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Aspergillus Species and Human Papillomavirus Infections in Epithelial Tumors of Nasal and Paranasal Cavities

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Abstract

Background: There are diverse lesions originating from the paranasal sinuses and nasal cavity. Tobacco use, alcohol consumption, and malnutrition have been identified to play a role in the development of head and neck carcinomas. Recently, fungi and viruses have been recognized as potential causes of nasal cavity and paranasal tumors.
Objectives: This study aimed at specifying the prevalence of *Aspergillus* and human papillomavirus (HPV) infections in the epithelial tumors of nasal cavity and paranasal sinuses.
Methods: In this cross-sectional study, 57 paraffin-embedded tissue samples of malignant and benign lesions of the paranasal sinuses and nasal cavity were evaluated for the presence of *Aspergillus* and HPV DNA by nested polymerase chain reaction (nPCR) technique with specific primers.
Results: Despite the absence of angular hyphae (acute angle) of the fungus on histopathological slides, overall, 10 (17.54%) out of 57 paraffin-embedded samples were found to be positive for *Aspergillus* species. However, HPV-DNA was not found in any of the samples.
Conclusions: Our data suggest that fungal infections (especially aspergillosis) as an etiological factor can be contributed to the development of sinonasal cancer and, therefore, they should be considered in the management of patients with sinonasal cancer. In addition, PCR can provide an alternative to culture-dependent identification methods.

Keywords: *Aspergillus*, Human Papillomavirus, Nasal Cavity, Nested PCR, Paranasal Sinuses

1. Background

Lesions in the paranasal sinuses (sphenoid, frontal, ethmoid, and maxillary sinuses) and nasal cavity are mucosal protrusions, which can be classified as malignant and benign tumors. Malignancies of the sinonasal tract account for 3% of all cancers arising from the upper respiratory tract and 1% of all human tumors (1). Occupational exposures, particularly wood dust, have been listed as the major risk factors for sinonasal cancers (2, 3). However, tobacco smoking, a primary risk factor for carcinoma in most areas of the head and neck, has only shown a minor connection with sinonasal carcinoma (4).

Human papillomavirus (HPV) and *Candida* infection have been identified as the major etiological factors of head and neck carcinomas (5). In large cohort research, HPV infection was detected in 25% of head and neck squamous cell carcinoma (SCC) (6). Moreover, numerous reports have also discovered HPV in sinonasal tract malignancies. However, highly variable detection rates have cast doubt on HPV as a carcinogen at this location (ranging from 0 to 100%) (7-9). Similarly, the coexistence of fungal infections such as aspergillosis and malignancies has been observed in the thoracic cavity, maxillary sinus, and brain in several reports (10-12). A study by Hongal et al. (13) revealed a significant association between the higher grade of epithelial dysplasia and the presence of fungal hyphae in patients with oral potentially malignant disorders (OPMDs), including leukoplakia, lichen planus, and submucosal fibrosis.

Up to now, the clinical significance of *Aspergillus* and HPV infections in sinonasal carcinomas has not been estab-

lished and insufficient data are available regarding these sinonasal carcinomas in Iran.

2. Objectives

Regarding the ability of some fungi to cause dysplastic changes, the presence of some fungi in a series of sinonasal neoplasms, and the role of HPV in the development of head and neck carcinoma, the present study aims at assessing the prevalence of *Aspergillus* and HPV infections in epithelial tumors of the paranasal sinuses and nasal cavity in an Iranian population by molecular methods.

3. Methods

This cross-sectional study was conducted on all formalin-fixed, paraffin-embedded (FFPE) tissue samples extracted from the epithelial tumors of the paranasal sinuses and nasal cavity. The samples were in the archives of Khalili Hospital and Oral and Maxillofacial Pathology Department, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran. The study was concordant with all relevant principles of the Helsinki Declaration and was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1395.S877). Written informed consent was obtained from each participant.

3.1. Sample Preparation and DNA Extraction

Four sections of 5 μm thick were cut and collected in microcentrifuge tubes for each case. The QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from the FFPE tissue sections. The extracted DNA was, then, eluted with 50 ml ATE buffer and stored at -70°C. A NanoDrop (ND-1000) spectrophotometer was used to measure the total amount of DNA (peQLab Biotechnologie, Erlangen, Germany). As indicated by Saiki et al. (14), the quality of the extracted DNA for each sample was assessed by polymerase chain reaction (PCR) amplification, using PC03, PC04, b-globin gene-specific primers, generating a 110 bp PCR product (Table 1). For further analyses, only b-globin-positive samples were included.

3.2. Detection of Aspergillus

Aspergillus DNA was evaluated by nested PCR (nPCR) with 2 sets of primers. It is worth mentioning that this type of PCR can identify all *Aspergillus* species. The first primers of *Aspergillus* species were AFU7S and AFU7AS (405 bp), and the second primers were AFU5S and AFU5AS (236 bp) (Table 1) (15). PCR was performed under the following conditions: 5 min at 94°C for the denaturation step, followed by 35 cycles of the 30s at 63°C, and 8 min at 72°C for the final extension step (16). The PCR products were visualized by electrophoresis on 2.5% agarose gel, and staining with ethidium bromide and UV light.

Table 1. The Sequence of Primers [a]

Primer Names	Sequences (5' to 3')	Product Size (bp)
PC03	ACACAACTGTGTTCACTAGC	110
PC04	CAACTTCATCCACGTTCACC	
AFU7S	CGG CCC TTA AAT AGC CCG	405
AFU7AS	GA CCG GGT TTG ACC AAC TTT	
AFU5S	AGG GCC AGC GAG TAC ATC ACC TTG	236
AFU5AS	GG RGT CGT TGC CAA YYCC TGA	
MY09	GCMCAGGGWCATAAYAAYTGG	452
MY11	CGTCCMARRGGAWACTGATC	
GP5+	TTTGTTACTGTGGTAGATACTAC	140 – 150
GP6+	GAAAAATAAACTGTAAATCATATTC	
16E6F1	CTGCGACGTGAGGTATATGACTTT	405
16E6R1	TTGTCTCTGGTTGCAAATCTAACA	
16E6F2	GGTCGGTGGACCGGTCGATG	129
16E6R1	TTGTCTCTGGTTGCAAATCTAACA	
18E6F1	CACTTCACTGCAAGACATAGA	322
18E6R1	GTTGTGAAATCGTCGTTTTTCA	
18E6F2	ATGCTGCATGCCATAAATGT	139
18E6R2	CACCGCAGGCACCTTATTA	

[a] Degenerate base code: H = A, T, or C; M = A or C; R = A or G; V = G, A, or C; W = A or T; Y = T or C.

3.3. Detection of HPV

An nPCR assay using MY09/MY11 degenerated primer set followed by GP5+/GP6+ consensus primers was performed for the detection of 23 mucosotropic HPV genotypes, as previously described (Table 1) (17, 18). All the primers were custom synthesized by the Bioneer Company (Daejeon, Korea). Briefly, for the first round of PCR, 0.5 - 0.8 μg of purified DNA was added to Taq DNA Polymerase 2x Master Mix RED (Amplicon, Denmark) in a total volume of 20 μL containing 0.4 μM of each primer. Amplifications were performed in an MJ mini thermal cycler (Bio-Rad. Laboratories, USA) with the following conditions: 95°C for 3 min, 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by a 10-min final extension at 72°C. The secondary round of the assay was performed according to the touchdown PCR protocol described by Evans et al. (19). Briefly, 1 μL of the primary PCR product was added

to the reaction mixture with the same primer and reagent concentrations. DNA was amplified through a touchdown PCR program consisting of initial denaturation for 3 min at 94°C followed by 1 min at 94°C, 1.5 min at 55°C to 40°C with 1.0°C decrements, 1.5 min at 72°C (16 cycles) continued for 1 min at 94°C, 1.5 min at 40°C, and 1.5 min at 72°C for 25 additional cycles, and 5 min at 72°C as the final extension step.

To reduce the possibility of missing an infection caused by the 2 most frequent oncogenic HPV types 16/18, a type-specific nPCR technique was performed for detecting each type. The primer sets used for detecting HPV-16 and -18 were, respectively, 16E6F1/16E6R1 and 18E6F1/18E6R1, as the outer primers for the primary round of nPCR, followed by 16E6F2/16E6R1 and 18E6F2/18E6R2 primers, as the inner primers for the secondary PCR (Table 1). PCR for both HPV-16 and -18 was carried out under the same reaction mixture concentrations and cycling conditions. Briefly, PCR was carried out, using 0.5 - 0.8 μg of the extracted DNA as the template, 0.5 μM of each primer, and Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Denmark) in a total volume of 20 μL. The first cycle of PCR was preceded by a 3 min denaturation step followed by 40 cycles at 94°C for 1 min, at 55°C, and 72°C for 1 min. The last cycle ended with a 7 min extension step at 72°C. For the second round of the assay, the 20 μL PCR solution contained 1 μL of the first round PCR product as the template, 0.4 μM of each primer, and the reagents used for the first step. The parameters applied for programming the thermal cycler for 40 cycles included 3 min at 94°C, 45 sec at 94°C, 45 sec at 60°C, 45 sec at 72°C, and 5 min at 72°C. The primers used in this study for the identification of HPV have been described by Farhadi et al. (20).

4. Results

A total of 57 available samples were analyzed in this research. The mean age of the patients was 52.14 ± 16.93 years (range: 19 - 87 years). The frequency of nasal cavity and paranasal sinuses specimens were 26 (45.61%) and 31 (54.39%), respectively. Among the cases, 29 (50.88%) were male and 28 (49.12%) were female. The frequency of various lesions of the sinonasal tract has been summarized in Table 2 based on the location of lesions and patients' demographics.

According to the findings, HPV was not detected in any of the available samples. On the other hand, 10 cases (17.54%) were positive for *Aspergillus*, while the presence of any angular hyphae (acute angle) of the fungus was not confirmed on their histopathological slides. These samples included nasopharyngeal carcinoma (n = 4), undifferentiated carcinoma (n = 2), SCC (n = 1), calcifying cystic odontogenic tumor (CCOT) (n = 1), small cell neuroendocrine carcinoma (n = 1), and melanoma (n = 1). The demographic characteristics of the infected patients with *Aspergillus* and the location of lesions have been presented in Table 3.

5. Discussion

The failure to identify the key etiologic factors has hampered efforts to understand, prevent, and cure sinonasal tract tumors. Evidence has indicated that cigarette smoking is not highly linked to carcinomas that originate from the sinonasal tract, despite other parts of the head and neck area, where carcinogenesis is predominantly related to tobacco use. Wood dust exposure has also contributed to a subgroup of sinonasal adenocarcinomas and is not responsible for the majority of carcinomas found in the area (2, 3). Thus, awareness of the etiological factors contributing to the development and spread of the disease can be helpful for the early diagnosis of the disease.

The possible role of HPV infection in sinonasal carcinomas has been extensively investigated and its prevalence has been reported to range from 0% to 100% (21-24). In a review study by Syrjänen (25), the involvement of HPV in sinonasal carcinomas was observed in a large number of investigations. Similarly, a review study of the related articles including 220 cases in 2005 - 2013 indicated that 39.7% of non-keratinizing SCCs, 3.4% of keratinizing SCCs, 41.7% of basaloid SCCs, 66.6% of adenosquamous carcinomas, and 6.5% of sinonasal undifferentiated carcinomas were the carriers of the high- risk HPV DNA (26). In the same line, in a meta-analysis conducted by Chang Sing Pang et al. (27), 60 eligible studies were reviewed and HPV prevalence rates ranged from 5.0% to 94.4%, with an overall HPV prevalence rate being estimated as 25.5%. This meta-analysis confirmed the causative role of HPV in a subset of sinonasal SCCs. In the current study, however, the nPCR with the GP5+/GP6+ and MY09/MY11 primer systems demonstrated that none of the samples were positive for HPV. These inconsistent results might be attributed to the differences in ethnicity, geographical region, HPV detection method, and sample size. Moreover, the current study included 57 biopsies of benign and malignant epithelial tumors of the sinonasal region, while most of the previous studies were conducted only on malignant lesions of this site.

Several molecular studies have found that the carcinogenic activities of the HPV oncogenes, E6 and E7, are primarily responsible for HPV-mediated carcinogenesis. E6 and E7 oncoproteins can induce the inactivation and degradation of the tumor suppressor p53 and the retinoblastoma family of proteins (pRb, p107, and p130), respectively (28). Cellular tumor suppressors, p53 and pRb

Table 2. The Frequency of Various Lesions Based on Location and Patients' Gender and Age

Lesions	Frequency of Lesions	Age (Mean ± SD)	Gender (%)		Location (%)	
			Female	Male	Nasal Cavity	Paranasal Sinuses
Dysplasia	13	46 ± 16.30	6 (46.15)	7 (53.85)	7 (53.85)	6 (46.15)
Melanoma	10	64.7 ± 11.11	6 (60)	4 (40)	5 (50)	5 (50)
Undifferentiated carcinoma	9	57.22 ± 18.47	5 (55.56)	4 (44.44)	4 (44.44)	5 (55.56)
SCC	5	59.80 ± 16.96	2 (40)	3 (60)	1 (20)	4 (80)
Nasopharyngeal carcinoma	5	35.40 ± 12.53	2 (40)	3 (60)	3 (60)	2 (40)
Adenoid cystic carcinoma	4	47.25 ± 1.78	2 (50)	2 (50)	2 (50)	2 (50)
Papillary adenocarcinoma	3	44.66 ± 2.35	0	3 (100)	3 (100)	0
Mucoepidermoid carcinoma	2	61 ± 2	1 (50)	1 (50)	0	2 (100)
Small cell neuroendocrine carcinoma	2	54 ± 18	1 (50)	1 (50)	0	2 (100)
PLGA	1	74	1 (100)	0	1 (100)	0
Ameloblastoma	1	33	1 (100)	0	0	1 (100)
CCOT	1	40	0	1 (100)	0	1 (100)
Basal cell adenocarcinoma	1	36	1 (100)	0	0	1 (100)
Total	57	52.14 ± 16.93	28 (49.12)	29 (50.88)	26 (45.61)	31 (54.39)

Abbreviations: SCC, squamous cell carcinoma; PLGA, polymorphous low-grade adenocarcinoma; CCOT, calcifying cystic odontogenic tumor.

Table 3. Location, Gender, and Age of Patients with *Aspergillus*-Positive Samples

Lesion	Age	Sex	Location
Nasopharyngeal carcinoma	44	M	Sphenoidal sinus
Nasopharyngeal carcinoma	37	F	Nasal cavity
Nasopharyngeal carcinoma	24	M	Ethmoid sinus
Undifferentiated carcinoma	30	F	Nasal cavity
Small cell neuroendocrine carcinoma	72	M	Sphenoid sinus
SCC	66	M	Nasal cavity
CCOT	40	M	Maxillary sinus
Nasopharyngeal carcinoma	53	M	Nasal cavity
Undifferentiated carcinoma	78	F	Paranasal sinus[a]
Melanoma	61	F	Nasal cavity

Abbreviations: SCC, squamous cell carcinoma; CCOT, calcifying cystic odontogenic tumor.
[a] The name of paranasal sinus has not been provided.

are also involved in DNA repair, differentiation, apoptosis, senescence, and cell cycle progression (29, 30). Additionally, multiple reports have demonstrated that HPV can bind to specific receptors on the cell surface, including the a6 integrin and heparin sulfate proteoglycans (31, 32).

In the present study, an nPCR test was used to identify the *Aspergillus* fungus. Despite the absence of the angular hyphae (with acute angle) of *Aspergillus* fungi on the histopathological slides, 10 samples (17.54%) were positive for *Aspergillus* fungi. Thus, our findings indicated a relatively high prevalence of *Aspergillus* infection in sinonasal tumors and also suggested that males were mostly infected. Consistently, Shobana et al. (33) found 40 cases of fungal infections out of 2800 sinonasal tract specimens (1.43%). Among the cases, 77.5% were male and the most common type of fungal infection was aspergillosis (35%). The presence of *Aspergillus* fungi in nasal cavities and paranasal sinuses in multiple studies confirms the tendency of this opportunistic pathogen towards epithelial structures (12, 34, 35). Moreover, the coexistence of *Aspergillus* and SCCs in the maxillary sinus has been observed in 2 studies (12, 36). Besides, studies have reported that *Aspergillus fumigatus* can release proteases, which have a key role in cell desquamation, morphologic changes, and the production of cytokines such as IL-6 and IL-8 (37, 38). IL-6 and IL-8 levels are correlated to a higher tumor stage and lymph node metastasis (39). *Aspergillus fumigatus* also can produce a class of mycotoxins known as gliotoxins, which

interact with numerous immune system cells involved in fungal infection resistance and inhibit critical neutrophil functions such as degranulation of myeloperoxidase activity, phagocytic activity, and production of reactive oxygen species (ROS) (40-42). Gliotoxin also promotes the apoptosis of dendritic cells and monocytes, thereby reducing antigen presentation, ROS production, cytokine production, and phagocytosis (43, 44). Overall, further studies are required to determine whether HPV and aspergillosis are contributed to the development of sinonasal carcinomas.

5.1. Conclusions

According to the findings of the current study, the possible role of fungal infections, especially aspergillosis, should be considered in the pathogenesis of malignant and benign tumors of the paranasal sinuses and nasal cavity. For better management of patients, the application of molecular methods to diagnose possible infections is recommended.

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Footnotes

Authors' Contribution: A.D.N. conceived and designed the evaluation. G.F. drafted the manuscript and helped with critical revision of the manuscript for important intellectual content. A.F. participated in designing the evaluation, performed parts of the statistical analysis and A.D.N helped with the data interpretation. P.B. re-evaluated the clinical data, and performed the statistical analysis. H.K. collected the clinical data, interpreted them and revised the manuscript. M.J.A. and F.P. re-analyzed the clinical and statistical data. All authors read and approved the final manuscript.

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Data Reproducibility: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

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