



Multiple DNA Viruses and HPV Integration in Inverted Papilloma and Associated Sinonasal Carcinoma

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Objectives: Sinonasal inverted papilloma (IP) has a locally destructive growth pattern, can relapse, and can undergo malignant transformation (IP-associated sinonasal squamous cell carcinoma [IP-SNSCC]). Human papillomaviruses (HPV)-6 and -16 are frequently detected in IPs. To clarify the possible roles of other DNA viruses in IPs, we explored viruses not studied in this context before. With the setting of pre- and post-malignant transformation samples, we investigated HPV genomes in depth to assess the integration of HPV into the human genome and the presence of minor intratypic variants.

Materials and Methods: We analyzed 35 IP samples representing 28 individuals, of which six had IP-SNSCC. For virus screening, we applied qPCR to detect 16 different DNA viruses in three virus families, comprising herpesviruses, parvoviruses, and polyomaviruses. In addition, targeted next generation sequencing (NGS) was used for detailed HPV analysis.

Results: We detected herpes-, parvo-, and polyomaviruses in 13/28 (46%) patients, with codetections of multiple viruses in six (21%) patients. NGS revealed HPV16 DNA in 2/6 IP-SNSCC and in their respective earlier benign IP samples, as well as in a plasma sample from one of these patients. HPV6 was detected in two IP samples without subsequent malignant transformation. We identified sequence reads containing junctions of HPV6 and HPV16 and host genome suggestive of viral integration. HPV6 and HPV16 minor intratypic variants were present across pre- and post-malignant transformation, with mostly non-synonymous mutations.

Conclusions: Multiple DNA viruses were present in IPs. HPV16 was detected only in IP-SNSCCs or in tumors that later underwent malignant transformation.

Key Words: carcinoma, herpesvirus, inverted papilloma, papillomavirus, parvovirus, squamous cell of head and neck.

Level of Evidence: 3

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INTRODUCTION

Sinonasal inverted papilloma (IP) is a benign epithelial tumor of the nasal cavity and paranasal sinuses with an annual incidence of 0.2–1.5 per 100 000 persons.^{1–3} Although benign, IP can grow locally aggressively and recur after resection. Progression to dysplasia or even malignant transformation, most commonly into squamous cell carcinoma (SCC), has been reported in approximately 9% of cases.⁴

The etiology of IP is unresolved. The role of human papillomaviruses (HPV), double-stranded DNA viruses of *Papillomaviridae*, has been studied intensively. Over 200 HPV types exist to date and may present, depending on the type, a high-risk (HR), or low-risk (LR) oncogenic potential in humans, the most common types being HPV6 (LR) and HPV16 (HR).⁵ The overall prevalence of HPVs in IP varies largely within studies (0%–62%),⁶ and meta-analyses present HPV prevalence rates of 23%–38%.^{6,7} HR HPV types 16 and 18 significantly increase the risk of

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IP-associated sinonasal squamous cell carcinoma (IP-SNSCC), and HPV16 is an independent risk factor for malignant transformation of IP.^{8,9}

The ability of HPV to cause cancer relies on its oncoproteins (E5, E6, E7) increasing the proliferation of epithelial cells. This can be accomplished by, for example, HPV integration to the host genome, which affects cell cycle regulation, leading to inactivation of *TP53* (E6) and *RB* (E7) tumor suppressor genes.¹⁰ During infection, HPV utilizes the host cell's DNA-replication machinery and proofreading capabilities, enabling low rates of nucleotide polymorphism.¹¹ Still, changes among HPV sequences ("variants," meaning smaller genetic variations in the viral DNA sequence) occur, leading to isolated genetic variation, some with respective amino acid changes (non-synonymous mutations). It has been proposed that these changes may influence the clinical outcome in HPV-induced cervical cancer.^{12,13} So far, the limited data on HPV intratypic variants within HPV-related head and neck cancer have not yet shown clinical relevancy.¹⁴

Due to the anatomical location, the exposure to microbes in the sinonasal area is remarkable. Only a few studies regarding the presence of Epstein-Barr virus (EBV, 0%–64%) in IP exist.^{15–17} Viruses may persist in tissues for decades with unresolved consequences. On the contrary, viruses with potential oncolytic capabilities, such as parvoviruses, may have an opposite role in disease and even provide tools for disease therapies.^{18,19}

The purpose of this study was to investigate, by qPCR, the presence of a broad range of DNA viruses (members of the *Orthoherpesviridae*, *Polyomaviridae*, and *Parvoviridae* families) in IP and in IP-SNSCC. With targeted next generation sequencing (NGS), we aimed to gather detailed information on HPV integration into the host genome and identify changes in HPV sequences before and after malignant transformation.

MATERIALS AND METHODS

Ethics

The research ethics committee at the Helsinki University Hospital approved the study (§31/07.03.2019), and the Institutional Review Board granted the study permission (HUS/332/2019, HUS/295/2024–15).

Patients and Clinical Specimens

The cohort comprised 28 sequential patients treated during 2017–2019 for IP and all IP-SNSCCs diagnosed between 1993 and 2019. The patients were treated at the Departments of Otorhinolaryngology–Head and Neck Surgery and Oncology, Helsinki University Hospital, Helsinki, Finland. We recorded the patient-, tumor-, and treatment-related parameters including follow-up information from hospital charts (Table I).

We collected IP samples and plasma samples for patients with IP-SNSCC that were available at the Helsinki Biobank. Altogether, we had 38 formalin-fixed, paraffin-embedded (FFPE) samples representing 28 patients (named IP01–IP28, Tables I–III). Twenty-nine samples were benign IP, six were IP-SNSCC, and two samples were taken from the healthy tissue surrounding the IP (Patient IP02, sample 2; Patient IP03, sample 4). Additionally, one sample was scar tissue, taken after

surgical treatment of earlier IP and subsequent IP-SNSCC (Patient IP03). The Helsinki Biobank provided plasma DNA from two patients (IP03 taken simultaneously with sample 3; IP28 taken 4 years later than sample 2) and one plasma sample from one patient (IP03 taken simultaneously with sample 2). All FFPE samples were collected in a PCR-sterile manner from paraffin tissue blocks as 2-mm punch biopsies in 1.5-mL microcentrifuge tubes.

DNA Extraction

We extracted DNA from the FFPE biopsy samples with QIAamp DNA FFPE Tissue Kit (Qiagen, Heiden, Germany), according to the manufacturer's protocol, with slight modifications as described previously.²⁰ From one plasma sample (patient IP03), we extracted DNA with QIAamp DNA blood Mini Kit (Qiagen), according to the manufacturer's protocol. We evaluated the DNA yields and human cell quantity by comparing viral loads with that of the human reference single-copy gene *RNase P* by qPCR.²¹

Virus DNA Detection by qPCR

We used qPCR to detect herpesviruses, parvoviruses, and polyomaviruses: herpes simplex-1 and -2, varicella zoster, EBV, cytomegalovirus (CMV), human herpesvirus (HHV)-6A, -6B, and -7, Kaposi's sarcoma-associated herpesvirus, human parvovirus B19 (B19V), bufavirus, cutavirus, tusavirus, BK and JC polyomaviruses, and Merkel cell polyomavirus (MCPyV).^{21–25} All qPCR reactions contained Maxima probe qPCR Master Mix (Life Technologies Thermo Fischer Scientific, Pittsburgh, PA, USA) or for herpesvirus analysis TaqPath ProAmp Multiplex Master Mix (Thermo Fisher Scientific). We performed all qPCR assays with AriaMx Realtime PCR System (Agilent Technologies, Santa Clara, CA, USA).

Virus DNA Detection by NGS

For detection of HPVs, we applied custom-targeted enrichment. With NGS, we focused on HPV genomes (HPV2, 6, 11, 16, 18, 31, 45) to assess the presence of minor variants and to explore integration sites of HPV in the human genome. We prepared the libraries with unique double indexes and performed in-solution capture-targeted enrichment of the viral DNAs.^{20,26} We quantified and pooled the enriched libraries for sequencing on NovaSeq 6000 (S1, PE1.51 kit; Illumina).

Sequence Data Analysis

We reconstructed viral genomes from the sequencing data with TRACESPipe.²⁷ Viral genomes with low coverage breadth (<15%) were manually inspected, and the viral origin of individual reads was confirmed by BLAST (Basic Local Alignment Search Tool; NIH National Library of Medicine, Rockville, Bethesda, MD, USA). In-depth genomic analyses were conducted for HPV DNA from three samples with average coverage breadth and depth of 99.8% and 94.8X, respectively. Integration junctions were screened with SurVirus default settings using the human reference GRCh38/h38.²⁸ Chimeric reads, mapping to both HPV and the reference human genome, were manually inspected. The host side of the junction was evaluated with Genome Browser to assess whether integration had occurred within or between genes. Minor variants from HPV reads were called with iVar²⁹ using a *p*-value of <0.05 and an allele frequency of ≥3% as cut-off.

TABLE I.
Clinical Characteristics.

Sample Number	Age (y)	Sex F/M	Smoking	Immunosuppression	Primary Tumor PAD	Relapse	Carcinoma [‡]	Location at Diagnosis			Radiation	
								Nasopharynx	Paranasal sinuses	Nasal Cavity		Chemotherapy
IP01	64	M	Ex	NO	IP	NO		1		1		
IP02	62	F	YES	NO	IP + D	YES		1		1		
IP03	81	M	YES	NO	IP	YES	YES			1		YES
IP04	60	F	NO	NO	IP	YES		1		1		
IP05	61	F	YES	NO	IP	NO		1		1		
IP06	52	F	NO	NO	IP	NO		1		1		
IP07	58	M	Ex	NO	IP	NO		1		1		
IP08	63	M	YES	YES*	IP	YES		1		1		
IP09	53	F	NO	NO	IP	NO				1		
IP10	31	M	NA	HCV+ [†]	IP + D	YES		1		1		
IP11	17	M	NA	NO	IP	YES		1		1		
IP12	58	F	NA	NO	IP	NO		1		1		
IP13	70	F	NO	NO	IP	NO		1		1		
IP14	69	M	NO	NO	IP	NO		1		1		
IP15	86	M	YES	NO	IP	NO		1		1		
IP16	50	M	ex	NO	IP	NO				1		
IP17	69	F	ex	NO	IP	NO				1		
IP18	54	M	NO	NO	IP	NO				1		
IP19	47	M	NA	NO	IP	YES		1		1		
IP20	49	M	NO	NO	IP	NO		1		1		
IP21	53	F	NO	NO	IP	YES		1		1		
IP22	60	M	YES	NO	IP	YES		1		1		
IP23	54	M	NA	NO	IP	YES				1		
IP24	38	M	NA	NO	IP	YES	YES	NA		NA		
IP25	53	M	NA	NO	IP + D	YES	YES	1		1		YES
IP26	44	M	YES	NO	IP + CA	YES	YES			1		YES
IP27	49	M	YES	NO	IP + CA	NO	YES			1		YES
IP28	79	F	NO	NO	IP	YES	YES			1		YES

CA = carcinoma; D = dysplasia; ex = ex-smoker; NA = not available; Relapse = any relapses detected.

*Methotrexate.

[†]Hepatitis C-virus infection.

[‡]Carcinoma detected in IP sample at any point of the disease.

RESULTS

Patient Characteristics

The mean age of the patients at the first IP diagnosis was 56.5 years (range 17–86). Eighteen patients were male, and 10 were female. The immunosuppressive state was normal in 26/28 patients. The clinical characteristics are presented in Table I.

Presence of Virus DNA by qPCR

By qPCR analysis, altogether 24 (distinct) findings of herpes-, parvo-, and polyomavirus DNAs were present in all tumor samples, and multiple viruses were present in six samples. The viruses were detected in 15/35 (43%) tumor samples, representing 13/28 (46%) patients (Table II). Across all samples, the viral loads ranged between 18 and 1.3×10^5 copies per 1 million cells (cpm, Table II). Eight (23%) tumors were positive for EBV. HHV-7 was detected in six (17%) tumors and in one IP-surrounding sample. HHV-6B was detected in four (11%) tumors, and B19V in three IPs and in one surrounding tissue sample. CMV was present in two (5.3%) IPs and MCPyV in one (2.6%) IP. No samples were PCR-positive for bufavirus, tusavirus, cutavirus, BKPyV, or JCPyV. The differences in virus DNA prevalence between IPs and IP-SNSCCs were minor, but IP-SNSCC exhibited proportionally more EBV (50%) findings than IP (18%), whereas HHV-6 was present only in IPs. Analysis of *RNase P*, expressing the cell count and indicating a successful DNA extraction, resulted in a mean of 9.9×10^3 cells/ μ L (range 1.0×10^2 – 6.4×10^4 cells/ μ L).

HPV Detected by NGS

HPV detection was performed with NGS to get, in addition to prevalence, information about the genomic integration junctions and intra-host diversity of HPV in our cohort. HPV16 was present in 2/28 (7.1%) patients (IP03 and IP28), in altogether six HPV16-positive FFPE samples. Both patients had a malignant tumor (IP-SNSCC) in their sample series (2/6 of IP-SNSCC; 33%; Table III), and the previous benign IP samples were HPV16-positive as well. Patients with IP only were all negative for HPV16. Patient IP03 had IP even after the surgical removal of IP-SNSCC, and this IP, and the tissue surrounding the IP, was HPV16-positive. In addition, we detected HPV16 DNA in one plasma sample (IP03; 12 individual reads), taken at the same time as the biopsy of IP03 sample 2 (IP-SNSCC), whereas the plasma sample taken together with IP03 sample 3 was negative. The plasma from patient IP28, in who's tissue also HPV16 was detected, was negative. HPV6 was present in 2/28 (7.1%) patients (IP13, IP16). The HPV6 positivity was observed only in patients without malignant tumors (2/22; 9.1%). When considering the herpes-, parvo-, and polyomavirus analyses by qPCR and the papillomavirus analysis by NGS, viral DNA positivity was detected in 20/35 tumor samples with altogether 31 virus findings, representing 15/28 (54%) patients.

HPV Integration in Host Genome

We analyzed HPV integration in three samples (two from patient IP03 and one from IP16), from which we had the highest sequencing coverage (Table III). Together, we detected five junctions of HPV6 and HPV16 into the host genome, with breakpoints listed in Figure 1 and Table IV. The virus junctions were supported on average by five read pairs and located in the genes *E1*, *E1^E4* of HPV16; and in *E5b* and *E1* (two locations) of HPV6 (Table S1).

HPV Subpopulations

From the same samples from which integrations were analyzed, we performed minor-variant analysis to identify low-frequency mutations and variations that may contribute to viral heterogeneity and potential pathogenicity. Two of these samples belonged to the same patient (before and after the malignant transformation IP03, samples 1 and 2; HPV16), while the third sample belonged to the patient with an HPV6-positive IP. We identified 87 sites with alternative nucleotides in addition to the consensus nucleotides across the 3 samples. All minor variants were manually inspected and verified. Out of the 87 sites, 67 were taken for downstream validation while 20 excluded, due to their location near the ends of the reads where there is a higher likelihood for artefacts. Details are listed in Table V and Figure 2. The number of mutations had doubled in the *E1* gene in the IP-SNSCC when compared to the preceding IP sample, and the increase of nonsynonymous mutations was three-fold. Two mutations were detected in the pre-oncogene *E5* in IP-SNSCC, whereas the benign IP had no mutations present. HPV6 nonsynonymous mutations were present in *L1*, *L2*, *E1*, *E5b*, and in oncogene *E6*.

DISCUSSION

We screened for DNA viruses in 35 FFPE tissue biopsies from 28 patients with either IP or IP-SNSCC. When combining the results from qPCR and NGS analyses, virus positivity was detected in 20/35 tumor samples with altogether 31 virus findings, representing 15/28 (54%) patients.

Apart from HPVs, only a few studies exist on the presence of DNA viruses in sinonasal IP. Nukpook *et al.* (2020) investigated the presence of EBV by qPCR in nasal polyps, sinonasal papillomas, and SNSCCs: EBV infection was detected in the infiltrating lymphocytes in 34%, 64%, and 38% of the samples, respectively, and authors suggested that EBV might enhance the tumorigenesis among IP and IP-SNSCC.¹⁶ Similar prevalence of EBV was detected by Schindele *et al.* in a study of 53 IP patients by EBER-ISH.¹⁷ Our results are in line, with the EBV-DNA prevalence being 18% in patients with IP, and 50% in those with IP-SNSCC.

Regarding other herpesviruses, we detected HHV-7, HHV-6B, and CMV in IP tumors. No reference data for these viruses are available for IP. In nasal polyposis ($n = 23$) and sinonasal lymphomas ($n = 26$) the

TABLE II.
Virus Presence in Tumors by qPCR.

Patient ID	Sample Series*	Histology	RNase P (cells/ μ L)	EBV (cpm)	HHV-7 (cpm)	HHV-6B (cpm)	B19V (cpm)	CMV (cpm)	MCPyV (cpm)	Total No of Viruses
IP01		IP	2.3×10^4	1.4×10^3						1
IP02	1	IP	1.3×10^4							0
	2	S	4.6×10^2		4.4×10^3					1
IP03	1	IP	5.7×10^3	3.3×10^3						1
	2	IP-SNSCC	1.0×10^4	3.4×10^3						1
	3	IP	1.8×10^3		1.4×10^3					1
	4	S	2.3×10^2				1.3×10^5			1
	5	C	5.6×10^2							0
IP04		IP	2.6×10^3							0
IP05		IP	3.0×10^3							0
IP06		IP	5.7×10^3							0
IP07		IP	6.4×10^4		2.7×10^2	4.1×10^1	2.1×10^2			3
IP08		IP	3.3×10^3		6.2×10^1		3.6×10^1			0
IP09		IP	4.0×10^4							2
IP10		IP	6.1×10^3							0
IP11		IP	1.0×10^2							0
IP12		IP	2.2×10^4			3.8×10^2				1
IP13		IP	1.2×10^4							0
IP14		IP	2.4×10^3	2.9×10^2						1
IP15		IP	1.6×10^4							0
IP16		IP	1.5×10^4						3.1×10^2	1
IP17		IP	1.8×10^4		5.6×10^1	1.8×10^1				2
IP18		IP	2.7×10^3	1.4×10^3						1
IP19		IP	3.2×10^4					2.3×10^1		3
IP20		IP	4.7×10^3		3.3×10^1	2.9×10^1				0
IP21		IP	2.0×10^3							0
IP22		IP	1.7×10^3							0
IP23		IP	3.4×10^3	5.0×10^2						1
IP24	1	IP	3.6×10^3	3.1×10^3				2.9×10^2		2
	2	IP-SNSCC	1.3×10^4							0
IP25	1	IP	4.3×10^2							0
	2	IP	6.3×10^3							0
	3	IP	2.5×10^3							0
	4	IP-SNSCC	1.6×10^2							0
IP26		IP-SNSCC	2.1×10^4							0
IP27		IP-SNSCC	5.6×10^3	1.6×10^3	1.4×10^2		9.0×10^1			3
IP28	1	IP	3.6×10^3							0
	2	IP-SNSCC	1.5×10^3							0

No samples were PCR-positive for bufavirus, tusavirus, cutavirus, or BK and JC polyomaviruses.
 C = cicatrix; IP = inverted papilloma; IP-SNSCC = IP-associated sinonasal squamous cell carcinoma; S = healthy tissue surrounding the IP (taken from the same block as the IP).
 *Different samples of the same patient either taken at the same time (IP + S) or in chronological order.

TABLE III.
HPV Detection by NGS.

Patient ID	Sample Series*	Histology	HPV Presence	Breadth	Depth
IP03	1	IP	HPV16	99.5	15.9235
	2	IP-SNSCC	HPV16	100	175.5283
		Plasma	HPV16	8.91 (12 reads)	0.1781
	3	IP	HPV16	64.9	1.6139
	4	S	HPV16	52.0	1.1499
IP13	5	C	Negative		
		IP	HPV6	3.81 (2 reads)	0.07452
IP16		IP	HPV6	100	204.8003
IP28	1	IP	HPV16	40.2	1.1721
	2	IP-SNSCC	HPV16	17.92	0.2592

C = cicatrix; IP = inverted papilloma; IP-SNSCC = IP-associated sinonasal squamous cell carcinoma; S = healthy tissue surrounding the IP (taken from the same block as the IP).

*Different samples of the same patient either taken at the same time (IP + S) or in chronological order.

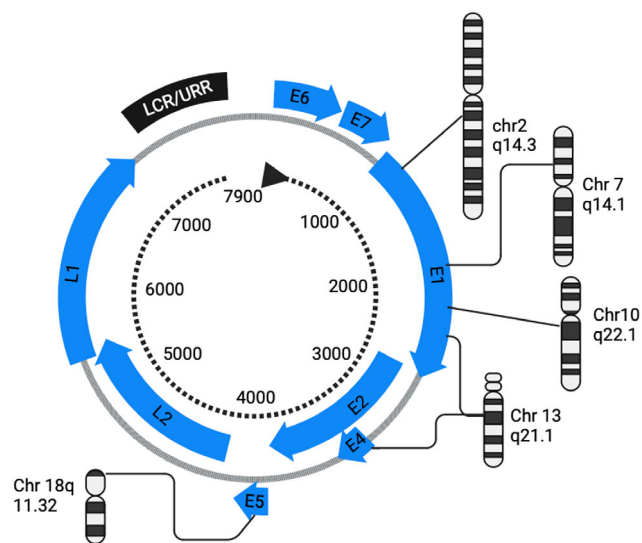


Fig. 1. HPV16 genome. The 7.9-kb genome with three reading frames are presented in three tracks. In the same diagram, HPV16 integration sites in human chromosomes 2 and 13 are shown together with schematic HPV6 integration sites in chromosomes 7, 10, and 13. Figure created with BioRender.com. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

prevalence of HHV-6B and -7 varies between 0%–25% and 0%–17%, respectively,^{30,31} and in healthy individuals' swab samples, a presence of up to 50% was observed.³² Latent HHV-6B and -7 infections have not directly been associated with disease development, but the nature of these viruses may be more complex, for example, by modulating the host-cell immune system.^{33–36} Within IPs, however, a relevant role of HHV-6 or HHV-7 seems unlikely considering their wide distribution.^{31,35,37}

The presence of parvovirus B19 in three samples does not suggest a major etiological role in IP. B19V was

detected in both IP and IP-SNSCC with low copy number. The prevalence of B19V in samples from healthy patients and in patients with head and neck SCC ranges between 6.5% and 37.9%.^{38,39} B19V DNA is known to persist for decades in various human tissues.^{37,39–41} It is noteworthy, that the only sample in our cohort with high B19V copy number (10^5), was taken from the healthy tissue surrounding the IP (patient IP03, sample 4). The potential oncolytic activity of parvoviruses is interesting but remains inconclusive.¹⁸

We used target-enrichment NGS for HPV detection. HPV16 was present in patients with IP-SNSCC throughout the course of the disease but not in patients with IP without associated malignancy. Interestingly, in the two patients with IP-SNSCC, HPV16 DNA was detected in the IP that preceded the malignant transformation. Moreover, for patient IP03, all subsequent samples even after resection of the malignant tumor and its radiotherapy were also positive for this virus DNA. However, when no more IP or IP-SNSCC was present, and the patient was cured, the final cicatrix tissue harbored no longer HPV16 (Table III). In this individual, we found evidence of virus DNA integration into the host chromosome, in ORF *E1* both before and after malignant transformation, a potential path for HPV-driven oncogenesis,^{12,42,43} and in ORF *E1*^{E4} (*E4*), responsible for genome amplification, virion release, and cell cycle arrest.^{12,44} HPV integration, known to promote malignant transformation, is present in up to 70%–80% of the events of cervical and head and neck cancer,^{45–47} while in IP-SNSCC integration is poorly documented. Overall, these findings contribute to the suggestion of HPV16 being a risk factor for IP-SNSCC malignant transformation, as observed in a meta-analysis by Stepp *et al.* and Ding *et al.*^{8,9} and hints to the persistence of the oncogenic virus in the surrounding tissue.

Because the lifecycle of HPV is intraepithelial, the viremia detected in our study is an uncommon finding. In a study of 136 patients by Adrian *et al.*⁴⁸ circulating

TABLE IV.
HPV16 and HPV6 Integration Details.

Patient/sample	Histology	Integration Point within Host	Gene/Area and Function*	HPV Type	Virus Position	Virus Protein Function†
IP03 sample 1	IP	Chr2 (q14.3)	ENSG00000286481 Long non-coding RNA (lncRNA). Not within a gene.	HPV16	E1	Genome replication.
IP03 sample 2	IP-SNSCC	Chr13 (q21.1)	Between RNY4P29 and ENSG00000288015 (lncRNA). ENSG00000122641.11. A TGF-beta family.		E1. E1*E4	Genome replication. Genome amplification and virion release. Cell cycle arrest.
IP16	IP	Chr7 (p14.1) Chr 10 (q22.1)	ENSG00000107736.22. cadherin CDH23. Cell adhesion. ENSG00000107738. mRNAV-set immunoregulatory receptor (VSIR). Telomere region. Prior to lncRNA (LINC02564).	HPV6	E1	Genome replication.
		Chr18 (p11.32)			E5b	Accessory oncoprotein. Control of cell growth and differentiation. Immune modulation

IP = inverted papilloma; IP-SNSCC = IP-associated sinonasal squamous cell carcinoma.

*Adapted from Nassar *et al.*⁵²

†Adapted from Nelson *et al.*^{12,49}

tumor HPV16 DNA load in oropharyngeal carcinoma significantly correlated with disease burden and associated with overall survival, and could function as an independent prognostic factor for disease. The HPV16 DNA in plasma at the time of IP-SNSCC could relate to a similar phenomenon, and plasma analysis of IP-SNSCC patients would be warranted.

Some HPV16 variants may result in a higher carcinogenic outcome in cervical cancer, especially in combination with a specific genetic background of the patient.¹⁴ Although HPV is accepted as an etiological factor in some oropharyngeal cancers and its association with IP-SNSCC has been proposed, the roles of HPV variants are not thoroughly documented. We observed a two-fold increase in HPV16 minor intratypic variants across pre- and post-malignant transformation samples (Fig. 2, Table V). While the role of the cumulative changes in the malignant transformation remains to be assessed, our findings are in line with those reported in the meta-analyses by Stepp *et al.*^{8,9}

HPV6 is a low-risk HPV type, the major cause of anogenital warts and laryngeal papillomas. In our cohort, IPs from two patients were positive for HPV6. In one of them, we identified integration of HPV6 DNA in a subset of viral reads at three sites in the host genome. Overall, integration of HPV6 genomes is rare and has only been reported anecdotally in respiratory papilloma related to laryngeal carcinoma.⁷ The virus integration junctions were present in genes *E1* and *E5b*, well-characterized sites in HPV16 but with inconclusive biological functions within low-risk HPVs, possibly participating in the viral replication, virus release, and immunosurveillance avoidance.⁴⁹ Further, a nonsynonymous nucleotide change was present in oncogene *E6*, whereas the others were in structural genes. Overall, the large number of mutations is noteworthy. Contrary to our findings, Jelen *et al.* noted a lower diversity in HPV6 than in HPV16.⁵⁰

This study provides valuable insights to the prevalence of herpes-, parvo-, and polyomaviruses in IP and IP-SNSCC. However, the impact of this study regarding HPV prevalence is minor due to the small cohort. The overall HPV prevalence of patients with IP or IP-SNSCC in our cohort (14%, 33%) is roughly in range with that of a meta-analysis comprising 1416 patients with IP (23%) and within patients with IP-SNSCC (24%).⁶ Moreover, this study provides novel understanding on the genomic diversity and integration status of HPV6 and 16. Limitations of this study include the cohort size and the use of FFPE tissue samples. Fresh tumor material could provide greater sensitivity. Nevertheless, our methods have been validated in formalin-fixed samples, supporting the accuracy of our findings.⁵¹ It is crucial to specify that the presence of a virus does not imply a role in tumor development, and in this study, viruses were sometimes present in low copy numbers. Hence, in addition to further virus search, in-depth studies are needed. Due to the rarity of follow-up tumors of IP and IP-SNSCC, larger multicenter studies would be beneficial.

TABLE V.
Minor Variants in HPV6 and HPV16 Sequence Data in Two Patients.

	ORF (5'-3')	CDS Start-End for HPV16/HPV6	Key Protein Functions*	Sample Information and No. of Variants			
				IP03, Sample 1 (HPV16)	IP03, Sample 2 (HPV16)	IP16 (HPV6)	
				Total No.: 8	Total No.: 15 (4 Outside ORF)	Total No.: 44 (1 Outside ORF)	
No. of SNVs/No. of Indels/No. of Nonsynonymous Changes							
Oncoprotein	E6	104-559/1-453	Degradation of p53. Inhibits apoptosis and differentiation.	0	0	0	1/0/1
Replication, release, genome maintenance	E7	562-858/429-725	Degrades pRb. Cell cycle control.	0	0	0	0
	E1	865-2814/731-2680	Helicase. Genome replication.	1/2/1	6/0/6	14/2/12	0
	E2	2756-3853/2622-3728	DNA-binding protein. Genome replication. Cell cycle and apoptosis regulation.	1/0/1	1/0/0-1 [†]	0	0
	E4 (E1+E4)	3358-3620/3154-3483	Genome amplification and virion release. Cytokeratin network remodeling. Cell cycle arrest.	1/0/1	1/0/0-1 [†]	0	0
Accessory oncoprotein	E5 a, b	3850-4101/3786-4276	Control of cell growth and differentiation. Immune modulation.	0	2/0/1	4/0/4	
Structure	L2	4236-5657/4322-5701	Minor capsid protein.	3/0/1	1/0/1	10/1/5	
	L1	5560-7155/5688-7190	Major capsid protein. Defines HPV type.	0	0	8/4/7	

Patient IP03, sample 1, is pre-malignant transformation (histology: inverted papilloma), and sample 2 is post-malignant transformation (histology: IP-associated sinonasal squamous cell carcinoma). Patient IP16 has inverted papilloma.

CDS = protein coding sequence; ORF = open reading frame; SNVs = single nucleotide variants.

*Modified from Nelson *et al.* and Graham.^{12,14}

[†]The nonsynonymous mutation is located either in E2 or E4 (overlapping reading frames).

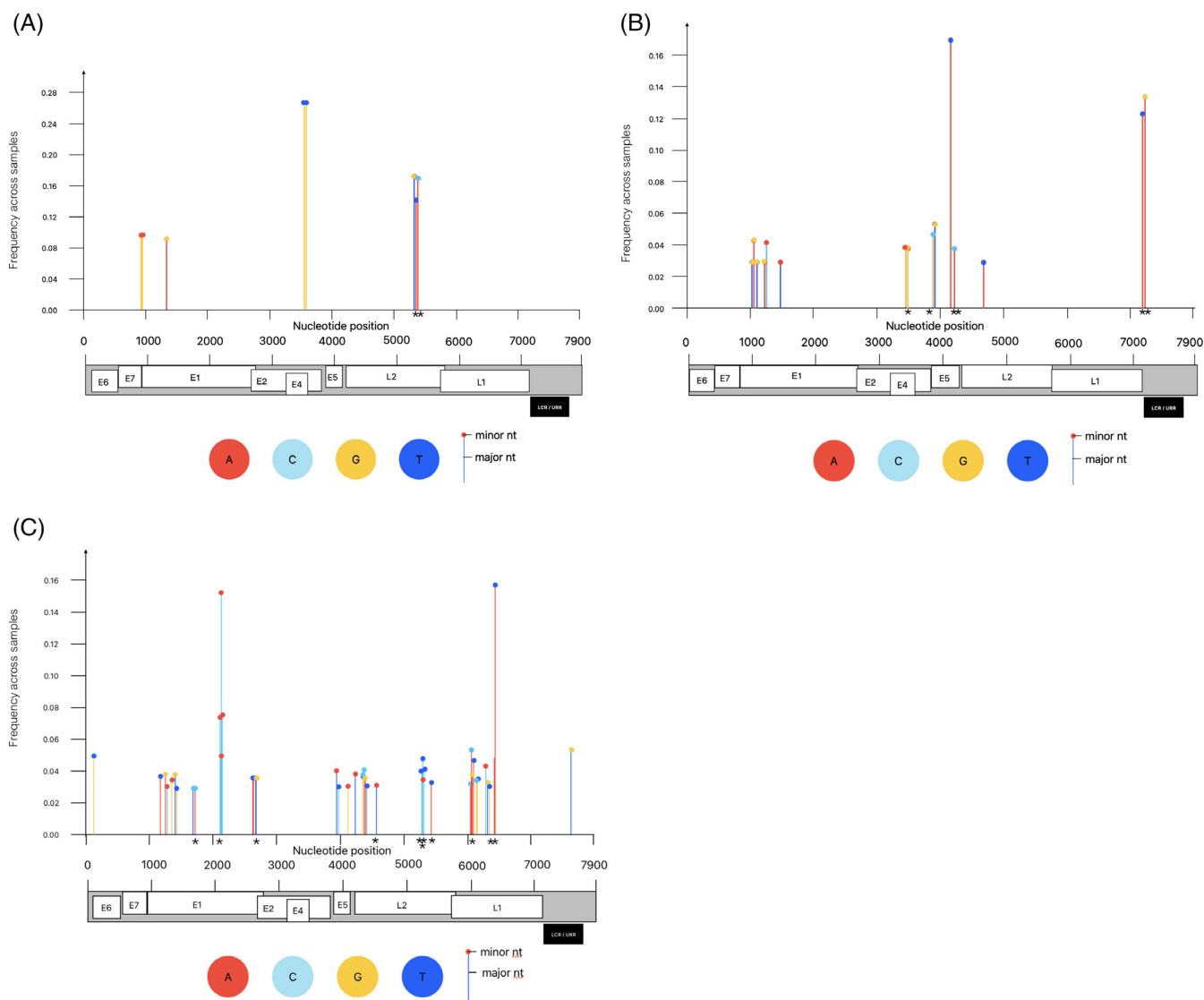


Fig. 2. Diagram of minor variants in HPV6 and 16 sequence data. The height of the bar represents the frequency of the alternative/minor nucleotides in the sample. The variant is labeled with the gene ID and the nucleotide change. (A) HPV16-positive patient IP03, sample 1, pre-malignant transformation (histology: inverted papilloma). (B) HPV16-positive patient IP03, sample 2, post-malignant transformation (histology: IP-associated sinonasal squamous cell carcinoma). (C) HPV6-positive patient IP16 (histology: inverted papilloma). [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

CONCLUSION

We investigated the presence of DNA viruses in IPs and IP-SNSCCs. Multiple DNA viruses were present, and they tended to accumulate in certain samples. Differences in the HPV behavior in pre- and post-malignant transformation samples were observed both within the integration and within intratypic nucleotide variance. A marked increase in viral diversity was present, supported by accumulation of mutations overtime. Our findings suggest HPV contributing to the malignant transformation of IP, although larger studies are warranted to characterize the changes potentially contributing to this process. The overall pathogenesis might be more versatile and not only limited to the presence of HPV or its integration.

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