

Molecular Epidemiology of High-Risk Types of Human Papillomaviruses (16, 18) in Pap-Smear, the North East of Iran

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Abstract

Background: Cervical Cancer is one of the most important and commonly diagnosed types of cancer in females. There are different causes of cervical cancer, amongst which recurrent and persistent infection with HPV types 16 and 18 are the most renowned ones. These genotypes are the main factors in 99 % of cases in developed countries and 70 % in developing ones. Due to the importance of these viruses in cervical cancer, molecular detection of HPV and its high risk genotypes in Gorgan was designed.

Methods: Pap smears and swabs specimens were taken from 308 women. Papanicola staining method and cytology were used. Nucleic acid was extracted by proteinase K phenol-chloroform standard method and then assessed by using beta-globin primer. Polymerase Chain Reaction (PCR) was then performed for papillomaviruses on all patients and positive cases from both types, including HPV 16 and 18 genotypes, were detected.

Results: Three hundred and eight women (15-75 years old) with mean age of 37.54±10.6 were recruited. Seventy six cases (20.1%) of whom were infected with HPV and 48.6% with HPV16 or 18 positive. Normal cytology was seen in 226 cases and 41 patients (18.1%) were HPV positive. Amongst those 152 cases with inflammation or abnormal cytology, 35 cases (23%) were HPV positive. No significant relation was reported between different variables and HPV infections.

Conclusions: Due to high rate of HPV infection, as well as its high risk genotypes in different studies, more careful screening of women by Pap smear is recommended.

Key words: HPV-16; HPV-18; Papanicolaou Smear

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Introduction

Cervical cancer is one of the most prevalent and important cancers amongst women. In some studies, it is reported as the second most common cancer after breast cancer while others consider it as the third most common, after breast and lung cancer [1-2]. About 465,000 new cases of cervical cancer are diagnosed annually, all over the world, and kill more than 200,000 people. The incidence rate is variable in different population and depends on different causes as described below [1]:

- 3.8/100,000 in Palestine
- 48.2/100,000 in Colombia
- 9.8/100,000 (15,700 new cases annually) in United States

- Finally in Latin America is reported 4 times more than USA and other developed countries.

This variation demonstrates the role of different etiologies, amongst which recurrent and persistent infection with Human Papillomaviruses type 16 and 18 (HPV-16, 18) are the most important and well-known causes [2-10].

In some studies DNA of high-risk Human Papillomavirus was detected in more than 95% of cervical biopsies. The prevalence of high risk HPVs in premalignant stage is more than low risk HPVs i.e. (HPV-6, 11) [3]. Other risk factors in cervical cancer are as follows: a) initiation of sexual activity at

younger age, b) multiple partners, c) long term usage of oral contraceptive (OCP) and d) cigarettes [1].

Given the importance of Human Papillomavirus role in cervical cancer and high frequency of HPV genital infection, detecting high-risk genotype of HPV infection is the best approach to prevent malignancy. Unlike other sexually transmitted diseases (STD), HPV infection can be asymptomatic without causing specific genital lesions (condyloma acuminata). Due to this feature, the patient might not be referred for treatment, giving the virus enough time to replicate in cells and to proceed to malignant stages in epithelial cells. On the other hand, transformation of dysplastic lesions into invasive cervical cancer is gradual and without any alarming symptoms in most cases [6].

Several methods have been employed to detect HPV infection, amongst which polymerase chain reaction (PCR) is considered the most accurate with a very high specificity and sensitivity. PCR can also detect HPV in cervical biopsy before any cellular changes and positive Pap smear.

This study was designed for molecular detection of HPV and its high risk genotypes in the North East of Iran.

Materials and Methods

Study Population

We studied 378 women in need of cervical Papanicolaou examination in the Academic Gynecology Hospital of Gorgan, located in the North East of Iran.

DNA Extraction from Pap Smears

Two Arye spatulas were used to collect samples: One to prepare cervical Pap smear and another for cervical sample, which was placed in a tube with PBS. The first spatula used for Pap smear was washed in the same tube.

Smears were fixed in alcohol, stained by Papanicolaou stains (Orange G, Hematoxylin, and EA Polychrome solution consisting of light green solfonated acryl-methane dye, eosin, and Bismark brown). They were covered by glass slips and finally evaluated by a pathologist.

The second set of samples containing exfoliated cervical cells were centrifuged at 3000g per 10 minutes. The resulting pellet was transferred into a 1.5 ml Eppendorf microtube containing 200 µl digestion buffer (0.2 mol Tris-HCL [PH 7.5], 25 mmol EDTA, 0.3 mol NaCl, 2% SDS). Subsequently, 20 mg of proteinase K was added, and the cells were digested at 60°C for 2 hours. The digested samples

were extracted twice with phenol-chloroform and once with chloroform.

The DNA was precipitated by adding 50 µl (ml) 3 mol sodium acetate and 1 ml ethanol. After 1 hour at -70°C, samples were centrifuged for 10 min at 14000 rpm. The DNA pellet were dried and dissolved in 100 µl TE (10 mmol Tris-HCl [PH 7.5], 0.1 mmol EDTA). The DNA samples were then tested by agarose gel electrophoresis to ensure that no degradation has occurred during the extraction.

In addition, in order to analyze the quality of the target DNA, beta-globin (positive samples) gene-specific primers [PCO3, 5'-ACA CAA CTG TGT TCA CTA GC-3' and PCO4, 5'-CAA CTT CAT CCA CGT TCA CC-3'] were used.

PCR Amplification

A slight modification was made to the Polymerase Chain Reaction (PCR) method as described by Saiki et al [11]. The PCR was performed in a total volume of 50 µl containing 100 ng of DNA extracted from paraffin-embedded tissue, 50 mmol KCl, 10 mmol Tris-HCl pH 8.3, 200 µM of each dNTP, 2 to 4 mmol MgCl₂, 1 U Taq polymerase and 50 picomol of each primer. The primers used in this study were GP5+/GP6+ pairs with the following sequences [de Roba Husman et al: 1995 modified in German Cancer Research Center]:

GP5 + 5' TTTGTTACTGTGGTA GATACTAC

GP6 + 5' GAA AAA TAA ACT GTA AATCATATTC

The mixture was denatured at 94°C for 5 min, followed by 40 cycles of amplification using a PCR processor Bio-Med (Perkin Elmer Cetus USA). Each cycle consisted of 94°C for 1.5 min, 40°C for 2 min, and 72°C for 1.5 min. The final elongation step, lasting 4 minutes, was to ensure the complete extension of the amplified product. Before and after amplification reactions, samples were processed in separated rooms to avoid contamination by PCR products. Samples containing distilled water were used as negative controls. Ten µl of each PCR mixture was finally analyzed by 1.5% agarose gel electrophoresis. All positive HPV DNA PCR samples were further analyzed for genotyping of HPV. The presence of genotypes 16 and 18 were investigated. HPV genotyping PCR was carried out using specific primers for amplification of the given sequence consisting of E6 region of HPV 16 and 18. The utilized sequences are: HPV 16 forward, 5'-TCA AAA GCC ACT GTG TCC TG-3', reverse 16, 5'-CGT GTT CTT GAT GAT CTG CA-3', HPV 18, forward, 5'-GAC ACA TTG GAA AAA CTA AC-3', reverse 18, 5'-TAG TGC CCA GCT ATG TTG TG-3'. The

amplified products correspond to 139bp for HPV 16 and 119bp for HPV 18.

Results

In this study, 378 women between 15 and 75 years old were recruited with mean age of 37.45 (± 10.6). Seventy six (20.1%) were infected with HPV and one patient was diagnosed with Invasive carcinoma. Inflammatory or abnormal pap-smears were reported in 151 patients and a past history of abortion was reported in 110 patients; amongst whom 18 cases of HPV infection (5 cases with HPV-16, 4 cases with HPV-18 and 9 cases with unknown specific primers) were detected (Table 1).

The relation between HPV infection and the number of intercourse in a week, as well as the results of cytological examination and the number of pregnancies are shown in table 2. Three hundred and forty nine individuals with a history of at least one pregnancy were identified. First pregnancy under the age of 19 was reported in 171 cases, amongst which 30 cases (17.5%) of HPV positive and 7 cases of HPV-16 and 3 of HPV-18 were detected.

Amongst those with the first pregnancy between 20-29 years old ($n=163$), 36 cases (22.1%) of HPV positive (10 cases of HPV-16 and 10 cases of HPV-18) were identified. Among 10 pregnancies, between 30-39 years old, only one case of HPV infection was detected. Given 13 smoker patients 3 were HPV positive and 2 out of 3 were HPV-18 positive. The correlation between HPV infection and contraceptive are shown in table 3.

Amid 18 widows, (4.8%) 3 were HPV positive and 2 out of 3 were HPV-16 positive. For those ($N=341$ (90.2%)) who were married once, 70 of them were HPV positive (20 with HPV-16 and 15 with HPV-18). However, amongst those (5% of patients ($N=19$)) who married twice 3 were diagnosed with infection. For those ten individuals whom their husband had a history of genital infection, 4 were infected and 2 out of 4 were HPV-16 positive. Finally for those 44 individuals who have used vaginal shower, 4 were diagnosed with HPV positive and 2 out of 4 with HPV-16 positive.

Discussion

Cervical cancer is the second cause of mortality amongst women all over the world [12]. Generally in 60-70 % of cases, genital infection occurs with either HPV-16 or HPV-18. In 70 % of cancer cases, these two genotypes were responsible and in the remaining 30 %, other genotypes were present [13]. In the USA, 99% of cervical cancers are due to HPV-16 and 18 [14]. The American Association of Cancer

suggests assessment of cervical discharge in parallel with pap-smear [15].

In the present study, 378 females, between 15 and 75 years of age, were evaluated; 20.1% of whom were infected with HPV (5.8% HPV-16, 4% HPV-18 and 10.3% unknown type specific primers).

In a study in USA the reported incidence rate was 26.8 %¹³, while in Taiwan and Poland it was reported as 19.85% and 25.4% respectively [16,17]. Although the HPV incidence rate in our country seems to be similar to the ones mentioned previously but some lower rates, such as 4.8 % in Mexico, were reported [18].

Our findings have shown a rate of 18.1% of infection amongst subjects with normal pap-smear and 23% amongst subjects with inflammatory or abnormal cytology. In a study in Argentina, the HPV infection rate was reported as 64.2% amongst subjects with premalignant and malignant lesions [2], but other reports did not support this high incidence rate [16-17]. Our study has detected an infection rate of 23.4% amongst subjects with 25-34 years of age and a rate of 11.5% for those older than 54 years of age. However the incidence rate was reported as 12% and 2.4% respectively in Poland [10], lower than the present study. In Brazil the rate was reported as 32.5% [19] which is more than our findings.

The subjects with highest infection rate were under 34 years old (23.6%), amongst whom 44.7% were diagnosed with HPV-16 and 18. Thus it can be assumed that HPV-16 and 18 can be present in people with normal and inflammatory cytology [9, 20]. For those (29.7%) with history of abortion, 16.3% were diagnosed with HPV (50% with either HPV-16 or 18. Other investigators reported HPV infection in 53.5% of whom with a history of abortion in contrast to 33.3% in others [21-22]. It was mentioned that HPV infection rate is 3 times higher in abortion [23-24].

Our result confirms that infection rate increases as the rate of sexual intercourses increases. Other investigators suggested that transmission occurs along with the sexual contacts and most of the people get rid of the virus except the persistent HPV-16 or 18 infections, which lead to cervical cancer [11].

Another study in Poland, illustrated that 16-19 years old women with more than one sexual partner, who have started the sexual activity before age of 16, have an infection rate of 27% [25].

Risk factors of HPV are reported as follow: a) sexual contact in younger age, b) multi-partnership, c) no use of condom, d) and more than two vaginal deliveries [16]. But we reported no significant

Table 1. Frequency and distribution of HPV and HPV genotypes in the studied subjects in Gorgan city

Age Groups	HPV Positive			HPV Negative (%)	Total (%)
	HPV-16(%)	HPV-18(%)	Non-HPV-16, 18 (%)		
15-24	1 (3)	1 (3)	6 (18.2)	25 (75.8)	33 (8.7)
25-34	8 (6.3)	7 (5.4)	15 (11.7)	98 (76.6)	128 (33.9)
35-44	7 (5.8)	4 (3.4)	12 (6.2)	96 (80.6)	119 (31.5)
45-54	5 (6.9)	34.2)	4 (5.6)	60 (83.3)	72 (19)
>54	1 (3.8)	0	2 (7.7)	23 (88.5)	26 (6.9)
Total	22 (5.8)	15 (4)	39 (10.3)	302 (79.9)	378 (100)

Table 2. Distribution of HPV genotypes with number of intercourse in a week, results of cytological exam and number of pregnancies in the subjects in Gorgan city

		HPV Positive			HPV Negative (%)	Total (%)
		HPV-16 (%)	HPV-18 (%)	Non-HPV-16, 18 (%)		
*Number of intercourse in a week	One time	2 (1.4)	2 (1.4)	6 (4.1)	136 (93.1)	146 (100)
	2-4 time	7 (5.9)	10 (8.4)	22 (18.5)	80 (67.2)	119 (100)
	>4 time	10 (11.9)	3 (3.6)	10 (11.9)	61 (72.6)	84 (100)
Cytological exam	Normal	9 (4.2)	7 (3.2)	25 (11.6)	175 (81)	226 (100)
	Abnormal & inflammatory	15 (9.9)	6 (3.9)	14 (9.2)	117 (77)	152 (100)
Number of pregnancy	non	3 (10.3)	2 (6.9)	1 (3.4)	23 (79.3)	29 (100)
	1-5	15 (5.3)	12 (4.2)	31 (10.9)	227 (79.6)	285 (100)
	>5	4 (6.1)	1 (1.5)	7 (10.6)	54 (81.8)	66 (100)

* Numbers of persons answered the question

Table 3. Frequency of HPV and genotypes and contraceptive methods in study subjects in Gorgan city

	HPV Positive			HPV Negative (%)	Total(%)
	HPV-16(%)	HPV-18(%)	Non-HPV-16, 18(%)		
IUD	0	0	1 (9.7)	12 (90.3)	13 (3.4)
Natural	4 (4.8)	1 (1.2)	7 (8.5)	71 (85.5)	83 (22)
Pill	1 (2)	3 (6.1)	5 (10.2)	40 (81.5)	49 (13)
Condom	0	1 (4)	4 (16)	20 (80)	25 (6.6)
Multiple	0	1 (6.2)	3 (18.8)	12 (75)	16 (4.2)
Don't Use	17 (8.6)	9 (4.8)	19 (9.9)	147 (76.7)	192 (50.8)
Total	22 (5.8)	15 (3.9)	39 (10.4)	302 (79.9)	378 (100)

relationship between HPV infection and these factors. Although 18.1% of those with normal cytology in pap-smear were infected with HPV (amongst whom 39% are diagnosed with HPV-16 and 18), this rate increases up to 23% and 60% respectively for those with inflammatory and abnormal cytology. This finding is confirmed in other studies as well [2]. Amongst those subjects, with 29 years of age and no

history of pregnancy, 20.7% were infected with HPV (83.3% were HPV-16 and 18 positive). Eighteen of them were widows and 16.3% of them were infected with HPV (66.7% were HPV-16). The subjects between 15 to 24 years of age face higher risk of infection, due to more sexual contacts. No significant differences were detected in regards to HPV infection and genotypes 16, 18 in different gravids.

In an investigation in Bulgaria, HPV infection was reported as 17.7% amongst pregnant women and 13.1% amongst non-pregnant [26]. In Japan, infection rate amongst pregnant women, younger than 25 years old, was reported as 22.6% [27]. HPV infection, especially HPV-16 and 18, in Gorgan city can be considered very important, amongst which genital cancer is the third cancer. It seems necessary to evaluate HPV-16 and 18 in all women referred for pap-smear.

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Conflict of Interest

No conflict of interest existence.

Authors' Contribution

AM designed the study and analyzed the data. SBN contributed to the literature review and writing-up process. SB contributed to the data entry and writing the paper. All authors read and approved the final manuscript.

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