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Original Article

The Common Human Papillomavirus Genotypes among Cervical pre-cancerous and cancerous samples in Kermanshah Province (2013)

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Abstract

Introduction: It is important to have data on human papillomavirus (HPV) from different geographical regions of Iran for health policymakers to make decisions on national programs of HPV vaccination. Because no study was found on HPV genotyping in Kermanshah Province, this study appeared necessary.

Methods: Fifty-three paraffin-embedded cervical tissue samples were collected. All samples were evaluated by polymerase chain reaction (PCR) or nested-polymerase chain reaction assays with specific primers designed for L1 gene. Positive samples were subjected to sequence and phylogenic tree analysis.

Results: Thirty-one out of 53 samples (58.5%) were found to be HPV positive. Prominent HPV genotype was HPV16 followed by HPV6, 31 and 53. Based on the histology of cervicitis, only HPV16 was found both in cervicitis and adenocarcinoma while HPV6 and 16 were found in cervical intraepithelial neoplasia I, and HPV6 and 53 were found in cervical intraepithelial neoplasia II. HPV16 and 31 were also found in squamous cell carcinoma.

Discussion: Findings from this study support a strong correlation of HPV16 as a leading cause of cervical cancer in this region. It appears that HPV vaccines could drastically reduce the incidence of cervical cancer.

Introduction

Cervical cancer is the fourth most common cancer in the world, with 528,000 new cases and 266,000 deaths in 2012 (1). Human infection with the papillomavirus causes this cancer (2). Studies on normal cervical samples indicate that high-risk infections are common in women, and most of these infections are cleared by the immune system within 6-12 months after the infection. In a few cases, these infections can stabilize and progress to type 3 dysplasia, which may ultimately lead to invasive cervical cancer (3).

Human papillomaviruses (HPV) is classified into three categories based on the potential for carcinogens: (1) low risk (such as types 6 and 11), (2) medium risk (such as types 53 and 66) and (3) high risk (including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 73) (4). Several studies indicate that the most common types of HPV in pre-cancerous and cancerous samples are the 14 high-risk types outlined above, among which type 16 is the most common, as it is detected in most cervical samples in the world (5-7).

Most studies in Iran indicate that two high-risk types 16 and 18 are common in various types of cervical samples (normal, pre-cancerous and cancerous), but interestingly, two low-risk types 6 and 11 are also common in some studies after types 16, and 18, while in other studies types 31 and 33 are more common after types 16 and 18 (8-14). The problem with determining the HPV types in Iran is that most studies did not use sequencing to determine the type of virus, and determined the types of positive samples using specific primers of types 16 and 18. Therefore, further studies are needed to determine the HPV types using the viral genome sequencing method, especially in areas where no information is available.

Information on the spread of common HPV genotypes in different parts of Iran can be very important for screening and prevention programs because the results of such studies can be useful for health policymakers for the national vaccination program against HPV. Because no study was found on HPV genotypes in Kermanshah Province, a study appeared necessary to determine the common genotypes of HPV.

Materials and Methods

A total of 53 paraffin-embedded tissue samples from cervical biopsies were collected from Razi Pathology Lab and Imam Reza Hospital of Kermanshah in 2012-

2013. Histologic evaluations showed some degrees of neoplasia. However, samples were re-examined by a pathologist and stained with hematoxylin-eosin to examine their neoplasticity.

First, thin layers were prepared from all the samples using disposable blades and placed in 2 ml sterile tubes. The paraffin was removed from the samples using xylene and organic material was removed from the environment using 100% and 75% alcohol and the tissues were dewatered. The tissues were digested at 37 °C for one night using a digestive buffer (50 mMTris (PH 8.5), 1 mM EDTA, 0.5% Tween 20) containing 200 g/mlμ of proteinase K. In the next step, samples were extracted using the phenol-chloroform method and DNA was precipitated with absolute cold alcohol (15). In order to control the quality of the extracted samples, PCR was performed using specific primers for the human β-globin gene (PCO3 and PCO4 primers) (16). In order to perform PCR on HPV, the primer pair of $GP5^{+}/GP6^{+}$ was used which replicated a fragment of 150 base pairs of ORF-L1 genome of HPV. Negative samples were re-tested by the Nested-PCR method using MY09/MY11 primers for the first stage (a fragment of 450 base pairs) and $GP5^{+}/GP6^{+}$primers for replication in the second stage (a fragment of 150 base pairs). PCR reaction mixture in both stages was prepared with a volume of 50 μl containing 2.5 mM MgCl2, 50 μMdNTP, 20 pmol primers and 2 U HotstartTaq DNA Polymerase (Qiagen, GmbH, Hildelberg, Germany), and genome replication was done according to the temperature conditions mentioned in Table 1. The PCR products were transferred to 2% agarose gel and then the gel was stained by safe stain and the desired bands were observed under a transluminator (Fig. 1).

Table 1. Primer sequence, number of cycles, and temperature conditions of different PCRs

		Primer sequence	Number of cycles	Temperature conditions
PCR for HPV		GP5+: 5'-TTTGTTACTGTGGTAGATACYAC-3' GP6+: 5'-GAAAAATAAACTGTAAATCATATTC-3'	40	95°C 50s, 55°C 50s, 72°C 1 min
Nested-PCR For HPV	First stage	MY09: 5'-CGTCCMARRGGAWACTGATC-3' MY11: 5'-GCMCAGGGWCATAAYAATGG-3'	40 times	95°C 50s, 55°C 50s, 72°C 1 min
	Second stage	GP5+: 5'-TTTGTTACTGTGGTAGATACYAC-3' GP6+: 5'-GAAAAATAAACTGTAAATCATATTC-3'	30 times	95°C 30s, 50°C 45s, 72°C 40s

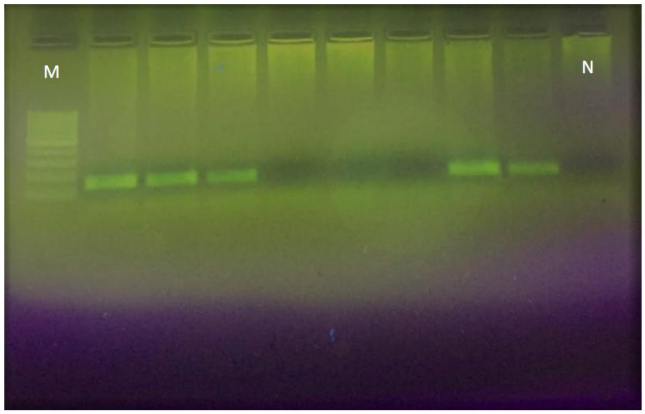

Figure 1. Electrophoresis gel to detect a fragment of 150 base pairs of HPV gene L1, left to right, well 1: marker of 100 base pairs indicated with the letter M; wells 2-9 of patient samples; and well 10 negative controls indicated with the letter N. It should be noted that in samples loaded in the wells 2-4 and 8-9, HPV was detected but not in the samples loaded in the wells 3-7.

Next, the sequence of PCR products was determined using the ABI Genetic Analyzer 3130 in the Virology Department, Faculty of Health, Tehran University of Medical Sciences, Tehran, Iran. The sequences were then edited with the Bioedit 7.0.5.2 software. Edited viral sequences and reference sequences obtained from the gene bank using the Bioedit 7.0.5.2 software were aligned. The phylogenetic tree was then drawn with 6.06 Mega software using the Neighbor-joining method and Bootstrap 1000 value (Fig. 2).

Results

This study was performed on cervical biopsy samples. The histological findings revealed cervicitis in 20 samples (37.7%), precancerous lesions type 1 (CIN I) in 8 samples (15.1%), precancerous lesions type 2 (CIN II) in 2 samples (3.8%), squamous cell carcinoma in 21 samples (39.6%), and adenocarcinoma in 2 samples (3.8%) (Table 2). According to Table 2, 41.5% of the subjects were 40 or younger and 58.5% of them were older than 40. The mean age ± standard deviation in patients with cervicitis, pre-cancerous and cancerous cervical lesions was 39.8 ± 9.1, 35.9 ± 9.8 and 55 ± 7.6 years, respectively.

PCR test on human β-globin gene showed that all samples had desirable conditions for tracing HPV genome. Among the 53 samples isolated from the patients, 31 (58.5%) were HPV positive. The prevalence of HPV in patients with cervicitis was 10%, in patients with CIN I was 50%, and in the rest of the patients including CIN II and both types of cervical cancer was 100% (Table 2). The sequencing findings indicate that type 16 was the dominant genotype in the samples, followed by types 6, 31 and 53 (Fig. 1). As Table 3

shows, based on the pathology, only type 16 in cervicitis, types 6 and 16 in in CIN I, types 6 and 53 in in CIN II, types 16 and 31 in squamous cell carcinoma, and type 16 in adenocarcinoma cases were detected. By age, types 6, 16 and 53 were detected in the under-40 age group and types 16 and 31 in the over-40 age group.

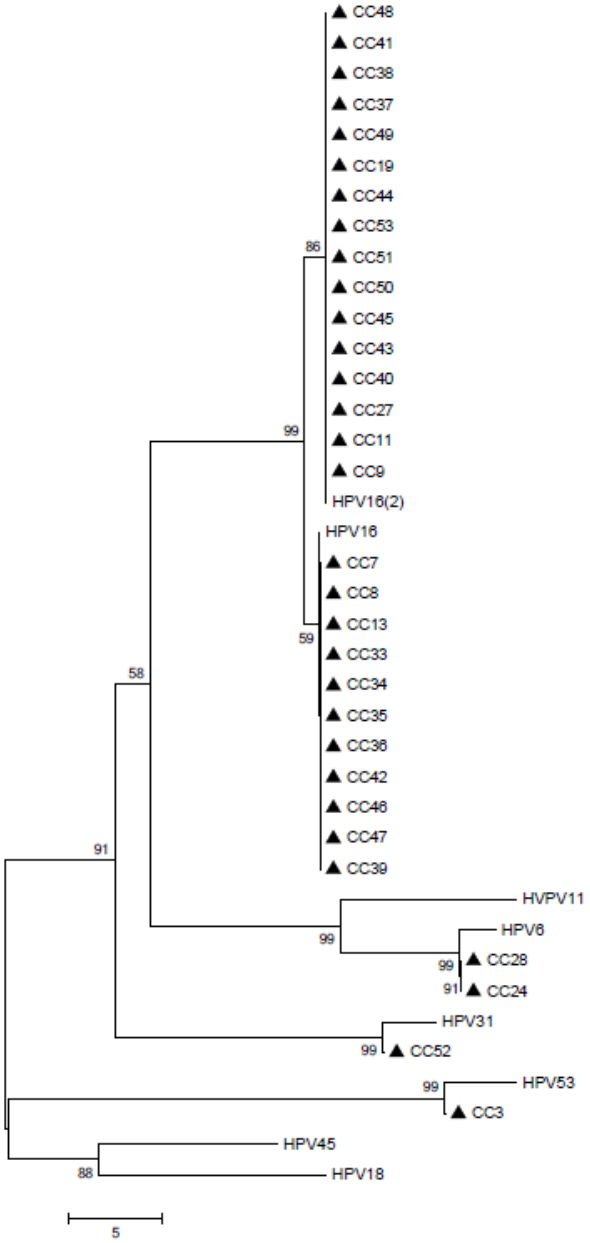

Figure 2: The phylogenetic tree drawn based on area ORF L1 of HPV in precancerous and cancerous cervix samples from Kermanshah province using Mega 6.06 software using the Neighbor-joining method and Bootstrap value 1000. Examples of the present study are shown with black triangles.

Table 2. The prevalence of HPV cases according to age and pathologic status in cervical samples, Kermanshah province

		The status of HPV		Total	P value
		Positive Number (percent)	Negative Number (percent)		
Age (years)	<= 40	5 (22.7)	17 (77.3)	22	0.00002
	> 40	26 (83.8)	5 (16.2)	31	
Pathologic status	Cervicitis	2 (10)	18 (90)	20	0.0000001
	*CIN I	4 (50)	4 (50)	8	
	CIN II	2 (100)	0 (0)	2	
	Squamous cell carcinoma	21 (100)	0 (0)	21	
	Adenocarcinoma	2 (100)	0 (0)	2	
Total		31 (58.5)	22 (41.5)	53	

*CIN: Cervical intraepithelial lesion

Table 3. Distribution of HPV genotypes based on pathologic status in cervical samples, Kermanshah Province

Pathologic status	HPV genotypes				Total
	6	16	31	53	
Cervicitis		2			2
*CIN I	1	3			4
CIN II	1			1	2
Squamous cell carcinoma		20	1		21
Adenocarcinoma		2			2
Total	2	27	1	1	31

*CIN: Cervical intraepithelial lesion

Discussion

Various studies suggest that the combination of screening and vaccination programs can be effective in controlling cervical cancer worldwide. There are currently three vaccines against the virus called 4 valent Gardasil, 9 valent Gardasil, and Cervarix. The 4 valent Gardasil vaccine creates immunity against types 6, 11, 16 and 18; the 9 valent Gardasil creates immunity against type 6, 11, 16, 18, 31, 33, 45, 52 and 58; and the Cervarix vaccine creates immunity against types 16 and 18. These vaccines have been used in some countries in the world for the prevention of cervical cancer for several years (17, 18). Determining the prevalence of various types of HPV in normal, pre-cancerous and cancerous lesion samples from different parts of Iran can be a step toward recognizing its status in Iran to guide major health policymakers and healthcare providers to determine which of the common vaccines could be more effective in Iran if HPV vaccination enters the national vaccination program in near future.

According to a study in Iran, the incidence of cervical cancer in Kermanshah Province is relatively low, such that its age-standardized incidence rates (ASR) was 1.1 per 100,000 people (19). The findings of this study indicate that the mean age of patients with cervical cancer was 55 years old. Cervical cancer usually occurs 20 years after HPV infection; therefore, it is known to occur in middle age (4, 20). A review study in Iran based on the 2007 Cancer Registry estimated that the mean age of patients with cervical cancer was around 60 years old (19).

Findings of the present study on determination of the prevalence of different types of HPV indicated that only high-risk types 16 and 31 were detected in the cancerous samples, while in pre-cancerous and cervicitis samples, in addition to the high-risk types, the medium-risk (type 53) or low-risk (type 6) types were also detectable. These findings were consistent with other studies from Iran and from different parts of the world. Various studies indicate that in addition to infection with high-risk types in normal and pre-cancerous samples, infection with medium- and low-risk types is also common, while in cancerous samples only high-risk types are detected (2, 21). Several studies in Iran have shown that HPV type 16 is the most common type in cervical cancer samples. A study in Tehran indicated that 85% of the squamous cell carcinoma cases were due to type 16 followed by types 18 and 31 (22). Another study in Tehran showed that the most common types of HPV in cervical cancer samples were type 16 (74%), followed by types 18 and 33 (14). A study in Isfahan also suggested that 73.1% of cases of cervical cancer originated from type 16 (23). The absence of type 18 in the present study might be due to the small sample size, or that the type had a low prevalence in the province.

Conclusions

The results of this study showed a strong relationship between HPV type 16 and the occurrence of cervical cancer. Therefore, it appears that all three types of HPV vaccine can significantly reduce the incidence of this cancer in Kermanshah Province.

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