HPV Typing in Women with Cervical Precancerous and Cancerous Lesions in Northwestern Iran

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along with rather different circulating HPV types in the study population. Hence, relevant HPV typing information in cervical carcinoma is very important for planning more efficient screening programs and for further HPV vaccine design.

Key Words
Human papillomavirus • Cervical cancer • PCR • Iranian population

Abstract
Background/Aims: The role of human papillomavirus (HPV) in the etiology of cervical cancer is now well established. This investigation was designed to study the prevalence of the four most common high-risk HPVs in the archival tissues with precancerous and cancerous lesions from patients from northwestern Iran. Methods: 133 formalin-fixed paraffin-embedded tissue specimens were tested for HPV DNA by using GP5+/6+-based general PCR and two type-specific PCRs. Results: In total, 84 (64%) out of 131 amplifiable samples were positive for HPV DNA. The most prevalent oncogenic HPV was type 16 (67.6%) followed by types 31 (22.8%), 18 (7.6%) and 33 (1%). Multiple HPV infections were present in 20 (15.3%) of the 131 samples. Notably, of these 20 cases with multiple infections, 15 were from patients with invasive cervical cancer. Conclusions: The multiplicity of HPV genotypes was noted in invasive cervical carcinoma samples, along with rather different circulating HPV types in the study population. Hence, relevant HPV typing information in cervical carcinoma is very important for planning more efficient screening programs and for further HPV vaccine design.

Introduction
Cervical cancer is the second most common cancer in women worldwide [1] and the role of sexually transmitted human papillomavirus (HPV) in its etiology is now well established [2]. More than 200 types of HPV have been recognized [3], with more than 30 different types infecting the cervix via sexual transmission [4]. Based on the epidemiological and molecular biological studies, 15 genital HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), so-called high-risk HPVs (hrHPVs), have the potential to induce invasive cancer [5].

Most HPV-induced cervical cell changes are transient, and 90% regress spontaneously within 12–36 months as the immune system eliminates the virus [6]. Persistent
infection with hrHPV types can progress into cervical intraepithelial neoplasia (CIN) and invasive cancer within several years [7]. Cofactors such as oral contraceptives, parity, and smoking have been identified [8–10]. Given the fact that early detection and subsequent early treatment of HPV in precancerous lesions can prevent progression to cancer [11], establishing a place for hrHPV testing in cervical screening programs and clinical practice seems to be necessary [12].

According to studies carried out in various countries by the International Association for Research in Cancer (IARC), the most prevalent reported hrHPV types, which infect the uterine cervix, are: HPV-16 (53%), HPV-18 (15%), HPV-45 (9%), HPV-31 (6%), and HPV-33 (3%) [13]. Reports on the prevalence of genotypes indicate that HPV-16 is the most prevalent [13, 14]. Nevertheless, the frequency of other high-risk types may vary according to geographic, demographic and clinical-pathological factors [15–17] and may also be influenced by the methods used for detection [18, 19]. Indeed the accurate knowledge on the circulation of different genotypes in a population is needed for (1) planning more efficient screening programs, (2) management of the disease (e.g. follow-up studies for monitoring persistent infections and for checking clearance of the treated cases), and finally (3) vaccination of target population against prevalent HPV types.

The present study was designed to analyze the prevalence of the four hrHPV types in the cervical precancerous and cancerous lesions of patients from northwestern Iran, a putatively high-risk area with no official report on the rate of HPV infection.

Materials and Methods

Clinical Specimens
A total of 133 formalin-fixed paraffin-embedded (FFPE) tissue specimens of patients with cervical precancerous and cancerous lesions were obtained from the Pathology Department, Alzahra Hospital, Tabriz University of Medical Sciences, during a period of 6 years (2000–2006). Eight negative control samples with benign lesions of the female genital tract were also included in the study.

DNA Extraction
Serial sections of 20 μm thickness were sliced from each FFPE block using disposable blades and placed in sterile 1.5-ml microtubes. HPV-negative control tissues were used to verify that no cross-contamination occurred between samples. Then DNA was extracted from the sections as described previously [20].

HPV DNA Detection
Consensus PCR primers GP5+/6+ were used for amplification of a broad spectrum of HPV types as described elsewhere [21] with slight modifications in lowering stringency of the reaction. The samples were genotyped by using two type-specific multiplex PCRs (TSM-PCR). In the first TSM-PCR, HPV-16 and HPV-18 were detected as described earlier [22]. The amplified fragments were resolved by electrophoresis on 2.5% agarose gel and ethidium bromide staining. The 6% polyacrylamide gel electrophoresis, however, may be used for better resolution of the bands. In the second TSM-PCR, HPV-31 and HPV-33 were identified according to the new type-specific primers and PCR conditions described by Baay et al. [23] with slight modifications in the post-PCR detection. Seven microliters of the amplification products were run on 15% polyacrylamide gel electrophoresis (100 V, 5 h) to separate type 31 (110 bp) from type 33 (117 bp). To control for cross-contamination during PCR runs, negative controls and distilled water were used.

Connexin 26 PCR
Connexin 26 PCR was performed using 35delG normal set primers spanning 202 bp [24] to assess the quality of the DNA in the samples with negative results.

Results

Table 1 shows the characteristics of the studied specimens and the detailed results of general and TSM PCRs. On the basis of histological criteria, the samples fell into four main categories: (1) ICC (invasive cervical carcinoma), (2) in situ carcinoma, (3) HSIL (high-grade squamous intraepithelial lesions), and (4) LSIL (low-grade squamous intraepithelial lesions). Of the 70 invasive cancer cases, 65 were squamous cell carcinomas and 5 cases were adenocarcinoma. There were also 4 patients with in situ carcinoma, 34 HSIL and 23 LSIL in the studied specimens. The mean age of the cases at diagnosis was 51.1 years (range 29–79). 58 samples were positive for both general and TSM PCRs, while 25 cases were only positive for TSM PCRs. The later cases include 15 samples with type 16, 5 with type 31, 4 samples with types 16 and 31, and one double infected sample with types 16 and 18. Only one GP+/TSM– sample was found suggesting that in this case infection with another HPV type is implicated. Among the 84 HPV-positive samples, the most common type was type 16 (67.6%). The prevalence of other types was as follows: HPV-18, 7.6%; HPV-31, 22.8%; HPV-33, 1%, and unknown type, 1% (table 1). Multiple HPV infections could be detected in 20 (15.3%) of the 131 amplifiable samples. Of these 20 cases with multiple infections, 19 were positive for double infections and the most common co-infections were HPV types 16/31 (13 cases), followed by HPV types 16/18 (3 cases) and HPV types 18/31 (3 cases). In the majority of the cases (16/19) of double infections, HPV types 16 and 31 were involved.
Triple infection with HPV types 16/31/33 was found in only 1 case (table 1). Among the 49 negative samples, 2 were also negative in the connexin 26 PCR and were therefore excluded from the study. None of the negative controls were positive for HPV. The amplified products of the PCRs are depicted in figure 1.

**Discussion**

Understanding the importance of HPV infection in the pathogenesis of cervical neoplasia has been accompanied by the increased number of studies in screening patients with cervical cancer. On the basis of further epidemiological studies, hrHPV types are found in human cervical cancers at considerably different prevalences and with significant interregional variation [25]. The present study is the first report on the prevalence and distribution of four hrHPV types in cervical precancerous and cancerous lesions from patients from the northwest of Iran. The rate of HPV infection among northwestern Iranian cervical cancer patients was found to be 64%, which is lower than that reported from other populations [16, 17, 23, 25]. However, it is comparable to the results of some other studies [25–27]. Such low percentage of HPV infection in this population may suggest differences in ethnic groups and involvement of other genetic mechanisms in cervical carcinogenesis. Moreover, general primers appeared to have lower sensitivities for certain HPV types [28]. Utilizing type-specific primers from E6 region for HPV types 16 and 18 and from E4 and E1 regions for HPV types 31 and 33, respectively, enabled to detect HPV DNA in 25 (35%) of 72 originally HPV-negative and amplifiable specimens. In fact, only in 1 of the studied cases was an unknown HPV type present.

**Table 1.** Prevalence of hrHPV types in cervical lesions of different histological grades

<table>
<thead>
<tr>
<th>Cytological diagnosis</th>
<th>General PCR positive</th>
<th>TSM-PCR positive</th>
<th>Overall positive result</th>
<th>16 18 31 16, 18 16, 31 16, 31, 33 18, 31 Not detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>37/65</td>
<td>42/65</td>
<td>43/65</td>
<td>24 2 2 3 9 0 2 1</td>
</tr>
<tr>
<td>SCC</td>
<td>1/5</td>
<td>2/5</td>
<td>2/5</td>
<td>2 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
<td>1 0 0 0 0 1 0 0</td>
</tr>
<tr>
<td>In situ carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSIL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN3</td>
<td>9/15</td>
<td>13/15</td>
<td>13/15</td>
<td>9 0 0 0 3 0 1 0</td>
</tr>
<tr>
<td>CIN2</td>
<td>8/19</td>
<td>15/19</td>
<td>15/19</td>
<td>9 0 5 1 0 0 0 0</td>
</tr>
<tr>
<td>LSIL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN1</td>
<td>2/23</td>
<td>9/23</td>
<td>9/23</td>
<td>9 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Total</td>
<td>59/131 (45%)</td>
<td>83/131 (63%)</td>
<td>84/131 (64%)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Results of HPV PCR amplification. a Lane 1 is a positive case for general PCR. Lanes 2–4 are positive samples for types 16, 18, and both types (16 and 18), respectively. b Lane 1 is a positive case for both types 31 and 33, while cases 2 and 4 are positive only for type 31. Lane 3 is a negative case for types 31 and 33. D = Distilled water, L = 100-bp ladder (Fermentas) as DNA size marker.
whereas roughly 20% of all carcinomas contain types other than the ones listed [13]. These results indicate that the amplification of the more specific regions such as E6/E7 genes may increase the sensitivity of HPV detection in the studied specimens.

HPV-16 was the most frequently detected viral type in all cervical lesions. This result is consistent with previous reports which also indicated a high occurrence of HPV-16 in cervical carcinoma [13, 14, 25]. Our results also indicate that the prevalence of type 31 is higher than that of type 18 in the HPV-infected cases, leading to the hypothesis that circulating types of HPV may be different in this population. A similar observation has also been reported [26].

We found that multiple HPV infections were present in about 15% of our samples. The majority of these multiple infected samples were from women with invasive cervical cancer. Previous studies have shown that multiple infections differ considerably between clinical groups. An international survey of invasive cervical carcinomas by the International Biological Study on Cervical Cancer revealed that 4% of specimens harbored double HPV infections [14]. The proportion of multiple HPV genotypes in HPV-positive cervical carcinomas varied across countries and in relation to the HPV detection assays used. These results have crucial implications for inducing therapeutic responses against HPV-induced neoplasia, since the strategy for targeting multiple genotypes of HPV should be a priority in the development of HPV vaccines [29].

Since the samples were collected from one of the most referred gynecologic centers in the northwest of Iran, the obtained results implicate the importance of screening and management of this sexually transmitted disease in this border region with a high rate of immigration.

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References


