Prevalence of human papillomavirus (HPV) and HPV-16 genotyping by real-time PCR in patients with several cervical pathologies

ABSTRACT

Purpose: this study was planned to evaluate the prevalence of HPV (excepting type 16) and HPV 16 by real-time PCR in colposcopy patients and to interprete the results with age, age of first sexual intercourse (FSI), parity and Pap smear results. **Methods:** one hundred and two colposcopy patients (50 and 52 of the patients were classified as colposcopy positive and negative, respectively) applying to Gynecology clinic were included. HPV (excepting type 16) and HPV 16 were detected by real-time PCR using the L1 region. Real-time nested amplifications of MY09/11 products were done by GP5+/GP6+ primers and Cyanine-5 labeled HPV and HPV 16 DNA specific probe after HPV DNA extraction by phenol chloroform isoamylalcohol. **Results:** HPV (excepting type 16) and HPV 16 were positive in 12% and 18% of the colposcopy positive patients respectively. HPV (excepting type 16) and HPV 16 were positive in 5.7% and 3.8% of the colposcopy negative patients, respectively. **Conclusion:** there was a statistically significant difference between colposcopy positive and colposcopy negative patients comparing HPV 16 with total HPV positivity ($p = 0.021$ for type 16 and $p = 0.010$ for total HPV) but there was not a statistically significant difference between colposcopy positive and colposcopy negative patients when we compared HPV (excepting type 16) positivity ($p = 0.314$). In conclusion, HPV detection and typing may be helpful for cervical cancer screening and prevention.

Keywords: HPV type 16, real-time PCR, colposcopy.

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INTRODUCTION

Cervical cancer is globally the second most common cancer in women with approximately 493,000 new cases annually.[1] Also cervical cancer is an important public health problem in developing countries. There is a statistically significant relationship between cervical cancer and the number of sexual partners of a woman. These data direct us to sexually transmitted agents, especially viruses.[2,3] As a result of the epidemiological studies on cervical cancer, there is no doubt about the importance of human papillomavirus (HPV), especially type 16 and 18, as an aetiological agent.[2-5] As a consequence, the detection and treatment of HPV infection can be an important stage in diagnosis and treatment of cervical cancer. The Pap smear test, as the most widely used cancer screening test in the world, is cost effective and organised screening provided a decline in cervical cancer mortality. However, in developing countries, where screening programmes are uncommon, cervical cancer still remains as one of the most important causes of death among women. HPV can only be reliably detected by DNA based tests since morphological changes on cytology such as koilocytosis are not specific for oncogenic HPVs. These observations underscore the need to develop more effective diagnostic methods.[6,7] PCR based methods amplifying nucleic acids of HPV are commonly used because of the limited sensitivity of Pap smear test leading to sample preparation and interpretation problems, insufficient serological tests and impossibility of in vitro cultures. Detection of HPV DNA, especially in latent infections, can be helpful in detecting cancer and precursor lesions.[8] This study was designed to evaluate the effect of HPV, especially type 16 in colposcopy patients, both with and without cervical pathologies and to associate it with the clinical findings in our hospital.

MATERIAL AND METHODS

Patients: from December 2003 to October 2004, patients directed to colposcopy were included in the study. After acetic acid application,

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observation of cervical acetowhite epithelium, punctuation, atypical vascularisation or mosaic pattern in colposcopic examination were classified as colposcopy positive patients, and patients with normal colposcopic findings were classified as colposcopy negative. All colposcopy patients were screened by Pap smear test and replied a questionnaire including questions, such as age, age of FSI, and parity.

Samples: cervical smear samples were collected in tubes containing 3-5 mL sterile phosphate buffered saline (PBS) in Gynecology Clinic of Gazi University Medical Faculty from colposcopy patients before application of acetic acid. After transporting to molecular diagnosis laboratory, all samples were vortexed and aliquoted in 1.5 mL eppendorf tubes and frozen at -86º C until DNA extraction.

DNA extraction: the cervical samples were digested in a buffer containing 20 mg/mL proteinase K (20 mM $(NH_4)_2SO_4$, 75 mM Tris HCl [pH 8,8] 0,1% Tween 20) at 55° C for 3 hours; followed by 10 minutes at 95°C. DNA isolation was performed by phenol-chloroform extraction and ethanol precipitation. DNA was then suspended in sterile distilled water and stored at -86° C until amplification.

DNA amplification: nested real-time PCR method was used for the analysis of HPV DNA and HPV 16 positivity. MY09/11 primer set (5'-CGTCCMARRGGAWACTGATC-3'), (5'-CMCAGGGWCATAAYAATGG-3', Tib Molbiol, Germany) was used for PCR amplifications following the extraction of the DNA. Real-time nested amplifications of MY09/11 products were done by GP5+/GP6+ primers and Cyanine-5 labeled HPV 16 DNA specific probe [Primer F 5' TTTGTTACTGTGGTAGATACTAC 3', Primer R 5' GAAAAATAAACTGTAAATCATATTC 3', Cy5.0 signal probe 5' Cy5-GTTTCTGAAGTAGATATGGCAGCACA-biotin 3' (Tib Molbiol, Germany)]. Real-time PCR product analysis was done by melting curve analysis on LightCycler Software version 3.5.3 (LC 2.0 Roche Diagnostics, Germany). Melting peaks of 78-82° C showed the detection of HPV DNA in the sample. Probe melting peaks of positive samples have been analyzed in the same run and HPV 16 positive samples yielded peaks around 68° C.

Ethical review of the proposal and the consent

The research proposal was approved by the ethical review board of the Faculty of Medicine, Gazi University. Informed consent was obtained from all women prior to the sample collection.

RESULTS

A total of 50 colposcopy positive women (18-63 years old; mean age ± SD: 39 ± 7) and 52 colposcopy negative women (17-65 years old; mean age ± SD: 40 ± 9) were included in the study. There was a statistically significant difference between colposcopy positive and colposcopy negative patients when we compared HPV 16 and total HPV positivity by Pearson chi-square test ($p = 0.021$ for type 16 and $p = 0.010$ for total HPV) but there was not a statistically significant difference between colposcopy positive and colposcopy negative patients when we compared HPV (excepting type 16) positivity by Fisher's exact test ($p = 0.314$) (Table 1).

According to age of women included in the study there was not a statistically significant difference between patients age ≤ 34 and ≥ 35 when we compared HPV 16 and HPV (excepting type 16) positivity by Fisher's exact test ($p = 0.154$) for type 16 and $p = 0.240$ for HPV (excepting type 16) but there was a statistically significant difference between patients age ≤ 34 and ≥ 35 when we compared total HPV positivity by Pearson chi-square test ($p = 0.036$) (Table 2).

There was no statistically significant difference between FSI in patients ≤ 19-year old and FSI in patients ≥ 20-year old when we compared HPV 16, HPV (excepting type 16) and total HPV positivity by Fisher's exact test ($p = 0.505$ for type 16, $p = 0.159$ for HPV (excepting type 16) and $p = 0.650$ for total HPV) (Table 2).

Parity seems statistically significant between 0-2 parity patients and ≥ 3 parity patients when we compared HPV 16, HPV (excepting type 16) and total HPV positivity by Fisher's exact test ($p = 0.037$ for type 16 and $p < 0.001$ for HPV (excepting type 16) and $p < 0.001$ for total HPV) (Table 2).

Table 1. Distribution of HPV DNA PCR results according to patient groups

	Colposcopy positive (n = 50)	Colposcopy negative (n = 52)	X^2
HPV 16 (+)			
n (%)	9 (18%)	2 (3.8%)	p = 0.021
HPV (+)			
n (%)	6 (12%)	3 (5.7%)	p = 0.314[a]
Total HPV (+)			
n (%)	15 (30%)	5 (9.5%)	p = 0.010

[a]:Fisher's exact was used.

Table 2. Distribution of HPV positivity according to variables

Number of patients(n)	HPV 16 (n = 11)		HPV (n = 9)		Total HPV (n = 20)	
	n	%	N	%	n	%
Age						
Age ≤ 34 (n = 75)	6	54.5	5	55.5	11	55
Age ≥ 35 (n = 27)	5	45.5	4	44.5	9	45
X^2	p = 0.154[a]		p = 0.240[a]		p = 0.036	
Age of FSI						
Age ≤ 19 (n = 67)	6	54.5	8	88.2	14	70
Age ≥ 20 (n = 35)	5	45.5	1	11.8	6	30
X^2	p = 0.505[a]		p = 0.159[a]		p = 0.650[a]	
Parity						
0-2 (n = 82)	6	54.5	2	22.2	8	40
≥ 3 (n = 20)	5	45.5	7	77.8	12	60
X^2	p = 0.037a		p < 0.001[a]		p < 0.001[a]	
Pap smear						
Normal (n = 74)	6	54.5	5	55.5	11	55
Pathological (n = 28)	5	45.5	4	44.5	9	45
X^2	p = 0.169[a]		p = 0.254[a]		p = 0.050	

FSI: first sexual intercourse.
[a]:Fisher's exact was used.

According to examination of Pap smear results, there was not a statistically significant difference between the patients with normal Pap smear and pathological Pap smear when we compared HPV 16 and HPV (excepting type 16) positivity by Fisher's exact test (p = 0.169 for type 16 and p = 0.254 for HPV (excepting type 16) but the p value was 0.050 by Pearson chi-square test which is a borderline value when we compared total HPV positivity (Table 2).

DISCUSSION

Because HPV, especially type 16, is reported as an important risk factor for development of cervical dysplasia and cancer, and 99.7% of the cervical cancers can be proved related to HPV, one of the main goals of preventing cervical cancer is screening for HPV.[9]

Tuncer *et al.*[10] detected in their study 12.5%, 19.4%, 46.3% and 83.3% of HPV type 16 positivity in CIN I, CIN II, CIN III and invasive carcinoma specimens, respectively, by PCR in Turkish patients. Also Onan *et al.*[11] from Turkey found 4.2%, 14.8%, 45% HPV positivity in CIN I, CIN II, and CIN III specimens, respectively, and HPV positivity in patients with CIN III was significantly higher than in patients with CIN I and CIN II.

In 102 colposcopy patients (both colposcopy positive and negative), a 19.6% of positivity in favour of total HPV was detected in our study, 9.5% positivity and 30.0% positivity in colposcopy negative and positive groups, respectively. This result indicates that there is a statistically significant difference between two groups (p = 0.010).

In joint assessment of HPV (excepting type 16) and HPV 16 we found a 55% of positivity in patients younger than 34 and a 45% of positivity in patients older than 35. Our results correlate with the studies indicating a decrease in HPV infection as the age increases,[12-15] although Ko *et al.*[16] in their study found that women between ages 30-69 had lower HPV positivity rate (ranging from 14-34%) compared to women with younger than 30 and older than 70 (ranging from 47-52%).

Considering total HPV, we found 70% positive cases in the group who experienced their FSI at the age of 19 or before, and 30% positive cases in the group who experienced

their FSI at the age of 20 or after. Although there is not a statistically significant difference between the two groups and HPV results, our findings confirmed the findings of previous studies[12,17,18] that HPV infection is more common among women who experienced their FSI in the early ages. Consequently, Flores *et al.*[19] indicate in their study that older age at FSI is significantly associated with a decreased risk of high grade CIN or cancer in HPV(+) women.

There are contradictory results in the studies evaluating HPV-cervical cancer and parity relationship, some of which suggest that there is no association with parity and HPV-cervical cancer.[20,21] Considering all HPV types, 40% positive cases in the group who gave two or less births and 60% positive cases in the group who gave three or more births were found in our study and HPV seems statistically significant between 0-2 parity patients and ≥ 3 parity patients when we compared HPV 16, HPV (excepting type 16) and total HPV positivity. Shields *et al.*[22] and Castellsague *et al.*[23] suggest that HPV exposed women with high parity are at increased risk for cervical cancer. Our study suggests that parity seems a risk factor for HPV and consequently for cervical cancer. On the contrary, Sellors *et al.*[17] in Canada detected a positivity of 17.1% in women who never gave birth, 12.7% in women who gave one birth, 8.7% in women who gave two births and 6.5% in women who gave three or more births.

We first performed colposcopy and cytology and than detected HPV DNA by real-time PCR. It is suggested that, as compared to Pap testing, HPV testing has greater sensitivity for the detection of CIN,[24] and the addition of a HPV test to a Pap test to screen women in their mid 30's for cervical cancer reduces the incidence of grade 2 or 3 CIN or cancer.[25] Cuzick[26] emphasizes in a review that detection of HPV DNA by molecular tests is more sensitive but less specific and therefore the use of these methods together must be the gold standard in cervical screening programs.

Grce *et al.*[27] detected HPV DNA in colposcopy positive patients in Zagreb. The results were 64.4% positive. As they detected the smears cytologically, they came to the conclusion that as the grade of SIL increased there was an increase in the prevalance of high risk HPVs (HPV DNA type 16 prevalance was 8.5% and 17.1% in LSIL and HSIL cases, respectively). The results were found to be statistically significant. Fife *et al.*[28] found 35% HPV 16 positivity in women with pathological Pap smear results in their study by PCR. All ASCUS, LSIL, HSIL, CIN I, CIN II, CIN III cases in our study were considered as pathological Pap smear results. We found 45.5% HPV 16 positivity in women with pathological Pap smear results. While 45% positivity (total HPV) was found in the group with pathological Pap smear results, the ratio of HPV positivity (total HPV) was 55% in the group with normal Pap smear results. Our results are not correlated with literature results. The reason for this declination may be due to sample preparation or smear results interpretation.

HPV DNA, especially type 16, followed by type 18, 45, 31 and 33 is diagnosed in more than 99% of cervical cancer biopsies. Although the ratios change according to the diagnosis method, HPV type 16 is detected in 33-50% of cervical cancer tissues and this is the main reason to focus on type 16.[29] Antonishyn *et al.*[30] found in their study that the most commonly identified genotype in patients with cervical intraepithelial neoplasia grade 2 or worse was HPV-16 (46.7%), followed by HPV-31 (14.7%) and HPV-18 (3.9%) by real-time polymerase chain reaction. They found that HPV-31 is contributing significantly to the proportion of women with cervical intraepithelial neoplasia in their study population and shows a higher prevalence than HPV-18 in high-grade lesions.

We studied smear samples and found a 19.6% positivity in all colposcopy patients. This result shows the high sensitivity of real-time PCR. Real-time PCR, the most advanced and sensitive of the molecular methods, was used in our study. Since the experiment occurred in a closed environment, there was minimal contamination compared to conventional methods and the sensitivity is higher due to one more step in amplification.[31,32]

We detected both HPV and HPV 16 positivity by real-time PCR. In order to detect types other than 16, sequencing can be performed.

In conclusion, we aimed to detect the prevalence of HPV, especially type 16 in colposcopy patients as a specific group with cervical complaints and this is the first study reflecting the HPV results of colposcopy patients in our country. Since cervical cancer is an important public health problem among women and HPV, especially type 16 is significantly related with cervical cancer, detection of HPV will be helpful for designing effective cervical cancer prevention programs. We think that further studies including large numbers of colposcopy patients must be performed to evaluate the prevalance of HPV in this patient group.

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