Human papillomavirus load and physical status in sinonasal inverted papilloma and squamous cell carcinoma*

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Summary

Background: This study investigated prospectively the role of human papillomavirus (HPV) in paranasal inverted papilloma (IP).

Methods: HPV presence and viral load and physical status of HPV-16 were examined by polymerase chain reaction-based methods using fresh frozen samples obtained from 13 patients with IP (IP group), 11 with squamous cell carcinoma in the maxillary sinus (SCC group) and 39 with chronic inflammatory lesions (inflammatory group).

Results: The presence of the HPV genome was detected in 46.1%, 27.3% and 7.6% of patients in the IP, SCC and inflammatory groups, respectively. The IP group showed significantly higher HPV-positive rates than the inflammatory group. All types of HPV detected were high-risk HPV, especially HPV-16. The relative HPV-16 copy numbers varied from 2.5 to 1524.1 per 50 ng genomic DNA. The viral load was higher in the IP and SCC groups than in the inflammatory group. In the IP group, no significant relationship was found between HPV-16 viral load and clinical characteristics, or between physical status and clinical characteristics. One patient with IP and concomitant squamous cell carcinoma, however, showed high viral load and integration.

Conclusions: HPV infection is involved in the pathogenesis of IP, and high viral load and integration of HPV have an important role in malignant lesion in association with IP.

Key words: inverted papilloma, integration, viral load, human papillomavirus, paranasal sinus

Introduction

Inverted papilloma (IP) of the paranasal sinus is a benign neoplasm with unique clinical characteristics, particularly a high recurrence rate and the association with malignant lesions. The recommended treatment for IP is complete surgical excision. Recently, endoscopic tumour resection has become the standard surgical approach for the management of IP. According to several meta-analyses of IP treatment, local recurrence of endoscopic resection and malignant lesion in association with IP is estimated to be approximately 11.8 – 15.0% and 8.0 – 9.3%, respectively [1-3]. In certain cases, it is very difficult to diagnose and control tumour recurrence and/or concomitant malignant lesions due to anatomical alterations from previous operations, such as bony hypertrophy and substantial scarring. Therefore, effective diagnostic markers to predict local recurrence and malignant lesion are needed.

There have been several reports regarding the close relationship between IP and human papillomavirus (HPV) infection. Beck et al., not only found that 63% of IP cases were positive for HPV DNA, but also that the presence of HPV sequences predicted recurrence of IP [4,5]. They reported recurrence in 13 of 15 patients whose tumours were positive for HPV, whereas only one of 10 patients with HPV-negative tumours experienced

recurrence [4].
Moreover, they found that patients with HPV types 16 or 18 had a higher rate of associated malignancy than patients with HPV types 6 or 11 [5]. However, other authors have not found a significant correlation between malignant lesions in IP and the HPV type [6,7].

Many methods are available to detect HPV DNA in samples and include Southern blotting, in situ hybridisation (ISH) and polymerase chain reaction (PCR) [4-7]. As IP is a relatively rare tumour, most previous studies have used formalin-fixed paraffin-embedded archival tissue. PCR amplification is more efficient on frozen samples than on paraffin-embedded samples, as it is known that the fixation procedure leads to DNA fragments that are often shorter than 200 bp [8]. In the present study, to clarify the clinical importance of HPV in IP, viral load and physical status of HPV were prospectively examined using fresh frozen samples of IP, squamous cell carcinoma (SCC) of the maxillary sinus and sinonasal inflammatory mucosa.

Subjects and methods

Study design

A total of 13 patients with IP in the paranasal sinus (IP group; 5 males, 8 females; age range 46 – 76 years; median age 57 years) participated in this study. All patients underwent surgery (conducted by M.H. and M.S.) between 2008 and 2010. IP specimens obtained during surgery were stored in liquid nitrogen for further analysis.

For comparison, specimens from 11 patients with SCC in the maxillary sinus (SCC group) and inflammatory nasal or paranasal specimens from 39 patients with chronic sinusitis, maxillary cyst or allergic rhinitis (inflammatory group) were also collected and stored in liquid nitrogen for later analysis. The inflammatory group consisted of 34 patients with chronic sinusitis and 5 patients with post-operative mucoecele in the paranasal sinus. Specimens in the inflammatory group were sampled from nasal polyps and hypertrophic mucosa around cysts.
The study protocol was approved in advance by the Institutional Review Board of the University of the Ryukyus. All participants provided written informed consent according to the guidelines of the Ethics Committee of the University of the Ryukyus. This study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient characteristics

The following data were noted in and later summarized from the clinical records of patients with IP: Krouse's stage [1], tumour location, history of previous surgeries, surgical procedures undertaken, recurrence and malignant lesion.

Detection of HPV genome and identification of HPV types

Fresh frozen samples from the three groups were analysed to determine the prevalence of HPV and HPV types. A Gentra Purification Tissue kit (Qiagen, Germantown, MD, USA) was used to isolate DNA from the specimens according to the manufacturer's specifications. The presence and integrity of DNA was verified in all samples by PCR β-globin gene amplification using the primers PC04 and GH20 [9]. Negative controls using water and positive controls using DNA of HPV-16-positive CaSki cells were included in each amplification series.
The presence of HPV DNA was analysed by PCR using the general consensus primer sets GP5+/GP6+ and MY09/11 [10,11]. DNA samples that were negative in the GP5+/GP6+ or MY9/11 PCR were re-amplified in a nested PCR using the GP5+/GP6+ primer pair as previously described [12], which can increase the sensitivity of HPV detection.
PCR was performed in a programmable thermal cycler (VeritiTM 96-Well Thermal Cycler, Applied Biosystems, Carlsbad, CA, USA). Subsequently, 10 µL reaction mixture was electrophoresed through 2% agarose gel containing 0.5 µg/ml ethidium bromide and visualised by ultraviolet transillumination.
After purification of positive PCR products, sequence analysis was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Obtained sequences were aligned and compared with those of known HPV types available from the GenBank database using the BLAST program.

Viral load and physical status of HPV-16

To investigate HPV-16 viral load and physical status, quantitative real-time PCR using HPV-16 DNA-positive samples was performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems) and TaqMan PCR Master Mix II (Roche Molecular Systems, Foster City, CA, USA). Primers and TaqMan probes that target the HPV-16 E6 and E2 open reading frames were used, as previously described [13]. Both E6 and E2 probes were labelled with FAM at the 5′ end and with TAMRA at the 3′ end (Applied Biosystems Japan Ltd, Tokyo, Japan). E2 primers and probe recognise the E2 hinge region, which was deleted on HPV-16 integration. Amplification conditions were 2 min at 50°C, 10 min at 95°C and a two-step cycle of 95°C for 15 s and 60°C for 60 s, for a total of 50 cycles [13]. Two standard curves for the E6 and E2 genes were generated by amplification of serial 10-fold dilutions (10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 viral copies) of a plasmid pβ-actin carrying the complete HPV-16 early region (Addgene, Inc., Cambridge, MA, USA). For specificity and validity testing, DNA samples from the SiHa and the CaSki cell lines were examined. For cellular DNA quantification, an external standard curve was generated using known serial dilutions (0.3, 3, 30 and 300 ng) of human genomic placental DNA (Sigma-Aldrich, St. Louis, MO, USA) [14], and β-globin was amplified as described by van Duin et al., [15]. The amount of DNA (ng) was calculated by plotting the Ct (threshold cycle) values against the logarithm of the standard curve. The relative viral load was

identified by calculating copies of specimen E6 in 50 ng cellular DNA.

The physical status of HPV-16 was determined according to a previously described method [13]. The integrated E6 was calculated by subtracting the copy numbers of E2 (episomal) from the total copy numbers of E6 (episomal and integrated). Ratios of E2 copy number/total E6 of < 1 indicate the presence of both integrated and episomal forms. The ratio of E2 to integrated E6 represents the amount of the episomal form in relation to the integrated form. An E2/E6 ratio nearly equal to 1 indicates predominance of the episomal form, whereas a ratio of 0 indicates the presence of only the integrated form [13].

Results

Patient characteristics

According to Krouse's classification, T2, T3 and T4 stage tumours were observed in 2, 9 and 2 patients, respectively. Patients with T4 tumours included one with IP with massive extension to the pterygoid fossa and another with IP and concomitant SCC. Figure 1 shows the histological findings in the latter case with IP and SCC. Although the majority of the tissue specimen had the characteristics of IP, the invasive SCC was present, in part, in IP. The observation period following surgery ranged from 5 to 41 months, with a median of 17 months. At the first clinic visit, 7 patients had a previous history of sinus surgery. Tumour locations were widely distributed within the nasal and paranasal sinus, and 10 of 13 patients with IP had lesions in the maxillary sinus. The main surgical procedure carried out at our facility to treat IP was endoscopic resection, that is, standard endoscopic sinus surgery or transnasal endoscopic medial maxillectomy (Table 1). Local recurrence after surgery was observed in 2 patients (15.4%), both of whom had a previous history of multiple sinus surgeries, with accompanying marked fibrosis and proliferation of osseous tissue in the nasal and paranasal cavity. Recurrent lesions were successfully salvaged through external approaches, such as external frontoethmoidectomy and lateral rhinotomy. Malignant lesion during the follow-up period was not observed in any patients with IP.

HPV detection

Figure 2 shows representative examples of the PCR results. Of 13 patients with IP, the HPV genome was detected in 6 patients (46.2%, Table 2). By contrast, 3 of 11 (27.3%) patients with maxillary SCC and 3 of 39 (7.7%) patients with inflammatory diseases were positive for HPV. Patients in the IP group showed significantly higher HPV-positive rates than those in the inflammatory group ($p < 0.01$, chi-square).

Of 6 HPV-positive IP patients, the types of HPV included HPV-16 (n = 4) and HPV-33 (n = 2). All HPV-positive specimens obtained from patients in the SCC and inflammatory groups contained the HPV-16 genome. Multiple infections were not detected in the IP, SCC or inflammatory groups. In the IP group, there was no obvious relationship between HPV presence and clinical features of IP (Table 3, chi-square).

Localisation of HPV genome

Samples from 3 of the 6 HPV-positive IP patients in whom the inferior turbinates adjacent to the IP were not affected by IP, were subjected to PCR to determine the presence of HPV. As a control, samples from 4 of 7 HPV-negative IP patients with inferior turbinates adjacent to the IP were also examined. No positive PCR reactions were observed in HPV-positive or -negative IP patients.

Viral load and physical status of HPV-16

As the most common HPV type detected was HPV-16, viral load and physical status of HPV-16 were investigated in the IP, SCC and inflammatory groups. Both validated assays of the real-time PCR for E2 and E6 genes using serially diluted HPV-16 early region plasmid DNA showed similar amplification efficiencies, as reflected by the almost identical slopes of the amplification curves.

Viral load and physical status of HPV-16 in IP, paranasal carcinoma and inflammatory diseases are shown in Table 4. E6 copy number per 50 ng genomic DNA in HPV-16-positive specimens varied from 2.5 to 1524.1. Although statistical analysis cannot be carried out because of the small number of cases, the IP and SCC groups showed relatively high viral load, compared with the inflammatory group. According to physical status, all specimens in the SCC group showed mixed type integration of HPV-16. By contrast, the IP group had 1 case of mixed type with concomitant SCC and IP and 3 cases of episomal types, and the inflammatory group had 1 case of mixed type and 2 of episomal types. There were no significant relationships in the IP group between HPV-16 viral load and clinical characteristics, for example, tumour staging and recurrence, or between physical status and clinical characteristics. However, the patient with IP and concomitant SCC showed a high E6 copy number and mixed type integration.

Discussion

To our knowledge, this is the first prospective study to simultaneously evaluate HPV presence, HPV viral load and physical status in IP, cancerous lesions and inflammatory lesions using fresh frozen samples. The results of previous studies to determine HPV presence in IP have been inconsistent [16,17]. In the present study, HPV was detected in 46.1% of patients with IP, 27.3% with maxillary SCC and 7.6% with inflammatory diseases. Although the reason for the differences in HPV presence among studies remains unclear, sample preparation (i.e. fresh frozen or paraffin embedded), methods for HPV detection (i.e. PCR, Southern blotting or ISH) and geographic factors may be the

Table 1. Summary of patient characteristics.

Case	Sex	Age	HPV detection	Krouse's stage	Previous surgery	Previous surgical procedure(s)	Tumour location	Concomittant malignancy	Current surgical procedure	Observation period (months)	Malignant transformation	Recurrence	Salvage operation
1	F	49	none	T2	none	none	MW	-	ESS	9	-	-	
2	M	46	HPV16	T3	2	ESS (2 times)	Et, FS, MW	-	ESS	41	-	+	External frontoethmoidectomy (34 months)
3	M	54	HPV16	T3	1	ESS	SS, SupT	-	ESS	18	-	-	
4	F	52	none	T3	none	none	MW, IW	-	ESS, Luc	15	-	-	
5	F	63	HPV33	T3	1	ESS	MW, IW	-	TEMM	17	-	-	
6	F	60	none	T3	none	none	MW, IW, Et	-	TEMM	31	-	-	
7	F	48	none	T3	none	none	MW, IW, PW, AW, Et	-	TEMM	24	-	-	
8	M	62	HPV16	T2	none	none	MW, Et, SupT, MidT	-	ESS	19	-	-	
9	M	75	none	T4	2	Luc, lateral rhinotomy	MW, SW, PW, Et, Ptery	-	Luc	18	-	+	Lateral rhinotomy (13 months)
10	F	58	HPV16	T4	none	none	MW, IW, SW, AW, LW	+	TEMM, Luc	7	-	-	
11	F	49	none	T3	1	ESS	MW, IW, SW	-	TEMM	6	-	-	
12	M	58	none	T3	1	ESS	SS, SupT	-	ESS	6	-	-	
13	M	76	HPV33	T3	1	ESS	Et, SS	-	TEMM	10	-	-	

ESS: endoscopic sinus surgery; TEMM: transnasal endoscopic medial maxillectomy; Luc: Luc's operation; MW: medial wall of maxillary sinus; IW: inferior wall of maxillary sinus; PW: posterior wall of maxillary sinus; AW: anterior wall of maxilary sinus; LW: lateral wall of maxillary sinus; Et: ethmoid sinus; SS: sphenoid sinus; FS: frontal sinus; SupT: superior turbinate; MidT; middle turbinate; Ptery: pterygoid fossa

source of the contradictory results. Despite a few studies that used fresh frozen samples of IP [18], the majority of previous studies to investigate HPV presence in IP employed formalin-fixed paraffin-embedded archival tissues [16,17]. Specimen storage with or without fixation, and the duration of tissue storage are important factors for HPV detection because of their influence on DNA preservation and retrieval efficiency for PCR [8,19]. DNA extraction from frozen samples generally leads to better DNA extraction and thus greater sensitivity for molecular analysis. Therefore, fresh frozen samples were used for prospective HPV analysis in the present study. The use of varying methods for HPV detection is also a possible cause of the wide range of observed HPV rates. PCR is a suitable and economical assay that is comparable to ISH with regard to sensitivity, and may provide logistical advantages compared to ISH for assessing HPV status in oropharyngeal malignancies [20]. Indeed, a significantly higher rate of HPV positivity was detected among cervical cancer samples with PCR than with catalysed signal amplification ISH [21]. Geographic factors may also be important. Lawson et al., reported that HPV presence was not different among patients from North America, Europe and East Asia [17]. However, analysis of HPV infection in head and neck squamous cell carcinoma showed a geographic difference of HPV viral load and rate of HPV presence across East Asia [22]. More precise analysis of geographic factors that regulate specimen storage and detection methods is needed to clarify these geographic differences.

Table 2. Detection of human papillomavirus (HPV).

	Human papillomavirus		
	Positive	Negative	Positive rate (%)
IP group	6	7	46,2
n = 13	HPV16: 4 HPV33: 2		
SCC group	3	8	27,3
n = 11	HPV16: 3		
Inflammatory gorup	3	36	7,7
n = 39	HPV16: 3		

Table 3. Relationship between HPV presence and clinical characteristics.

	HPV(+) (n)	HPV(-) (n)	Total (n)
Sex			
Male	4	2	6
Female	2	5	7
Age			
<60	3	5	8
≥ 60	3	2	5
Krouse's stage			
T2	1	1	2
T3	4	5	9
T4	1	1	2
Previous surgery			
No	2	4	6
Yes	4	3	7

Table 4. Physical status of HPV16 in specimens.

Specimen	HPV type	E6 (copies/50 ng genomic DNA)	E2/E6	Physical status
IP group				
IP (case 2)	16	74	1	Episomal
IP (case 3)	16	24.2	1	Episomal
IP (case 8)	16	2.5	1	Episomal
IP + SCC (case 10)	16	1524.1	0.65	Mixed
IP (case 5)	33			
IP (case 13)	33			
SCC group				
Maxillary SCC	16	594	0.46	Mixed
Maxillary SCC	16	79	0.09	Mixed
Maxillary SCC	16	35	0.11	Mixed
Inflammatory group				
Sinusitis	16	11.6	1	Episomal
Sinusitis	16	5.1	0.12	Mixed
Sinusitis	16	6.8	1	Episomal

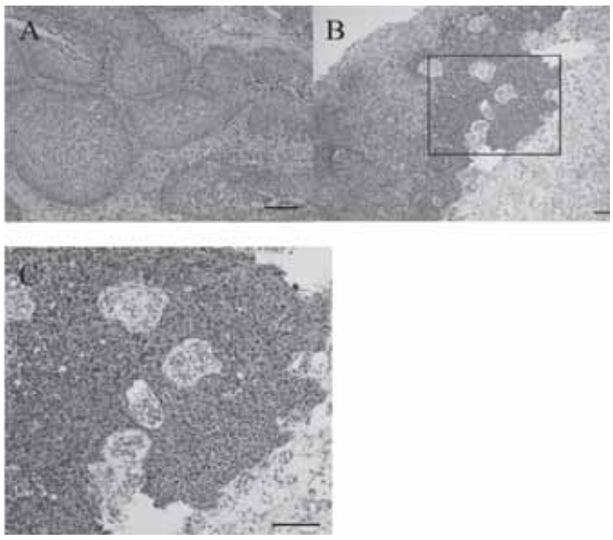

Figure 1. Histological findings of inverted papilloma with squamous cell carcinoma.
(A): The majority of samples obtained during sinus surgery showed inversion of the multilayer epithelium into the underlying oedematous stroma. Scale bar = 200 µm.
(B) Dysplastic squamous epithelium was transformed into invasive carcinoma with destruction of the basement membrane. Scale bar = 100 µm.
(C) Enlargement of the rectangular portion in (B). The tumour cells show marked atypia, an increased nuclear to cytoplasmic ratio, conspicuous nucleoli and a loss of polarity; these cells were diagnosed as non-keratinising squamous cell carcinoma. Scale bar = 100 µm.

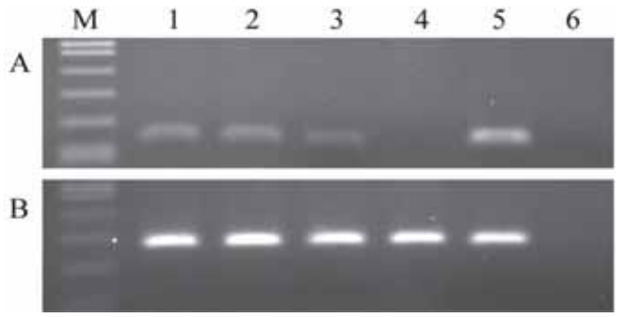

Figure 2. Representative examples of PCR electrophoresis using consensus primer set GP5+/GP6+.
(A): The 150-bp bands were observed as amplicons of HPV DNA.
(B): The 268-bp bands were observed as amplicons of beta-globin DNA to ensure the quality of the DNA.
M: DNA marker; lane 1: HPV-16-positive maxillary SCC; lane 2: HPV-16-positive inverted papilloma with SCC; lane 3: HPV-16-positive inverted papilloma without SCC; lane 4: nasal sinusitis; lane 5: PC: positive control (SiHa cell); lane 6: NC: negative control (no template DNA).

In the present study, the presence of HPV was limited to the tumour itself and did not spread to neighbouring areas. Hoffmann et al., reported that tissue derived from normal mucosa adjacent to the head and neck carcinoma showed no PCR amplification for HPV DNA [23]. Although the natural history of HPV has not been fully determined, generally this infection does not lead to visible lesions and may be cleared by the immune system within a short period of time [24]. The localisation of HPV in IP may reflect such a process.

The role of HPV in IP has not been well established. Several high- and low-risk HPV types have been reported in IP. In a study conducted in Japan, Katori et al., demonstrated the presence of low-risk HPV types (6 or 11) in 11 of 26 (42.3%) IP patients and high-risk HPV (16 or 18) in 8 of 26 (30.8%) patients [25]. In a US study, Beck et al., showed that 12 of 39 (31%) patients were low-risk HPV positive and 8 of 39 (21%) were high-risk HPV positive [5]. By contrast, Hoffmann et al., [18] and Kraft et al., [7] detected only low-risk HPV-6 and 11 in IP (11.5% and 3.0%, respectively). In general, low-risk HPV-6 and 11 exist in episomal forms independent of squamous intraepithelial lesion grade in the cervix [26]. In the head and neck region, low-risk HPV is frequently observed in benign lesions such as laryngeal papilloma and exophytic nasal papilloma. The overall detection rate of HPV in IP varies among studies, ranging from 0% to 72.4% [17]. Precise analysis of published reports demonstrated that HPV-6 and 11 were found in 17.8% of IP cases, whereas HPV-16 and 18 were present in 8.1% [17]. In the present study, 46.1% of IP contained a high-risk HPV genome and the prevalence of HPV in IP was significantly higher than in the inflammatory group. There have been a few studies of HPV prevalence in inflammatory disease. Hoffmann et al., reported that 1 of 39 (2.6%) patients had HPV-positive disease [27], and Zaravinos et al., showed that 3 of 23 (13.0%) patients were HPV positive [28]. Unfortunately, these studies did not provide information about HPV type. In the present study, we describe the HPV type in inflammatory nasal diseases. We found that the HPV prevalence (6.8%) in inflammatory diseases fell between the previously reported values, and surprisingly the type of HPV was high-risk HPV-16, as in IP and SCC. We previously reported an HPV prevalence of 2.1% and the presence of HPV-33 in chronic tonsillitis [22]. From these data, it appears that there is a low incidence of HPV, even the high-risk type, in the upper respiratory tract. Marked differences in the prevalence of HPV between IP and inflammatory nasal diseases suggest that HPV infection is involved in the tumorigenesis of IP.

Although no correlation was found between malignant lesion in association with IP and HPV presence in some studies [6,7], McKay et al., reported that HPV transcripts were present in

IP with SCC positive for HPV, using real-time PCR [29]. In the present study, the mixed or integrated form of HPV was found in 1 of 4 (25%) patients with IP and 1 of 3 with (33%) inflammatory diseases, compared with all 3 patients with maxillary SCC. No significant relationship between clinical features of IP and HPV integration was observed in patients with IP because of the small number of subjects. However, it is interesting that a patient with IP with integrated HPV-16 also presented with concomitant SCC. In the inflammatory disease group, despite carrying the high-risk HPV, the viral load of high-risk HPV was low. Given that the patient with IP with integrated HPV and concomitant maxillary SCC had high-risk HPV and a relatively high viral load, malignant lesion observed in IP may be related to a high viral load in addition to high-risk HPV integration to the host genome. Further prospective studies with large samples are needed to clarify the role of HPV in the tumorigenesis and malignant lesion in association with IP.

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Authorship contributions

MH obtained research grants for this study, acquired samples and clinical data, supervised the experiments and prepared the manuscript; DZ obtained research grants for this study, conducted the experimental studies and acquired data; HM, YY, SM, AK, SA and TU acquired samples and clinical data and performed data analysis; and MZ contributed to the study design, supervision of the experiments and manuscript review.

Conflicts of interest statement

There are no potential conflicts of interest relevant to this article to disclose.

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