepithelial neoplasia

Table 4. Frequency distributions of CIN groups in the anatomical locations on the cervix

	Anatomical Locations On The Cervix						
	Lower quadrant	Upper quadrant	Lower- upper quadrant	Unknown	ECC	Total	p
CIN1	8(53.3%)	8(50.0%)	9(60.0%)	11(44.0%)	5(55.6%)	41(51.2%)	0.729
CIN2	5(33.3%)	2(12.5%)	2(13.3%)	6(24.0%)	1(11.1%)	16(20.0%)	
CIN3	2(13.3%)	6(37.5%)	4(26.7%)	8(32.0%)	3(33.3%)	23(28.7%)	
Total	15(100.0%)	16(100.0%)	15(100.0%)	25(100.0%)	9(100.0%)	80(100.0%)	

(p> 0.05) ECC: endocervical curettage, CIN: cervical intraepithelial neoplasia

Discussion

Preinvasive cervical intraepithelial neoplasm (CIN) is the precursor of invasive cervical cancer.SIL is divided into the two-tiered degreeas low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL) in the latest classification system of the World Health Organization (WHO)[3]. Cervical SIL's generally affect women of reproductive age [28,29]. According to Heatley et al., correct colposcopy and histological evaluation of cervical precursor lesions are important to determine clinical management [5].

Since approximately two-thirds of the CIN1 lesion is expected to regress naturally, these lesions are usually followed up by cytology and colposcopy and it is not treated by excision or ablation methods (such as conization) [30,31]. According to Baak et al., excisional treatment or close surveillance approaches may differ between clinics due to the regression rate of CIN2 [8].

According to Vink et al, CIN2 and CIN3 lesions are at a higher risk of progression to cervical cancer if left untreated. Therefore, these lesions are usually treated with conization [31].So the treatment approach differs between low and high grade SIL.

The cytological approach also has some advantages, as long as it allows the patient to avoid surgical procedures [32]. Recently, immunochemical staining for p16INK4a in cytological smears has also been proposed [33].Therefore it is important to classify their malignant potential to accurately diagnose precancerous lesions, to apply the correct surgical approach, and to avoid unnecessary surgical treatment that may increase the risk of miscarriage or premature birth [34].

Kanjana et al emphasized the importance of performing the immunohistochemical evaluation with p16 in addition to H&E staining in the diagnosis of SIL [35].

In our study, the mean age of the patients in the CIN3 group was significantly higher than the patients in the CIN2 group. Since the progress from CIN1 to CIN3 is a dynamic process, this finding was considered to be compatible with the findings of the literature [5].

In our study, p16 scores of patients in the CIN2 and CIN3 group were found to be significantly higher than in the CIN1

group (p <0.001).The progression of CIN to cancer represents a morphological continuity [5]. In the progression from CIN1 to CIN3, since CIN3 is the most advanced precancerous lesion, high p16 scores in CIN2 and CIN3 have been interpreted to support the literature findings [1,24].

Despite well-defined criteria, histopathological diagnoses may show variations among pathologists [11].The immunohistochemical staining of P16INK4a allows precise identification of even small CIN or cervical cancer lesions in biopsy sections and reduces the inter-observer variation in histopathological interpretation of cervical biopsy specimens [36].

Most cancers of the cervix are squamous cell carcinoma originating from the transformation zone. As age increases, transformation zone sampling becomes less accessible [37].

Foote et al. found that biopsies from the anterior and posterior cervix are more likely to have more CIN grade 3 lesions than biopsies taken from lateral angles[38]. According to He et al, the distribution of CIN lesions was not randomly observed across the cervix. The most common CIN location was at 12 o'clock and the least common CIN location was at 2 o'clock [39].

According to Zhao et al, 4 o'clock and 7 o'clock sites should be preferred especially during biopsy[40]. However, to date, there is no clear consensus on the importance of the distribution pattern of the CIN throughout the cervix.

In our study, no significant result was found in cervical intraepithelial neoplasia cases in terms of cervical distribution. This was thought to be related to the small sample size.

Conclusion

Overexpression of p16 protein is considered one of the important prognostic factors for CIN.Grading of cervical intraepithelial neoplasia (CIN) with the p16 immunoscore system provides convenience for pathologists.Numerous prospective studies with p16 immune staining have shown a good agreement between p16-positive staining and an increased degree of the intraepithelial lesion.However, p16 staining is not sufficient in some cases to provide an accurate diagnosis and accurate differentiation.

It should be noted that CIN1 and CIN2 / 3 can sometimes show similar p16 expression and also p16 may be present in inflammatory cervical lesions.

Conflict of interests
The authors declare that they have no competing interests.

Financial Disclosure
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Ethical approval
Before beginning the study, permission was granted by the Clinical Research Ethics Committee dated 23/05/2016 and numbered 2016/48

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