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Human papillomavirus genotypes in Iranian patients with cervical cancer

Reza Shahsiah [a,b,*], Morteza Khademalhosseini [a], Nili Mehrdad [c], Fatemeh Ramezani [a], Seyed Alireza Nadji [d]

[a] Department of Pathology, Tehran University of Medical Sciences, Tehran, Iran
[b] Cancer Research Center, University of Medical Sciences, Tehran, Iran
[c] Department of Gynecologic Oncology, Tehran University of Medical Sciences, Tehran, Iran
[d] Virology Research Center, NRITLD, Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

The aim of this study was to determine the frequency of HPV genotypes isolated from cervical intra-epithelial neoplasia grade III and invasive carcinomas of Iranian patients.

A total of 94 cases were selected in five years from 2003 to 2007. After nucleic acid purification, real-time PCR was performed by means of GP5+/GP6+ primers. Subsequently, PCR products were sequenced, on the basis of which a phylogenetic tree was constructed. Negative samples and twelve randomly selected positive samples were also typed by reverse hybridization to increase the sensitivity and to confirm the results.

Of 94 evaluated samples, 7 were negative for internal control gene and were excluded from the study. The overall genotyping results of phylogenetic analysis and hybridization methods were as follows: HPV 16: 75% (65/87); HPV 18: 3% (2/87); HPV 31: 1% (1/87); HPV 45: 1% (1/87).

High frequency of HPV 16 and low frequency of HPV 18 were found in this study. Information about HPV genotype distribution is important in cervical cancer screening and prevention.

Introduction

During the last 50 years, the incidence of cervical carcinoma has declined because of the screening programs [11,25]. Nevertheless, this neoplasm is still the third most common malignancy in women after breast and colorectal cancer, and is a cause of mortality and mortality in women around the world despite the availability of screening [11]. Most cervical carcinomas are caused by human papillomavirus (HPV) [2,18].

HPVs are non-enveloped, double stranded DNA viruses of the *Papillomaviridae* family, which infects mucosal and cutaneous surfaces. Papillomavirus genome is organized into non-coding and coding regions. The former, long control region (LCR), regulates viral transcription while the latter codes early and late viral proteins. Encoding major capsid protein, L1 open reading frame (L1 ORF), is the most conserved part of the virus genome, and is employed for constructing the phylogenetic tree of papillomavirus types [7]. Viral oncoproteins E6 and E7 target p53 and retinoblastoma protein (RB), interfere with cell cycle, and initiate malignant transformation [8]. Usually associated with benign papillomas, mucosal HPV Infection is one of the most common sexually transmitted infections [2].

Mucosal infection with HPV can rarely progress to dysplasia and cancer, a process that takes a long time [8]. Fourteen HPV types that are commonly isolated from malignant lesions are classified as high risk types [18]. While the probability of tumorigenesis is different among various high risk HPVs, types 16, 18, and 45 are more strongly associated with invasive cancer [4]. Because the risk of tumorigenesis is different among various genotypes, the information on HPV genotype distribution is important in cervical cancer screening and prevention [21].

Appropriate HPV testing conditions are defined by Food and Drug Administration (FDA), and require analytical and clinical performance validation of the test [23]. Moreover, inappropriate testing conditions may do more harm than good for the patient [22]. Screening tests ought to be applicable to the target population; therefore, prevalence of specific high risk HPV genotypes, ethnicity, sexual activity, and other factors are important in this regard [1].

Because the purpose of a screening test is to detect and prevent cervical malignant neoplasms, screening tests ought to have high sensitivity for those genotypes isolated from these neoplasms. Meanwhile, information on HPV genotype distribution in cervical cancers is important in planning prevention programs. The aim of this study was to determine the frequency of HPV genotypes isolated from cervical intra-epithelial neoplasia grade III and invasive carcinomas in our region.

* Corresponding author at: Department of Pathology, Tehran University of Medical Sciences, Keshavarz Boulevard, Tehran, Iran. Tel.: +98 21 61192425; fax: +98 21 66581558.
E-mail address: shahsiah@yahoo.com (R. Shahsiah).

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Materials and methods

All uterine cervix samples with the following criteria were included in this retrospective cross-sectional study: (1) cervical intra-epithelial neoplasm grade 3/3; (2) invasive carcinoma of any type: squamous cell carcinoma (SCC), adenocarcinoma, and adenosquamous carcinoma. Consequently, 113 cases were chosen in a period of five years from 2003 to 2007, by searching the admission database of two university hospitals: Cancer Institute and Women's Hospital. Patients are admitted to these referral centers from all regions of Iran, representing a wide range of socioeconomic levels. H&E-stained slides of each paraffin block were reviewed by an expert pathologist (RS) to meet the above mentioned inclusion criteria. Demographic and pathological data, as well as PCR results of the cases, were analyzed by SPSS version 12.0 (SPSS Inc.).

Nucleic acid extraction

Two 5 μm sections of each paraffin block were made using disposable blades, and then the paraffin sections were transferred to DNAse/RNAse free 1.5-mL Eppendorff tubes by means of a disposable plastic applicator; a new blade and applicator were used for each block. After every 30 samples, a tube with distilled water was included as negative control. Following deparaffination by xylene and ethanol, nucleic acid was extracted using Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) as instructed by the manufacturer. Briefly, deparaffinized tissue was incubated with 200 μL tissue lysis buffer and 40 μL proteinase K in a dry block at 55 °C, and checked every day until macroscopic tissue particles disappeared completely. After addition of 200 μL binding buffer and 100 μL isopropanol, the mixture was brought to column. Subsequently, three rounds of washing with inhibitor removal or wash buffers were performed. DNA was eluted into a final volume of 200 μL and stored in −20 °C.

Polymerase chain reaction (PCR)

Serial dilutions of 1:1, 1:5, and 1:10 were prepared from extracted samples. Subsequently, the amount of DNA of each dilution was measured by a spectrophotometer (Specgene, Tehne, Duxford, Cambridge, UK), and 10 μL of the first dilution less than 10 μg/mL was used for PCR amplification. The internal control amplification was performed for a 119 base-pair region of *Homo sapiens* hydroxymethylbilane synthase (HMBS) gene, as previously described, with some modifications [16]. The PCR of internal control was performed in a 20 μL reaction containing 0.2 μM of each forward and reverse HMBS primers, and 10 μL SYBR Premix Ex Taq II (Takara Bio, Ostu, Shiga, Japan). The PCR conditions were as follows: initial denaturation at 95 °C for 1 min, and then 40 cycles of 95 °C for 10 s and 60 °C for 30 s, real-time acquisition of SYBR green fluorescence was performed during annealing/extension phase on green channel. GP5+/GP6+ primers were employed for amplification of a 150 base-pair region of HPV L1 gene [5]. The PCR for target sequence was performed in 40 μL reaction containing 0.5 μM of each forward and reverse primers, and 20 μL SYBR Premix Ex Taq II(Takara Bio, Ostu, Shiga, Japan). The PCR conditions were as follows: initial denaturation at 94 °C for 0.5 min, and then 45 touchdown cycles of 95 °C for 10 s, 55 °C–45 °C for 20 s and 72 °C for 34 s the real-time acquisition of SYBR green fluorescence was performed during annealing phase on green channel. The touchdown annealing phase was composed of 20 cycles of 55 °C–45 °C with 0.5 °C decrease in each cycle. Finally, a 5 min terminal extension was performed followed by melting curve construction by heating up the PCR product from 60 °C to 95 °C at a rate of 1 °C/s with aquisition on green channel [9]. The primers and probes for both internal control and target genes were synthesized by Bioneer Corp. (Alameda, CA). Primer characteristics and product sizes are shown in Table 1.

Table 1
Primers and PCR product characteristics.

Primer	Sequence (5′–3′)	Product size	Product melting (°C)
GP5+/GP6+	TTTGTTACTGTGGTAGATACTAC GAAAAATAAACTGTAAATCATATTC	150	80
HMBS	GCCTGCAGTTTGAAATCAGTG CGGGACGGGCTTTAGCTA	119	84

All PCR reactions were performed in a rotor-gene 300 real-time machine (Corbett Research, Mortlake, Australia). Chosen from a patient infected with HPV genotypes 6, 16, 39, 66, positive control was included in each run. Samples with less than 0.5 °C melting peak difference from positive control were considered positive and were sent for sequencing.

Genotyping

Positive PCR products were sent for purification and sequencing to Macrogen (Geum chun-gu, Seoul, Korea) where the sequencing was performed by ABI 3730 XL machine employing forward primer (GP5+). Subsequently, phylogenetic analysis was performed by means of MEGA software version 4.0.2 [24] by Neighbor-Joining method and bootstrapping of 500 replicates, the phylogenetic tree was constructed out of the sequence data of the samples and eight HPV genotypes that cause most cervical cancers (Fig. 1) [6].

Internal control positive samples that were not successfully genotyped by the above mentioned method, as well as other 12 randomly selected samples, were genotyped by means of GenoArray reverse hybridization (Hybribio, Two-china chm-plaza, 68-Conmaught Road Central, Central, Hong Kong) as instructed by the manufacturer.

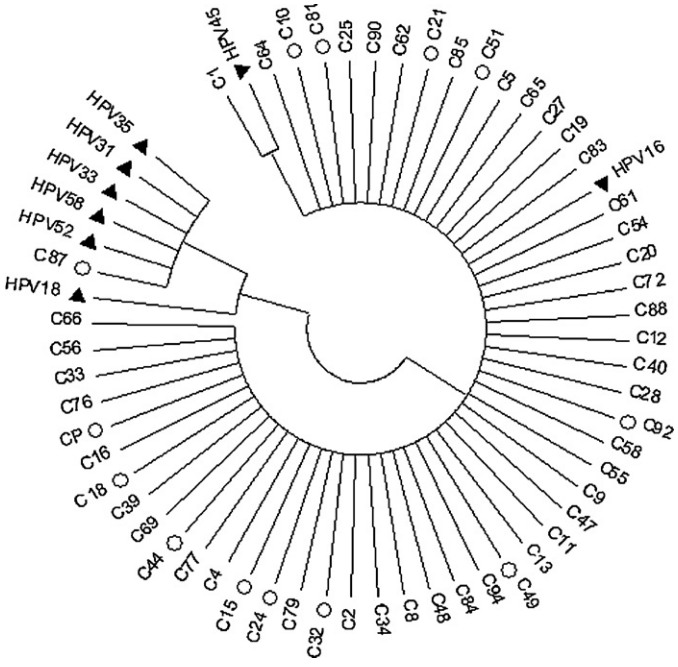

Fig. 1. Phylogenetic tree of HPV isolates. (○) Typed also by means of reverse blotting. (▲) Pubmed reference HPV type: HPV-16 [K02718]; HPV-18 [X05015]; HPV-31 [J04353]; HPV-33 [M12732]; HPV-35 [GQ479037.1]; HPV-45 [X74479]; HPV-52 [X74481]; HPV-58 [D90400].

Results

Age of patients at the time of admission ranged from 28 to 72 years, with a mean and standard deviation of 48 and 11 years, respectively. Of 94 evaluated cases, 13 were cervical intraepithelial neoplasia grade 3 (CIN3), one was adenosquamous carcinoma, and the rest were SCC. Of 94 evaluated samples, 7 were negative for internal control gene and were excluded from the study. Fifty-three out of 87 (61%) samples were genotyped by amplification of HPV L1 gene and subsequent sequencing. Of 34 remaining cases, 19 were successfully genotyped by GenoArray, and the remaining samples were negative. Only one sample (no. 53) was positive for both HPV 16 and HPV 18, and the overall genotyping results of phylogenetic analysis and hybridization methods were as follows: HPV 16: 78% (68/87); HPV 18: 3% (3/87); HPV 68: 2% (2/87); HPV 31: 1% (1/87); HPV 45: 1% (1/87). The overall sensitivity was 83% (72/87). Of 12 cases that were typed by both methods, sequencing and hybridization showed similar results (Fig. 1) except sample 1, which could not be typed with GenoArray.

Discussion

In the current study, polymerase chain reaction (PCR) and, subsequently, sequencing was employed for detection and genotyping of HPV DNA. The advantage of HPV L1 gene amplification followed by sequence analysis is that the method is capable of classification of a broad range of HPV genotypes [19]. Although the sensitivity of this method for detection of multiple HPV genotypes is low, the presence of multiple HPV genotypes in tumor samples is not common [17,20]. While hybridization is sensitive in the detection of multiple HPV genotypes, almost all tumor samples tested with this method showed only one HPV genotype. Studies have shown that the frequency of HPV 16, 18 is much lower among HPV-infected patients in the general population compared to the patients with HSIL or invasive tumors [3]. Meanwhile, the frequency of HPV types 16, 18 is lower in HSIL compared to invasive tumors [4,6,21]. The reason might be that HPV types 16, 18 are more oncogenic compared to other genotypes [21].

In the current study, the frequencies of HPV 16 and HPV 18 in invasive carcinomas were 75% and 3%, respectively. Although similar results regarding the frequency of HPV 18 were reported by Farjadian et al. in Iranian patients with cervical carcinoma, compared to our results, the frequency of HPV 16 was only 26.7% in their report [10]. The frequency of cervical SCC in Iranian patients has been reported to reach 88% [12]. This rate is higher than the rate reported by researchers in the United States and other developed countries, where SCC is decreasing because of the effectiveness of cervical cancer screening [25]. While HPV 16 is more prevalent in SCC, HPV 18 is more prevalent in adenocarcinoma [15,21]. Therefore, the reason behind the high frequency of HPV 16 and low frequency of HPV 18 might be that the majority of the selected tumors in the specified period of time were SCC. While all cases were included in the specified time according the inclusion criteria, sampling error and over-inclusion of SCC samples could not be completely excluded.

A source of bias could be the cases in which internal control was positive but no HPV was detected. If a specific type of HPV was highly prevalent in these cases, the overall prevalence of HPV types would change. Even in this scenario, HPV 16 would still be the most frequent type. Nevertheless, such a probability could not be dismissed even in centers with high experience in HPV testing [6]. Another limitation of this study was that with L1 being the target of GP5+/6+ primers, L1 deletion makes the PCR negative. A minority of tissue specimens with HPV incorporated into the host genome show deletion in L1 region of the viral genome during integration. Because the number of such cases is negligible as evidenced by a pervious study, it is unlikely that such a deletion significantly influences the results of the current study [17]. Moreover, employment of formalin-fixed specimens, in contrast to fresh tissue, is not optimal for molecular studies [13,14]. In order to minimize the effect of formalin-fixation on the results, inappropriate samples were recognized and consequently omitted when the result of internal control amplification was negative.

In conclusion, high frequency of HPV genotype 16 was found in Iranian patients with cervical neoplasms. Furthermore, this reveals the importance of vaccination for this genotype in the prevention of cervical neoplasms in our population. Moreover, any clinical HPV screening test employed for cervical cancer prevention ought to have good detection sensitivity for those HPV 16 types and their variants.

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