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High-Risk Human Papillomavirus Genotypes and Their Association with Nasopharyngeal Carcinoma: A Case-Control Study in Southeast Iran

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ABSTRACT

Introduction: Nasopharyngeal carcinoma (NPC) is a multifactorial malignancy whose etiology involves complex interactions among genetic, environmental, and infectious factors. Although Epstein–Barr virus (EBV) is a well-established etiological factor in NPC, the potential involvement of human papillomavirus (HPV) remains controversial. The aim of this study was to determine the prevalence and genotypic distribution of HPV in NPC patients in the Kerman Province in the south-east of Iran.

Methods: A case control study was performed with 100 tissue samples of formalin fixed paraffin seeded tissue collected between 2010 and 2018, including 50 NPC cases and 50 benign nasopharyngeal controls. Using real-time PCR, HPV DNA was detected and genotyped for HPV types 6, 11 (low risk) and 16, 18 (high risk). Phylogenetic analyses were carried out to evaluate the genetic diversity of the HPV strains identified. Statistical analysis has been performed using SPSS v.22.

Results: HPV DNA was detected more frequently in male patients with NPC, with the highest prevalence observed in the age group 20-60 years. Among the NPC cases, 31 (62%) were male and 19 (38%) female, compared with 38% for the control cases. Among HPV-positive NPC cases, the most frequently detected genotype was HPV-18. Phylogenetic analysis has shown that there is a significant genetic diversity of HPV strains circulating in this area. Statistical analysis showed a significant association between HPV presence and the presence of NPC ($p<0.05$).

Conclusion: These results affirm further molecular investigations to determine whether the observed HPV genotype distribution has implications for regional prevention strategies.

Keywords: Nasopharyngeal carcinoma, Human papillomavirus type, Phylogeny, RT-PCR, Kerman.

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Introduction

Cancer remains the world's leading cause of death, accounting for nearly 10 million deaths in 2024 (1). Nasopharyngeal carcinoma (NPC) is a rare cancer of the head and neck with unique epidemiological and etiological characteristics (2). According to the IARC GLOBOCAN Report 2022, which be published in 2024, there are an estimated 120,434 new NPC cases worldwide (3). Several demographic and environmental factors sucha as age, gender, genetic susceptibility, ethnicity and geographic location have been shown to be consistently associated with the risk of developing NPC in high-risk populations (4, 5). Furthermore, dietary factors such as consumption of fish preserved in salt and foodstuffs containing nitrosamines were identified as independent risk factors, especially in endemic areas (6). Occupational exposure to dust from wood fires, formaldehyde and other chemical carcinogens is also a factor (7).

Epidemiological studies have shown significant geographical variation in the incidence of NPC. Particularly high rates have been reported in the South China and Hong Kong, as well as in parts of Southeast Asia, including Malaysia, Indonesia, Brunei, Vietnam, and Singapore (7-10). An estimated 133,354 new cases and 80,008 deaths are expected worldwide by 2020, with almost half of these cases and deaths occurring in East Asia, mainly China. The incidence and mortality rates were approximately three times higher in men than in women, and the highest incidence and mortality rates were in Southeast (SE) Asia by age group (11). In addition, in 2022, the incidence and mortality of NPC was higher in men than women in all SE Asian countries, underlining the strong geographic and gender-specific variation in epidemiology of NPC in the region (12). In the Middle East, countries such as Afghanistan, Tajikistan, Azerbaijan and Lebanon report a slightly higher prevalence while in Iran the prevalence of NPC is relatively low and remained constant (13, 14). A comprehensive analysis of cancer registry data for Iran 2003-2017 showed a stable incidence trend with significant regional variation, with higher rates in the northern provinces than in the southern ones (13).

Viral oncogenesis is a major cause of cancer in humans and includes infectious agents such as hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus (EBV), human papillomavirus (HPV), and human T-cell lymphocytic virus type 1 (HTLV-1) (15, 16). Papillomaviruses belong to the HPV family and are non-enveloped, double-stranded DNA viruses that infect the skin, mucous membranes and basal epithelial cells. More than 450 HPV genotypes have been identified for their oncogenicity and are classified as either high-risk (HR-HPV) or low-risk (LR-HPV). HPV is causally associated with all anal cancer, including cervical, vulvar, vaginal, anal, penile and anal carcinomas (17). The biological plausibility of HPV involvement in NPC pathogenesis is supported by a number of lines of evidence (18, 19). An updated systematic review and meta-analysis of HPV prevalence in the NPC showed that HPV-16 and HPV-18 were the the most frequently detected genotypes in NPC (18). According to a retrospective study, the NPC in Ghana has a comparatively high prevalence of HPV-18 and HPV-31, and its histopathology is comparable to that found in endemic regions (20). Mirzamani. et al., (2006), in Iran, detected HPV sequences in only 20 % of Iranian NPC cases in Tehran by hybridisation with PCR-South Blot (21). In addition, in a case-control study in Kerman, Iran, HPV DNA was detected in 27 of 101 patients with head and neck squamous cell carcinoma, HPV-11 was detected in 14.9 %, and HPV-16 and HPV-18 were detected in 11.9 % of patients with head and neck squamous cell carcinoma (SCC) (22). However, the specific role of HPV in NPC is not clearly elucidated.

These significant geographical differences therefore highlight the importance of examining population-specific factors that may contribute to the susceptibility and burden of non-nepal carriers. However, although Iran is a country with significant regional variation in the incidence patterns of cancer, molecular epidemiological data on PN are still remarkably limited. Critically, no study has specifically examined the prevalence and distribution of HPV in NPC patients in South-Eastern Iran. The Kerman province, situated in south-eastern Iran, is a particularly important but under-exploited area. Understanding the molecular epidemiology of the NPC in this population may provide insight into regional variation in viral etiology and inform public health strategies. Therefore, this study was

designed to determine the prevalence of HPV DNA by PCR in real time, identify and characterise HPV genotypes, analyse demographic and clinical correlates of HPV positivity, and perform phylogenetic analysis to assess the genetic diversity and evolutionary relationship of the detected strains in the population.

Materials & Methods

Study Design and Setting

This case control study was conducted from 2010 to 2018 at the Shah Faha Hospital in Kerman, Iran, to investigate the prevalence and genotypic distribution of HPV in NPC.

Participants and Sample Selection

The population of this case-control study included all patients with suspected nasopharyngeal carcinoma who were admitted to Shafa Hospital in Kerman between 2010 and 2018 and who underwent a diagnostic biopsy. Tissue samples were taken from surgical samples stored in the pathology lab. Written informed consent has been obtained from these patients prior to the use of their tissue samples for research purposes. All participants have been assured of the confidentiality of their personal dataThe sufficient sample size for estimation of the difference in HPV prevalence in tissue samples was calculated by equation to determine the sample size for comparison, with a HPV prevalence assumption of 44% in the control group and 56% in the study group, error type 1 = 0.05 and power = 80. The minimum sample size for both the caseload and control groups was 76 samples, with 38 samples per group. However, a total of 100 samples were taken at random, 50 from each group.

Inclusion and Exclusion Criteria

Based on pathological findings, the patients were divided into two groups. The case group consisted of patients with a histopathologically confirmed diagnosis of nasopharyngeal carcinoma, while the control group included patients with no evidence of nasopharyngeal carcinoma as measured by pathology. In this case control study, inclusion criteria for NPC patients included a histologically confirmed diagnosis of nasopharyngeal carcinoma, availability of adequate tissue blocks of formalin-fixed paraffin of sufficient quality for DNA extraction, and age of the patient at least 18 years. Exclusion criteria included lack of tissue samples for molecular analysis, prior radiotherapy or chemotherapy before the biopsy, concomitant malignancies, and recurrent cases of NPC. The control group consisted of age (±5 years) and gender matched subjects with histologically non- malignant nasopharyngeal lesions, no history of malignancy of the head or neck and adequate samples of FFPE tissue.

Histopathological Assessment

A total of 100 tissue samples, including 50 malignant nasopharyngeal carcinoma samples (the case group) and 50 benign nasopharyngeal carcinoma samples (the control group), were analysed after being fixed with paraffin. All samples were sent to the virology lab at the Kerman Medical School for molecular analysis.

Ethical Considerations

The confidentiality of the participants was strictly preserved throughout the study and all personal identifiers were anonymised. The study was conducted in accordance with the Helsinki Declaration and was approved by the Kerman University Ethics Committee (IR.KMU.REC.1398.140). The Ethics Committee waived written informed consent due to the retrospective nature of the study using archived tissue samples.

FFPE Tissue Processing and DNA Extraction

Genomic DNA was extracted from tissue samples following deparaffinization with xylene. Tissue slices were treated with Gesylol in microtubes, followed by centrifugation, ethanol washing, and extraction using the PreP DNA-RIBO extraction kit. The quality and integrity of the extracted DNA were verified using agarose gel electrophoresis.

HPV Detection by Real-Time PCR

HPV DNA detection was performed using G5+ and G6+ primers (Pishgam Company, Iran), targeting the L1 region of the papillomavirus genome. Primer sequences and detailed information are presented in Table 1. Real-time PCR assays were conducted according to the manufacturer's protocols, and samples were analyzed in triplicate to ensure reliability.

Table 1. G5+, G6+, MY11 and MY09 primer sequences

target gene	primer sequence
G5+	TTTGTTACTGTGGTAGATAC
G6+	GAAAAATAAACTGTAAATCA
MY11	GCMCAGGGWCATAAYAATGG
MY 09	CGTCCMARRGGAWACTGATC

Table 2. Gender and age frequency in the case (NPC) and control groups

		Control group	NPC group
Gender	Female	22 (58%)	19 (38%)
	Male	28 (42%)	31 (62%)
	Total	50 (100%)	50 (100%)
Age	<20 Year	8 (16%)	6 (12%)
	20-40 Year	19 (38%)	10 (20%)
	40-60 Year	20 (40%)	28 (56%)
	>60 Year	3 (6%)	6 (12%)
	Total	50 (100%)	50 (100%)

* Data are presented as numbers and percentages. Comparisons between NPC and control groups were performed using the chi-square test for categorical variables (gender and age groups). No statistically significant differences were observed between the groups ($p > 0.05$).

HPV Genotyping and Sequencing

The L1 protein, critical in HPV infection and pathogenesis, was the target for genotyping and sequence analysis. Sequencing of the L1 region was performed, and chromatograms in .ab1 format were processed using Chromas software. Sequences were compared with reference sequences from the NCBI database using BLAST.

Phylogenetic Analysis

Phylogenetic trees were constructed with MEGA 4 software to assess genetic diversity, evolutionary relationships, and clustering of HPV strains. Branch lengths indicated sequence divergence, and bootstrap values were used to evaluate confidence in the groupings.

Statistical Analysis

Statistical analyses were performed using SPSS software version 22 (SPSS Inc., Chicago, IL, USA). The prevalence of HPV infection between NPC and control groups, as well as the distribution of HPV genotypes, was compared using the Chi-square test. Associations with clinicopathological variables (age, sex, histological type) were also assessed. 95% confidence intervals (CI) were calculated for prevalence estimates where appropriate. A *p*-value < 0.05 was considered statistically significant.

Results

Gender and age distribution in the case (NPC) and control groups

Table 2 shows demographic characteristics of the study subjects. The study included 50 patients with nasopharyngeal carcinoma (NPC) and 50 age-matched controls. Within the NPC group, men were the majority (31 out of 50, 62.0%), followed by 19 women (38.0%). A more balanced gender distribution was observed in the control group, with 28 males (56.0%) and 22 females (44.0%).

Regarding age distribution, both groups showed a predominance of participants in the middle-age range. In the NPC group, 28 cases (56.0%) were aged 40–60 years, followed by 10 cases (20.0%) aged 20–40 years, and 6 cases each (12.0%) in the <20 and >60 years groups. In the control group, the 40–60 years category also represented the largest proportion (20/50, 40.0%), although a higher %age of controls were younger: 19 (38.0%) were aged 20–40 years, 8 (16.0%) were <20 years, and only 3 (6.0%) were >60 years. The age distributions of cases and controls were generally similar, with most participants in both groups being between the ages of 20 and 60.

HPV DNA test distribution in the case (NPC) and control groups

Table 3 summarises the distribution of HPV DNA positivity in NPC cases and controls stratified by age group. HPV DNA was found in 28 out of 50 cases (56.0%) in the NPC group, whereas 22 cases (44.0%) had negative results. The age distribution of NPC cases revealed that the 40–60 age group had a clear predominance (28/50, 56.0 %), followed by the 20–40 age group (10/50, 20.0 %). <20 years (6/50, 12.0 %), and > 60-year-old groups (6/50, 12.0%). The >60 year group had the highest HPV positivity rate among NPC cases (4/6, 66.7 %), followed by the 20–40 year group (6/10, 60.0 %), the 40–60 year group (16/28, 57.1 %), and so on. <20 years (2/6, 33.3 %) groups. HPV DNA positivity was less frequently observed in patients >60 years of age (n=4; 8% positive), however, the small number of patients in this age group makes it difficult to make a definitive interpretation of trends in age.

Table 3. HPV DNA test in the case (NPC) and control groups

HPV distributions	Control group			NPC group		
	Negative N (%)	Positive N (%)	Total N (%)	Positive N (%)	Negative N (%)	Total N (%)
<20 Year	8 (16 %)	0 (0 %)	8 (16 %)	2 (4 %)	4 (8%)	6 (12%)
20-40 Year	19 (38 %)	0 (0 %)	19 (38 %)	6 (12%)	4 (8%)	10 (20%)
40-60 Year	18 (36 %)	2 (4 %)	20 (40%)	16 (32%)	12 (24%)	28 (56%)
>60 Year	3 (6 %)	0 (0 %)	3 (6%)	4 (8%)	2 (4%)	6 (12%)
Total	48 (96 %)	2 (4 %)	50 (100%)	28 (56%)	22 (44%)	50 (100%)

* Data are presented as numbers and percentages. HPV status was compared between NPC and control groups using the chi-square test. A statistically significant higher prevalence of HPV positivity was observed in the NPC group compared to the control group ($p < 0.05$).

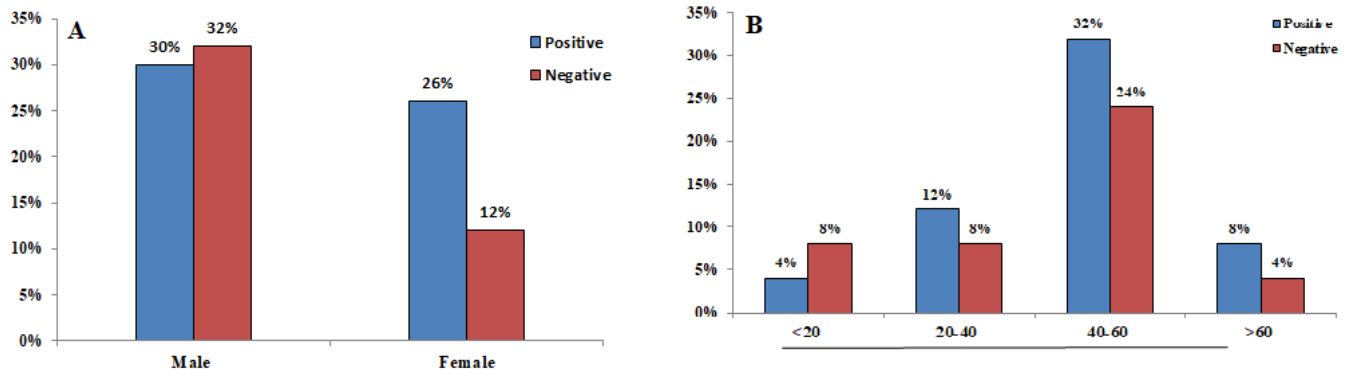

Figure 1. HPV distribution by gender (A) and age group (B) in patients with NPC.

In the control group, HPV DNA positivity was markedly higher, with 48 out of 50 individuals (96.0 %) testing positive and only 2 (4.0 %) testing negative. The age distribution in controls was relatively even across the middle age ranges, with 20 participants (40.0 %) in the 40–60 years group, 19 (38.0 %) in the 20–40 years group, 8 (16.0 %) in the <20 years group, and 3 (6.0 %) in the > group of 60 years. HPV positivity exceeded 94 % in all age strata among controls, with 100 % positivity observed in the <20, 20–40, and > groups of 60 years, and 90 % (18/20) in the group of 40 to 60 years. Overall, the prevalence of HPV DNA was significantly lower in cases (56.0 %) compared to controls (96.0 %) across all age groups, even though NPC cases were concentrated in the 40–60 age group.

HPV DNA distribution by gender and age in patients with NPC

Figure 1 shows the distribution of HPV DNA positivity among patients with nasopharyngeal carcinoma (NPC) by gender and age. As shown in Figure 1-A, HPV DNA was detected in 30% (n=15) of male patients and 26% (n=13) of female patients. Negative cases accounted for 32% (n=16) of males and 12% (n=6) of females. Overall, 56% of the study population tested positive for HPV DNA, with a slight male predominance among HPV-positive cases.

Figure 1 shows the distribution of HPV DNA positivity by gender and age in nasopharyngeal carcinoma patients. As shown in Figure 1-A, 30 % (n=15) of male patients and 26 % (n=13) of female patients had HPV DNA detected. Negative cases were found in 32 % (n=16) of men and 12 % (n=6) of women. Overall, 56 % of the study population tested positive for HPV DNA, with a slight male prevalence in HPV positive subjects. Figure 1-B shows the age distribution of the HPV DNA. The highest rate of HPV positive cases was observed in the age group aged 40-60 years (32%, n=16), followed by the age group aged 20-40 years (12%, n=6), the age group aged >60 years (8%, n=4), and the age group aged <20 years (4%, n=2). Within the negative group, the 40-60 years group was also the most frequent (24 % of n=12) while the <20, 20-40 and >60 years groups accounted for 8%, n=4, 8%, n=4, and 4%, n=2 of the negative cases, respectively. These findings suggest that most HPV positive and HPV negative NPC cases occurred in the middle age population, with a clear peak in the age range of 40-60 years.

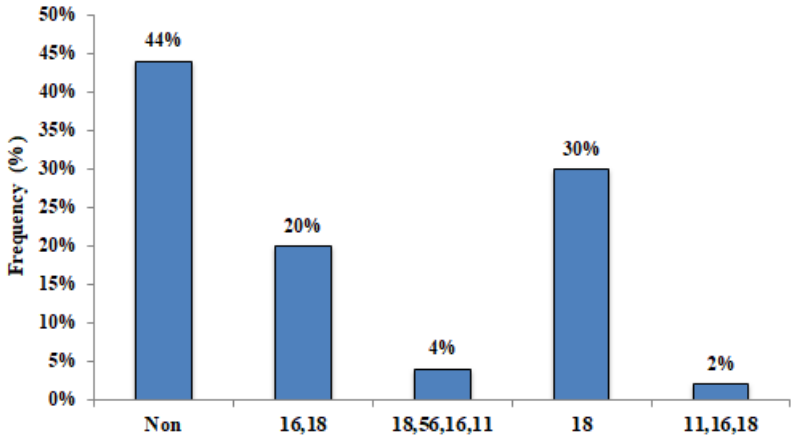

Figure 2. Distribution of HPV types among NPC patients. Data are presented as the number and percentage of patients infected with each HPV type.

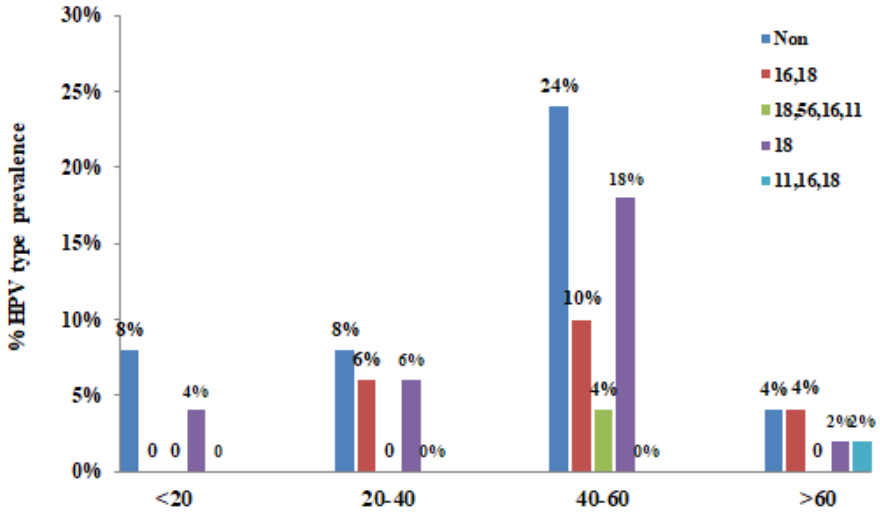

Figure 3. Prevalence of different HPV types stratified by age groups. Data are presented as the percentage of HPV-positive individuals within each age group.

Distribution of HPV genotypes in patients with NPC

The genotype distribution among HPV DNA-positive samples is presented in Figure 2. Of 76 HPV positive samples collected in the study (28 in the NCP group and 48 in the control group), the most common category was Non - representing infections with HPV genotypes other than the specifically tested or untyped strains (44 %, n=22). The most frequently high-risk genotype detected was HPV18, accounting for 30 % (n=15) of the total number of infections. 20 % (n=10) of the positive samples were observed to be infected with HPV16 and HPV18. Multiple infections with HPV18, 56, 16 and 11 were detected in 4 % of the cases (n=4), while combinations with HPV11, 16 and 18 were the least common (2 %, n=2).

Distribution of HPV genotypes by age in patients with NPC

Figure 3 shows a different pattern of prevalence in the four age groups, with the highest burden observed in middle-aged individuals (aged 40-60 years). The overall prevalence was low in participants under 20 years of age. The category of non (representing HPV types other than those identified or HPV negative samples) was 8 %, while the combination of 11,16,18 was detected in 4% of cases. No infections of genotypes 16, 18, 18, 56, 16, 11 or single 18 were observed in these groups. In the age group 20-40, the category of Non remained stable at 8. The prevalence of combination 16,18 and single 18 genotypes was 6 %. No other genotypic combinations have been identified.The highest prevalence was observed in the age group 40-60 years. The category of non-answers was 24 %, followed by genotype 18 at 18, 16,18 at 10 and 18,56,16,11 at 4 %. This age group was the highest prevalence of high risk HPV genotypes, in particular HPV18-associated infections. HPV

prevalence decreased significantly in the age group >60 years. A category of non-susceptible was detected in 4 % of samples, while a single 18 genotype, 18,56,16,11 and 11,16,18 combinations were both detected in 2 %. Overall, HPV genotype 18 (alone or in combination) was the most commonly detected high-risk type in all age groups, with the highest prevalence (18 %) in the age group 40-60 years. Among patients >60 years of age (n=9), HPV was detected in 3 cases and mixed genotypes were observed; however, due to the small size of the subgroup, interpretation is limited.

Phylogenetic analysis

To confirm the identity and phylogenetic placement of the detected HPV18 strains, sequencing of the L1 gene was performed on representative positive samples**.** The resulting phylogenetic tree (Figure 4) showed that the two sequences isolated in this study, identified as HPV18-HPF H08 08 Kerman and HPV18-HPF E09 05 Kerman, clearly clustered in the HPV18 clade. These sequences showed close genetic affinity for several reference HPV18 L1 sequences stored in GenBank, with nucleotide identicity between 91 and 99 % for the nearest reference strains. Sequencing of HPV-18 positive samples (n=15) revealed 6–7% nucleotide divergence from the HPV-18 reference sequence (GenBank accession X05015). Therefore, tree topology suggests that Kerman isolates belong to established HPV18 lineage and do not represent novel distinct variants.

Discussion

This study investigated the prevalence and molecular properties of HPV in cases of NPC. Our findings revealed a predominance of male patients (62%) and a clear peak incidence within the age group of 40-60 years (56%). HPV DNA in 28 of 50 (56%) NPC cases compared with 2 of 50 (4%) benign controls, indicating a statistically significant association between HPV prevalence and NPC development. Moreover, the genotyping analysis revealed that HPV18 was the most frequent high-risk genotype, accounting for 30% of all infections as monotype and contributing to

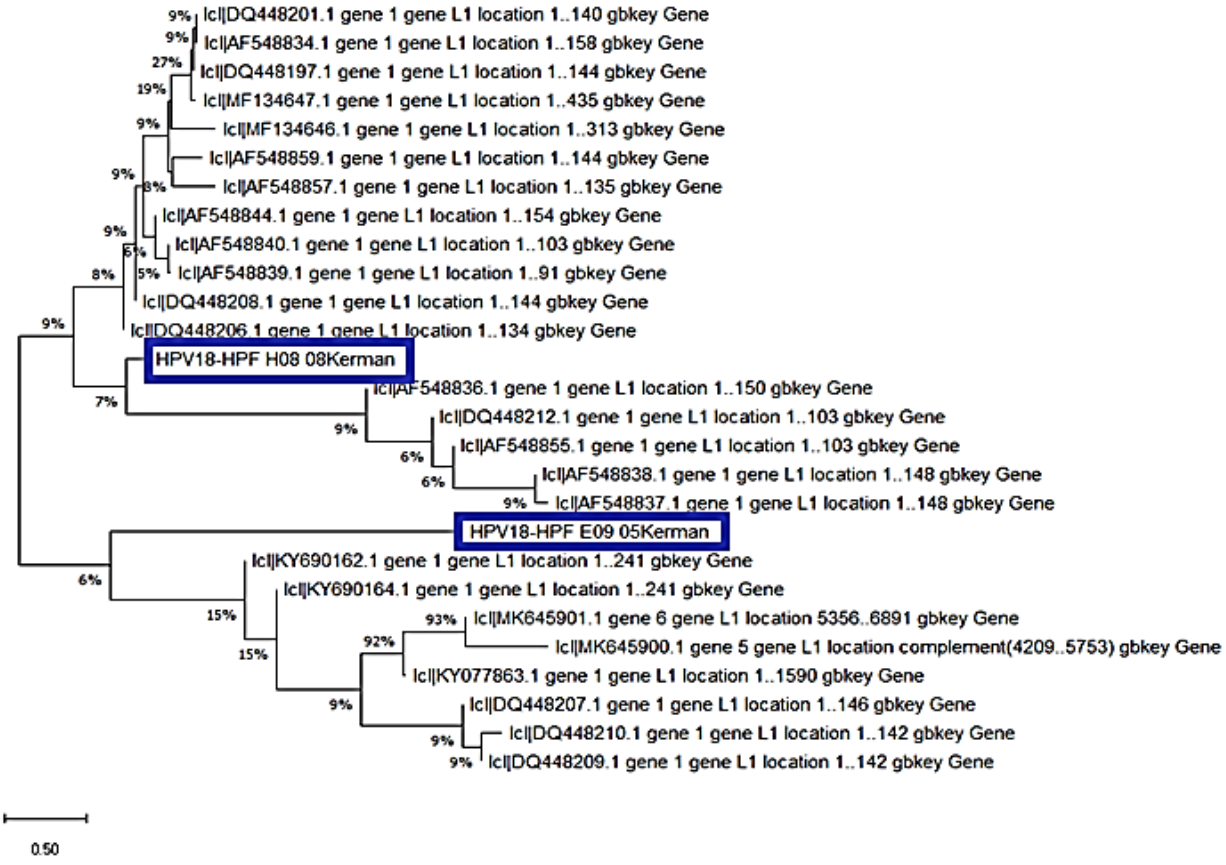

Figure 4. Phylogenetic tree of HPV L1 sequences from NPC patients, aligned with reference strains and constructed using Neighbor-Joining with 1,000 bootstrap replicates (values ≥70% shown). PCR products were purified, bidirectionally sequenced, and processed in Chromas. Sequences were compared to NCBI references via BLAST, and alignment and phylogenetic analyses were performed in MEGA X to assign HPV genotypes based on sequence similarity.

more than half of the positive results when cross-infection was included. Phylogenetic analysis of the L1 gene confirmed that two representative HPV18 isolates (HPV18-HPF H08 08Kerman and HPV18-HPF E09 05Kerman) in the population are closely related to established reference strains in the wild-type HPV18 family, indicating that they are not new strains but belong to the same family of the same strain.

Nasopharyngeal carcinoma (NPC) is one subgroup of head and neck cancers in the world. In 2018, NPC represented less than 1 percent of all cancers; more than 70 percent of the NPC incidence is reported in East and Southeast Asia alone (23, 24). However, the rarity of the NPC and the high geographical variability leave limited data compared to its EBV-associated counterparts. The lack of comprehensive data on NPC in specific populations highlights the importance of further epidemiological and molecular research in order to facilitate the early detection of the disease and the development of effective treatment strategies. Although HPV is a viral risk factor for NPC (25), the role of HPV-associated NPC is still limited and its pathological and epidemiological characteristics are not well defined.

The significant difference between NPC (56%) and controls (4%) patients in our study reinforces the evidence that HPV is an important etiological factor in the development of NPC. Moreover, the 56% HPV prevalence observed in our study of the NPC is significantly higher than the rates reported in the previous studies. Several factors, including geographic location and genetic susceptibility differences between populations, may explain regional differences in HPV prevalence. Moreover, the detection method (PCR) used in our study may have an effect on the observed prevalence. Comprehensive systematic review and meta-analysis by Zhao et al. (2024) reported the prevalence of HPV in the NPC variable according to geographical location, methods of detection and histological subtypes (18). Therefore, This technical consideration should be taken into account when comparing our results with those of studies using other detection platforms. However, previous studies in North America and Asia have shown that the prevalence of HPV is highest in North America (25%) and lowest in Asia (13%) in the non-NPC endemic region (18). Several reports on HPV-associated NPC in Japan indicate that although the incidence of HPV-associated oropharyngeal cancer has increased worldwide, HPV-associated NPC in Japan is uncommon and has a relatively stable epidemiological profile. This is an important finding of the study, which suggests that HPV plays a minor role in NPC pathogenesis in Japan (19, 26-28). In Iran, NPC is considered to be rare and its prevalence is still poorly characterised. Safavi and al. (2004-2009) reported an incidence rate of 0.38 per 100,000 people according to the National Cancer Database System registry (29). A newer study (2003-2017) showed that although the incidence of NPC showed an increasing trend in previous years, this trend has stabilised in recent years (30).

Our findings also indicate a higher prevalence of NPC in men, which is consistent with the established global epidemiological trend of male dominance (31-35). The previous literature has shown that men are disproportionately affected, with the incidence rate usually three to five times higher than that of women, and has highlighted the important gender gap in the burden of the disease (11, 34). For example, data from China for the year 2021 show a male incidence of 5.16 per 100 000 and a female mortality of 2.32 per 100 000 cases (36). Although the exact etiology of this difference remains unclear, it is thought to be a combination of lifestyle factors (e.g. tobacco and alcohol consumption, combined with genetic predisposition, hormonal effects and different occupational and environmental exposure (7, 32, 37-39). These findings underline the need for multidisciplinary research to further elucidate these complex biological, behavioural and social determinants.

Our findings show a clear non-modal pattern in the age distribution of the NPCs. Specifically, the highest frequency was observed in the control group in individuals aged 20-40 years, while the NPC group was highest in the age group 40-60 years (56%). This age distribution pattern is consistent with the natural history of HPV infection observed in other anatomical sites, where acquisition typically occurs in younger adults and malignant transformation may occur years to decades later. However, as sexual behavior data were not collected in this study, we cannot determine whether the observed age-related HPV prevalence reflects differences in exposure patterns, immune clearance capacity, or

time to oncogenic transformation. Future prospective studies incorporating behavioral risk factor assessment are needed to clarify the mechanisms underlying age-specific HPV prevalence in NPC. This is in line with previous reports by Safavi Naini et al. (2015), which also identified the age group of 40-60 as the most affected (29). In addition, recent national data show regional variations in the average age at diagnosis, from the lowest in the province of Semnan (36.9 years) to the highest in the province of Markazi (56.4 years) (29). Epidemiological data indicate that Iran, like other low-risk countries, has a bimodal distribution of NPC, with a minor peak in the 15-25 years range and a major peak in the 70 years range (30, 40). A recent study (https://doi.org) showed that the age effect has been demonstrated by an increase in the prevalence of NPC with age, which is highest in the 35-39 age group (41). This distribution may be due to a combination of genetic predisposition in adulthood and differences in exposure to environmental and viral risk factors. Regarding viral association, our study found 56 percent (28 out of 50) of the NPC cases to be HPV positive, compared with only 2 cases in the control group. This prevalence is notably higher than the prevalence reported in most recent studies (20, 42-45). For example, Du-Boi Asante and colleagues reported prevalence of HPV in China at 19.2% (14 of 72 cases) (20). These differences may be due to regional variation, sample characteristics or methodological differences in HPV detection.

Genotyping analyses revealed that the the most frequently detected genotype in our study was HPV-18 (30%), followed by combinations such as 16-18 ((20%), 11–16–18–56 (4%), and 6–11–18 (2%). These results are in line with previous studies identifying HPV18 and HPV16 as the primary high risk genotypes associated with NPC (20, 21, 36, 45-48). In the comparative genotyping studies of the PCN, the dominant genotypes were HPV 16 (44%) and HPV 18 (33%) genotypes (18).

In addition, phylogenetic analysis of the L1 gene of two HPV18 isolates identified in the Kerman population (HPV18-HPF H08 08 Kerman and HPV18-HPF E09 05 Kerman) revealed a nucleotide difference of 6-7% with the nearest reference sequences. Although these isolates are clearly clustered in the HPV18 clade, this level of variation represents considerable intra-type variability and is close to the 10% threshold used to differentiate HPV types from variants. This difference is notably higher than the 2-5% usually reported for global isolates of HPV18 (49, 50). This suggests a distinct regional evolutionary pathway, possibly due to geographical isolation, founder effects, or local host selection. This genetic diversity may have functional implications, including altered oncogenicity of E6 and E7 and potential effects on antigenic L1, which may influence the history of infection and the long-term effectiveness of current HPV vaccines against locally circulating strains.

The age-related patterns observed in this study have important clinical implications for optimising screening and prevention strategies. In particular, age-based risk stratification could improve the allocation of resources and support targeted HPV screening in middle-aged individuals at highest risk of HPV-related non-proliferation. In addition, these findings provide empirical support for early HPV vaccination programmes, which are a crucial precaution against infections that may become malignant decades later. The observed gender difference in the incidence of NPC can be attributed to several multi-faceted mechanisms. Hormonal factors represent a plausible biological explanation; indeed, some evidence suggests that oestrogens may play a protective role in women. However, it should be noted that many studies have not been able to establish a consistent association between specific environmental exposures and risk of NPD in females (32, 51, 52). In addition to hormonal effects, behavioural and occupational determinants contribute significantly to the observed gender differences. Established risk factors such as the prevalence of smoking and alcohol consumption in men play an important role in the pathogenesis of the disease. In addition, occupational exposure to carcinogens, including formaldehyde and wood dust, is more common in male-dominated sectors and is associated with an increased risk of NPD. Finally, genetic and immunological differences may further support the susceptibility of NPCs. This includes possible genetic factors related to the X chromosome and gender-specific variations in the immune response to viral infections (53-55). These interrelated biological, behavioural and

social determinants highlight the complexity of the epidemiology of NPCs and require ongoing multidisciplinary research.

Despite the findings presented, a number of limitations need to be recognised. First, the retrospective, single-centre design of this study may introduce selection bias and reduce the statistical power for the detection of small associations. Therefore, findings may not be fully representative of the wider population and have limited generalizability outside the area of study. Secondly, the design of the case control, combined with the fact that detection of HPV DNA alone cannot distinguish active oncogenicity from incidental viral presence, prevents the determination of a definitive causal relationship or time sequence. Thirdly, the study was limited in its assessment of potential confounding factors. In particular, the lack of data on sexual behaviour, tobacco and alcohol use, occupational exposure and socio-economic status prevents a reliable adjustment for variables that may independently influence the risk of NPC. Finally, while our HPV detection methodology has provided basic molecular data, the lack of integrated analyses - e.g. co-infection status with EBV or functional gene expression (e.g. E6 and E7) - limits our ability to fully characterise the complex viral pathology of the NPC in this population. However, these results provide a basic framework for future future multicentre investigations. A limitation of this study is the lack of detailed histopathological subtyping for all patients, as these data were not explicitly recorded in the retrospective pathology archives. Additionally, due to the retrospective nature of this study and the limitations of available archival pathological records, the exact histopathological subtypes of NPC were not explicitly documented. We acknowledge that the absence of detailed subtyping may limit our ability to further correlate HPV prevalence with specific histological patterns.

Conclusion

In conclusion, this study shows a strong association between HPV prevalence and NPC development, with a clear predominance of male patients. Our molecular analysis identified HPV18 as the primary high-risk genotype responsible for the emergence of these infections. Phylogenetic characterisation further confirms that the circulating HPV18 isolates are of established wild-type strains, which provides important epidemiological insight into the molecular structure of the NPC in this population. The lack of data on EBV co-infection, functional viral gene expression and possible environmental or behavioural confounds limits the interpretation of these findings. Therefore, these results should be considered hypothesis-based rather than definitive. Future research should use large-scale, multicentre longitudinal studies to characterise the co-infection pathways of EBV and HPV, to investigate functional viral activity and to assess the prognostic relevance of HPV status to clinical outcome. Finally, the regional distribution of high risk genotypes is essential for informing targeted public health interventions and optimising HPV vaccination strategies.

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Conflict of Interest:

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During the preparation of this work, the authors used Grammarly and ChatGPT to assist with editing and grammar. After reviewing and editing the content as necessary, the authors take full responsibility for the published article.

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