European Review for Medical and Pharmacological Sciences 2012; 16: 1588-1595

The immunohistochemical detection of P16 and HPV L1 capsid protein on cell block sections from residual PapSpin liquid-based gynecology cytology specimens as a diagnostic and prognostic tool

H. HALTAS, R. BAYRAK, S. YENIDUNYA, U. YILDIRIM

Department of Pathology, Fatih University Hospital, Ankara, Turkey

Abstract. – BACKGROUND: Immunohistochemical staining for p16INK4a (p16) and HPV L1 capsid protein (HPV L1) are a useful ancillary technique for diagnosing preneoplastic lesions of the cervix in histologic specimens. The purpose of the current study was to examine the usefulness of p16 and HPV L1 immunolocalization in PapSpin Liquid-Based Gynecology Cytology Specimens (PapSpin cytology) derived cell block material in the diagnosis and prognosis of preneoplastic lesions of the cervix.

METHODS: The cervical cytologic smears of 64 patients who underwent colposcopic biopsy our Center were retrospectively evaluated. The cervical cytologic smears have been previously assessed by the PapSpin cytology and cell blocks were prepared from these samples. Immunohistochemical staining p16 and HPV L1 was performed on paraffin-embedded cell blocks of 64 PapSpin cytology specimens.

RESULTS: The positive staining of the cell blocks for P16 was directly proportional to the degree of intraepithelial lesion. In cases with squamous cell carcinoma (SCC) and high grade squamous intraepithelial lesion (HSIL), the positive staining of p16 was found to be statistically significant. In contrast, with the increasing degree of the lesion, a statistically significant decrease was observed in the HPV L1 positivity.

CONCLUSIONS: Immunohistochemical p16 and HPV L1 studies on cell block may increase the diagnostic accuracy of cervical cytology. When p16 and HPV L1 are immunohistochemically applied together on the cell blocks, they can provide information about the prognosis of cervical intraepithelial lesions.

Key Words:

P16, HPV L1, Capsid protein, Cell block method, Cytologic screening programs, Cervical cytology, Preneoplastic and neoplastic lesions of the cervix.

Introduction

The early diagnosis of cervical intraepithelial neoplasia is vital for the prevention and the treatment of cervical tumors. The wide application of cytologic screening programs has led to a consistent reduction in the incidence of cervical cancer in the developed countries[1].

Despite a marked decline in the number of cervical cancer deaths over the last 50 years, the Papanicolaou (PAP) test has its own diagnostic limitations. In literature, the reported specifictiy and sensitivity of PAP test range from 31-97% and 50-90%, respectively[2,3]. The errors in conventional PAP smear testing occur as a result of variations in sampling, preparation, screening, and interpretation. To improve the Pap test the Bethesda System and the liquid-based cytology (LBC) have been introduced during the last two decades. The introduction of the Bethesta system has led to a more uniform interpretation of the findings and the liquid-based technology has decreased the false-negative results found in cervical cytology[4,5].

Shandon PapSpin (ThermoShandon, Pittsburg, PA, USA) liquid-based gynecologic system (PapSpin Cytology) is an alternative to the conventional smear. The PapSpin cytology uses the centrifugation and the fluid absorption principles and allows the deposition of a thin layer of cells on round or rectangular areas. The PapSpin cytology meets the need for an inexpensive processor with familiar cellular criteria[6]. This method also allows for the construction of the cell block, such as the LBC methods.

The cell block (CB) preparation has been used for a number of years next to the smears in the

Corresponding Author: Hacer Haltas, MD; e-mail: hhaltas@hotmail.com

diagnosis of potential lesions, in nongynecologic cytology. The CB sections showed clearly recognizable normal and abnormal cells with minimal shrinkage and aberration. The CB technique has an added advantage that multiple sections of the same material can be obtained for special stains and immunohistochemistry. The morphological details including preservation of the architectural pattern like cell balls and papillae and three dimensional clusters, excellent nuclear and cytoplasmic details, and individual cell characteristics can also be obtained with the CB method[7]. The tissue fragments can easily be interpreted in a biopsy-like fashion. Unfortunately, CB has been highly neglected in cervico-vaginal cytology. In the literature there are a few reports on its utility with regard to the cervical cytology[7-10].

Human papilloma virus (HPV) is the primary etiologic agent of the cervical cancer, and HPV DNA is detected in 93 to 100% of the cervical cancer cases and its precursor lesions[11,12]. Because the HPV cannot be cultured and the serological analysis of HPV is not convenient, the HPV infections are diagnosed molecular methods for detection of the HPV DNA[13,14]. The HPV DNA testing methods, used most commonly on residual liquid-based cervical cytology material, are the hybrid capture method or the polymerase chain reaction (PCR)[15,16]. Although these molecular methods are very delicate and sensitive, they do not provide a microscopic evaluation of the abnormal cells. In that respect, the immunohistochemical HPV staining of the cell blocks, by which the histology and the cytomorphology can be monitored, might be beneficial in diagnosis.

$p16^{INK4a}$ (p16) is a cell-cycle inhibitor that binds to the cyclin-dependent kinase 4 (CDK4)/CDK6 and prevents the phosphorylation and subsequent inactivation of the retinoblastoma protein (pRB)[17]. The p16 protein was found to be overexpressed in cervical preneoplastic and neoplastic lesions, in which high-risk HPV subtypes exist[18]. Although many reports have described the application of p16 immunohistochemistry to liquid-based and conventional cervical cytology, immunostaining of cytology smears may be hampered by technical difficulties, including control availability and procedure standardization[19-22].

The objective of the current study was to determine the usefulness of p16 and HPV L1 capsid protein (HPV L1) immunolocalization in PapSpin cytology derived cell block material in the diagnosis and prognosis of preneoplastic lesions of the cervix.

Materials and Methods

PapSpin cytology and cell block were obtained from the archives of the Department of Pathology, Fatih University Medicine Faculty. Only cases with adequate cell blocks and available histologic follow-up were included. The study was approved by Ethics Committee of our University (Registration/Approval no: 2012-04).

Cytology

Cervical cellular samples were taken from the cervical ostium by using the Papette™ (Wallach, Orange, CT, USA) brush. A cervical cytology was done for routine diagnosis. The brush head with residual material was ejected into a vial of PapSpin Collection Fluid™ preservative. The liquid-based cytology (PapSpin®) was performed by using the Cytospin 4 (Thermo Shandon), according to the manufacturer's protocol. The slides were stained by using the traditional Pap method and screened by three experienced pathologists.

Evaluation of Cytology

For the cytological diagnosis, we used the Bethesda system 2001: negative for squamous intraepithelial lesions or malignancy (NILM) atypical squamous cells cannot exclude high grade squamous intraepithelial lesion (ASCH), atypical squamous cells of undetermined significance (ASCUS), low and high grade squamous intraepithelial lesions (LSIL and HSIL).

Cell Block Preparation

The cytoblock technique was performed with the Shandon kit according to the manufacturer's instructions (Shandon Inc., Pittsburgh, PA, USA). We slightly modified the technique by using only one cytocentrifugation step. The button of cells formed was routinely processed and embedded in paraffin. The tissues were fixed in neutral formalin solution.

Evaluation of Cervical Biopsy Specimens

Colposcopically guided punch biopsies were fixed in neutral buffered formalin, embedded in paraffin, sectioned and then stained with hematoxylin-eosin. Histopathological specimens were categorized as negative (benign/chronic cervicitis), CIN I, CIN II, CIN III.

Immunohistochemistry

From each paraffin CB prepared in the study, 5-µm-thick sections were cut. The CB sections were

deparaffinized in xylene, washed in ethanol, and finally washed in phosphate-buffered saline (PBS), pH 7.4. To increase the specificity and the sensitivity, the sections were pretreated in a microwave for 20 minutes on high in 10 mM of citric acid at pH 6.0. After cooling to the room temperature and rinsing in distilled water, endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 minutes. The sections were subjected to immunocytochemistry with either $p16^{INK4a}$ (clone 16P07, LabVision/NeoMarkers) mouse monoclonal antibody (clone 16P07, LabVision/NeoMarkers) or HPV L1 (clone K1H8, LabVision/NeoMarkers) mouse monoclonal antibody (ready to use: Lab Vision, Fremont, CA, USA). The antibody recognized the major L1 including HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-42, HPV-51, HPV-52, and HPV-58.

The slides were incubated for 60 minutes with the primary antibodies to $p16^{INK4a}$ and HPV L1 at room temperature. The standard avidin-biotin-peroxidase complex (ABC) technique was performed using the LabVision Secondary Detection Kit (UltraVision Detection System Anti-polyvalent, HRP). AEC (animo ethyl carbazol) was used as chromogen. All slides were counterstained with Mayer's hematoxylin.

Evaluation of Immunohistochemically p16 and HPV L1

Positive p16 immunostaining cells appeared as a brown color within the nucleus with or without cytoplasmic staining. Positive immunostaining for HPV L1 was identified in the nuclei of infected cells. At least 10 cells stained for p16 was considered positive. Even if only one staining of the nucleus was considered positive for HPV L1.

Statistical Analysis

The software program SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Distribution of data was tested with Chi-square test was used for analysis of categorical variables, and p values < .05 were considered indicative of statistical significance.

Results

A total of 64 patients were included to the study, from whom cervical cytology and colposcopic biopsy were available. The mean age was 39.75 years (range 18-64 years). The difference among the groups in terms of age was statistically significant ($p < 0.05$).

Comparison Between PapSpin LB Gynecological Specimens with cell Block and Histologic Diagnosis

Of 27 patients who had LSIL on the Pap smear, 22 had CIN I, and 5 had CINII-III. Of the 14 patients who had HSIL on the Pap smear, 7 had CIN II, 5 had CINIII, 2 had SCC. Of the 9 patients diagnosed as ASCH on the Pap smear, 1 had CIN I, and 8 had CIN II-III (Table I). One patient who had NILM on the Pap smear, had CIN I on the final histology. The relationship between cytopathological and histopathological results was significant.

Immunohistochemistry for p16 and HPV L1 cell Block Preparation

The rates of positive staining for p16 in CB preparations that were diagnosed as CC, CIN-1, CIN-2/CIN-3, and SCC were 0%, 46 %, 81%,

Table I. Comparison of the PapSpin cytology with cell block and biopsy.

PapSpin cytology	Biopsy diagnosis					Total
	Chronic cervicitis	CIN I	CIN II	CIN III	SCC	
NILM	8	1	0	0	0	9
ASCUS	0	2	2	0	0	4
LSIL	0	22	4	1	0	27
ASCH	0	1	6	2	0	9
HSIL	0	0	7	5	2	14
SCC	0	0	0	0	1	1
Total	8	26	19	8	3	64

NILM, negativity for squamous intraepithelial lesions or malignancy; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of undetermined significance, CIN, squamous intraepithelial lesion; SCC, Squamous cell carcinoma.

Table II. p16 stain compared wih histological follow up results.

p16	CC[a]	CIN I[b]	CIN II	CIN III	SCC[c]	Total
Positive	0	12	15	7	3	37
Negative	8	14	4	1	0	27
Total	8	26	19	8	3	64

[a]CC Chronic cervicitis; [b]CIN squamous intraepithelial lesion; [c]Squamous cell carcinoma.

and 100%, respectively. The positivity of p16 staining was determined according to the increased pathological grade. We found significantly higher expression of p16 in the cytoplasm of dysplastic cells in CIN II/III compared with CINI (Table II). In contrast to p16 staining, the detecting rates for HPV L1 in CC, CIN-1, CIN-2/CIN-3, and SCC were 0%, 100%, 40.74%, and 0%, respectively, which decreased gradually according to the severity of cervical dysplasia (Figure 1 a-o) (Table III).

The differences in positive staining rates for p16 among CIN-1 and CIN-2/CIN-3 and for SCC were statistically significant. Positive rates for HPV L1 among specimens that were diagnosed as CIN-1 and CIN-2/CIN-3 on CB preparations were also significantly different.

The combined expression of p16 and HPV L1 in CIN and SCC was analyzed and shown in Table IV.

Discussion

Cervical cancer is a leading malignancy, threatening the health of women, especially in the developing and the underdeveloped countries. It is the second most common malignancy worldwide, while it is at the 6th place in the ranking in Turkey[23,24]. Papanicolaou (Pap) smear test has been used in the last 50 years for the diagnosis of cervical neoplasms, and is a screening method that has started a new era in this field. It led to a significant reduction in the incidence of cervical cancer, after it was started to be routinely used[25]. Pap smear screening programs are implemented in many developed countries. However, a high false negative rate and frequent interobserver variability remain limitations of conventional Pap tests[26,27]. Although some studies reported the sensitivity and the specificity the conventional Pap smear (CP) as 90% and 97%, respectively, the false negative rate has been reported to be as high as 6-15%, and in some instances even up to 50%[1,2,28-30]. In order to reduce the false-negative results, and improve the reliability of the smear, a liquid based cytology technique has been developed in the cytology practice in recent years. Currently, most laboratories in the developed countries are using a liquid-based collection method for pap testing.

However, the liquid based cytology methods are expensive when compared with the conventional smear. Some reliable and also more affordable systems compared to the conventional cervical smear have also been proposed. The liquid-based PapSpin gynecological system is a preparation method in cervical cytology, which is developed with a number of modifications to the Shandon Cytospin device, that is avaliable in most pathology laboratories. This method, which basically works with the principle of cytocentrifuge, gathers the cells in an rectangular area of 294 mm^2, providing a thinner cell preparate compared to the conventional cervical cytology and shortens the examination time significantly. At the same time, just like the LBC, it provides material for the molecular studies and for the cell block construction creating residual cell suspension.

Table III. Imunohistochemically HPV L1 Capsid protein compared wih histological follow up results.

HPV L1 capsid protein	CC[a]	CIN I[b]	CIN II	CIN III	SCC[c]	Total
Positive	0	24	10	1	0	35
Negative	8	2	9	7	3	29
Total	8	32	19	8	3	64

[a]CC Chronic cervicitis; [b]CIN squamous intraepithelial lesion; [c]Squamous cell carcinoma.

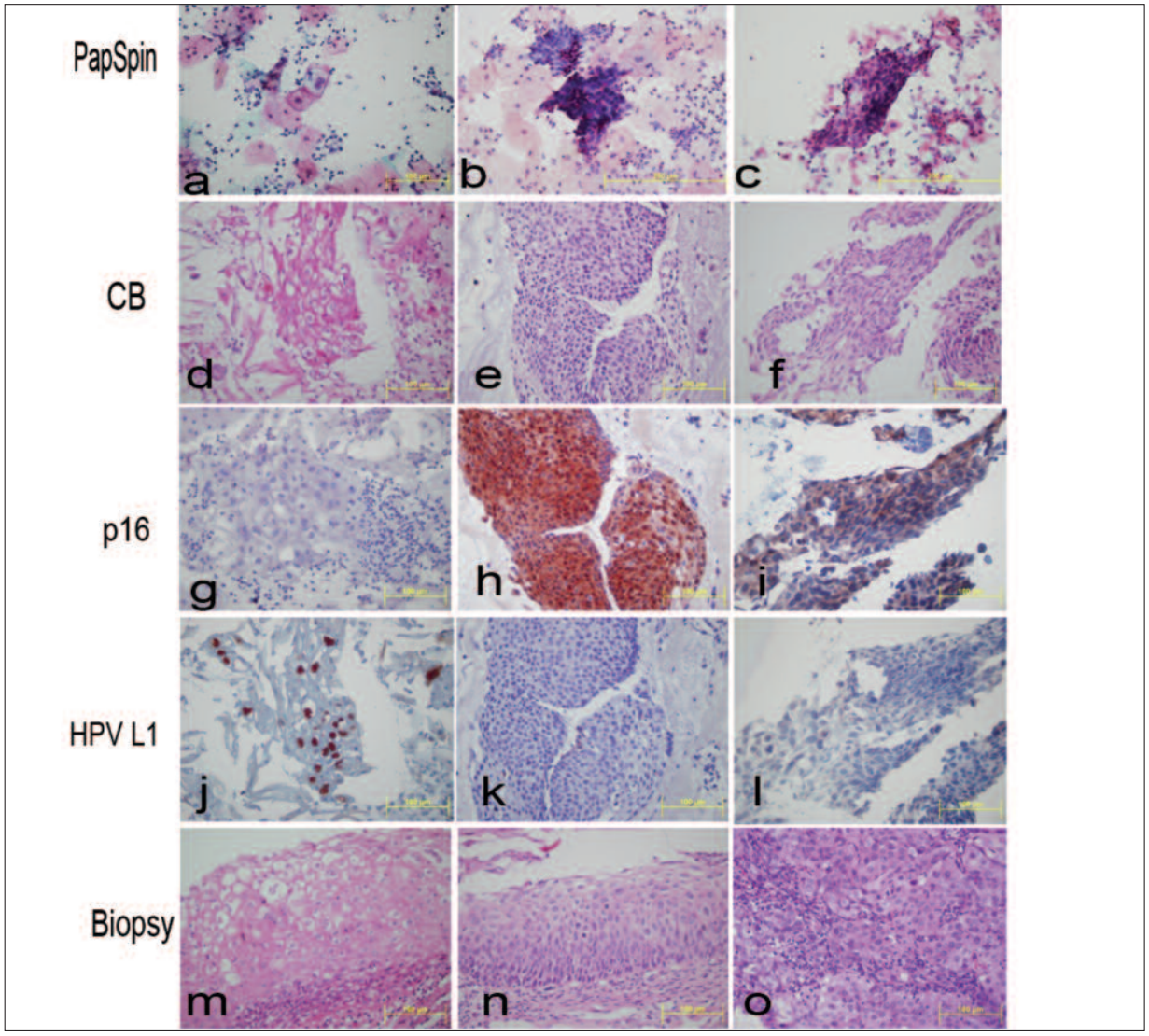

Figure 1. ***a,*** Low-grade squamous intraepithelial lesion (LSIL). The squamous epithelium of the cervix demonstrate the characteristic features of a productive human papillomavirus (HPV) infection. They show multinucleation, perinuclear halos, nuclear enlargement, and hyperchromasia. ***b,*** High-grade squamous intraepithelial lesion (HSIL) in PapSpin cervical cytology. The dysplastic cells are seen here in hyperchromatic crowded group. ***c,*** Malignant cell cluster in squamous cell carcinoma. Tumor diathesis in the background. ***d,*** In cases of low-grade squamous intraepithelial lesion, cell block showed HPV cytopathic effect. ***e,*** High-grade squamous intraepithelial lesion (HSIL) in cell block. ***f,*** Cell block preparation showing atypical cells with numerous mitosis in squamous cell carcinoma. ***g,*** Absence of P16 staining in koilocytic cells. ***h, i,*** There is strong p16 positivity in HSIL and SCC. ***j,*** Low-grade squamous intraepithelial lesion cells showing positive staining for human papilloma virus by Immunohistochemistry. ***k,*** A lack of HPV L1capsid protein immunstaining in HSIL and SCC. ***l, m,*** CIN I in cervical biopsy. This image shows classic koilocytic atypia. There is binucleation and nuclear hyperchromasia (HEX400). ***n,*** CINII in cervical biopsy. There is evidence of some maturation at the top. Occasional mitotic figures are seen in the lower half of the epithelium (HEX400). ***o,*** Invasive squamous cell carcinoma (HEX400).

The cell block (CB) method has lately been a procedure commonly used to help diagnosis especially in the practice of nongynecologic cytology. Although it is routinely used for nongynecologic cytology, it is almost completely ignored in cervical smears. In the CB method, the cells form an organoid structure, such as in the biopsy sections, providing tissue integrity, and a superior morphological assessment compared to cytology. In some investigations, the CB method is reported to be particularly useful in the diagnosis of high grade lesions, causing a decline both in the false positive and the false negative results[31,32]. In addition, the intensive collection of the material to be evaluated

Table IV. Combined expression of p16 and HPV L1 Capsid protein.

	No. of specimens				
	HPV-p16-	HPV+p16	HPV+p16+	HPV-p16+	Total
Biopsy chronic cervicitis	8	0	0	0	8
CIN I*	1	12	11	2	26
CIN II	0	4	6	9	19
CIN III	0	2	0	6	8
SCC**	0	0	0	3	3
Total	9	18	17	20	64

CIN squamous intraepithelial lesion; **SCC Squamous cell carcinoma.

on one side of the CB provides ease of rapid evaluation and appropriate material for the advanced diagnostic techniques, such as histochemical and immunohistochemical examination. Although many immunostaining markers have been reported recently in cytology smears, immunostaining of cytology smears may be hampered by technical difficulties, including control availability and procedure standardization.

In the previous studies, p16 has been proposed as a biomarker, helpful for identification of the dysplastic cervical cells. Recent reports have shown an increased immunoexpression of p16 in cervical intraepithelial neoplasia's and a positive correlation with the degree of the cervical neoplasia. The immunohistochemical detection of p16 expression could be useful in cervical dysplasia, where it could be used as an auxiliary indicator of progression[10,42,43].

Strong and full-thickness staining for p16 is observed in the CB preparations from all squamous cell carcinoma (SCC) sections and from nearly half of the CIN-2/CIN-3 sections (48%) in literature. Some studies show that p16 staining can be seen in tubal metaplasia, giant cells, malignant and benign endometrial cells, atrophic cell, and in atypical glandular cells[44-49]. In our study, p16 positivity was observed in all cases with SCC, in 81% of patients with CINII-III, and in 46% of CIN I cases. No p16 staining was observed in cases with chronic cervicitis.

The L1 capsid protein is the major target for the cell-mediated immune response to the HPV infections and normally is detectable during the productive stage of HPV disease. It is produced abundantly in mild-to-moderate dysplasia but rarely is observed in nonsuspicious Pap smears or severe dysplasia, and it is not produced in carcinomas[50-52]. The L1 capsid protein was positive in 30% of LSILs, 12% of HSILs, and 0% of SCCs in LBC and in 43.7% of LSILs and 33.3% HSILs in cervical smears[51]. In our study, a positive HPV L1 staining was observed at a rate of 100% in patients with CIN I, 40% in patients with CIN II-III and 0% in the patients with SCC.

The L1/p16 expression patterns are related to the severity of cervical lesions and may serve as a valuable index for predicting the prognosis and determining a follow-up strategy for the dysplastic lesions of the cervix, as proposed by previous studies[9,53]. In our study, we have determined an increase in the immunohistochemical p16 staining and a decrease in the immunohistochemical HPV L1 staining. These findings are in concordance with the literature.

Conclusions

Our preliminary data show that (1) the CB preparation, which is one of the possibilities provided by the PapSpin cytology, helped in improving the diagnostic accuracy of the smears; (2) p16 is a marker to confirm the diagnosis of HSIL; (3) the detection of immunohistochemically the p16 positivity and HPV L1 negativity may be related to the increasing severity of cervical dysplasia.

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