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Short Communication

Association of E6 gene expression of high risk human papillomaviruse HPV 18 in patients with Cervical squamous cell dysplasia and Cancerous Lesions

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Abstract

Background and Aims: Cervical cancer is among leading causes of cancer related death in women and human papilloma virus (HPV) is one of the important risk factor of this cancer. The aim of the present study was to develop a PCR method for identification of a high carcinogenic type of HPV, HPV 18 using E6 gene as a marker in patients with cervical cancer

Materials and Methods: 92 Formalin-Fix (FF) and Paraffin-Embedded (PE) tissues of premalignant and malignant lesions from cervical cancer patients were collected. DNA was extorted followed by PCR application in two steps using L1 and E6 consensus primers.

Results: Infection with HPV was observed in 68(73.91%) out of 92 samples by L1 region consensus primers, while 18 (26.47%) positive cases were detected to be HPV 18 using E6 type specific primer. Six of them were CINII and CINIII, and 12 cases were diagnosed as squamous cell carcinoma.

Conclusions: Our findings demonstrated that the assessment of HPV18 using E6 gene with a specific PCR can help in identification of high carcinogenic genotypes of HPV. Further studies are needed to assess the value of this method in a larger multicenter setting for establishing their values for early detection of cervical cancer patients.

Keywords: E6 papilloma virus, cervical cancer, HPV18.

Introduction

Human papilloma virus (HPVs) is the major risk factor of cervical cancer. Cervical cancers are the second

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leading cause of death in women worldwide (1-5). The America Cancer Society has predicted 12,990 cases of invasive cervical cancer in 2016 in the United States. Moreover, it is expected that more than 4,120 deaths from cervical cancer will occur in 2016 in US (6). Several studies have shown the association of high risk types of HPV with cervical cancer (7, 8). Cervical cancer which is caused by HPV infection can be consider as sexually transmitted disease (STD) (9). The probability of persistent infection is higher among individuals with multiple sexual partners (10, 11). HPV encodes three oncogenic proteins: E5 (activates PDGF receptor), E7 (inhibits Rb), andE6 (inhibits p53). The most important risk factor for cervical cancer is infection by the HPV (12-16) . Previous findings demonstrate that HPV-16 and -18 have a higher prevalence in our population than 31 and 51 genotypes.

Several methods have been developed for detection of high and low risk HPVs, including HPV genotyping based PCR or real time PCR methods. The aim of these methods is to recognize the DNA of this virus. Based on the studies conducted by the international center of cancer research in 22 different countries, 99.7% of 1000 samples with the history of invasive cervical cancer (ICC) were positive with HPV16 and 15% of viral infection were positive for HPV18 (15). Against this information, E6 and E7 are expressed in most of the malignant and premalignant lesions. These genes are responsible for cells transformation and are associated with HPV infection (10). E6 and E7 oncoproteins confer with the function of P53 and Retinoblastoma (Rb) tumor suppressors proteins that cause cellular division and genomic instability (14, 17). Several studies showed that E6 and E7 of high risk viruses are able to immortalize keratinocytes. Acting together, two viral oncogenes, E6 and E7, are sufficient to induce transformation in the absence of mutations in cell regulatory proteins. The E5 oncoprotein of HPV, which causes sustained activation of the PDGF receptor, enhances proliferation of the transformed cells. In this study we used a specific, sensitive, and simple method which can detect E6 of HPV18 from paraffin blocks. Currently, some methods are being used to detect HPV by PCR using general primers of MY09/MY11 and GP5+/GP6+, but a wide spectrum of HPV types can be detected as low risk ones (18, 19). Moreover there are a few reports available for the virus detection form paraffin block of cervical cancer (20).

Materials and Methods

Populations. 92 Paraffin- embedded cervical tissue samples with dysplastic and cancerous lesions of cervix were collected. Informed written consent was obtained from all participates, and the research protocol was approved by the local Ethics Committee. Informed written consent was obtained from all participates, and the research protocol was approved by the Ethics committee of Kashan university of medical sciences by No: 29/5/1/4222 on 6.Feb.2013. The tissue was cut at 4 mm and then stained with hematoxylin and eosin staining.

DNA extraction and HPV 18 screening. DNA extraction from the paraffin block was performed as follows: 1ml xylol 60˚C was added to the tissues, followed by incubation for 20 min at 60˚C and centrifuged for 15 min at 14000 rpm. Ethanol 99% was added to each sample and incubated at 60˚C for 20 min. Afterward, 300 µl digestive buffer (1.5 mg/ml Proteinase, 50mM NaCl, sodium dodesylsuflate 0.5%, 10mM EDTA, and 50 mM Tris-HCl pH=7.5) were added and incubated at 56˚C for 24 h. After centrifuging, the supernatant was transferred into new tubes. To have pure nucleic acid, the extraction process was followed by phenol/chloroform method. After DNA extraction, the existence of HPV genome was determined using two MY11 and MY09 primers. Specific E6 pair primers were used to determine the high risk type HPV18 (21). Since the expression of E6 and E7 oncogenes of HPV 16 and 18 has a strong positive correlation with the metastasis of invasive cervical cancer , specific primers (Table 1) was used for detection of E6 (22, 23). DNAs was amplified by PCR using following condition: PCR mixture volume was 50µl which contained 35µl H2O, 6µl 10X buffer, 2

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Table1: Primers used for the detection of HPV DNA from cervical cancer biopsy samples

Primer	Amplified Region	Sequences	Amplifier length	References:
HPV (MY09/MY11)	**L1**	5-CGTCC(A/C)A(A/G)(A/G)GGA(A/T)ACTGATC-3 or 5-CGTCCMARRGGAWACTGATC-3 5-GC(A/C)CAGGG(A/T)CATAA(C/T)AATGG -3 or 5- GCMCAGGGWCATAAYAATGG-3	450bp	(22)
HPV18 (E6 type specific primer)	**E6**	F: 5′-GCGCTTTGAGGATCCAACAC-3′ R: 5′-ACGAATGGCACTGGCCTCTA-3′	415 bp	(23)

Table 2 : PCR conditions

Stage	Temperature	Time	Number of Cycles
Primary denaturation	95 °C	5 sec	1
Denaturation	95 °C	30 sec	40
Annealing	55 °C	45 sec	
Elongation	72 °C	1 sec	
Final elongation	72 °C	5 sec	1

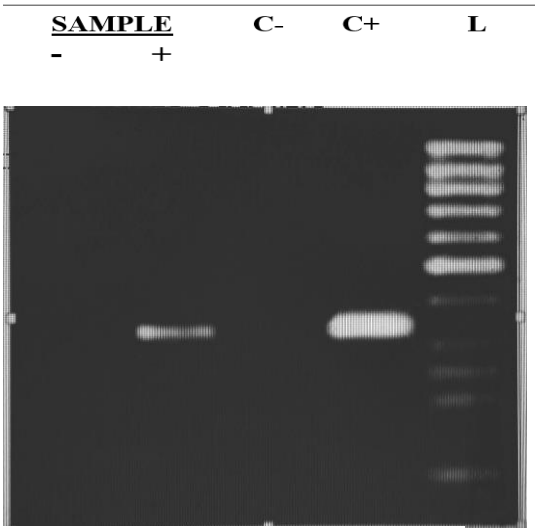

Fig. 1. Samples containing the E6 gene of HPV18: 415bp band related to the E6 gene of HPV18. L: ladder 100bp, C+: positive Control, C-: Negative Control.

µl MgCl2, 0.5 µl dNTP, 0.5 Primer F, 0.5 µl Primer R, 0.5µl Taq (CinnaGen company, Tehran, Iran), and 5µl DNA sample (~10ng/ul) (Table 2). The obtained product was run on 2% agarose gel and stained by ethidium bromide (Figure 1).

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Table 3 : Evaluation of the association of risk factors to cervical cancer and HPV frequency.

Variable	HPV / Groups	Positive N (%)	Negative N (%)	Total	Number (Percentage)
Age	Lower than 39	10(47.6)	11(52.4)	21(100)	92(100)
	40-59	11(22.4)	38(77.6)	49(100)	
	More than 60	3(13.6)	19(86..4)	22(100)	
Marriage age	Lower than 17	44(75.9)	14(24.1)	58(100)	92(100)
	Over than 18	22(64.7)	12(35.3)	34(100)	
Smoking	yes	37(100)	0(0)	37(100)	92(100)
	No	43(78.2.)	12(21.8)	55(100)	
OCP consumption	yes	43(72.9)	16 (27.1)	59(100)	92(100)
	not	23 (69.7)	10(30.3)	33(100)	
history of Delivery	less than 3	21(70)	9 (30)	30(100)	92(100)
	More than 4	19(73.1)	6(26.9)	26(100)	
	not	30(81.1)	7(8.9)	37(100)	

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Results

The clinical and general characteristics in patients with cervical cancer are reported in Table 3. The ranges of age were from 40 to 59 which accounts for 49% of our population. OCP consumption and history of child delivery were found in 59 and 56 patients respectively. The pathological data showed that 63.23% of CIN and SCC patients had OCP consumption, 54.41% had smoking history, and 27.94% had more than 4 deliveries.

Furthermore our data showed that 68 out of 92 samples (73.91%) were found to have positive results by using general primers for HPVs. From these, 17 cases (25%) were CINII and CINIII, and 51 cases (0.75) had SCC. By using HPV18 E6 specific primers, 18 cases (19.56%) of 92 paraffin blocks had positive results, and from these 12 patients, 6 were CINII and CINIII and 6 were SCC (Table 4).

Table 4 : Total and relative frequency distribution of studied samples based on HPV 16 and 18 infections.

		HPV		HPV18		Total
		Positive	Negative	Positive	Negative	N (%)
Diagnose	CIN	17	9	6	20	26 (100)
	SCC	51	15	12	54	66(100)
Total		68	24	18	74	92(100)

Discussion

Cervical cancer is among the major public health problem. Increasing evidence showing the association of HPV infection with the pathogenesis of cervical cancer (21) . There are a few studies evaluating the frequency of HPV18 in Iranian population and it possible association with pathological data. Therefore in the present study we evaluated the frequency of HPV18 by E6 gene in tumor tissue and investigated its association with pathological characteristics of patients. We found the important role of E6 expression for evaluation of HPV. This study from 92 patients, 73.91% were positive for HPV 18 which is in line with previous observations. Hamkar and colleagues in 100 biopsy showed that 16 and 18 genotypes were existent in 60.6% of cervix carcinomas (24). Farjadian et al., in 101 tissue samples revealed that HPV16 was found in 26.7% of the cervical cancer cases (25). Another study by Ghaffari et al., found 18 genotypes in 28% of cases with tumor and 8% of the total abnormal samples (26). Piroozmand et al., demonstrated that HPV-16 and -18 have a higher prevalence in Iranian population than 31 and 51 genotypes (27).

Additionally Niakan et al., evaluated the frequency of HPV in an Iranian population, showing, the existence of oncogenic papilloma in 24.7% and 65% of cases (28). Another research by Pavai in 2008 on 66 discharge samples, showed that HPV 18 and 16 had 46-63% and 10-14% frequencies respectively (29), which these frequencies are more or less similar with the study by Varnai in 2006 on 58 tissue samples in an German population (30).

In addition to the infection with HPV, which is the main cause of cervical cancer, age, smoking, OCP consumption, and incidence of cancer can also increased the risk of developing cervical cancer. In particular age is one of the factors for HPV infection. Some studies have shown that women under 25 years old are more susceptible for infection (29-31). Some studies have been reported the association of OCP consumers, and women with more than 3 deliveries with cervical cancer due to HPV18 infection. But, our data showed no significant correlation between

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frequency of HPV18 infection and number of delivery. Also evaluation of the data showed that the quality of the obtained product of extracted DNA from paraffin blocks was sufficient for PCR test. Considering the efficacy of this protocol, we might use this method for detection of HVP18.

Results of the study confirm the previous reports concerning the correlation between HPV and cervical cancer. In aggregate we illustrated that the application of this PCR method by the assessment of E6 gene in detection of cervical cancer could provide a useful method for evaluation of the patients. With attention to high prevalence of cervical cancer, promoting education and

Conclusions

knowledge about the importance and necessity of pap smear and risk factors of cervical cancer and encouraging married women to take part in screening programs is necessary. Further studies are needed to assess the value of this method in a larger multicenter trial setting for establishing their values for early detection in patients with cervical cancer.

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