ARTICLE

MOLECULAR DIAGNOSIS OF HUMAN PAPILLOMAVIRUS (HPV) GENOTYPES IN TEHRAN CITY

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ABSTRACT

***Background** and **objective:** The human papilloma virus is known as one of the cause's anogenital cancers, including cervical cancer there is a strong link between presences of different types of human papilloma viruses to the development of genital lesions. HPV16 and HPV18 were defined as most important etiologic agents for cervical dysplasia and carcinoma. The aim of present research was to find genital HPV genome in patients with cervical cancer and control group and to investigate incidence of HPV16, HPV18, HPV31, HPV33, HPV45 and HPV51 types among patients admitted to medical centers. **Materials and Methods:** Fifty samples of women cervical paraffin-embedded tissue, HPV positive from pathology department of Imam Khomeini (RA) were collected. For this purpose, DNA was extracted and purified using a DNA Extraction Kit (Cinnagen, Tehran, Iran] from 50 patients with cervical cancer attending the Imam Khomaini Hospital (Tehran, Iran) and amplified by PCR. The nucleotide sequences were analyzed using the BLAST program (http://www.ncbi.nlm.gov/ BLAST). **Results:** The molecular and pathological investigation of samples showed that %71 of cases were HPV-positive. Out of them, %41 of samples was determined HPV 16, %28 HPV18, %10 HPV31, %5 HPV33, %7 HPV45 and %9 HPV51. **Conclusion:** As a matter of fact, the virus is not related to significantly detectable symptoms, and also the extreme prevalence of HPV in Iran, early detection of HPV can be prohibit preventing cancer development from pre-cancer lesions. Therefore, using molecular techniques like PCR may contribute effectively to identifying HPV cases to diagnose in a proper time.*

KEY WORDS
Papiloma virus; cervical cancer; cancer diagnosis

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INTRODUCTION

The human papilloma virus is known as one of the cause's anogenital cancers, including cervical cancer [1]. Which approximately 30-40 percent of them are due to contamination of mucous, especially in the anogenital areas [2] lack of ability to viral culture, makes the initial identification of the virus problem. And up to now more than 100 different types of human papillomavirus have been identified with the help of molecular techniques [1]. PCR technique is the ability to replicate virus DNA using primers specific for the particular type of virus as one of the most sensitive methods to detect HPV in genital tissues are considered. This method along with cytological methods can increase the resolution and accuracy in investigating clinical patients. Papilloma viruses have circular double-stranded DNA genomes with sizes close to 8 kb. Due to their small size, their molecular biology is very complex [5]. Human papilloma virus genome consists of three initial proteins E1-E8, L1-L2 protein secondary and non-coding control region and the LCR or URR [6 and 7, 8, 9, 10, 11, 12]. Cervical cancer is the first epidemiological study was conducted by Rigoni-Stern in 1842 showed that the incidence of cervical cancer is more common in married women [13]. The HPV genotypes in terms of cancer risk can be grouped as high-risk (HR) or low-risk (LR): LR HPV includes types 6, 11, 40, 42, 43, and 44, whereas HR HPV includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66.

On the basis of all married women 20-65 years after first entering the screening Pap test every year once they are done [14 and 15]. HPV is the most common disease and showed that the incidence of cervical cancer is more common in married women [13]. HPV is the sexually transmitted disease (STD) acquired from the oncoviruses comes to the types 6 and 11 account not only the anogenital region but also in the respiratory tract, lungs, mucous membranes, skin, head and neck and mouth and the injury, including warts anogenital that an infectious transmitted disease in both sexes by sexuality [16]. Types 16 and 18 caused serious problem for women including cancers of the cervix, vulva, anus, vagina, etc. [17]. Anogenital infection in the form of warts, *Condylomata acuminata*, and mucosal lesions of the cervix (CIN) appears [18] for cervical lesions CIN1, CIN2, CIN3 or classified CIS [19, 20]. The lesion is usually diagnosed as CIN1 [21]. There lesion in the middle and upper thirds as CIN2, CIN3 is detected, indicating the progress of cervical lesions and cervical cancer [22]. Cervical cancer occurs most often between the ages of 30 to 55 years but has recently been observed in young women. Cervical cancers in developing countries, including Iran, are more common than in developed countries [23]. By early detection of the HPV virus can be prevent many cancers. All diagnostic methods has advantages and disadvantages, but new molecular methods of detection and genotyping of HPV have a higher sensitivity and specificity (28). PCR amplification of the target DNA using different today and Hybrid Capture HPV virus detection is done accurately [24, 20]. The objectives of this study were to investigate molecular diagnostics HPV genotypes (HPV) causes' cervical cancer in Tehran city.

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MATERIALS AND METHODS

Tissue samples

A total of fifty cervical paraffin-embedded (FFPE) tissue samples from 50 different women who were diagnosed as HPV positive were selected for DNA extraction and human papillomavirus (HPV) genotyping and were retrieved from the archives pathology department of Imam Khomeini hospital (Tehran, Iran), from October 2014 to February 2016.

Tissue retrieval and DNA extraction

For all 50 samples, paraffin-embedded blocks were examined for adequate tissue volume, defined roughly as an area at least twice that of a 1 mm micro-punch. Only one block per case was used, and within that block, one contiguous area of tumor was selected for analysis. One-millimeter disposable sterile micro-punches were used to punch tissue cores from marked paraffin embedded blocks. Between 3 and 7 1-mm cores per block were obtained. Tissue cores were first de paraffinized and proteinase K–digested according to the manufacturer's instructions. DNA was extracted using a commercially available DNA extraction Rima Pure FFPE kit (Recover all Total Nucleic Acid Extraction Kit for formalin fixed, paraffin-embedded Tissues, Life Technologies, Grand Island, NY) that uses a spin column–based extraction technique to maximize nucleic acid purity. Final DNA concentrations were determined using a Nano-drop 1000 spectrophotometer. Samples were included for PCR amplification and sequencing if the DNA concentration was greater than 6 ng/µL in the 50 µL DNA extraction volume.

PCR and Sanger sequencing

At least 200 ng of DNA was PCR amplified using forward and reverse primers that targeted HPV types 16, 18, 31,33,45,51 Primer sequences were as mentioned I [Table 1]. The PCR reactions were carried out in a reaction volume of 25 µl containing genomic DNA 30–40 ng, 1 mol/l dNTP 2 µl, 25 mM MgCl2 1.5 µl, 10x PCR Buffer 2.5 µl, 10 pM primer 0.5 µl each, 1 U Taq polymerase (CinnaGen) 0.4 µl, and DDH2O. The reactions were carried out in the following thermo cycler conditions: denaturation at 94 ^{o}C for 5 min, 36 cycles of 94 ^{o}C for 30 s, 63 ^{o}C for 30 s, and 72 ^{o}C for 30 s, and final elongation step at 72 ^{o}C for 10 min. PCR products were visualized by gel electrophoresis and staining with ethidium bromide in a digital camera system.

The presence of HPV different genotypes (HPV16, HPV18, HPV31, HPV33, HPV45 and HPV51) in patients were determined by using following primers sequence as mentioned in [Table 1].

Table1: Primers used of the detection of HPV genotypes 16, 18, and 31,33,45,51

Type	Primer	Sequences	Length *[bp]*	Annealing Temperature	Reference
HPV-16	F	ACCCAGTATAGCTGACAGT	252	48 ºC	**Raji, 2011 [32]**
	R	CTCGTTTATAATGTCTACACA			
HPV-18	F	ATAGCAATTTTGATTTGTC	455	44 ºC	**Raji, 2011 [32]**
	R	AAACTCATTCCAAAATATG			
HPV-31	F	CACAACATTTGATTTGTCCC	351	54/3 ºC	**Raji, 2011 [32]**
	R	CTCGTTTATAATGTCTACACA			
HPV-33	F	ATGCACAACTTGCAGATTC	449	57 ºC	**Raji, 2011 [32]**
	R	AAACTCATTCCAAAATATG			
HPV-45	F	GCTACAGCTGTTATTACGCAG	455	60 ºC	**Weyn, 2007 [33]**
	R	GCAATTGTGCAGGTTTAC			
HPV-51	F	CCTAAAACCTCAACGCGTGCTGCT	452	52 ºC	**Weyn, 2007 [33]**
	R	TTGTTGTGCATTGCCATTTGC			

After extracting DNA and insert samples in agars gel 5/1% and electrophoresis presence of HPV in the sample was determined. Next the samples with HPV-DNA typing kits for DNA Technology were selected and prepared by the reaction mixture to separate in micro tubes to which 10 ml and 5 ml sample buffer and the same amount as the reaction mixture or positive control or added based on the order of 45 cycles with temperatures kit (Denaturation 94 ^{o}C, 64 ^{o}C primer binding temperature of 70 ^{o}C Extension) PCR reactions done and in the end product by 2% agarose gel stained with Ethidium Bromide was observed and interpreted

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according to the kit manual. Symptoms of HPV type 33 bp 449 to get the band is. A band types 16 and 31 and 51 bp 642 signs are present. Size bp 285 bp 291 bands and HPV types 18 and 45 are respectively. Then the final PCR products were sequenced by Macrogen Company in South Korea.

RESULTS

A total of fifty cervical paraffin-embedded (FFPE) tissue samples from 50 different women who were diagnosed as HPV positive were collected for DNA extraction and human papillomavirus (HPV) genotyping and were retrieved from the archives pathology department of Imam Khomeini hospital (Tehran, Iran),
DNA was extracted and purified using a DNA Extraction Kit (Cinnagen, Tehran, Iran) from 50 patients with cervical cancer attending the Imam Khomaini Hospital (Tehran, Iran). The quality of DNA extraction method using electrophoresis in agarose gels and stained with ethidium bromide were studied. The mean age of the study population was 50 years and their age range 25 to 76 years old. The maximum number of patients at the age of 36 to 55 age category with the abundance of % 53.2 [Fig. 1].

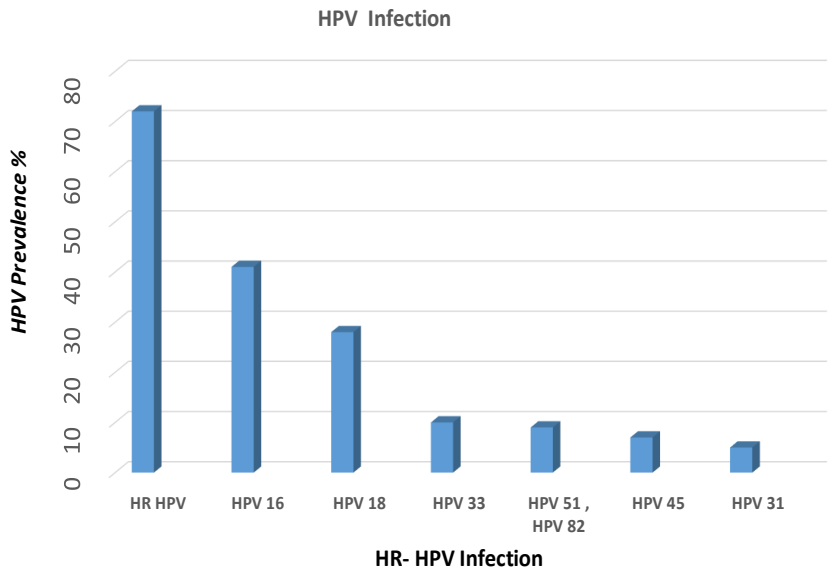

Fig. 1: Distribution as percentage of the most prevalent HPV genotypes in the selected population.

Results of the molecular and pathological investigation of samples showed that 36 women (%71) were positive for HPV. For HR HPV infection, HPV 16 (41%) was the most common type, followed by HPV 18 (28%), HPV 31 (10%), HPV 51 and HPV 82 (9%), HPV 45 (7%) and HPV33 (5%) [Fig. 2].

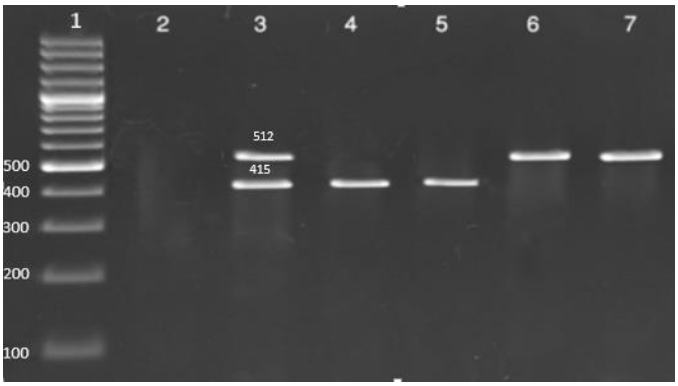

Fig. 2: Agarose gel electrophoresis of human papillomavirus (HPV) polymerase chain reaction amplification products HPV 16 and HPV 18.

Nucleic acid sequences of the different types of HPV were compared with those available in the GenBank database using NCBI/BLAST to search for related sequences.

Results of homology comparison in in the GenBank database using NCBI/BLAST shows that HPV 16 were 89% homologous with HPV16 from Germany; (Gene Bank ACCESSION Number: AJ313181) and 85% homologous with HPV16 from South Korea; (Gene Bank ACCESSION Number: KT428600) [Table 2]. HPV 18

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were 93% homologous with HPV16 from Brazil; (Gene Bank ACCESSION Number: KP965187.1) and 93% homologous with HPV18 from Brazil; (Gene Bank ACCESSION Number: KP965186.1) [Table 2]. HPV 31 were 89% homologous with HPV31 from Italy; (Gene Bank ACCESSION Number: JN041177.1) and HPV 33 were 92% homologous with HPV33 from Mongolia; (Gene Bank ACCESSION Number: KC862076.1) [Table 2] [Fig. 3]. HPV 45 were 94% homologous with HPV45 from USA; (Gene Bank ACCESSION Number: KC470254.1), HPV 51 were 98% homologous with HPV51 from USA; (Gene Bank ACCESSION Number: U45917.1) and HPV 51 were 98% homologous with HPV82 from USA; (Gene Bank ACCESSION Number: KF436795.1) [Table 2].

Table 2: Homology of the Nucleic Acid Sequences of the different types of HPV in the GenBank Database (NCBI)

HPV Types	Homology [%]	Accession No.	Country
HPV16	89%	AJ313181.1	Germany
HPV16	85%	KT428600.1	South Korea
HPV18	93%	KP965187.1	Brazil
HPV18	93%	KP965186.1	Brazil
HPV31	89%	JN041177.1	Italy
HPV31	88%	JN041176.1	Italy
HPV33	92%	KC862076.1	Mongolia
HPV33	92%	KC862075.1	Georgia
HPV33	92%	KC862074.1	Vietnam
HPV45	94%	KC470254.1	USA
HPV45	94%	EF202160.1	USA
HPV51	98%	U45917.1	USA
HPV51	97%	KF436874.1	USA
HPV82	82%	KF436795.1	USA
HPV82	82%	KF436794.1	USA

Fig. 3: Phylogenetic Tree of Human Papillomavirus type 31.

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DISCUSSION

The genotype-specific prevalence of HPV in Tehran city women, is essential for achieving further progress in cervical cancer prevention. According to our knowledge, this is the first validation set to comprehensively describe prevalence of HPV infection of Tehran city women. The overall prevalence of HPV infection was %71 (n= 36). The results of this study indicate a much higher prevalence of HPV than the overall prevalence worldwide [34]. Age-specific HPV prevalence estimates were highest in women younger than 34 years and prevalence decreased in the 35–44 year-group [35]. Age-specific HPV prevalence were highest in Tehran City women with 36 years old. For HR HPV infection, HPV 16 (41%) was the most common type, followed by HPV 18 (28%), HPV 31 (10%), HPV 51 and HPV 82 (9%), HPV 45 (7%) and HPV33 (5%). Differences in the prevalence of HPV genotypes might be related to the geographical location factors and biological interactions between different HPV types and Immunity system of host [36]. This is the first prevalence report of HPV 82 in Iran. Results of Bruni et al., [37] indicated that among the women with type-specific HPV data, the 5 most common types worldwide were HPV-16 (3.2%), HPV-18 (1.4%), HPV-52 (0.9%), HPV-31 (0.8%), and HPV-58 (0.7%). Identification of HPV infection is the central causal agent of cervical neoplasia has created new research fronts in primary and secondary prevention of this disease. Sequence similarity searching to identify homologous sequences is one of the first, and most useful informative, steps in any analysis of newly determined sequences. Homologous sequences have similar structures, and frequently, they have similar functions as well. Results of homology comparison in the GenBank database using NCBI/BLAST shows that HPV 16 were 89% homologous with HPV16 from Germany; Gene Bank ACCESSION Number: AJ313181 [38]. HPV 18 were 93% homologous with HPV16 from Brazil; Gene Bank ACCESSION Number: KP965187.1 [39]. HPV 31 were 89% homologous with HPV31 from Italy; Gene Bank ACCESSION Number: JN041177.1 [40]. HPV 33 were 92% homologous with HPV33 from Mongolia; Gene Bank ACCESSION Number: KC862076.1 [41]. HPV 45 were 94% homologous with HPV45 from USA; Gene Bank ACCESSION Number: KC470254.1 [41], HPV 51 were 98% homologous with HPV51 from USA; Gene Bank ACCESSION Number: U45917.1 [42]. Many studies have shown that some types of high-risk HPV types are known to play a major role in creating this type of cervical cancer so that more than 99 % of cervical cancers have been identified in the world [43].

CONCLUSION

This finding adds knowledge to molecular diagnosis of human papillomavirus (HPV) genotypes information in Tehran city, HPV epidemiological investigation, and addresses further studies aimed to consider public health for identifying groups at high-risk (HR) for cervical cancer. The relatively ease and economic accessibility of the PCR technique can potentially have an impact in HPV screening in Iran.

CONFLICT OF INTEREST

There is no conflict of interest.

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FINANCIAL DISCLOSURE

None

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