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Human Papillomavirus Infection and Biomarkers in Sinonasal Inverted Papillomas: Clinical Significance and Molecular Mechanisms

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Abstract

Background—The role of HPV in sinonasal inverted papillomas (IP) is controversial. Determining the prevalence of HPV infection and its impact on the molecular biology of these tumors is critical to characterizing its role in the pathogenesis of inverting papillomas.

Methods—A total of 112 paraffin embedded IPs from 90 patients were studied. A tissue microarray was constructed and stained for p16, p53, EGFR and cyclin D1. HPV presence and type were determined using PGMY 09/11 primers and integration using HPV 11 DIPS-PCR.

Results—HPV was detected in 11/90(12%) of patients. HPV 11 was found in 9 samples. HPV 6 and HPV 27 were found in one sample each. EGFR staining proportion was higher in HPV positive IPs vs. HPV negative specimens (56.2 vs. 23.6%; $p=0.009$). Differences in p16, p53 and cyclin D1 staining were not significant. HPV positive lesions tend to progress to malignancy ($p=0.064$). Three samples were analyzed for integration. Viral integration was found in both malignant tumors but not in the precursor IP.

Conclusions—Degradation of p53 and p16/cyclin D1 dysregulation are not important mechanisms in low risk HPV related IP. The low prevalence of HPV in this series indicates it is not a main etiological factor for IPs, however, when present, low risk HPV may contribute to the biology of IPs through an increase of EGFR expression and a predisposition for malignant progression by integration into the cellular genome.

Keywords

inverted papilloma; HPV; EGFR; p16; Cyclin D1; p53; DIPS PCR; integration

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Introduction

Sinonasal inverted papillomas (IPs) are benign but locally aggressive tumors ¹. With an incidence between 0.74 and 1.5 cases per 100,000 persons per year, IPs account for 0.4% to 4.7% of all sinonasal neoplasms.^{2,3} IPs are reported to have a substantial recurrence rate of up to 32%, and degenerate into or simultaneously harbor squamous cell carcinoma (SCC) in 15% of cases.^{3,4} The pathogenesis and etiology of IPs is still unclear. HPV has been implicated repeatedly in development of inverted papillomas, but the detection rates vary widely in the literature with a range of 0–72 percent and an average of about 25%.^{3,5}

HPV infection plays an important role in the clinical evolution of IPs. The reported detection rate of HPV is increased in IPs with high grade dysplasia and carcinoma when compared to IPs with no dysplasia or mild dysplasia. Similarly the HPV detection rate is higher in the IPs that recur.^{5–7} HPV types detected in inverted papillomas are most commonly low risk types HPV 6/11, and high risk types HPV 16/18. Of these subtypes, HPV 6/11 are more common than HPV 16/18, with an overall unadjusted ratio of low risk to high risk HPV of 2.8:1. When dysplasia or SCC is present, HPV typing reveals a higher proportion of high risk types.^{5,8}

The oncogenic mechanisms of high risk HPV in head and neck cancer have been extensively studied and described. The role of these mechanisms in the pathogenesis of IPs is still not clear. Previous studies have provided evidence of the presence of HPV 6 E6 and E7 transcripts in IPs.⁹ E6 has many biological activities that can lead to cellular transformation. One of the most studied mechanisms of this oncoprotein is its capability to cause the ubiquitination of p53. The ubiquitin conjugated p53 is exported from the nucleus and degraded by proteosomes.^{10,11} A study of IPs suggests that this E6 mediated mechanism takes place in these tumors, in that the levels of p53 and p21 are decreased in IPs with high risk HPV compared to the IPs without HPV infection.⁷ In contrast, in a previous study we reported that regardless of the HPV status high expression of p53 correlates with the presence of carcinoma in IPs.¹² Since decreased p53 levels appear to be a hallmark of high risk HPV induced tumors, this study's results do not concur with the hypothesis that IPs malignant transformation is an HPV driven event.

HPV is also reported to increase the expression of EGFR by a direct increment in its transcription mediated by E6¹³ or by recycling of EGFR mediated by E5.^{14,15} In IPs increased expression of EGFR correlates with high grade dysplasia and malignancy.^{16,17}

The HPV protein E7 is able to bind to the tumor suppressor protein Rb and decrease its activity. This depressed Rb activity is independent from its normal regulatory proteins cyclin D1 and p16. The transcription factor E2F, which is normally inhibited by Rb, drives expression of genes that cause cell cycle progression from G1 to S phase. The normal feedback inhibitory protein of this pathway p16INK4a is typically overexpressed in the presence of HPV transforming proteins. Overexpression of p16 has proven to be a specific and sensitive marker for HPV involvement in squamous oropharyngeal cancer.^{18,19} However in IPs p16 could not be established as a marker of HPV infection and low expression of p16 has been correlated with malignancy in IPs.^{12,20} The absence of an

elevated p16 expression in IPs harboring cancer does not support a model where high risk HPV E7 protein is a driver of malignant transformation.

Recent studies in head and neck cancer have demonstrated high risk HPV integration to the cellular genome with consequent disruption of important cellular regulatory pathways.²¹ Integration of low risk HPV has also been implicated in malignant progression of laryngeal papillomatosis.²² Using the E7/E5 transcript ratio as an indirect indicator of HPV integration, one study suggested the integration of HPV in IPs with synchronic SCC.²³

The purpose of this study is to describe the clinical and biological role of HPV infection in a group of 90 patients with IPs. We study the mechanisms of HPV pathogenesis in IPs using a detection of integrated papillomavirus sequences by ligation-mediated PCR (DIPS-PCR) assay modified for HPV 11 and complement this data with previously published immunohistochemistry for p53, p16, EGFR and cyclin D1.¹²

Methods

The study was approved by the University of Michigan Institutional Review Board (IRB). Patients with IPs were identified by searching the University of Michigan Pathology Archive from the year 1996 to 2011. Other types of Sinonasal papillomas were excluded (i.e. fungiform and oncocytic/cylindrical papillomas). IPs with dysplasia or carcinoma were included in the study.

P53, p16, EGFR and Cyclin D1 immunohistochemistry

The p53, p16, EGFR and cyclin D1 immunohistochemistry performed on these specimens was previously described and published.¹² A tissue microarray (TMA) was constructed, deparaffinized and rehydrated. The immunohistochemistry staining was then performed. The tumor area stained was scored as a proportion on a scale of 1 to 4 (Grade 1 = less than 5%; Grade 2 = 5–20%; Grade 3 = 21–50%; Grade 4 = more than 50%). The Intensity of staining was also scored on a scale of 1 to 4 (1=no staining; 2=low intensity; 3=moderate intensity; 4=high intensity). Tumors with a staining intensity score 2 or higher were considered to stain positive for the biomarker.

DNA Extraction from Paraffin Embedded Inverted Papillomas

A pathologist marked representative areas of tumor in the paraffin blocks. Cores were taken from the marked areas, deparaffinized with xylene and digested with proteinase K. DNA quantity and quality were corroborated with UV-Vis spectrophotometer using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

HPV Detection

The PGMY 09/11 L1 consensus primer set was used to detect HPV as described.²⁴ The isolated DNA from each sample was amplified in the presence of 4 mM MgCl₂, 200μM dNTP mix, 0.2 μM PGMY09/11 primer mix, 0.05 μM β-globin primers (PCO4 and GH20), 1x PCR Buffer II, and 7.5U of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, New Jersey USA). Amplification was performed in an Eppendorf

Mastercycler Gradient thermal cycler (Eppendorf Scientific, Hamburg, Germany) at 95°C for 9 minutes and 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. This was followed by a final extension at 72°C for 5 min, and the amplification reaction mixtures were stored at 4°C. For the samples that showed an L1 PCR product, a new PGMY 09/11 PCR reaction was performed excluding the β -globin primers. The DNA was purified using the QIAquick PCR Purification Kit as described by the manufacturer (QIAGEN Sciences, Maryland, USA) and Sanger sequenced in the University of Michigan Genomics Core. The sequences were aligned with known HPV types using the NCBI Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

All samples were also analyzed with a second sensitive method, HPV PCR-MassArray, for the detection and identification of high risk HPV types as previously described.²⁵

HPV 11 DIPS PCR

To determine the physical state and site of integration of high risk HPV-16 a Detection of Integrated Papillomavirus Sequences by Polymerase Chain Reaction (DIPS-PCR) based method was used.^{26–28} We modified this method and designed a new set of primers that will cover the entire low risk HPV 11 genome (Table 1 and Figure 1). The Genomic DNA (800ng) was digested using 20 units of TaqI (New England Biolabs, MA, USA) in a volume of 20 μ L at 65°C overnight in an thermal cycler (Eppendorf Mastercycler Gradient, Eppendorf Scientific, Hamburg, Germany). The reaction was heat inactivated at 80°C for 20 minutes. A double stranded annealed adapter was then ligated to the digested DNA. The adapter consists of a long strand (5'-gggcatcagtcagcagtcgtagccggatccagacttacagttg-3') and a short strand (5'-PO₄-cgcaacgtgtaagtctg-NH₂-3'). The ligation was performed using 3 units of T4 DNA Ligase (Promega Corporation, WI, USA), 50pmol annealed adapter and the digested DNA in a 20 μ L reaction at 14°C overnight. The product was then diluted to 40 μ L. A first linear PCR reaction was performed using 2 μ L of the previously digested and ligated DNA, 1x PCR buffer, 1.5mM MgCl₂, 200 μ M dNTP mix, 0.2 μ M viral linear primer and 1 unit Platinum Taq DNA Polymerase (Invitrogen, Life Technologies, NY, USA) in a total volume of 25 μ L. The reaction parameters were an initial denaturation at 96°C for 2 minutes, then 40 cycles of 96°C denaturation of 30 seconds, 62°C primer annealing for 30 seconds and 72°C primer extension for 3 minutes followed by a final 72°C extension for 7 minutes. For the second exponential PCR reaction we used 2 μ L of the first linear PCR product, 1x PCR buffer, 1.5mM MgCl₂, 200 μ M dNTP mix, 0.4 μ M viral exponential primer, 0.4 μ M adapter-specific primer (5'-ggccatcagtcagcagtcgtag-3') and 1 unit Platinum Taq DNA Polymerase in a total volume of 25 μ L. The reaction parameters were an initial denaturation at 96°C for 2 minutes, then 30 cycles of 96°C denaturation of 30 seconds, 62°C primer annealing for 30 seconds and 72°C primer extension for 3 minutes followed by a final 72°C extension for 7 minutes. The PCR products were analyzed using electrophoresis in a 2% agarose gel. The resulting bands were cut from the gel and purified using the QIAquick Gel Extraction Kit as described by manufacturer (QIAGEN Sciences, Maryland, USA). The purified DNA was sequenced, and the integration sequences were detected using the NCBI Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical Analysis

Mean percentage staining by tumor surface area for HPV positive and HPV negative IPs were compared using two-tailed T-test. Staining proportions were compared using a two-tailed Z-test for proportions. All p values < 0.05 were considered as statistically significant, and values between 0.05 and 0.10 as indicating a statistical trend.

Results

A total of 162 paraffin embedded specimens from 147 patients were collected and studied. Twenty two specimens (13.6%) harbored carcinoma. 111 patients (75.5%) were male. The population characteristics have been described before.^{12,29}

HPV incidence and types

DNA was successfully extracted for analysis from 112/162 IP samples corresponding to 90 patients. HPV detection was carried out using the PGMY PCR based detection method²⁴. Co-amplification of β -globin was used as a reaction positive control. Eleven (12.2%) of the 90 patient tumors studied were HPV positive (Table 2.). One patient with an HPV positive tumor had two specimens collected one year apart. Both samples were positive for the same HPV type, and the second sample contained SCC. From the 11 HPV positive tumors 9 contained HPV 11, one HPV-6 and one HPV-27. There were no high risk HPV types detected in this patient cohort as assessed by the PGMY L1 assay and confirmed by HPV PCR-MassArray.

HPV Status and expression of EGFR, p16, p53 and cyclin D1 Immunohistochemistry

The HPV status was compared to p53, p16, EGFR and Cyclin D1 protein levels (Table 3. and Table 4.). Immunohistochemistry data was available for 77 tumors, 8 HPV positive and 69 HPV negative. HPV infected IPs stained for EGFR in 87.5% (7/8) tumors, while only 58% (40/69) of HPV negative IPs stained for EGFR. This difference was not statistically significant ($p = 0.11$). However, when the mean percentage area of the tumor stained for EGFR was considered, HPV positive tumors had increased staining compared to the HPV negative tumors, with a stained mean percentage area of 56.2% vs 23.6% respectively ($p = 0.009$).

Positive p16 immunohistochemistry was observed in 50% (4/8) of the HPV infected IPs versus 71% (49/69) of HPV negative IPs although the difference was not significant ($p=0.22$). The tumor mean percentage area stained for p16 was 11.7% vs 10.3% for the HPV positive and HPV negative tumor respectively ($p=0.858$).

Positive staining for p53 was present in 62.5% (5/8) of HPV positive specimens vs 50.7% (35/69) of HPV negative samples ($p=0.52$). The mean area stained for p53 was 11% for HPV infected specimens and 8.5% for HPV free specimens ($p=0.612$).

Cyclin D1 immunohistochemistry was positive in 75% (6/8) of HPV infected and 80% (57/71) of HPV negative samples ($p=0.72$). Tumor mean percentage areas were 17.08% and 17.23% for HPV positive and HPV negative samples respectively ($p=0.983$).

HPV in cancer progression and papilloma recurrence

Progression of IPs to SCC occurred in 36.36% (4/11) HPV positive patients, and 14.1% (11/78) HPV negative patients ($p = 0.064$). All HPV positive patients who presented with SCC had HPV 11 infection. Also, IPs recurred in 63.64% (7/11) of HPV positive versus 45.33% (34/75) of HPV negative lesions ($p=0.256$).

Integration of HPV 11 in inverted papillomas that progressed to SCC

From the HPV 11 positive specimens only three had sufficient DNA for HPV 11 DIPS PCR analysis. Two of the specimens were from the same patient. The first specimen was an IP, and one year later a specimen containing SCC was obtained from this patient. The specimen with SCC showed that HPV 11 had one integration site in an intergenic region of chromosome 5q31. The HPV 11 insertional breakpoint was found in L1. The IP obtained one year earlier from the same patient did not show any HPV integration events.

The third specimen analyzed with HPV 11 DIPS PCR is a squamous cell cancer that contained three integration events. The integrations were found in intergenic regions of chromosomes 18q11.1, 5p13.2 and 2p22.1. The HPV integration breakpoints were in E6, L1 and L1 respectively.

Discussion

Many etiological agents have been proposed for IPs. HPV has been proposed as the main etiological factor. However, in our dataset only a low proportion of IPs, 11/90 or 12.2% of our patients, harbor HPV in their lesions. A recent meta-analysis reported that HPV has been found in 25% of IPs with high variability between studies.⁵ The low HPV presence suggests that it is only one of the etiological agents for IPs.

Interestingly, we did not find any high risk HPVs in the IPs studied. We used two robust assays, one based on consensus L1 PCR primers PGMY 09/11²⁴ and the HPV multiplex PCR MassArray analysis based on the viral E6 region.²⁵ Even without the presence of high risk HPVs we noted a trend for low risk HPV positive samples to have a greater proclivity for malignant progression to invasive squamous carcinoma than HPV negative samples IPs. However this trend was not statistically significant. This observed tendency to progression was further studied in a meta analysis and proved that HPV positive samples progress more frequently than HPV negative IPs. They also found that HPV positive IP's have a higher recurrence rate than the HPV negative tumors, a correlation that was not found in our study. This discrepancy could be caused by our lower statistical power due to our low number of HPV positive cases, or by a difference in the biological activity of high risk HPV versus low risk HPV. In contrast to our study, in the cited meta-analysis high risk HPV 16/18 were detected in 3.5% (0–33.3%) IPs with no or low grade dysplasia and 33.8% (0–100%) IPs with SCC.⁵ The role of low risk HPV as a prognostic marker has not been confirmed.

The variability of HPV detection between studies can be a reflection of different patient characteristics and may also include varying levels of HPV detection expertise. Sample sets that include a higher number of dysplastic and malignant lesions and higher numbers of recurrent cases are more likely to have higher HPV incidence.^{30,5} Laboratories with greater

experience with HPV detection and strong quality control measures may have more accurate HPV detection rates and lower false positive rates.^{31–33}

The oncogenic mechanisms of HPV have been studied extensively. Most high risk HPV induced cancers are driven by the viral oncogenes. HPV positive tumor cell lines are dependent on E6 and E7 expression for proliferation and survival.^{34–36} Whereas most attention has been given to these HPV-mediated effects on the p53 and pRb pathways, this study's results indicate the importance of HPV induced upregulation of EGFR expression.^{13–15} EGFR mean percentage area staining was the only biomarker that was significantly increased in our low risk HPV positive set compared to the HPV negative cases. Cyclin D1, p16 and p53 expression showed no difference between the two groups. These results provide a rationale to test EGFR inhibitors for the treatment of low risk HPV-positive IPs. The Radiotherapy plus Cetuximab multicenter study of locoregionally advanced head and neck cancer reported by Bonner et al. revealed that overall survival was only modestly increased in the RT plus cetuximab arm over radiation alone. However, in a subsequent follow-up at 5 years the survival advantage for the cetuximab arm became significant. In subgroup analysis, male patients with less than 65 years, treated in the USA with T1-3, N1-N3 oropharynx cancer exhibited the greatest benefit from the addition of Cetuximab to the treatment.³⁷ This effect could be attributed to the presence of HPV, considering that it has been established that HPV is now the main etiologic agent for oropharynx cancer,³⁸ while in head and neck cancers at other sites the role of HPV is not as significant.^{39,40} Furthermore, Kies et al. treated oropharynx cancer with paclitaxel and carboplatin with and without cetuximab and found that survival was better for the HPV positive patients in the cetuximab group.⁴¹ Thus, it is conceivable that there may be a role for anti-EGFR treatment in the management of HPV-related sinonasal tumors.

Recent studies indicate that integration of high risk HPV in human genomic DNA from SCC can disrupt important cell regulatory genes.²¹ Here we designed a DIPS-PCR assay modified to detect the integration of low risk HPV 11 into human genomic DNA. Interestingly we found that the low risk HPV 11 all integrations were in intergenic DNA regions and did not directly alter the sequence of any gene. This is in contrast to gene disruption by intragenic high risk HPV integration as a secondary factor in oncogenesis.²¹ However, Schmitz et al.⁴² observed that in a large number of cervical carcinomas, intergenic integration also frequently occurs in the vicinity of microRNAs which could also alter host cell gene expression and foster malignant progression. In their work Schmitz et al. noted that the integration process of high risk HPV's was directed by cellular homologies with the viral E5 and L2 sequences. These homologies targeted 9 genes that were commonly disrupted.⁴² In this study we found that the low risk HPV 11 was frequently integrated in the L1 sequence. This finding suggests that integration of HPV 11 is also a targeted process, but more studies are necessary to confirm this.

In one subject an initial IP sample and a second sample that was obtained when the lesion progressed to SCC were available for evaluation. There was no HPV11 integration present in the IP sample although an HPV 11 intergenic integration was present in the SCC sample one year later. Huebbers et al.²² reported similar findings in a case of a young woman with HPV6 positive recurrent respiratory papilloma that progressed to SCC. HPV integration was

not found in two respiratory papilloma specimens but in the later SCC sample from this patient, HPV6 was integrated into aldo-keto reductase 1C3 resulting in gene disruption and loss of function.²² These results indicate that HPV integration occurs concurrently with malignant transformation, but it is still unclear if the viral integration is a driver of malignancy or a consequence of genomic instability caused by HPV independent mechanisms.

Conclusions

The low frequency of HPV in IPs in this series suggests that this virus is not a major etiological agent for this disease. We identified a trend to progression to carcinoma in the presence of HPV, an observation that has been confirmed in a recent meta-analysis.⁴ EGFR was the only biomarker tested that was overexpressed in low risk HPV positive samples, suggesting that anti EGFR therapy could have a role for reducing risk of progression in HPV positive IPs. Intriguingly our results suggest that integration of HPV into the cellular genomic DNA occurs with malignant transformation but this requires further study. It is unclear if viral integration is the cause or a consequence of malignancy.

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Figure 1.

Position of TaqI restriction sites and DIPS-PCR primers on HPV 11 genome. Upstream (reverse) primers are indicated by grey arrowheads labeled R1-5. Downstream (forward) primers are indicated by black arrowheads labeled F1-5. TaqI restriction sites are indicated by dashed lines.

HPV 11 DIPS PCR Primers

Table 1

Linear PCR				Exponential PCR				Target TagII Restriction Site	Product Size (bp)	Detection Length (bp)
Primer Name	Direction	5' Binding Nucleotide	5'-3' Sequence	Tm (C°)	Primer Name	5' Binding Nucleotide	5'-3' Sequence	Tm (C°)		
F1L	Forward	463	TGGGAAAGGCACGCTTCATA	59.67	F1E	504	GGTCGTTGCTTACACTGCTG	59.49	3211	1473
F2L	Forward	1857	GGCCAGACCGTTATTGAAC	59.90	F2E	1977	GGGTGGAGACTTTGACTCCA	59.97	1738	1648
F3L	Forward	3729	ATAGCAGTGAGGAACAACGTCA	59.70	F3E	3788	AGGCATAAAGGTGGGGTTTATGT	59.41	1121	1031
F4L	Forward	5051	TTCTCTCGTGCTTTTCCTCGG	60.32	F4E	5104	CAGGTACAGGTTACGGACCC	59.47	2166	630
F5L	Forward	5537	CCCTTTCACAAATCGTGGGGTA	60.00	F5E	5734	CGCAGACGCCGTAAACGTAT	61.14	1536	1446
F6L	Forward	7185	GACGGACGTCGTCTCGTACA	61.90	F6E	7203	CAGGTATAAAGCGCCACAGCT	60.18	4445	1234
R1L	Reverse	790	TAGTGTGCCACAGCAAAAGGT	59.82	R1E	758	TGATGTCTCCGTCGTGCAC	60.04	1600	1511
R2L	Reverse	3591	TGCAGTTGCACTATAGGCGT	59.75	R2E	3470	GGGTGTTGTTAGTGGACGGT	59.89	4312	2712
R3L	Reverse	4465	ATAGTTGTGTGGCTGACGCA	59.97	R3E	4435	TGCGTGCCCTAGGTTTCATT	59.96	899	810
R4L	Reverse	6072	CCTGTGCACGCCCCATACTAA	60.11	R43	6051	CGCTGTGTAGTGGGGTCAAA	60.25	1321	1232
R5L	Reverse	6997	TCTACCATTTGGTGGAGGCG	60.32	R5E	6870	TCAAACTCCTCCACATGGCG	60.32	2140	819

Abbreviations: Tm, Melting Temperature.

Table 2

HPV Detection in Patients with Sinonasal Inverted Papillomas

HPV Type	Proportion
HPV negative	79/90 (89%)
HPV 11	9/90 (10%)
HPV 6	1/90 (1.1%)
HPV 27	1/90 (1.1%)

Table 3

Average percentage area stained for p16, EGFR, p53 and Cyclin D1 for HPV positive vs HPV negative IPs

	HPV +	HPV –	P value
P16 (% Avg.)	11.67	10.27	0.858
EGFR (% Avg.)	56.25	23.65	0.009
P53 (% Avg.)	11.04	8.49	0.612
Cyclin D1 (% Avg.)	17.08	17.23	0.983

Abbreviations: Avg, average.

Table 4

Biomarker staining for HPV positive vs HPV negative IPs

	HPV +	HPV –	P value
p16 positive	4/8 (50%)	49/69 (71%)	0.22
EGFR positive	7/8 (87%)	40/69 (58%)	0.11
p53 positive	5/8 (63%)	35/69 (51%)	0.52
Cyclin D1 positive	6/8 (75%)	57/71 (80%)	0.72