

HPV-DNA testing and Ki-67 immunocytochemistry in liquid based cervical cytology in prostitute women

Hayat kadınlarında sıvı bazlı servikal sitolojide HPV-DNA testi ve immünsitokimyasal Ki-67 indeksi

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ABSTRACT

HPV causes several changes in the function of host genes, and these interactions cause deregulation of the cell cycle manifested by abnormal expression of cell cycle associated proteins, such as Ki-67. The detection of Ki-67 can play a role in screening and diagnosis of HPV infection with risk of progression towards dysplasia and carcinoma. To show this relation in prostitute women, cervical cells were collected in the PapSpin Collection Fluid. A starting volume of 1000 µl for each sample, and a 200 µl cell suspension were used to prepare each sample for thin layer liquid based cytology and then they were stained by Papanicolaou method. The cytological results were classified according to the Bethesda 2001 system. From the remaining cell suspension of 800 µl, a 400 µl sample was used for HPV-DNA detection by PCR, a 50 µl aliquot was used to make thin layer preparations for immunocytochemistry. Single antigen staining was performed with Ki-67 protein. Cells were considered immunopositive if the nuclei were stained. All cells in one high power field (x400) were counted, and the fraction of immunopositive cells on the slide was calculated. This fraction was expressed as the number of positive cells per 1000 cells to facilitate comparisons of differential cell counts. HPV types 6 and 32 in the study, and HPV types 6 and 51 in the control group were detected. The mean Ki-67 values were 2.7±1.2 and 3.6±4.1 in HPV positive and negative cases respectively. There was a positive correlation only with nuclear changes and HPV positivity ($\chi^2=28.8$, $p<0.001$). There was not any significant correlation between HPV or Ki-67 and leukocytosis. An association with HPV and contraception, smoking, and concurrent genital infection was not found. The prevalence of HPV types in different geographical locations and races may indicate different etiologies of cervical cancer. Our results suggest that Ki-67 immunocytochemistry is not useful as a surrogate marker for HPV types of 6, 32 and 51.

Key words: HPV, Ki-67, thin layer liquid based cytology, immunocytochemistry

ÖZET

HPV konakçı genlerinde birtakım değişikliklere sebep olur, bu değişiklikler Ki-67 gibi hücre döngüsü ile ilişkili proteinlerin anormal ekspresyonu ile kendini gösteren hücre döngüsü düzensizliklerine yol açar. Ki-67'nin saptanması displazi ve karsinoma ilerleme riski olan HPV enfeksiyonunun taranması ve tanısında rol oynayabilir. Biz genelev kadınlarında bu ilişkiyi göstermeyi amaçladık. Servikal hücreler PapSpin biriktirme sıvısında toplandı. Her örnekten alınan 1000 µl hücre süspansiyonunun 200 µl'si ile ince tabaka sıvı bazlı sitoloji preparatı hazırlandı ve Papanicolaou ile boyandı. Kalan 800 µl hücre süspansiyonunun 400 µl'si PCR'da HPV-DNA saptanması için kullanıldı. Kalan 400 µl hücre süspansiyonunun 50 µl'si immünsitokimya için ince tabaka preparatı hazırlamada kullanıldı. Ki-67 proteinini ile antijen boyanması yapıldı. Nükleer boyanma gösteren hücreler immün pozitif olarak kabul edildi. Temsil olarak seçilen bir alandaki hücreler büyük büyütmede (x400) sayıldı ve lamın üzerindeki pozitif hücrelerin oranı hesaplandı. Bu oran pozitif hücre sayısı/1000 şeklinde ifade edilip tüm örneklerin sonuçları kıyaslandı. Çalışma grubunda HPV tip 6 ve 32, kontrol grubunda HPV tip 6 ve 51 saptandı. Ortalama Ki-67 değerleri HPV pozitif ve negatif olgularda sırasıyla 2.7±1.2 ve 3.6±4.1 olarak bulundu. HPV pozitifliği ile sadece nükleer değişiklikler arasında pozitif bir korelasyon saptandı ($\chi^2=28.8$, $p<0.001$). Diğer sonuçlarımız istatistiksel olarak anlamlı değildi. Lökositoz ile HPV ya da Ki-67 arasında önemli bir ilişki bulunmadı. Ayrıca HPV ile doğum kontrol uygulaması, sigara içimi, tekrarlayan genital enfeksiyon arasında da bir ilişki saptanmadı. HPV tiplerinin prevalansı farklı coğrafi konumlar ve ırklarda serviks kanserinde farklı etyolojileri düşündürmektedir. Bizim sonuçlarımız Ki-67 immünsitokimyasının HPV tip 6, 32 ve 51 için faydalı bir yöntem olmadığını göstermektedir.

Anahtar sözcükler: HPV, Ki-67, ince tabaka sıvı bazlı sitoloji, immünsitokimya

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INTRODUCTION

The causal relation between genital human papilloma virus (HPV) infection and cervical dysplasia/carcinoma is well established (1-4). However, even though the prevalence of cervical cells infected with HPV can be as high as 60%, only 10% of infected women will develop cervical dysplasia (4-8). The infection of cervical cells by HPV manifests itself by changes in the function or expression of host genes, and the detection of these alterations can play a role in screening and diagnosis (2,4). These interactions cause deregulation of the cell cycle manifested by abnormal expression of cell cycle associated proteins, such as Ki-67. The detection of abnormal expression can identify clinically important cases of HPV infection with risk of progression towards dysplasia and carcinoma (4).

Over the past decade, many large prospective studies have investigated the prognostic value of Ki-67 in outcome of cervical carcinoma. Ki-67 has been used in routine cervical pathology to assess cervical intraepithelial neoplasia (CIN) and to differentiate between postmenopausal atrophy and CIN (4,9). However few studies have investigated the application of Ki-67 in cervical cytology using classic Papanicolaou (Pap) smears (4,9).

We investigated the possibility of applying Ki-67 and polymerase chain reaction (PCR) to liquid based cytology samples. We aimed to show the relationship between cervical carcinoma, some types of HPV and Ki-67 in prostitute women.

MATERIALS and METHODS

Study population

There were 20 prostitute women as a study group and 20 ordinary women as a control group sampled from the out-patient clinic of Gynecology Department of Mustafa Kemal University, Medical Faculty with a mean age of

32.6±5.78 and 32.9±5.32 respectively. The cervical cytology samples were obtained from all women.

These samples were then stained for Ki-67 and Papanicolaou. All participants were asked to complete a self-administered confidential questionnaire, which included questions about contraception, age at first sexual intercourse, smoking, pregnancies, concurrent genital infections, and number of male partners. Investigations were performed in accordance with the guidelines of the Local Ethics Committee. Although no risks were attributable to our study, informed consent was obtained from all the patients.

Cytology

Cervical cells were collected using the PAPETTE™ (Thermo Shandon, Pittsburg, USA) cervix brush and placed immediately into the vial of PapSpin Collection Fluid (Thermo Shandon, Pittsburg, USA). Thin layer liquid based cytology (LBC) preparations were made with the Cytospin 4 cytocentrifuge (Thermo Shandon). From a starting volume of 1000 µl for each sample, a 200 µl cell suspension was used to prepare each LBC.

All slides were manually screened by a pathologist after training in the evaluation of thin layer slides. Abnormal or dubious cases were reviewed again by the pathologist for final diagnosis.

The cytological results were classified according to the Bethesda 2001 system (10), using the following categories: Within normal limits (WNL), atypical squamous cells of undetermined significance (ASC-US), low grade squamous intraepithelial lesions (LSIL), high grade squamous intraepithelial lesions (HSIL), and atypical glandular cells (AGC).

HPV- DNA testing by PCR

As reported previously, from the remaining cell suspension of 800 µl, a 400 µl sample was saved for HPV- DNA detection by PCR.

All samples (200 µl) were tested with Qiagen, PureArt DNA Mini Kit (Qiagen GmbH, Hilden/Germany) for DNA extraction. After that, PCR was performed by using “MY09/11” primers which could amplify approximately 400 base pairs of L1 gene location of the virus. These primers were obtained from LCD-Array HPV 2.5 (Chipron GmbH, Germany) kit and included all types of HPV. Amplification was run as follows: initial denaturation of 96°C for 2 minutes, followed by 45 cycles of 94°C/40 sec, 48°C/40 sec and 72°C/40 sec, respectively. For HPV positive samples, hybridization and staining procedures were performed. Briefly, hybridization was performed to on PCR products reproduced by using type specific probes which were fixed to “LCD-array”. The spots formed after staining procedure that showed hybridization, were evaluated by automatic analyse system “SlideReader software” (LCD-Analysis package, Chipron, Germany) was used for determining HPV types.

Immunocytochemistry

From the remaining 400 µl cell suspension after HPV DNA detection, 50 µl was used to make thin layer preparations with the Cytospin 4 cytocentrifuge (Thermo Shandon) on poly-L-lysine coated glass slides. Preparations were fixed in a methanol+acetone mixture at -20°C. Single antigen staining was performed with Ki-67 protein, and Ultra Vision Polyvalent, Rabbit, HRP-AEC kit (Neomarkers-Biogen, Lab Vision Corp. USA). Cells were considered immunopositive if the nuclei were stained. Evaluation of the immunocytochemistry was done by an Olympus Bx51 light microscope. All cells in one high power field (x40), which was considered to be representative of the whole cell area, were counted, and the fraction of positive cells on the slide was calculated. This fraction was expressed as the number of positive cells per 1000 cells to facilitate comparisons.

The statistical procedures were carried out by the software of Epi INFO version 3.3.2 CDC (Centers for Disease Control and Prevention,

USA). $p < 0.05$ was considered statistically significant. For the comparison of the findings t-test and chi-square test were performed.

RESULTS

Immunoreactive cells showed dark brown homogeneous or punctate nuclear staining by Ki-67 (Figs 1, 2).

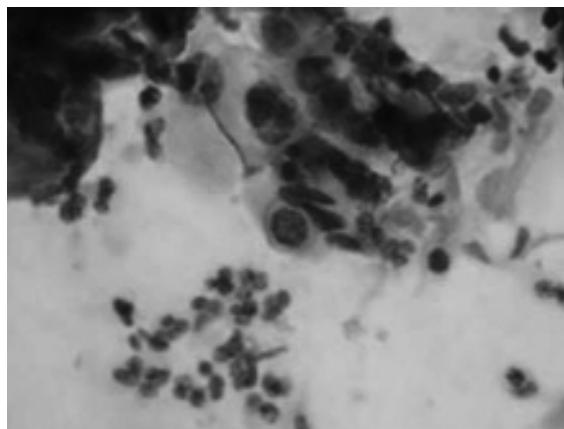


Figure 1. The cervical cells with pleomorphic, hyperchromatic nuclei and leucocytosis in a HSIL cytology (Pap x400).

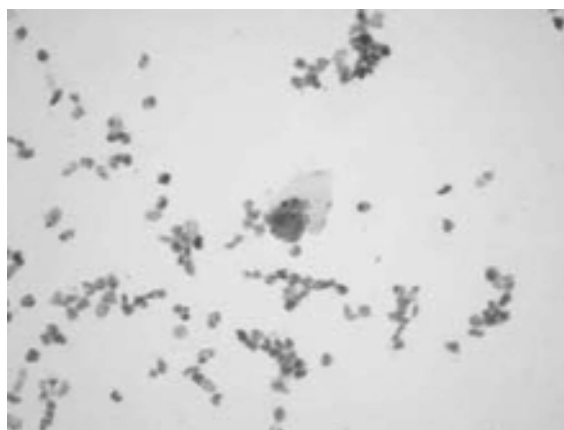


Figure 2. Ki-67 positive stained cervical cells in a cervical cytology (Ki-67 x100).

The mean Ki-67 values were found as 4.7 ± 5.21 in the study and 2.5 ± 1.31 in the control group, respectively. Leukocytosis was detected as 1.6 ± 826 cells/ml in the study and 1.2 ± 1.206 cells/ml in the control group, respectively (Table 1).

Table 1. The distribution of Ki-67, age, first sexual experience age and leucocytosis according to groups (Group statistics, T test).

Group		n	Mean	Std. Deviation	T test
Ki-67	Study	20	4.7000	5.21233	t=0.45
	Control	20	2.5000	1.31789	p=0.6
Age	Study	20	32.6000	5.78928	t=0.75
	Control	20	32.9500	5.32595	p=0.45
First sexual experience age	Study	20	16.7500	2.31414	t=1.31
	Control	20	20.3000	2.00263	p=0.19
Leucocytosis	Study	20	1.6000	.82078	t=1.72
	Control	20	1.2500	1.20852	p=0.09
					t=1.80
					p=0.14

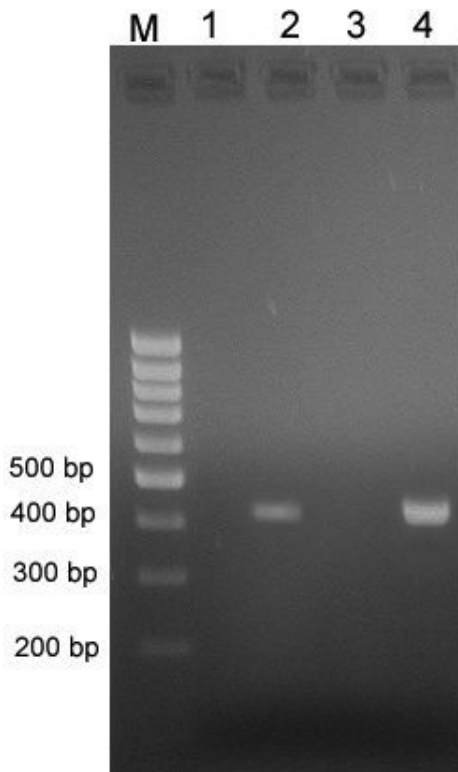


Figure 3. Lane M is molecular size marker 100 bp DNA ladder (Biotools, Spain).
 Lane 1 is a negative patient
 Lane 2 is a positive patient
 Lane 3 is a negative control
 Lane 4 is a positive control (approximately 450 bp)

The mean age at the first sexual experience was 16.7 ± 2.31 years in the study and 20.3 ± 2.00 years in the control group, respectively.

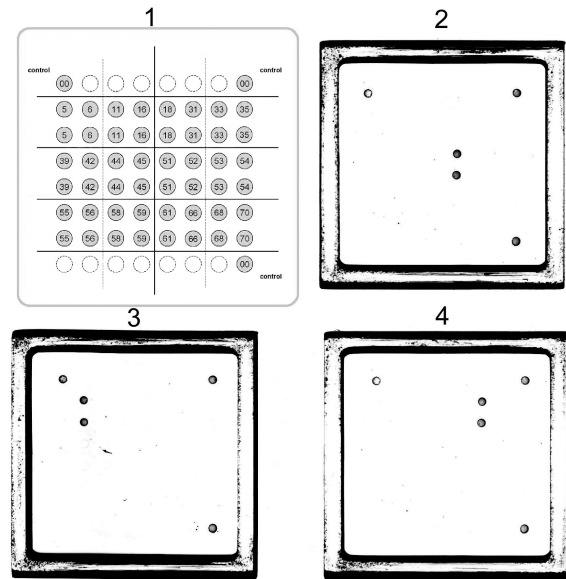


Figure 4. 1-Schematic diagram of LCD-Array. Arrays were spotted in duplicate. The different regions explored by the probes tiled on the array.
 2-HPV-Tip 51 positive patient
 3-HPV-Tip 6 positive patient
 4-HPV-Tip 31 positive patient

There were 2 patients with nuclear enlargement, one patient with LSIL and one patient with HSIL in the study group and also 2 patients in the control group. There was a positive correlation between nuclear changes and HPV positivity ($\chi^2=28.8$ $p<0.001$).

Two patients were detected as HPV positive in each group; Type 6 and 32 positivity in the study and Types 6 and 51 positivity in the control group (Figs 3, 4).

The mean values of Ki-67 were found as 2.7 ± 1.2 and 3.6 ± 4.1 in HPV positive and negative patients respectively. Leucocytosis was calculated as 2.2 ± 0.95 6 cells/ml in HPV positive and 1.3 ± 1.01 6 cells/ml in HPV negative women, respectively.

There was a positive correlation between nuclear changes and HPV positivity ($\chi^2=28.8$ $p<0.001$), other results were not statistically meaningful. There was no relationship between HPV, Ki-67 and leukocytosis. Also there was also no relationship between HPV and contraception, smoking, or concurrent genital infections (Table 2).

Table 2. The distribution of contraception, nuclear changes, smoking and genital infection according to HPV positivity (Chi square test).

		HPV (-)		HPV (+)		Total		X ²	P
		n	%	n	%	n	%		
Contraception	Yes	29	93.5	2	6.5	31	77.5	1.9	0.213
	No	7	77.8	2	22.2	9	22.5		
Nuclear changes	WNL	34	100	0	100	34	85	28.8	<0.001
	Nuclear enlargement	2	50	2	50	4	10		
	LSIL		0	1	100	1	2.5		
	HSIL		0	1	100	1	2.5		
Smoking	Yes	24	92.3	2	7.7	26	65	0.4	0.602
	No	12	85.7	2	14.3	14	35		
Genital infection	Yes	6	100	0	0	6	15	0.8	1.00
	No	30	88.2	4	11.8	34	85		
Total						40	100		

LSIL=Low grade squamous intraepithelial lesion

HSIL=High grade squamous intraepithelial lesion

WNL= Within normal limits

DISCUSSION

Papanicolaou (Pap) cervical cytology screening has helped to reduce cervical cancer rates dramatically since its implementation in 1950s (11). Pap test reporting classifications have evolved and been refined with the Bethesda system. At the same time there is an increased interest in using the detection of HPV-DNA as an adjunct to classic cytological evaluation (2,11,12).

Since the introduction of programs for preventing cervical cancer, the incidence of this type of cancer has declined appreciably in developed countries. In contrast, cervical cancer is still a problem in developing countries. Strategies for preventing cervical cancer in these countries should overcome barriers such as inadequate medical infrastructure and poor rates of participations in screening programs (8,13).

Detection of some specific types of HPV provides strong evidence implicating the role of HPV in cervical carcinogenesis (13). Most authors agree with the hypothesis that the integration of the HPV genom occurs very early in the development of cancer (2). We also found a po-

sitive correlation between nuclear changes and HPV positivity ($\chi^2=28.8$ $p<0.001$).

We detected nearly equal HPV-DNA loads either in the control and in the study group which had all risk factors for the development of cervical carcinoma.

The use of Pap test alone is not enough to eliminate cervical carcinoma (14). Liquid based cytology has some added advantages like immunocytochemistry and PCR besides classical examination and also it has a favourable cost-effectivity (15). Molecular epidemiologic studies have elucidated the central role that specific HPV types play in the development of cervical neoplasia (16). The increased sensitivity afforded by liquid based preparations is a peculiar advantage of HPV testing over repeat Pap tests (17).

We detected HPV 6 and 32 in prostitute women and HPV 6 and 51 in the control group in this study. Different genetic, racial and also environmental factors such as geographical locations may contribute differently to the mechanism of cervical cancer induction by different types of HPV (8,18). Recent studies have shown that HPV types 16 and 18 were prevalent in Western countries, but HPV 18 has shown pro-

found variations in different geographical regions and HPV 16 is the most prevalent type associated with cervical malignancy worldwide (18). Other HPV types such as 31, 33, 35, 52b and 58 have been frequently reported especially in Japan (18).

On the other hand PCR is an extremely sensitive method and it is being applied in diagnosing a wide range of clinical conditions (19). Therefore PCR technique and laboratory contamination might account for these differences (18).

Although several studies have been performed to detect HPV in cervical smears in Turkey, this is the first immunocytochemical study conducted in prostitute women in Turkey.

We could not find a statistically significant relationship between contraception, age at the first sexual experience age, smoking, pregnancies and concurrent infections with HPV and Ki-67 protein expression rates. However this study might have given different results if it were done with a large study population consisting of similar patient groups.

Ki-67 is a nuclear antigen expressed in all phases of the cell cycle excluding the G0 phase (20). The cytological diagnoses of ASCUS or HSIL with a high count of Ki-67 immunopositive cells could be useful for the follow up of these women, whose cell cycles might be disrupted. The detection of deregulation of the cell cycle identifies those infections at risk of progression towards dysplasia and carcinoma (4).

Ki-67 expression is normally confined to the basal and parabasal layers of the normal cervical squamous epithelium in histologic sections. This expression extends above the basal one third of epithelium and the number of positive cells increases in dysplasia and carcinoma (4).

The overlap in immunopositive counts in the different groups could be explained by the fact that normal proliferating cells from the basal layer of the epithelium could be assessed as positive cells (4). Mean Ki-67 values were de-

tected as 4.7 ± 5.21 and 2.5 ± 1.31 in our study and in the control group respectively. This study reveals that when used alone, Ki-67 is not a satisfactory method for the differential diagnosis of these cases.

Shabeli et al. used Ki-67 marker in liquid based cytology samples and showed strong Ki 67 positive cells in HSIL ve HPV16 positive cases (4).

We did not detect HPV 16 DNA and there is not a statistically significant relation between Ki-67 and HPV types in our study. This may suggest that Ki-67 immunocytochemistry could be useful as a surrogate marker of HPV-16 infection but not for other types of HPV.

Ki-67 immunocytochemistry is an easy and fast technique which could be an attractive method especially for smaller or less specialized laboratories.

Since the origin of Ki-67 antigen has not been known yet, the false positive results cannot be excluded. At the same time, Ki-67 immunoreactivity is highly dependent on fixation time, specimen storage conditions and antigen retrieval techniques (20).

Ki-67 is a very popular laboratory technique, however, this method has some serious limitations and its cytochemical importance needs to be studied in a prospective situation (20).

A combined approach including routine cytological parameters, immunocytochemistry and other molecular techniques such as PCR may be more useful. However this combined approach should be evaluated in large scale prospective studies.

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