

## ORIGINAL ARTICLE

## The characteristics of human papillomavirus DNA in head and neck cancers and papillomas

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**Aim:** To determine the prevalence, type, physical state, and viral load of human papillomavirus (HPV) DNA in cases of head and neck cancer and recurrent respiratory papillomatosis (RRP).

**Methods:** The prevalence and type of HPV DNA was determined in 27 fresh frozen tissue specimens from patients with head and neck cancers and 16 specimens from 10 patients with RRP by MY09/MY11 and GP5+/GP6+ nested polymerase chain reaction (PCR) and subsequent restriction enzyme cleavage. The physical state of HPV DNA was analysed by E1, E2, and E1E2 specific PCRs and Southern blot hybridisation (SBH).

**Results:** HPV DNA was detected in 13 of 27 cancers and 10 of 10 papillomas. Both low risk HPV-6 and HPV-11 and high risk HPV-16 were present in cancers in low copy numbers, whereas papillomas exclusively harboured low risk HPV-6 and HPV-11. E1E2 PCRs failed to determine the physical state of HPV in cancers except one case where HPV-6 DNA was integrated. In contrast to cancers, all papillomas showed the episomal state of HPV DNA and a relatively higher viral load.

**Conclusions:** Based on the prevalence, type, physical state, and copy number of HPV DNA, cancers and papillomas tend to show a different HPV DNA profile. The 100% positivity rate of low risk HPV types confirms the role of HPV-6 and HPV-11 in the aetiology of RRP.

The association of high risk human papillomavirus (HPV) types with anogenital cancers is well established. In the past two decades, several authors have detected HPV DNA in head and neck cancers by Southern blot hybridisation (SBH) or polymerase chain reaction (PCR).<sup>1–10</sup> Among head and neck cancers, HPV DNA positivity tends to show site dependence, with the tonsils, oral cavity, and larynx being the most common sites.<sup>1–7 10</sup> High risk HPV types 16 and 18 are by far the most predominant types at all sites.<sup>3 7 10 11</sup> However, epidemiological data reveal that the role of HPVs in the aetiology of head and neck cancers is rather controversial: the reported frequency of HPV DNA even in the often studied laryngeal site varies between 3% and 85% in the literature.<sup>8</sup> In addition, the prognostic value of HPV DNA positivity is also equivocal.<sup>2 9</sup>

Recurrent respiratory papillomatosis (RRP) is the most common benign tumour of the laryngeal epithelium.<sup>12</sup> Low risk HPV-6 and HPV-11 are the most frequently detected types and are accepted as aetiological agents in RRP.<sup>13–16</sup>

“The product of the E2 open reading frame has a regulatory role in the transcription of the transforming E6 and E7 viral genes”

HPV DNA may exist either in the episomal form or integrated into the host's genome.<sup>17–20</sup> In the episomal form, the circular double stranded papillomavirus DNA is intact. In contrast, integration results in disruption of the circular viral genome, mainly in the E1E2 open reading frames (ORFs).<sup>17 21</sup> The product of the E2 ORF has a regulatory role in the transcription of the transforming E6 and E7 viral genes. Disruption of this regulatory role at integration results in increased concentrations of transforming viral proteins E6 and E7, which interact with the p53 and retinoblastoma tumour suppressor proteins, respectively, and therefore alter cell cycle control.<sup>21</sup> The physical state of HPV DNA tends to correlate with histopathological findings in the uterine cervix. Viral DNA is present as an episome in low grade cervical intraepithelial lesions, whereas the integrated form is predominant in high grade cervical intraepithelial lesions and

invasive tumours, although integration is not an essential prerequisite for malignant progression.<sup>17 18 22</sup> Thus, the prognostic role of integration in cervical cancer remains unclear.<sup>23 24</sup>

The physical state of HPV DNA in head and neck tumours has not been widely studied.<sup>1 25 26</sup> Results of integration studies indicate that HPV DNA can exist either in an integrated or episomal form, or both, in head and neck cancers, and integration is a rare event compared with cervical cancers.<sup>1 26</sup> The prognostic relevance of HPV DNA integration in head and neck cancers is unknown.

In our present study, we attempt to determine the prevalence, type, physical state, and copy number of HPV DNA in our study population in the northeast region of Hungary. We must emphasise that both the age standardised incidence and mortality rates of laryngeal cancer in Hungarian men are still the highest in Europe.<sup>27</sup>

## MATERIALS AND METHODS

## Patients and clinical specimens

Fresh frozen tissue samples from 27 patients (26 men, one woman; age, 43–72 years, mean age, 57) with cancers of the larynx and pharynx were collected over a two year period in the clinic of otorhinolaryngology and head and neck surgery, University of Debrecen, Hungary. Written informed consent was obtained in all cases. Biopsies for HPV DNA examination were taken from the centre of the lesions. The initial tumour site was laryngeal in 16 cases, hypopharyngeal in four cases, laryngo-hypopharyngeal in two cases, and mesopharyngeal in five cases. According to the TNM criteria, two patients had T1, eight patients had T2, 15 patients had T3, and two patients had T4 disease in the primary tumour site. The lymph node status was N0 in 15 cases, N1 in seven cases, and N2 in five cases. Haematoxylin and eosin stained sections of all cases were reviewed and the histopathological diagnosis of squamous cell cancer was confirmed.

**Abbreviations:** HPV, human papillomavirus; ORF, open reading frame; PCR, polymerase chain reaction; SBH, Southern blot hybridisation; RRP, recurrent respiratory papillomatosis

**Table 1** E1, E2, and E1E2 specific PCRs for HPV types 6, 11, and 16

HPV type	Primer designation	Sequence and genomic position	PCR product	PCR conditions
6	E1	E1/1: 5'-GGC ACA GCA GGA ATA TTG GA-3' (nt 1403-1422)	1130 bp	1 cycle: 94°C, 2 min. 40 cycles: 94°C, 1 min; 50°C, 1.5 min; 72°C, 1.5 min. 1 cycle: 72°C, 2 min
		E1/2: 5'-GAT GTT ACT AGC AGA GGT GG-3' (nt 2513-2532)		
	E2	E2/1: 5'-TTA GAT GCG TGC CAG GAA CA-3' (nt 2745-2764)	845 bp	
		E2/2: 5'-TGC ACT ATA GGC GTA GCT GA-3' (nt 3570-3589)		
	E1E2	E1E2L: 5'-CAG GCA CAG CAG GAA TAT TG-3' (nt 1250-1269)	2206 bp	
		E1E2R: 5'-TTG GAC TCC TCG TGC TCG TT-3' (nt 3436-3455)		
11	E1	E1/1: 5'-GGC ACA GCA GGA ATA TTG GA-3' (nt 1688-1707)	1081 bp	As for HPV-6 E1E2 PCR, but annealing T=52°C (E1E2 PCR); 35 cycles
		E1/2: 5'-GAT GTT ACT AGC AGA GGT GG-3' (nt 2749-2768)		
	E2	E2/1: 5'-TTA GAT GCG TGC CAG GAA CA-3' (nt 2704-2723)	1050 bp	
		E2/2: 5'-TGC ACT ATA GGC GTA GCT GA-3' (nt 3734-3753)		
	E1E2	E1E2L: 5'-CAG GCA CAG CAG GAA TAT TG - 3' (nt 1391-1410)	1935 bp	
		E1E2R: 5' - TTG GAC TCC TCG TGC TCG TT-3' (nt 3306-3325)		
16	E1*	E1/1: nt 1796-1815	1086 bp	As for HPV 6 E1E2 PCR, but annealing T=55°C; 35 cycles
		E1/2: nt 2863-2881		
	E2†	E2/1: nt 2734-2753	1139 bp	
		E2/2: nt 3853-3872		
	E1E2‡	E1E2L: nt 1808-1827	1744 bp	
		E1E2R: nt 3532-3551		

\*Chen and colleagues<sup>29</sup>; †Das and colleagues<sup>22</sup>; ‡Szarka *et al.*<sup>24</sup>  
HPV, human papillomavirus; PCR, polymerase chain reaction; nt, nucleotides.

Sixteen samples from 10 patients with RRP formed the second study group. Three patients had juvenile onset and seven patients had adult onset RRP. Two samples were available from four patients and three samples were available from one. Disease was limited to the larynx in eight patients, whereas in two patients mesopharyngeal papillomas were also seen in addition to the laryngeal disease. Distal spread to the trachea or bronchi was not diagnosed.

### PCR amplification and HPV typing

After DNA extraction with the standard phenol/chloroform/isoamylalcohol method the control of DNA integrity was performed using a PCO3/PCO4 PCR protocol, which amplified a 110 bp sequence of the human  $\beta$  globin gene. The MY09/MY11 consensus PCR amplifies an approximately 450 bp sequence from the conserved L1 region of a large number of mucosotropic HPVs.<sup>8</sup> After initial denaturation (95°C for two minutes) the MY09/MY11 PCR consisted of 40 cycles of denaturation at 96°C for 20 seconds, annealing at 50°C for five seconds, followed by 52°C for 15 seconds, and extension at 72°C for 90 seconds. A final extension lasted for 90 seconds at 72°C.

**Table 2** The results of HPV prevalence and typing in the cancer group

Site	HPV positivity	HPV type			
		6	11	16	NT
Larynx	8/16	2	3	2	1
Hypopharynx	2/4		1	1	
Larynx + hypopharynx	2/2	1			1
Mesopharynx	1/5	1			
Total	13/27 (48%)	4	4	3	2

HPV, human papillomavirus; NT, could not be typed by the methods used.

The GP5+/GP6+ consensus PCR, which is also capable of detecting a wide range of HPV types, amplifies an approximately 145 bp sequence inside this PCR product. Initial denaturation (94°C for four minutes) was followed by 35 cycles of PCR: denaturation at 94°C for 60 seconds, annealing at 48°C for 90 seconds, and extension at 72°C for 90 seconds, with a final extension for two minutes at 72°C.

The PCR products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide, at 100 V for one hour and visualised under ultraviolet light.

For HPV typing, 5  $\mu$ l PCR products of the HPV positive samples were mixed with 2  $\mu$ l (10 U/ $\mu$ l) RsaI and 1 $\times$  restriction enzyme buffer to a final volume of 20  $\mu$ l. After incubation for two hours at 37°C, the resulting fragments were run on a 21% non-denaturing polyacrylamide gel and detected by silver staining. Reference plasmids containing the entire HPV-6, HPV-11, HPV-16, and HPV-18 genome were used as standards.

### Determination of HPV DNA physical state by E1, E2, and E1E2 specific PCR

Table 1 provides a summary of the primers, products, and conditions of the E1, E2, and E1E2 specific PCR for HPV types 6, 11, and 16. The PCR amplification mixture consisted of 1 $\times$  RedTaq PCR buffer (Sigma, Poole, Dorset, UK) with 1.1mM MgCl<sub>2</sub>, 100  $\mu$ l of each dNTP (Promega, Mannheim, Germany), 0.2  $\mu$ g of each primer, 2.5 U of RedTaq polymerase (Sigma), and 2  $\mu$ l (0.1-0.3  $\mu$ g) of DNA in a final volume of 50  $\mu$ l. Amplified products were electrophoresed in 1% agarose gel (at 100 V for one hour), stained with ethidium bromide, and visualised under ultraviolet light.

### Determination of HPV DNA physical state by SBH

SBH was used to determine whether integrated HPV DNA was present in samples positive for the E1E2 specific PCRs. The SBH method used in our study has been published elsewhere in detail.<sup>24</sup> Briefly, for SBH of HPV-6 and HPV-11 DNA 5  $\mu$ g of genomic DNA was treated as follows. Each

**Table 3** Patient data in the RRP group

N	Age	Sex	Age at onset	No. of excisions	Follow up time	Disease aggressiveness <sup>28</sup>	HPV type (serial no of excision)	Range of viral load
1	11	M	2 years 2 months	7	9 years 6 months	NA	11 (7th)	ND
2	9	M	1 year 6 months	24	4 years 5 months	A	11 (21st) 11 (22nd) 11 (23rd)	10 <sup>5</sup> 10 <sup>2</sup> 10 <sup>7</sup>
3	5	M	3 years 1 months	1	2 years 2 months	NA	6	ND
4	20	F	18 years 3 months	4	1 year 11 months	NA	11 (3rd) 11 (4th)	ND 10 <sup>7</sup>
5	33	M	28 years	5	5 years 4 months	NA	6 (4th) 6 (5th)	10 <sup>5</sup> -10 <sup>6</sup> 10 <sup>4</sup> -10 <sup>5</sup>
6	29	M	26 years	4	1 year 8 months	NA	6 (3rd) 6 (4th)	ND 10 <sup>4</sup> -10 <sup>5</sup>
7	40	M	37 years	3	1 year 4 months	NA	6 (2nd) 6 (3rd)	10 <sup>5</sup> -10 <sup>6</sup> <10
8	36	M	30 years	2	1 year 1 month	NA	6 (2nd)	ND
9	37	M	33 years	5	3 years 10 months	NA	11 (4th)	10 <sup>6</sup>
10	72	F	71 years	4	1 year 1 month	NA	6 (4th)	ND

Viral load refers to the HPV DNA copy number of 1 µg sample DNA.  
A, aggressive disease; F, female; HPV, human papillomavirus; M, male; NA, non-aggressive disease; ND, not done; RRP, recurrent respiratory papillomatosis.

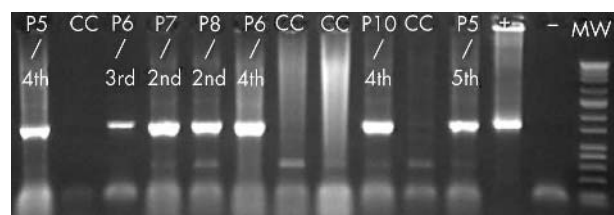
sample was digested by EcoRI (non-cut for HPV-6 DNA) and BamHI (one cut) restriction enzymes separately and an undigested control was also included. The episomal and integrated physical states of HPV DNA are distinguished by their different SBH patterns.

**The estimation of viral load in cancers and papillomas**

For calibration, serial dilutions of pBR322 plasmid DNA harbouring HPV-6, HPV-11, and HPV-16 genomes were prepared so that 1 µl of the diluted sample contained 2 × 10<sup>9</sup>, 10<sup>9</sup>, 10<sup>8</sup>, 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, and 10 copies of the HPV genomes. Subsequently, the MY09/MY11 PCR was performed on the calibrating serial dilutions. To estimate the viral load of papillomas and cancers the same PCR was performed on 1 µg of the sample DNA. In the cancer group, three HPV-6 positive and three HPV-11 positive samples were studied, whereas in the papilloma group, five HPV-6 positive samples of four patients and five HPV-11 positive samples of three patients were studied. After agarose gel electrophoresis of the PCR products, the viral load was estimated by comparing the density of the sample bands to the densities of the calibrating serial dilutions (using Biorad Gel Doc™ 2000 Gel Documentation System as hardware and Quantity One version 4.0.3 as software; Biorad, Hemel Hempstead, Hertfordshire, UK). In this case viral load refers to the HPV copy number in 1 µg of sample DNA.

**Comparison of the sensitivities of the MY09/MY11 PCR and the E1E2 PCR**

Both MY09/MY11 PCR and E1, E2, and E1E2 PCRs were performed on 10 fold serial dilutions of the prepared DNA of an HPV-6 positive papilloma.



**Figure 1** E2 specific polymerase chain reaction for human papillomavirus type 6 (HPV-6) DNA positive samples. Samples P5-P8 and P10 are derived from papillomas and harbour the examined 845 bp sequence of the HPV-6 E2 gene (for patient and excision number identification see table 3); the remaining samples are from cancers (CC); +, plasmid vector containing HPV-6 DNA; -, negative control; MW, Sigma wide range molecular weight marker.

**RESULTS**

The overall HPV DNA positivity was 48%. Because of the low sample number from different sites we did not correlate HPV positivity rates or certain HPV types with the initial tumour site. Low risk HPV types 6 and 11 and the high risk HPV-16 showed an approximately even distribution in our carcinoma samples. In two cases, the nested consensus PCR yielded a positive result for the L1 region of HPV but the viral type could not be ascertained by our restriction enzyme cleavage method. No multiple infections were found. Table 2 contains the results of HPV prevalence and typing in the cancer group.

The E1, E2, and E1E2 PCRs were able to determine the physical state in only one case: this specimen was derived from the HPV-6 positive mesopharyngeal cancer and the viral DNA was in the integrated physical state. Based on the positivity of the E1 PCR and the negativity of the E2 and E1E2 PCRs, the integration site was probably in the E2 ORF. All other HPV DNA positive cancer specimens were negative for E1, E2, and E1E2 PCRs.

In the papilloma group, the HPV positivity rate was 100%. Six cases were positive for HPV-6 and four cases were positive for HPV-11. Tissue specimens from consecutive papilloma excisions of the same patient consistently showed the same viral type. Neither high risk types nor multiple infections were found (table 3).

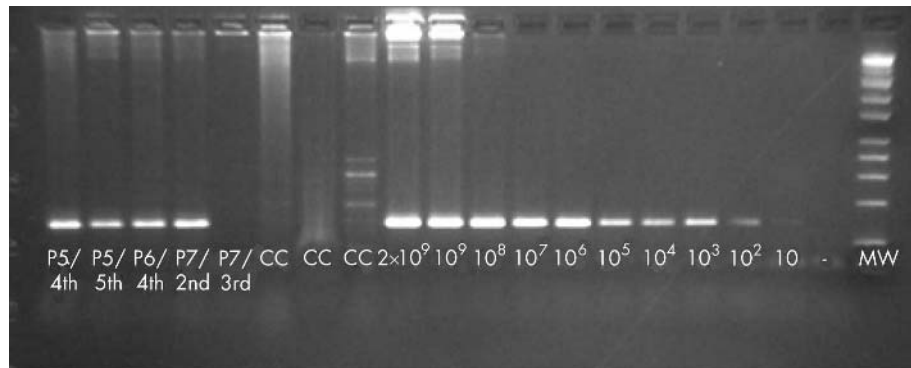
In contrast to the cancer group, all papilloma samples were positive by E1, E2, and E1E2 PCR, indicating an intact E1E2 region (see an example in fig 1).

Figure 2 shows an example of the estimation of viral load in HPV-6 DNA positive samples using the calibrating serial dilutions. All examined HPV positive carcinoma samples harboured less than 10 copies of HPV DNA in 1 µg of sample DNA. Table 3 summarises the overall results for the viral load of papillomas. In two papilloma cases, two subsequent samples were available for the estimation of viral load, whereas in one case three subsequent samples were available. The HPV DNA copy number tended to vary by several orders during the course of the disease.

Figure 3 shows the comparison of the different sensitivities of the MY09/MY11 PCR and the E1, E2, and E1E2 PCRs. Based on serial dilutions of the PCR products of the different PCRs, the consensus PCR is more sensitive than the E1E2 specific PCRs by approximately three orders of magnitude.

**DISCUSSION**

The 48% overall HPV DNA positivity rate in the cancer group is similar to that seen in other studies.<sup>10</sup> Differences between



**Figure 2** The estimation of viral load in human papillomavirus type 6 DNA positive samples using calibrating serial dilutions. Samples P5–P7 are derived from papillomas (for patient and excision number identification see table 3); CC: cancer samples;  $2 \times 10^9$ – $10$ , the calibrating serial dilutions; –, negative control; MW, Sigma wide range molecular weight marker.

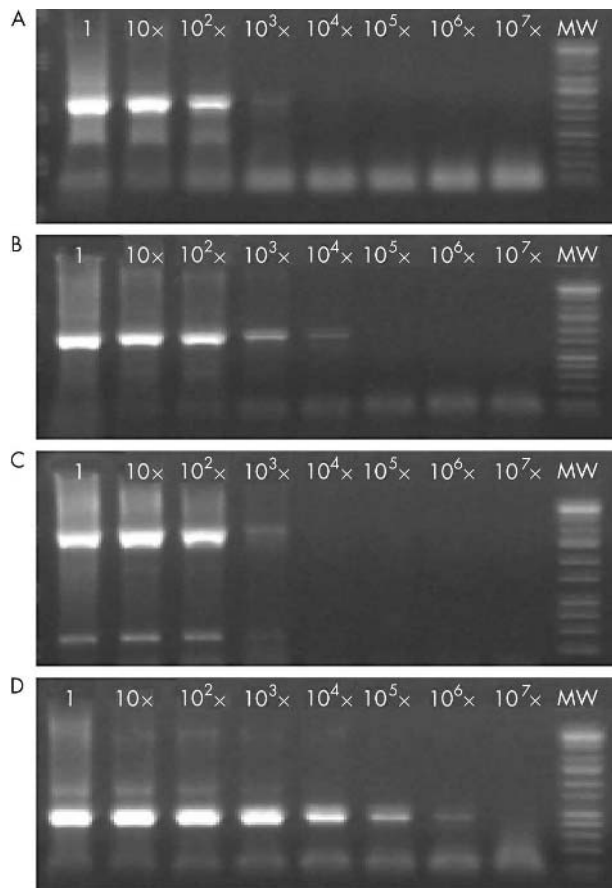
tissue specimens (fresh frozen versus archival), the properties of study populations, the anatomical site, the sensitivity of DNA detection methods (PCR versus SBH or in situ hybridisation), and the differences between statistical methods may account for the highly variable frequency of HPV positivity.<sup>7 10 26</sup> Even in PCR studies on laryngeal cancer, the HPV DNA frequency varies between 3% and 85%, which is

attributable to differences in primers (consensus versus type specific), genomic localisation and length of PCR products, PCR conditions, and false positive results originating from the contamination of samples with HPV DNA.<sup>8</sup>

The presence of low risk HPV DNA in cancer samples has already been described.<sup>1 6 26</sup> Venuti *et al* reported five HPV-6 DNA positive cases among 25 laryngeal cancers and one of these five cases harboured the integrated form of viral DNA. The surrounding mucosa contained HPV DNA exclusively in episomal form, even in the case where the tumour itself showed the integrated form. Using the reverse transcriptase PCR technique, no HPV-6 specific mRNA was detected.<sup>26</sup> Badaracco *et al* identified 10 HPV-6 positive and three HPV-11 positive tumours excised from different head and neck sites, but the integrated form of the virus was present in only one HPV-6 positive laryngeal verrucous cancer.<sup>1</sup> Of the four HPV-6 positive and four HPV-11 positive cancers in our study, we were able to determine the physical state in only one HPV-6 positive mesopharyngeal case, where the viral DNA was integrated. Matsha *et al* report HPV-11 as the most predominant type in oesophageal cancers.<sup>6</sup> The low risk types detected in cancers may either be aggressive mutants or HPV may be only a passenger in neoplasms developed independently from papillomaviruses.<sup>26</sup>

In our opinion, the inability to determine the physical state by E1, E2, and E1E2 specific PCRs in most cancers may be attributable to: (1) the extremely low viral load in cancers (fig 2) and (2) the different sensitivities of the nested PCR and the E1E2 PCRs (fig 3). However, the viral load of the one cancer specimen in which the physical state of HPV DNA could be determined did not differ from that of the other cancer specimens. Detection of the episomal physical state by the E1E2 specific PCRs does not exclude the simultaneous presence of the integrated state. This would have been detected with a characteristic SBH pattern.<sup>24</sup>

There is a consensus in recent studies that by using optimised PCR techniques the frequency of HPV DNA in recurrent respiratory papillomatosis approaches or reaches 100%.<sup>14 16 31</sup> Our results in the RRP group reaffirm this consensus. In addition to the 100% HPV DNA positivity the consistent presence of a certain low risk HPV type in serial specimens from one patient also supports the viral aetiology of RRP. The exclusive presence of low risk types HPV-6 and HPV-11 is also widely accepted.<sup>14 16</sup> In contrast, Peñalosa-Plascencia *et al* report seven different HPV types in patients with juvenile onset RRP. The high frequency of multiple HPV infections and the predominance of HPV-16 DNA in their study might be explained in part by the properties of the study populations.<sup>30</sup>



**Figure 3** A comparison of the sensitivities of the (A) E1, (B) E2, (C) E1E2, and (D) MY09/MY11 PCRs performed on the same human papillomavirus type 6 (HPV-6) DNA positive papilloma specimen (fourth excision of patient 6 in table 3). Numbers indicate the degree of dilution. MW, Sigma wide range molecular weight marker. E1, E2, and E1E2 PCRs amplify a 1130 bp, 845 bp, 2206 bp, and 450 bp sequence of the HPV-6 DNA, respectively (table 1).

### Take home messages

- Low copy numbers of both low risk human papillomaviruses (HPVs) HPV-6 and HPV-11 and high risk HPV-16 were present in about half of the head and neck cancers studied, whereas all 10 papillomas exclusively harboured low risk HPV-6 and HPV-11
- The physical state of HPV could not be discerned in the cancers, except for one case where HPV-6 DNA was integrated, whereas all papillomas showed the episomal state of HPV DNA and a relatively higher viral load
- Thus, cancers and papillomas tend to show a rather different HPV DNA profile
- The 100% positivity rate of low risk HPV types confirms the role of HPV-6 and HPV-11 in the aetiology of recurrent respiratory papillomatosis

There is growing evidence that HPV-11 is associated with a more aggressive clinical course of RRP, including longer periods of disease activity, more surgical treatments needed for each patient, and an increased risk of distal spread and developing squamous cell carcinoma.<sup>15 16</sup> Doyle *et al* define the criteria of aggressive RRP as 10 or more total procedures, with three or more procedures within a one year period and/or spread of disease distal to the subglottis.<sup>28</sup> In our RRP population, one patient with HPV-11 fulfils these criteria (table 3). However, the low patient numbers and the relatively short follow up time do not allow an evaluation of the prognostic relevance of certain HPV types.

“The consistent presence of a certain low risk human papillomavirus type in serial specimens from one patient also supports the viral aetiology of recurrent respiratory papillomatosis”

In contrast to cancers, the physical state of HPV DNA could be determined in RRP by the E1E2 specific PCRs. SBH also yielded patterns characteristic of the uniform episomal physical state. The episomal state, together with a high viral load, might refer to a productive viral infection. The viral load of papillomas can change during the course of disease, as shown by the serial samples of patients 2, 5, and 7 (table 3). These alterations might reflect the antiviral immunity. The prognostic or therapeutic value of viral load needs further investigations on larger study groups.

In conclusion, head and neck cancers and papillomas tend to show a different HPV status. Cancers were characterised by an approximately 50% HPV DNA prevalence and a low viral load. In contrast, papillomas harboured higher copy numbers of episomal low risk HPV DNA, confirming the role of human papillomaviruses in the aetiology of RRP. Finally, the very low patient numbers must be emphasised, especially in the determination of viral load. Further studies are needed with higher numbers of patients with cancer and RRP to confirm our findings.

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