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Table of Contents

Original Articles

Review Article

Case Reports

Conference Highlights/Scientific Contributions

Original Article

Human Papilloma Virus (HPV) in Sinonasal Papillomas and Squamous Cell Carcinomas: A PCR–based Study of 60 cases

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Abstract

This study was carried out to observe the association of Human Papilloma Virus (HPV) with papillomas and squamous cell carcinomas of the sinonasal region. The present study was a hospital–based study conducted over a period of three years from May, 2014 to May, 2017 in the Department of Pathology, Government Medical College, Srinagar. A total of 196 cases of non–neoplastic and neoplastic lesions of nasal cavity and paranasal sinuses were observed during the study period. Out of total 196 cases, 102 were non–neoplastic and 94 were neoplastic. Of the 94 neoplastic lesions, 58 were benign and 36 were malignant. A total of 60 cases which included 38(63.33%) inverted papillomas, 12(20%) exophytic papillomas and 10 (16.66%) squamous cell carcinomas were included in the present study for HPV association. We studied the association of HPV with sinonasal papillomas and squamous cell carcinomas by polymerase chain reaction (PCR). HPV positivity was seen in 5(13.16%) out of 38 cases of inverted papillomas, whereas 4 out of 12(33.33%) exophytic papillomas tested positive for HPV. Out of 10 squamous cell carcinomas HPV positivity was seen in 2(20%) cases. Low risk HPV types 6 and 11 showed an association with sinonasal papillomas and oncogenic HPV types 16 and 18 with squamous cell carcinomas.

Keywords: Sinonasal, inverted, exophytic, Human Papilloma Virus (HPV).

Introduction

Papillomas of the nasal mucosa have been recognized since 1854 when Ward first described the name inverted papilloma[(1)]. The term "papilloma" describes a benign epithelial neoplasm with a finger–like or verrucous projection over a fibrous stalk. There are three histological subtypes of sinonasal papilloma – cylindrical papilloma (Oncocytic cell papilloma), everted papilloma (Exophytic, squamous or Fungiform papilloma), and inverted papilloma (Transitional cell or Ringertz papilloma)[(2–6)]. However, many studies refer papilloma only to inverted papillomas. Fungiform papillomas arise most commonly from the anterior part of nasal septum and are exophytic, whereas, inverted papillomas arise most commonly from the lateral wall of the nose.

Papillomas, though being benign lesions have the tendency to recur even after total excision and even have a risk of malignant transformation.

The first evidence regarding the etio–pathological role of HPV in causation of both benign respiratory papillomas and squamous cell carcinomas was provided in 1980's. Since then a rapidly growing interest in tumor of this region has resulted in the accumulation of substantial number of papers exploring such evidence[(7,8)]. Human papillomavirus (HPV) is a small DNA virus showing an affinity to the stratified squamous epithelium found on the mucosa and skin. Approximately 120 subtypes of human papillomaviruses have been identified9. Some types are associated only with benign squamous papillomas[(10)], but some human papillomaviruses have also been implicated in the genesis of several squamous cell cancers[(11,12)].

Studies have identified HPV genome in 50–100% of tested exophytic papillomas[(13)] and in 0.86% of inverted papillomas[(14)]. Some studies suggest that most HPV positive cases of sinonasal papillomas are of inverted type[(15)]. Benign papillomas are preferentially associated with the low–risk HPV types 6 and 11 whereas their malignant counterparts are exclusively positive for HPV 16 DNA[(16)]. HPV DNA has been demonstrated in the lesion by molecular hybridization and polymerase chain reaction[(17)]. So far, HPV types 6,11[(13,16,18–26, 5727,28)], and 16/18 (in cancer associated cases)[(14,15,18,20,23,24,26)] have been detected in

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nasal papillomas. The frequency of HPV DNA detection in nasal papillomas varies from 6–89% according to the detection method used, the particular DNA probes or primers, and the patients examined[14,16,17–27]. In addition, geographic differences also exist.

PCR is the most sensitive method for demonstrating HPV–DNA.A consequence of this high sensitivity results in false positive results due to contamination with minute amounts of HPV–DNA during the collection of samples or in PCR laboratory. DNA in situ hybridization, on other hand, is less sensitive and may yield false negative results[29].

Materials and Methods

This is a hospital–based study conducted over a period of 3 years from May 2014 to May 2017 at the Department of Pathology, Government Medical College Srinagar. During this period, a retrospective study was undertaken for a period of 1 year and 7 months from May 2014 to December 2016 and a prospective study was carried out from December 2016 to May 2017 for a period of 5 months. A total of 196 cases of sinonasal masses were observed. Complete history of the patients was recorded. Proper inclusion and exclusion criteria were met. The histopathological reports of the samples received in our Department from May 2014 to December 2016 were reviewed and wherever necessary, blocks were re–cut, stained with Hematoxylin and Eosin (H&E) stain. The tumors were classified as per WHO classification (2005) and observations were compared with other studies. After histopathological diagnosis was made, association of Human Papilloma Virus type 6, 11, 16 and 18 with sinonasal papillomas and squamous cell carcinomas was analyzed by DNA amplification using Polymerase chain reaction (PCR). The genetic studies were performed on the formalin–fixed, paraffin–embedded material in the Department of Biochemistry, Government Medical College Srinagar.

HPV analysis

Extraction of genomic DNA:

For the purpose of extracting genomic DNA, a kit–based method was used. The kit used was Quick–g DNA Mini Prep supplied by ZYMORESEARCH. The DNA eluted was stored at –20°c for longer duration storage until further downstream processes.

Qualitative and Quantitative Analysis of Genomic DNA

The quality and quantity of the DNA was determined by measuring optical density at 260nm and 280nm by double beam spectrophotometer (Evolution 60S from Thermo Scientific). DNA was aliquoted so as to protect damage from frequent freeze–thawing and stored at –20°c for longer duration of time.

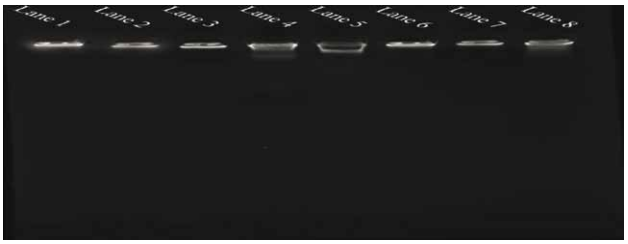

Figure 1: Representative gel picture showing the integrity of the genomic DNA on 1.0% agarose gel. Lane 1 to 8 contains the isolated genomic DNA.

The representative gel picture of the isolated genomic DNA is given in Figure 1.

DNA Amplification by PCR

Amplification kit supplied by GeNei™ that contained all the reagents required for the amplification of the said strains of HPV was used and the protocol followed was as per the kit. Both positive and negative controls for HPV DNA were used. The amplification was performed using Thermal Cycler (Eppendorf). The amplified products were analyzed by gel electrophoresis after staining with ethidium bromide.

Statistical Analysis

Statistical analysis was done with the help of SPSS version 17.0 software. Categorical variables are expressed as frequencies and percentages. Nominal categorical data between the groups were compared using Chi–square test or Fisher's exact test as appropriate. $p<0.05$ was considered statistically significant.

Results

Of the total 196 sinonasal lesions, 60 cases of sinonasal papillomas and squamous cell carcinomas were selected to determine the role of human papillomavirus virus (HPV) in their etiology. 38 inverted papillomas, 12 exophytic papillomas and 10 squamous cell carcinomas were examined for the presence of HPV DNA by polymerase chain reaction (PCR). Out of 38 cases of inverted papillomas HPV positivity was seen in 5 (13.16%) cases whereas, 4 out of 12 (33.33%) exophytic papillomas tested positive for HPV. Out of 10 squamous cell carcinomas HPV positivity was seen in 2 (20%) cases.

Overall, HPV–6 was detected in 8 cases (16%) of sinonasal papillomas (4 exophytic papillomas and 4 inverted papillomas) and HPV–11 was detected in 6 cases (10%) sinonasal papillomas (4 inverted papillomas and 2 exophytic papilloma) whereas, none of 10 cases

S. No.	Histologic Diagnosis	No. of Cases	% of HPV Positive Cases
1.	Inverted papilloma	38	13.15 (5cases)
2.	Exophytic papilloma	12	33.33 (4cases)
3.	Squamous cell carcinoma	10	20 (2cases)

Table 1: Frequency of HPV Positivity in Sinonasal Papillomas and Squamous Cell Carcinomas

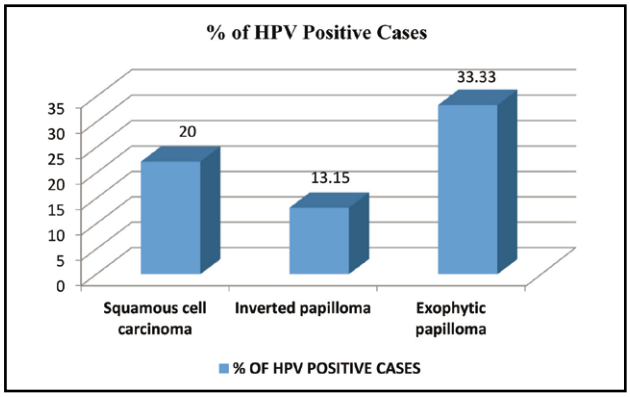

Figure 2: Frequency of HPV Positivity in Sinonasal Papillomas and Carcinomas

HPV Type	Squamous Cell Carcinoma (n=2)	Inverted Papilloma (n=5)	Exophytic Papilloma (n=4)
HPV 6	0	4 (80%)	4 (100%)
HPV 11	0	4 (80%)	2 (50%)
HPV 16	2 (100%)	0	0
HPV 18	1 (50%)	0	0

Table 2: Relationship of Different HPV Strains with Squamous Cell Carcinoma and Histologic Sub–types of Sinonasal Papillomas

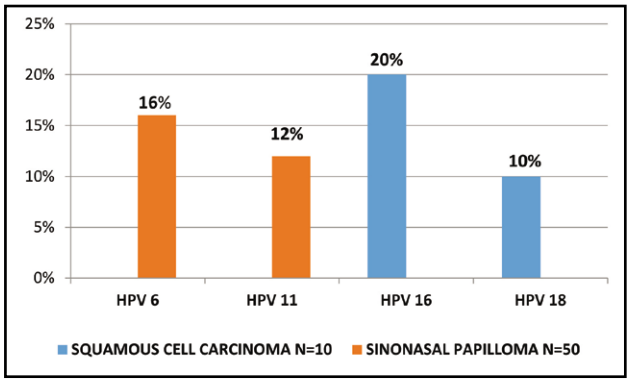

Figure 3: Relationship of HPV Strains with Sinonasal Papillomas and Squamous Cell Carcinomas

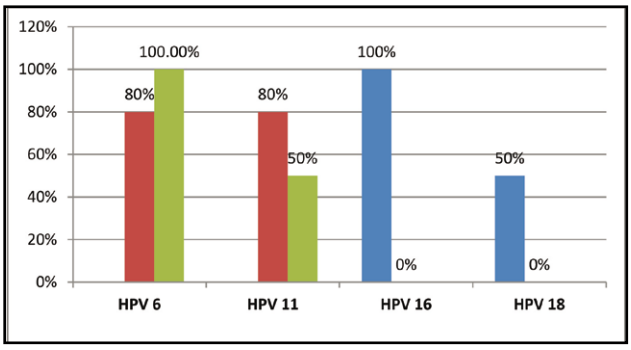

Figure 4: Relationship of Different HPV Strains with Squamous Cell Carcinoma and Histologic Sub–types of Sinonasal Papillomas

of squamous cell carcinomas showed positivity for HPV –6 or HPV–11. HPV–16 positivity was seen in 22.22% (2 cases) and HPV–18 in 11.11% (1 case) of squamous cell carcinomas. None of 50 sinonasal papillomas showed positivity for HPV –16 or 18.

The size of amplified products ranged between 215 to 278 bp and indicated infection with low risk and high risk HPV. The product size of different HPV types obtained with the primers supplied with the kit are as shown in table below;

Discussion

Genotype	HPV 6	HPV 11	HPV 16	HPV 18
Bp	215	278	238	268

Table 3. Size of HPV Subtypes

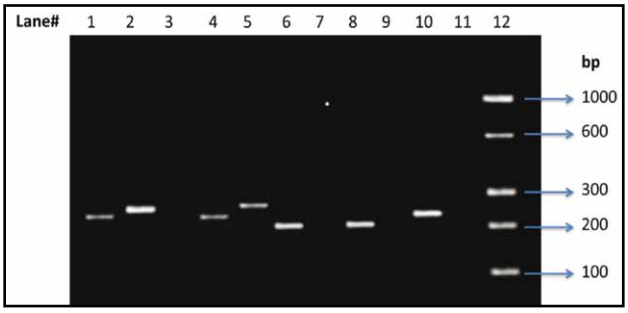

Figure 5: Representative gel picture of the PCR amplified products run on 3.0 % agarose gel.

Lane 1: Sample Positive for oncogenic HPV 16
Lane 2: Sample Positive for oncogenic HPV 18
Lane 3: Sample Negative for oncogenic HPV
Lane 4: Sample Positive for oncogenic HPV 16
Lane 5: Sample Positive for oncogenic HPV 11
Lane 6: Sample Positive for oncogenic HPV 6
Lane 7: Sample Negative for oncogenic HPV
Lane 8: Sample Positive for oncogenic HPV 6
Lane 9: Sample Negative for oncogenic HPV
Lane 10: Positive control
Lane 11: Negative control
Lane 12: DNA Molecular Weight Marker (Ready to use)

Several DNA viruses have been implicated in causation of tumors in humans. Of the various human DNA viruses, human papillomavirus (HPV) is one of the most important tumorigenic factor. HPV cannot be propagated in cell culture, and therefore, in most cases its identification is based upon molecular biology techniques. HPV is a double–stranded DNA virus comprising of a genome of about 8000 base pairs (bp) and has a well–organized physical structure. The tests of choice for detecting HPV in clinical specimens are based on nucleic acid probe technology[30].

Currently, HPVs are accepted as tumour–inducing and tumour–promoting agents involved in the benign and malignant tumors of mucosal tissues of upper aero–digestive tract. The evidence supporting the role of HPV in the etiology of sinonasal cancer is derived from two major lines of research; 1) the reports on malignant transformation of benign (HPV associated) papillomas, and 2) direct detection of HPV DNA in sinonasal carcinomas by hybridization assays and PCR15. More than 80 HPV subtypes have been identified to date on the basis of differences in their nucleotide sequence. Initially, HPV types 6, 11, 16 and 18 were identified in lesions of genital tract. These same HPV types have been identified in lesions of upper respiratory tract, especially in respiratory papillomas (HPV 6 and 11)[31]. The early onco–proteins of HPV 16 are encoded by the genes E6 and E7. The E6 protein targets the tumour suppressor gene p53 for ubiquitination and degradation[32]. The E7 protein is involved in suppression of RB function.

The molecular PCR–based techniques are highly sensitive, specific, and widely used and even detect HPV DNA in formalin–fixed, paraffin embedded tissue. In a conventional PCR, the thermostable DNA polymerase recognizes and extends a pair of oligonucleotide primers that flank the region of interest. In the final process, the PCR can generate one billion copies from a single double–stranded DNA molecule after 30 cycles of amplification[33].

The primers target conserved regions of the HPV genome, such as the L1 capsid gene[34]. After amplification, the HPV genotypes are determined separately, using techniques such as direct sequencing, linear probe assays, restriction–fragment length polymorphism (RFLP), or genotype–specific primers[35]. Some researchers have used a type–specific PCR, with primers that amplify the long control region L1 and E6/E7[36].

In our study genotype specific primers provided alongwith HPV detection kit supplied by GeNei™ were used. Proper protocol was followed. HOT START PCR was performed to prevent non–specific amplification. As per manufacturer, this technique increases both sensitivity and specificity.

HPV association with benign condylomas, flat lesions, and precancerous and cancerous lesions[37–39] of genital tract was discovered since 1970's, which in–turn led to significant interest worldwide to explore the evidence for possible involvement of HPV at other mucosal sites[9,10] including that of sinonasal region[40]. Evidence on the involvement of HPV was immediately obtained in benign sinonasal papillomas[29], and a few years later also in sinonasal SCC's[41].

In our study, we detected HPV positivity in 5 (13.16%) out of 38 cases of inverted papillomas whereas, 4 out of 12 (33.33%) exophytic papillomas tested positive for HPV. Out of 10 squamous cell carcinomas HPV positivity was seen in 2 (20%) cases. Out of 2 squamous cell carcinomas positive for HPV, 2(100%) were found to be positive for HPV 16 and 1 (50%) for HPV 18.Out of 5 HPV positive inverted papillomas, 4 (80%) were positive for HPV 6, 4 (80%) for HPV 11 and 1 (20%) for both HPV 6 and 11. Out of 4HPV positive exophytic papillomas, 4 (100%) were positive for HPV 6 and 2 (50%) for both HPV 11.We found in our study 20% positivity rate of high risk HPV subtypes in tissue samples from patients with squamous cell carcinoma which is similar to other studies[42,43].

Kashima et al 1992[42] observed HPV in 7 (24%) of 29 inverted papillomas, 4 (15%) of 24 squamous papilloma and 1 (4.16%) of 24 squamous cell carcinomas. Of these HPV 6 were identified in 5 specimens (3 exophytic and 2 inverted papillomas), HPV 11 in 6 specimens (1 exophytic and 5 inverted papillomas) and HPV 18 in 1 of 24 squamous cell carcinomas. HPV 16 was not identified in any of the specimens.

Furuta Y et al 1990[44] had observed HPV 16 and HPV 18 in 4 cases (10%) of inverted papilloma and in one case (2.5%) of squamous cell carcinoma respectively. Also HPV 16 was detected in 2 of 7 cases in which IP was associated with SCC.

Buchwald et al (1995)[26] found HPV in 6% of 52 inverted papillomas and 69% of 16 exophytic papillomas which are similar to our observations. HPV 6/11 was identified in all of these HPV positive cases. In SCC's, HPV was detected in 2 (1 HPV 6/11 and 1 HPV 18).

Kraft et al (2001)[45] detected HPV in 60% of exophytic papillomas and 3% of 29 inverted papillomas. In particular, HPV –11 was found in 3 lesions (2 exophytic papillomas, 1 inverted papilloma) (8%) and HPV 6b was detected in one lesion 1 exophytic papilloma (3%). No HPV was detected in any of four carcinomas in their study.

In addition to epidemiological factors, differences in techniques used would possibly explain the disparity in results of different group of researchers. Although many studies have investigated the role of HPV in inverted

papilloma, most of them only detected the HPV genome in inverted papillomas and a few studies have characterized HPV in its "transcriptionally–active" form in IP[15,46,47]. In our study, we observed that 20% of sinonasal SCC patients harbor HPV infection.

PCR is highly sensitive in detecting HPV–DNA, even in formalin fixed and paraffin embedded tissues. However, in as much as only type specific probes for HPV –6, –11, –16, and –18 were used in this study, other HPV types may have been overlooked. From our study we suggest that HPV6 and 11 may be involved in the development of IP, but high risk HPV subtypes may have an important role in the development of squamous cell carcinomas of this region.

Conclusion

Low risk HPV types 6 and 11 show an association with sinonasal papillomas and oncogenic HPV types 16 and 18 with squamous cell carcinomas. Further studies with large cohort of patients in this population is needed to study the role of HPV in the transformation of inverted papillomas to squamous cell carcinomas as determined by other studies in other populations.

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