DETECTION OF HUMAN PAPILOMAVIRUS TYPES 16 AND 18 IN PATHOLOGIC SAMPLES FROM PATIENTS WITH CERVICAL CANCER BY PCR AND RFLP METHODS

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ABSTRACT

Infection with human papiloma virus (HPV) is the most frequent sexually transmitted disease worldwide. HPV types 16, 18, 31 and 33 are considered as the most important types in the cervical cancer.This study was undertaken on 64 samples of archival cervical carcinoma pathologic to assess the rate of HPV infection (HPV16,18) in cervical carcinoma among Iranian patients. HPV DNA was detected by polymerase chain reaction (PCR) and typing by restriction fragment length polymorphism (RFLP) analysis.The total prevalence of HPV in this study (HPV16,18) for all cases was 59.4% (38/64). HPV type 16 was the most common one (22/64, 34%) followed by HPV type 18 (16/64, 25%). On the basis of the rate of HPV (16,18) which were detected in squamous cell carcinoma and adenocarcinoma,only women with HPV18 infection showed a statistically significant risk for development of cervical cancer (P=0.019) while P value for HPV16 was 0.47 .

Keywords: HPV, Cervical cancer, PCR, Iran

INTRODUCTION

In developing countries, cervical cancer is the most frequent female malignancy after the breast cancer (1). However, the prevalence of cervical cancer in Iran is much lower than other parts of the world (2).

Clinical and epidemiological studies have shown that papiloma viruses (HPV) play a major role in the development of different types of cervical lesions, and are therefore considered as the major infectious aetiological agents of genital lesions as well as cancers (1). The other known risk factors for cervical cancer are high parity, smoking, sexual behavior and hygiene (1,3,4,5).

To date, more than 85 HPV types have been characterized (6). HPV types 16, 11, 42, 43 and 44 are associated with benign lesions (condylomas), whereas HPV 31, 33, 35, 51, 52 and 58 are detected more frequently in low grade squamous intraepithelial lesions (LSIL) and HPV 16, 18 are predominant in high grade LSIL and invasive carcinomas (7).

Human papiloma viruses (HPV) are causative agents of cervical cancer in women and some other genital mucosal and skin lesions. Cervical cancer takes many years to develop, and changes can be detected in the cervix for some time before the appearance of cancer. In principle, screening of women for these changes may allow treatment of those with early signs of developing disease, like cancer (8).

The lack of standardized detection methods represents a major problem for monitoring HPV in developing countries. However, sensitive and special methods, based on the detection of HPV DNA, are available, of which, the polymerase chain reaction (PCR) technique is the method of choice for epidemiological studies (2,8,9,11). In this study the presence of HPV nucleic acid sequences in samples was investigated by PCR method. The identification of the HPV types was performed using restriction fragment length polymorphism (RFLP) analysis.

MATERIALS AND METHODS

Tissue samples

Formalin fixed paraffin-embedded samples from patients with cervical cancer (n=64) were processed (archival pathologic specimen of Mahdie and Loghaman Hospitals).

The mean age of patients was found to be 52.5 (32-73) years.

Processing of samples

In the first step a thick section from samples was provided and placed in sterile 2 ml eppendorf

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tubes. Paraffin was removed with warm xylene extraction (60°C) followed by washing with 90% ethanol two times. Samples were dried in thermomixer for 30 minutes and were then incubated in digestion buffer (10 mM Tris-HCl, , 25 mM EDTA, 0.5% SDS, 2 mg/mol proteinase K) pH= 8.5 at 37°C.
After centrifugation at 5000 rpm, light phase was used for DNA extraction.

DNA extraction
An equal volume of phenol and chloroform were then added to samples, mixed and centrifuged.
Light phase was transferred to a microtube and mixed with an equal volume of chloroform and centrifuged. It was then mixed with two volume of ethanol and freezed at -20°C over night.
The formalin was removed from the pelletted DNA by drying. The amplification reaction was performed in a final volume of 50µl containing 40 pico mol of each primer, HP 168F (5'GAATATGATTTRCAGTTTATTT3') and HP168 R (5'TCTYKGAAACTTTCCTT3'), 0.25 unit Taq DNA polymerase, 10mM dNTP, 50 mM $MgCl_2$, 5µl 10x PCR buffer and the mixture denatured at 94°C for 5 minutes.
These sequence primers was designed by oligos program from sequence of strains HPV 16 (Gene bank Accession number S71514)and HPV 18 (Gene bank Accession number U 45893).
These primers were amplified a 269 bp fragment. Depending on the type of virus, the amplicons had either *Bam* H1 restriction site for HPV 18 and *Eco* R1 restriction site for HPV 16 .
Then, 35 cycles of PCR were performed by denaturation at 94°C for 30s, annealing at 52°C for 30s, and extension at 72°C for 30s.
At the end of last cycle, mixture was incubated at 72 °C for 5 min.
The PCR was performed in a eppendorf thermocycler. For every reaction, negative control (master mix and sterile distilled water), a positive control (HPV18 DNA, HPV 16 DNA) were employed. PCR product (269 bp) were analysed by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide and visualized on a UV transilluminator (Fig 1, 2).
RFLP method(Restriction Fragment Length Polymorphism Analysis) permits reliable identification of HPV types. The PCR products and DNA from positive control (HPV16,18) were digested with 20 units of endonuclases *Eco*R I for HPV16 and *Bam* H I for HPV18 . The fragments subsequently were separated in polyacrylamide gel electrophoresis and DNA patterns were visualized by UV transillumintor after staining the gel with ethidium bromide. Restriction fragments were seperated on polyacrylamide gel and were visualized by UV transilluminator after ethidium bromide staining.

Statistical analyses
Te relationship between HPV (16, 18) infection, age of patient, and two types of cervical cancers (adenocarcinoma, squamous cell carcinoma) were assessed by Fisher's test.
All data were analysed using [1] SAS version 8.00 and were two-sided where $P< 0.05$ was regarded as significant.

RESULTS

The mean age of the patients was 52.5 years, ranging from 32 to 73 years. The majority of patients were in the range of 30 to 40 years (Fig. 3). The total prevalence of HPV in the study(16,18) for all cases was 59.4% (38/64) (Fig. 3).
The prevalence of HPV type 16 and type 18 were 34.4% (22/64) and 25% (16/64) for all cases respectively (Fig. 4).
DNA from HPV type16 was detected in 22 cases, of which 19 cases had squamous cell carcinoma (86.4%) (19/22) and remaining (3) had adenocarcinoma (14%) (3/22) (Fig. 4).
DNA from HPV type18 was detected in 16 cases of which 11 cases had squamous cell carcinoma (68.8%) (11/16) and 5 cases had adeno carcinoma (31.2%) (5/16) (Fig. 4). On the basis of the rate of prevalence rates of HPV (16/18) which were detected in squamous cell carcinoma and adenocarcinoma only women with HPV18 infection showed a statistically significant risk for development of cervical cancer ($P= 0.019$) while p value for HPV16 was 0.47.

DISCUSSION

Cervical cancer is the second most prevalent cancer among women worldwide (9).
Currently there is compelling evidence that the development of human cervical cancer without involvement of the specific human papiloma virus (HPV) is exceptional or impossible. In this study pathologic blocks were used for HPV testing by PCR and RFLP methods. Today, the diagnosis of HPV infection is based on the detection of viral DNA (11,18,19).
Because of the sensitivity of the methods, samples containing very low levels of HPV DNA, are becoming viable HPV sample sources and in this study presence of HPV types 16,18 in samples (59.4%) were demonstrated.
HPV type 16 was the predominant infection (34.4%) in this study. In another study in Iran, the prevalence rate of HPV 16 was reported 6.7% (2). The prevalence of HPV 16 among Iranian patients with cervical cancer is lower than those in Croatia (50%), Australia (53%), Thailand (41%) Colombia (69.9%), Spain (66.4%), Italy (32.6%)

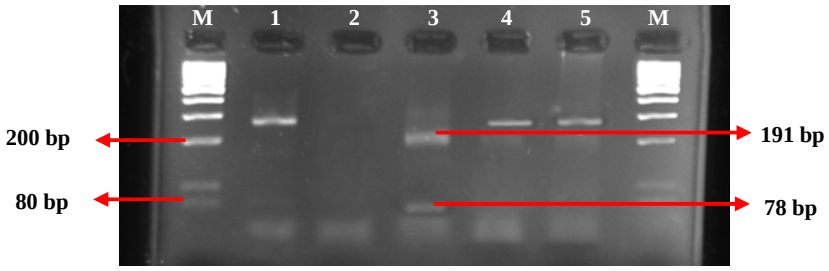

Figure 1. 1.2% gel electrophoresis of HPV 16 PCR product restriction analysis
M : 100 bp DNA ladder marker; Lane 1 :Postive control; Lane 2 :Negative control; Lane 3 :Digested PCR product by *Eco*R1(there is one Restriction site for *Eco*R1 on HPV 16 sequence); Lane 4 : Digested PCR product by *Bam*HI; (there is no restriction site for *Bam* HI on HPV16 sequence; Lane 5 : Uncut PCR product

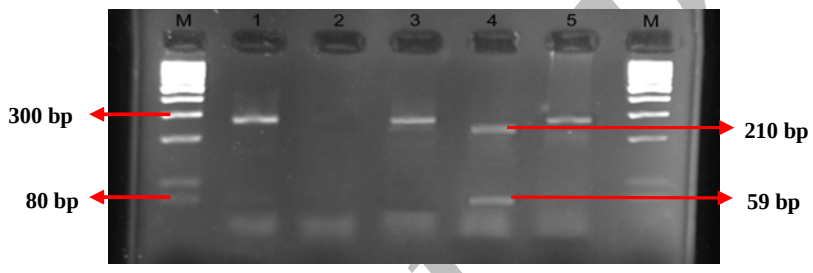

Figure 2. 1.2% gel electrophoresis of HPV 18 PCR product restriction analysis
Lane M : 100 bp DNA ladder marker; Lane1 : Positive control ; Lane2 : Negative control; Lane3 : Digested PCR product by *Eco*RI(there is no restriction site for *Eco* RI on HPV 18 *sequence*); Lane4 : Digested PCR product by *Bam* HI; (there is one restriction site for *Bam* HI on HPV 18sequence **);** Lane5 :Uncut PCR product

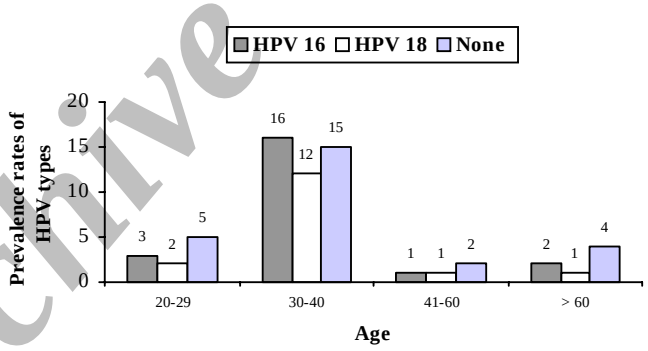

Figure 3. Prevalence rates of HPV types detection in various age group

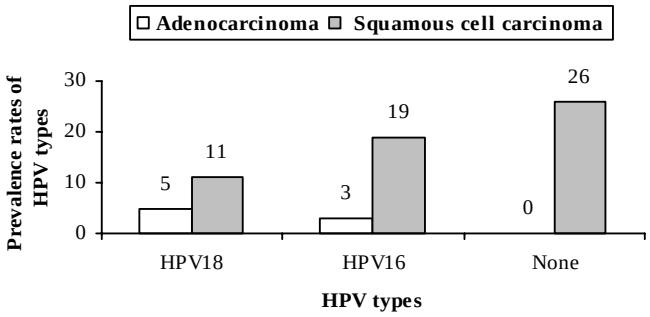

Figure 4. Prevalence rates of HPV types detected in adenocarcinoma and squamous cell carcinoma

and China (48.8%) (12-16,19).While the prevalence rate of HPV18 was 25% in this study, other study results of another study in Iran has been reported 0% (2). This type has been reported from Australia (17.2%), Thailand (21%) and South Africa (34%) (13,14,17). The distribution of HPV 16,18 in squamous cell carcinoma (30/38) were higher than adenocarcinoma (8/38) (Fig.4).
There was no statistically significant association between HPV 16 and development of squamous cell carcinoma and adenocarcinoma (P= 0.47). However a statistically significant relationship was found for HPV18 (P= 0.019).
In summary, our study suggests that HPV typing is an important factor for cervical cancer diagnosis, and multiplex PCR, and RFLP are effective methods for detection of HPV types.

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