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Frequency of E6 and E7 Oncogenes of Human Papillomavirus Types 16 and 18 in Cervical Cancer Patients in Pakistani Women

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ABSTRACT

Prevalence of E6/E7 oncogenes of Human Papillomavirus (HPV) types 16 and 18 have been studied in cervical cancer patients in Pakistani population. DNA extracted from 83 formalin fixed paraffin embedded cervical cancer samples was used for PCR amplification using primers specific for E6/E7 oncogenes of HPV type 16 and 18. Sixty five out of 83 cervical cancer samples were found to be positive for E6 and E7 oncogenes in HPV 16/18 subtypes (78.31%). Out of HPV positive samples, 63 (96.92%) cases were positive for HPV 16 and only 2 (3.07) % were positive for HPV 18. In HPV16 positive cases, mean age was found to be 35.753±11.231 and 63.5% cases were $\geq$ 40 years. Regarding HPV 18 positive samples, one was 40 years of age with moderately differentiated histological grade and the other was 60 years of age with poorly differentiated cervical cancer. In HPV 16/18 positive samples, 52.31% were of moderately differentiated histological grade, 27.69% were of poorly differentiated histological grade and 20% were of well differentiated histological grade. In HPV 16 positive cases, 57 were of squamous cell carcinoma type and seven cases were adenocarcinoma. Two HPV 18 positive cases were of adenocarcinoma variety. It is concluded that frequency of E6 and E7 oncogenes in HPV 16/-18 subtypes in cervical cancer patients is about 78% of which 97% were positive for HPV 16 and only 3% were positive for HPV 18.

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Authors' Contribution
AZ and ARS conceived and designed the project. AZ collected samples, performed the experimental work and wrote the article. ARS supervised the study.

Key words
Cervical cancer, E6 and E7 oncogenes, HPV 16 and 18

INTRODUCTION

Cervical cancer is one of the most common cancers among women worldwide and the commonest cancer cause of death in women in developing world (WHO, 2016; Deny, 2010). Approximately 500,000 new cases of cervical cancer are diagnosed annually in developing countries with an increase in incidence by 10-folds and 250,000 deaths per annum (Yousuf *et al*., 2010). It is 2nd most common cancer among Pakistani women aged between 15-44 years of age. According to Information center on HPV and cancer (ICO), cervical cancer in Pakistan ranks the 3rd most frequent cancer among women after breast and oral cavity and every year, 5233 women in Pakistan are diagnosed with cervical cancer, while 2876 die of this lethal disease (Gul *et al*., 2015). Early (E) proteins, particularly E6 and E7, of high risk subtypes of HPV 16 and 18, play an important role in the development of cervical cancer.

Human Papillomavirus infection is considered to be the most important risk factor in the development of cervical cancer. Sexual transmission is the predominant route of HPV infection, although HPV is not always spread through sexual intercourse. Women with high-risk HPV infection had a nearly 33-fold increased risk of cervical cancer compared to HPV-negative women (Munoz *et al*., 2003). Certain types of HPVs, such as HPV-16, and HPV-18, have been recognized as high risk subtypes and causative agents of cervical cancer. Together, HPV16 and 18 are the cause of nearly 70% of cervical cancers across the world. Early protiens particularly E6 and E7, of HPV 16/18 subtypes play a crucial role in cervical cancer development. The pathogenesis of cervical cancer is thought to occur through a multistep process involving HPV infection. (Lipari *et al*., 2001). The viral oncogenes E6 and E7 of HPV functionally interfere with cell cycle control by inactivating tumor suppressor gene p53 and the retinoblastoma protein respectively (Yang *et al*., 2016; Basu *et al*., 2016; Sandhu and Shivakumar, 2016; Kaufmann *et al*., 2002). The E6 protein is thought to promote cell proliferation by stimulating degradation of the tumor suppressor p53 protein via the formation of a trimeric complex comprising E6, p53 and the cellular ubiquitination enzyme E6-AP. E6-stimulated degradation interferes with such biological functions of p53; thus perturbing the control of cell cycle progression, leading finally to increased tumor cell growth (Yim and Park, 2005). In addition, one function of E6 is to activate telomerase, and E6 and E7 cooperate to effectively

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immortalise human primary epithelial cells (Narisawa-Saito and Kiyono, 2009; Yugawa and Kiyono, 2009)

The E7 protein is primarily localized to the nucleus and has been shown to cause cellular proliferation, immortalization and transformation (McMurray *et al*., 2001). It is hypothesized that E7 binds to the retinoblastoma protein (pRb), which inhibits its normal function resulting in uncontrolled cell proliferation. (Doorbar, 2005; Stoler, 1996). Hence E6 and E7 oncogenes are involved in host cell proliferation (Fujii *et al*., 2006; Duvlis *et al*., 2015). The virulence of HPV is mainly exhibited by E6 and E7 encoded oncoprotein that cause low to high-grade cervical lesions (CIN-1, 2, 3), leading to cause 99.7% of squamous cell and 89% of adenocarcinomas cervical cancer worldwide (Ramakrishnan *et al*., 2015). In most of cervical cancer cases, the HPV viral genome is found to be integrated into the host cell genome, which commonly results in deletion of portions of the viral genome but in the majority of cases, the E6 and E7 open reading frames (ORFs) remain intact which drive carcinogenesis in host cells (Siddiqa and Bhatti , 2016).

In short, E6 and E7 are active in transmembrane signaling, regulation of the cell cycle, immortalization of primary cell line, transformation of established cell line and regulation of chromosomal stability. The viral E6 and E7 oncoproteins are necessary for malignant conversion of cells (Lipari *et al*., 2001). Cervical cancer is a slow progressive disease and there is a gap of 10–20 years between pre-cancer and cancer which provides an opportunity to screen, detect and treat pre-cancer and timely prevent its progression to invasive cancer (Ahmad *et al*., 2015).

There is very little data available to identify the burden of HPV and HPV-associated cervical carcinoma in Pakistan, due to the fact that all matters pertaining to sex are considered as social taboo in this region. These socio-cultural prohibitions create a barrier to the investigation of issues concerning sexually transmitted diseases. As majority of cervical cancer is caused by high risk types of human papillomavirus (HPV), reliable detection has considerable diagnostic and prognostic relevance (Bosch *et al*., 1995; Walboomers *et al*., 1999).

As E6 and E7, are invariably expressed in HPV-positive cervical cancer cells, HPV PCR strategies directed at the E6/E7 region are preferable because they are the oncogenic regions of HPV and are retained after infection. Moreover E6 and E7 exhibit strong sequence conservation, unlike other regions such as L1 and E2 (Morris, 2005).

The present study was designed to determine the frequency of E6/ E7 oncogenes of high risk HPV 16 and 18 in cervical cancer samples in Pakistani population.

MATERIALS AND METHODS

Specimen collection

A total of 83 formalin fixed paraffin-embedded tissue blocks of cervical cancer patients were obtained from the Allama Iqbal Medical College and Fatima Jinnah Medical College Lahore. The information provided with each sample was age of patient and histological grade of cancer type. All patients were females with ages from 19-70 years. These samples were in the form of formalin fixed paraffin embedded (FFPE) tissue blocks. Three 10µm thick sections of each formalin fixed paraffin embedded tissue blocks were taken in separate 1.5ml eppendorf tubes and stored at -4°C for isolation of DNA.

DNA extraction

DNA extraction was done by using master pure ™DNA purification kit -cat. No MCD 85201 Epicentre Biotechnologies. First step was removal of paraffin wax, so the excess paraffin was trimmed from the tissue sections and cleaned sections were placed in eppendorfs. Then 1ml of xylene was added to extract paraffin and incubated at room temperature for 10 min. Solvent was discarded. This xylene wash was repeated twice. Next was ethanol wash, for which 1ml of 100% ethanol was added and incubated at room temperature for 10 min. Ethanol was then discarded. Two ethanol washes were given. Second step was tissue lysis, for which above treated samples were taken and 2µl of Proteinase K @ 50µg/µl was added along with 300µl of tissue and cell lysis solution (provided in the kit).The tube was homogenized and vortexed. Incubated at 65°C for one hour. The tubes were incubated at 65°C for 15 min. The samples were then allowed to cool down over ice for 10 min. The third step was DNA precipitation. 175µl of MPC protein precipitation reagent (provided in the kit) was added to 300µl of above lysed sample and vortexed vigorously (for 10 sec). This was followed by centrifugation at 13000xg for 10 min at 4°C. Supernatant was transferred to new micro centrifuge tube. Isopropanol (500µl) was added in the supernatant. Contents were mixed by inverting tubes several times (30-40 times), and centrifuged at 13000xg for 10 min. The pellet containing DNA was rinsed with 70% ethanol twice and then air dried. The pellet was suspended in 35µl of TE buffer (provided in the kit).

Polymerase chain reaction

Polymerase chain reaction (PCR) was used for the amplification of a region in the HPV-E6/E7 gene for the general detection of the Human Papillomavirus and for the genotype specific detection of high risk HPV 16 and

18 using the GP5/GP6 primers and genotype specific primers, respectively (Andersson *et al*., 2005).

HPV 16
F 5' AAGGCGTAACCGAAATCGG 3' 206 bp
R 5' CATATACCTCACGTCGCAG 3'

HPV 18
F 5' AAGGCATAACCGAAATAGG 3' 418 bp
R 5'TTCTGCTGGATTCAACGGT3'

PCR reaction mixture (30 µl) comprised 1x PCR reaction buffer, 3 mM $MgCl_2$, 0.24 mM dNTPS, 100 pmole/µl of each primer, 2.5 U of Taq polymerase, 1.5 µg genomic DNA.

Statistical analysis

Statistical analysis was done by using SPSS 15.0 software. Identification of E6 and E7 oncogenes of HPV 16 and 18, the age of patient, histological grade of cancer, were described by using frequencies and percentages. The association of cancer with age of patient and histological grade of cancer was studied.

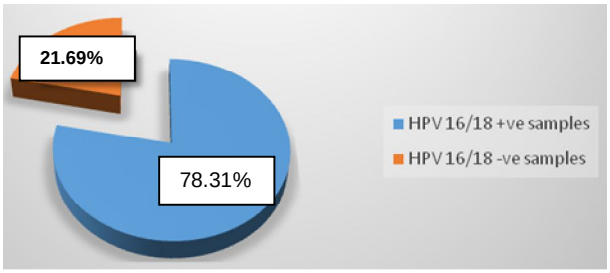

Fig 1. Frequency of E6 and E7 oncogenes in HPV subtypes 16 and 18 in cervical cancers in Pakistani population (n=83).

RESULTS

It was found that out of 83 cervical cancer samples, 65 were found to be positive for HPV 16/18 (78.31%) (Fig. 1). Out of HPV positive samples, 63(96.92%) cases were positive for HPV 16 and only 2 (3.07%) were positive for HPV 18 (Figs. 2, 3). It was found that 42 (89.4%) HPV 16/18 positive samples were of age less than or 45 years. Results of present study also showed that among HPV 16/18 positive samples, 81.2% were of well differentiated histological grade, 82.9% were of moderately differentiated grade and 69.2% cases were of poorly differentiated grade (Table I). When further explored it was noted that in HPV16 positive cases, mean age was found to be 35.75±11.231 (Mean years ±SD). Regarding HPV 18 positive samples, one was 40 years of age with moderately differentiated histological grade and the other was 60 years of age with poorly differentiated cervical cancer. In HPV 16 positive cases, 57 were of squamous cell carcinoma type and seven cases were adenocarcinoma. Two HPV 18 positive cases were of adenocarcinoma variety (Fig. 4)

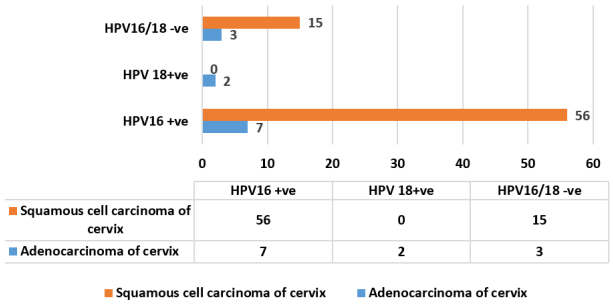

Fig. 4. HPV 16/18 types in different histopathologic types of cervical cancer among Pakistani population.

DISCUSSION

HPV16/18 are the oncogenic genotypes predominantly, causing approximately 70% of global cervical cancer cases (Ogembo *et al*., 2015; Smith *et al*., 2003; Clifford *et al*., 2003). HPV type 16 (HPV-16) is most commonly linked with cancer, since it is present in 50 percent of cervical cancers and high-grade cervical intraepithelial neoplasias (Koutsky *et al*., 2002; Bosch *et al*., 1995; Moscicki *et al*., 2001) and in 25 percent of low-grade cervical intraepithelial neoplasias (Kulasingam *et al*., 2002). HPV-16 and -18 are the prevalent genotypes in cervical lesions isolated in Pakistan (Siddiqa *et al*., 2014; Khan *et al*., 2007).

In present study, it was found that out of 83 cervical cancer samples of Pakistani women, 65 were found to be positive for oncogenes E6 and E7 in HPV 16/-18 (78.31%). Out of HPV positive samples, 63 (96.92%) cases were positive for HPV 16 and only 2 (3.07) % were positive for HPV 18. Bachtiary *et al*. (2002) found that HPV16 was the most commonly found genotype and was detected in 71.9% of HPV-positive tumors. In a study on Pakistani population, high-risk HPVs (16 and 18) were found in 69% of cervical carcinoma cases (Anwar *et al*., 1991). Evidences showed that the prevalence of HPV 16 and 18 in cervical cancer are high in China. In Asia, a comparative data indicated that HPV 16 and 18 are more prevalent in India, Sri Lanka and Bangladesh, additionally this study concluded that HPV-16 and 18 were detected in 80% of patients with cervical cancer in India (Sankaranarayanan *et al*., 2008; Schwartz *et al*.,

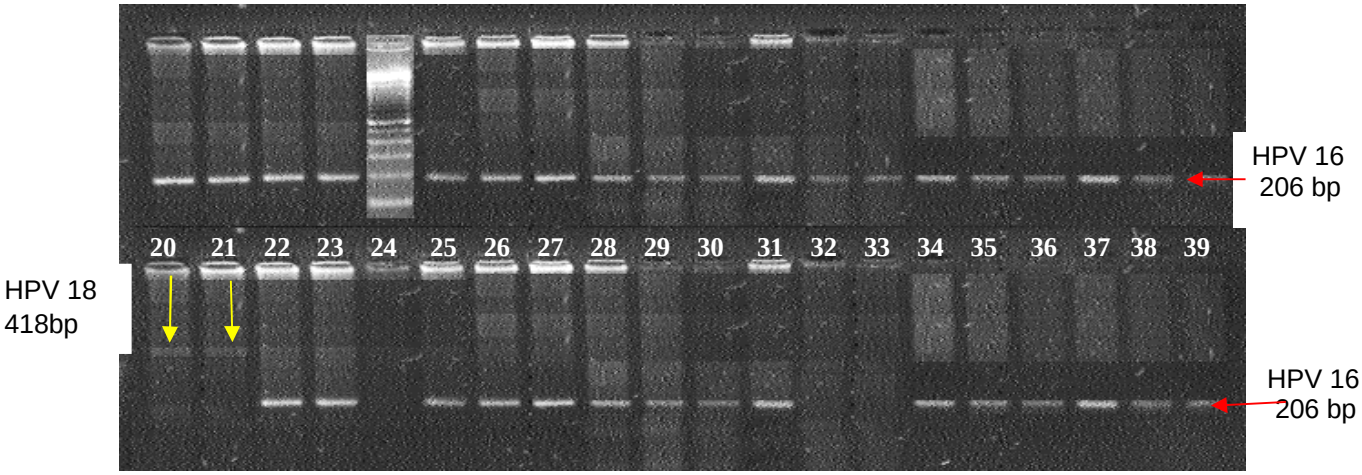

Fig. 2. Representative Agarose gel electrophoresis image showing PCR products for E6/E7 oncoprotein of High risk HPV 16/18. All lanes showing positive for HPV 16 at 206 bp samples except 20, 21, 24, 32 and 33. Lanes 20 and 21 showed HPV 18 at bp 418).

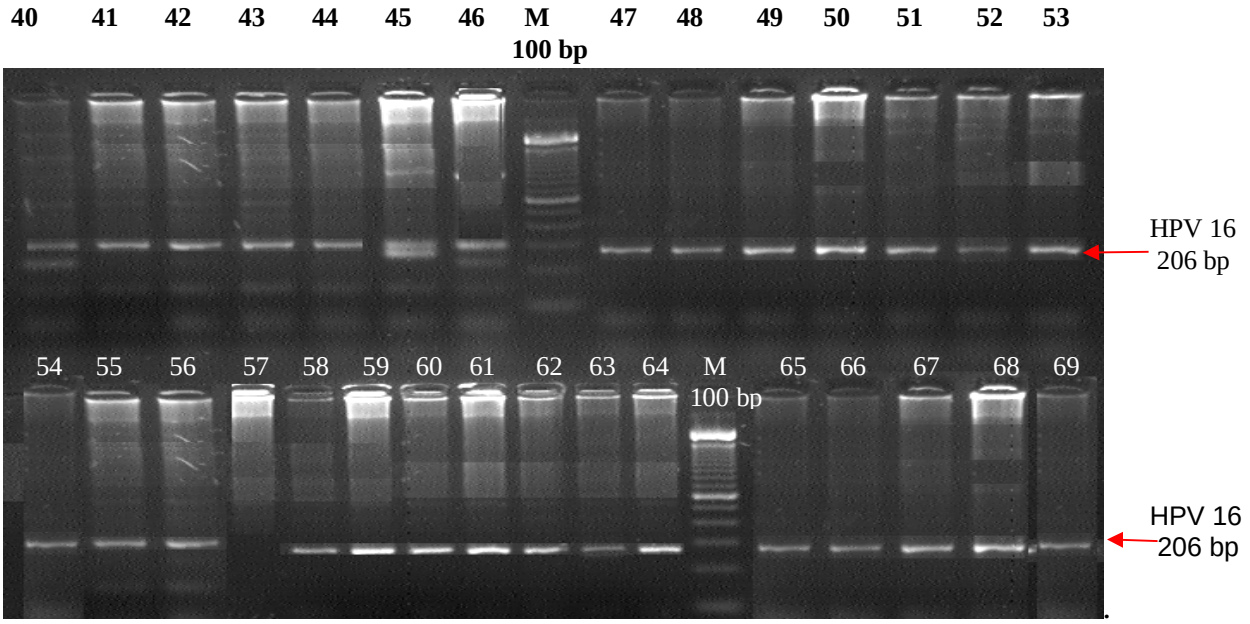

Fig. 3. Representative Agarose gel electrophoresis image shows the PCR products for of E6 and E7 oncogenes in High risk HPV 16/-18. All lanes showing positive HPV 16 at bp 206 except 57. All samples are HPV 18 negative

2001). The HPV16 accounted for three-quarters of Invasive cervical cancer in Pakistan, confirming the high prevalence of HPV16 observed in ICC from the Indian subcontinent compared with other world regions (Raza *et al*., 2010).

Siddiqa *et al.* (2014) in a study conducted in Punjab, Pakistan, found that HPV18 alone is as common as infection with HPV16 alone contrary to the present study in which, 96.92% cases were positive for HPV 16 and only 3.07% were positive for HPV 18. The results of another study on Pakistani population showed that HPV was not found in majority (82%) of the cases and was detected only in 18% out of fifty paraffin embedded tissues specimens of squamous cell carcinoma of cervix. Out of the cases that were positive, 55.6% were infected with HPV16, while in 44.4% of cases, the genotype could

Table I.- Association of HPV 16 and 18 with age and histological grade of cervical cancer in Pakistani women.

		HPV16/18						Chi-sq.	P-value
		Positive		Negative		Total			
		n	%	n	%	n	%		
Age(yrs)	≤ 45	42	89.4	5	10.6	47	100.0	7.99	0.005
	> 45	23	63.9	13	36.1	36	100.0		
	Total	65	78.3	18	21.7	83	100.0		
Histological grade	Grade - I	13	81.2	3	18.8	16	100.0	1.86	0.395
	Grade- II	34	82.9	7	17.1	41	100.0		
	Grade - III	18	69.2	8	30.8	26	100.0		
	Total	65	78.3	18	21.7	83	100.0		

Grade-I, well differentiated; Grade, II moderately differentiated; Grade III, poorly differentiated.

not be identified (Yousuf *et al*., 2010). Another study on Pakistani population showed that the HPV induced cervical cancer rate is higher in the age group of 41-60 years. HPV 16 was found to be comparatively more prevalent than HPV 18 among the women aged between 21 and 60, while HPV 18 was found in patients older than 60 (Gul *et al*., 2015), the results comparable to the present study in which it was noted that in HPV16 positive cases, mean age was 35.75 years and 63.5% cases were ≥ 40 years of age. Out of 83 cervical cancer samples only two were HPV 18 positive for E6 and E7 oncogenes, one was 40 years of age and the other was 60 years of age.

An increase in HPV infection with high risk types, emphasizes an immediate need of a public health policy implementation for HPV screening and its vaccination in Pakistani women (Raza *et al*., 2010). Cervical cancer is a preventable disease. A combination of HPV DNA and Papanicolaou testing had almost 100% sensitivity and negative predictive value (Lörincz and Richart, 2003). Prophylactic and therapeutic vaccines are available in the developed countries. These vaccines are highly effective and if HPV is considered to be the major etiological factor for development of cervical cancer in the Pakistani females, these vaccines can be introduced for Pakistani population as well (Yousuf *et al*., 2015).

CONCLUSION

It is concluded that frequency of E6 and E7 oncogenes in HPV 16/-18 subtypes in cervical cancer patients.is about 78% of which 97% were positive for HPV 16 and only 3% were positive for HPV 18.

Statement of conflict of interest

The authors have declared no conflict of interest.

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