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Short Communication

Phylogenetic Analysis of *E6* and *E7* Gene of Human Papillomavirus 16 Variants Isolated from Pakistani Population

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ABSTRACT

HR-HPVs, or high-risk human papillomaviruses, are the cause of cervical cancer, ranks as the second leading cause of female mortality worldwide, following breast cancer. HPV16 is globally recognized as the most common high-risk type. This research sought to examine sequence differences between the local HPV16 genotypes' E6 and E7 oncogenes and those with foreign genotypes of HPV16. Additionally, it aimed to establish phylogenetic relationships according to the nucleotide sequence comparisons between the variants found in the present study and isolates from different parts of the world that have already been reported.Cervical cancer patients provided samples for the study. Following DNA extraction, HPV16 E6 and E7 amplification and sequencing were performed. Using the obtained E6 and E7 sequences, HPV16 phylogenetic trees were generated. The analysis revealed that Pakistani isolates belong to the A1 sub lineage within the European branch. The study incorporated locations of the first, second and third codons. The final dataset encompassed 477 positions for E6 and 297 positions for E7. Phylogenetic analysis confirmed that Pakistani isolates are situated within the sub lineage A1 of HPV16's lineage A.

Papillomaviruses (PV) are tiny, round, non-enveloped, double-stranded DNA viruses that have a diameter of around -55 nm and a size of about 8 kb (Zheng and Baker, 2006; Fernandes and de Medeiros-Fernandes, 2012; Amador-Molina *et al*., 2013). A number of illnesses, from benign warts to aggressive cervical cancer, are caused by human papillomaviruses (HPVs). It has been established that HPV is the causative agent of oropharyngeal and urogenital malignancies. 120 types of HPV have been characterized (Morshed *et al*., 2014; Graham, 2010). Among these HPV genotypes, only 12, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, are considered types of high risk HPV (HR-HPV) (Van Doorslaer et al., 2012).

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The HPV genome comprises three regions: First, the early region, which comprises over half within the genome and consists of E1, E2, E4, E5, E6, and E7; second, (L) the late region that represents 40% of the genome, comprising L1 and L2; and third, the genomic regulatory area, that accounts for the remaining 10% of the genome (Hafkamp *et al*., 2004). Viral oncoproteins E6 and E7 are responsible for the proteolytic cleavage of p53 and the inactivation of retinoblastoma (pRB) protein, respectively (Dunne and Park, 2013).

Approximately 70% of all cases of cervical cancer cases worldwide are caused through HPV 16 and 18 types. The true incidence of cervical cancer among Pakistanis remains undetermined. The HPV positivity rate among cervical carcinoma patients varies from 18% to 98.33% (Yousuf *et al*., 2010; Khan, 2010; Dunne and Park, 2013). This variability is attributed to poor documentation regarding screening, vaccination, and epidemiology of the disease (Asiaf *et al*., 2014; Gul *et al*., 2015). Cervical cancer is reported to be the third most prevalent type of cancer in Pakistan's female population. About 0.5% of women have HPV 16/18 at any given moment, and types 16/18 are responsible for 88.1% cases of extremely

transmissible cervical cancer (Bruni *et al*., 2023) Additional data regarding the effectiveness and safety of HPV vaccinations remain unavailable in Pakistan and Asia. Four separate lineages A, B, C, and D are used to further classify the HPV16 intratypic variants according to the geographic origin of the individuals which they have been isolated. Lineage A is categorized as three Europeans sub lineages i.e. A1, A2, A3 and One Asian sub lineage (A4). And lineage B is further subdivided in two sublineages of Africans (B1 and B2), While lineage C likewise contains African Sequences, sequences from Asia and North America are found in D1, D2 and D3 sub lineages that make up Lineage D (Ramas *et al*., 2018).

This study's main goals were to assess the sequence variations among HPV16's E6 and E7 oncogenes by comparing the sequences found in our investigation to previously published sequences from various parts of the world and figuring out the phylogenetic relationship between sequence variants from Pakistan and those from other countries.

Materials and methods

Cervical swabs were obtained from 132 cervical cancer patients aged 35-72 years at various hospitals in Lahore, Pakistan. These samples were processed at the Centre of Excellence in Molecular Biology's Virology Laboratory at Lahore.

HPV DNA extraction was conducted using ethanol precipitation. A real time PCR method, employing th14 HPV types of detection kit (Healgen) was used to identify high-risk HPV types. This kit can detect 14 HPV types (16, 18, 31, 33, 35, 39, 45, S1, 52, 56, 58, 59, 66 and 68). Samples testing positive for HPV-16 were chosen for further sequence study.

The specific coding area primers were used to amplify the HPV E6 and E7 genes independently (Table I) at an annealing temperature of 58°C. Samples negative for both E6 and E7 were excluded from subsequent analysis.

Table I. Primer sequences for the amplification of *HPV16 E6* and *E7* genes.

Primer	Primer sequence
HPV-16 E6	F 5′ -ATGCACCAAAAGAGAACTGC- 3′
	R 5′-TTACAGCTGGGTTTCTCTACG-3′
HPV-16 E7	F 5′ - ATGCATGGAGATACACCTAC- 3′
	R 5′ -TTATGGTTTCTGAGAACAGATGGG-3′

PCR products underwent purification using the PureLink PCR purification kit. Sequencing was performed bidirectionally using the BigDye Terminator Sequencing Kit on an ABI PRISM 3100 genetic analyzer (applied biosystems) to confirm the PCR-amplified product. The resulting sequences were submitted to the *NCBI* gene bank database.

Sequence analysis was conducted using BLAST from PubMed. Phylogenetic trees for HPV16 were constructed using E6 and E7 sequences acquired throughout this investigation.The evolutionary history was inferred using the Maximum Likelihood method based on the general time reversible model (Nei, 2000). Reference sequences for 10 sublineages were taken from the Gene Bank database (www.ncbi.nlm.nih.gov). These included: A1: K02718 and KU298880, A2: AF536179, FJ610152 and KU053892, A3: HQ644236, A4: AF534061, HQ644234 and HQ644261, B1: AF472508, AF536180 and HQ644296, B2: HQ644298, C: AB818690 and AF472509, D1: HQ644257, D2: Ay686579, D3: AF402678, HQ644285 and HQ644289.

Results and discussion

Among 132 women with cervical cancer, HPV16 was identified as the most common type, present in 61 cases. Pakistani HPV16 isolates E6 and E7 gene sequences were analyzed for similarity and submitted to the NCBI Genebank database. The local E7 gene was assigned the accession number MH884521, while MT955329 was given to the local E6 gene.

The maximum likelihood method and general time reversible model (Nei, 2000) were employed to infer evolutionary history. Figure 1 illustrates potential nucleotides at each ancestral node based on their inferred likelihood. The heuristic search's initial trees were obtained using the Maximum Parsimony approach. Site rates were treated as a Gamma distribution with invariant sites, utilizing 5 Gamma categories. The analysis involved 21 nucleotide sequences, including all codon positions. The final dataset contained 477 positions for E6 and 297 for E7. MEGA11 (Tamura *et al*., 2021) was used for evolutionary analyses.

The Papillomaviridae family includes HPV, comprising 29 genera with 189 PV types isolated from various species (Morshed *et al*., 2014). The double stranded DNA that makes up HPV genome of approximately 8kb, containing 8 genes: E1, E2, E4, E5, E6, E7, L1, and L2 (Zheng and Baker, 2006; Morshed *et al*., 2014; Amador-Molina *et al*., 2013; Hafkamp *et al*., 2004). Oncoproteins, E6 and E7 remain crucial for initiating plus maintaining HPV-associated malignancies and, are expressed themselves in cells that have undergone transformation (Yang *et al*., 2016).

Identifying variants of HPV are crucial for creating testing methods, vaccinations, and therapies (Pande *et al*., 2008). Previous research has linked HPV variant

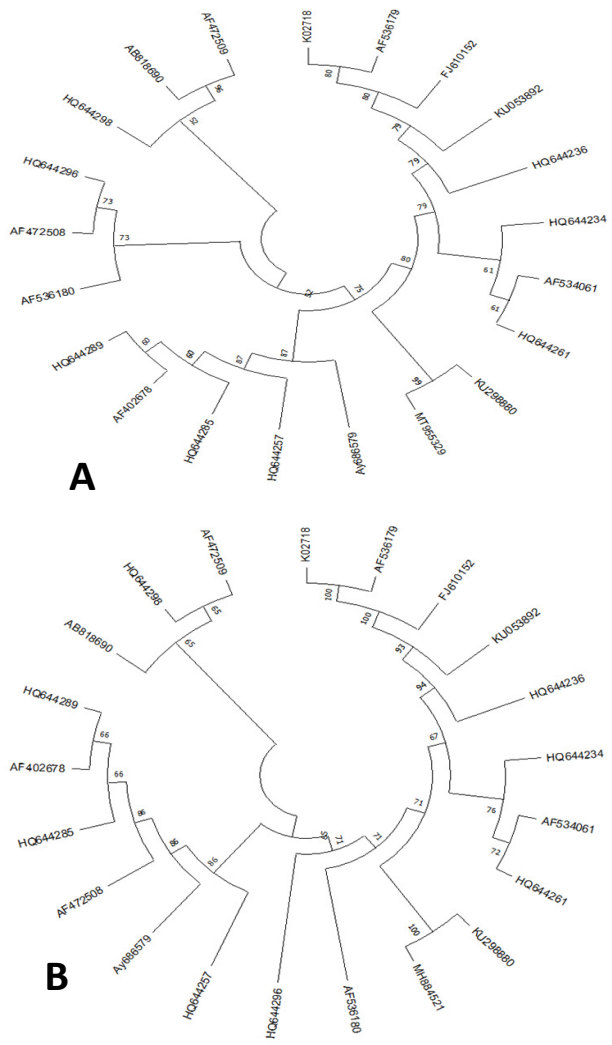

Fig. 1. Phylogenetic tree of *E6* gene (A) and *E7* gene (B) of different HPV16 isolates.

distribution to geographic or racial factors (Xi *et al*., 2006; de Araujo Souza *et al*., 2009). The purpose of this study was to show how various HPV16 lineages are distributed throughout the Pakistani population, following Yamada *et al*. (1997) classification.

Cervical cancer ranks as the third most prevalent cancer among women globally, with an estimated 569,847 new cases and 311,365 deaths in 2018. Squamous cell carcinoma is the most common type, followed by adenocarcinomas (Forman *et al*., 2012). In Pakistan, 2012 estimates revealed that the prevalence of HPV related cancers was 7.9%, whereas the combined prevalence of ano-genital and head and neck malignances was 0.5% (De Martel *et al*., 2017). The ICO/IARC HPV information centre reports that approximately 5,601 new cervical cancer cases are diagnosed annually in Pakistan. Cervical cancer is the third leading cause of cancer and cancer-related deaths among Pakistani women overall, and the second most common in the 15-44 age group (Ferlay *et al*., 2018).

While HPV is a significant factor in cervical cancer development, it is not the sole cause. Several other contributing factors have been identified, including cigarette smoking, extended use of hormonal contraceptives, multiple childbirths, and HIV co-infection. Additional risk factors encompass co-infections with Chlamydia trachomatis and herpes simplex virus type-2, compromised immune systems, and specific nutritional deficiencies. The development of cancer can also be influenced by genetic and immunological host factors, as well as viral characteristics such as the type of virus, viral load, and viral integration (Muñoz *et al*., 2006).

The samples we collected were classified within the sublineage A1 of HPV16's alpha9 species. The Pakistani HPV16 isolate showed 99% sequence similarity with Brazilian isolates (the sequence of reference KU298880 for the A1 sublineage). This grouping was supported by a strong 91% bootstrap value. In Pakistan, HPV16 was also categorized into the alpha9 species group in a previous comprehensive study on HPV classification (Abdullah *et al*., 2016).

Given that sexual contact remains the primary mode of HPV transmission, socio-cultural obstacles hinder the accurate assessment of HPV prevalence in Pakistan. Limited awareness results in few women opting for HPV screenings, allowing HPV infections to go undetected until they progress to more severe conditions. Phylogenetic analyses play a crucial role in mapping the groups to which HPV isolates belong, as they can help identify regions that may be potential sources of HPV spread within a country.

Conclusion

The main cause of CC in females, HPV, is estimated to be responsible for 3,000 fatalities annually. A phylogenetic study of the genes E6 and E7 sequences about Pakistani HPV16 isolates of CC associated with HPV were this study's findings. The HPV16 strain from Pakistan shared 99% of its sequence with isolates from Brazil. In order to create tools for molecular diagnosis in the Pakistani population, phylogenetic analyses are essential.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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