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ESOPHAGEAL SQUAMOUS CELL CANCER IN PATIENTS WITH HEAD AND NECK CANCER: PREVALENCE OF HUMAN PAPILLOMAVIRUS DNA SEQUENCES

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An etiologic role for human papillomavirus (HPV) infections in either head and neck (HNC) or esophageal carcinogenesis remains debatable. Patients with head and neck cancer are at high risk for developing a second esophageal squamous cell cancer (ESCC). The aim of our study was to determine whether HPV infections play a role in this multifocal carcinogenesis. Samples from 2 groups of HNC patients were studied: Random esophageal biopsies were collected from the first group of 60 patients who had been screened for asymptomatic ESCC. The second group consisted of 21 patients with pairs of HNC and ESCC. Both the fresh frozen biopsy samples of the first group and the paraffin-embedded specimens of the second group were evaluated for the presence of HPV DNA sequences by PCR amplification, cloning and sequencing. HPV DNA sequences were detected in 66.7% of normal/inflammatory (34/51) and dysplastic and malignant (6/9) esophageal tissues from HNC patients being screened endoscopically. Similarly, in the second group of 21 patients with both HNC and ESCC, HPV DNA sequences were demonstrated in 13 (61.9%) of the HNC biopsies and in 14 (66.7%) of the ESCC biopsies. The prevalence of high-risk-type HPV 16 was low (5/51, 9.8%) in normal/inflammatory esophageal mucosa but higher (10/24, 47.6%) in ESCC. The low-risk HPV 11 was present in 37.3% (19/51) of normal/inflammatory, 66.7% (4/6) of dysplastic and 28.9% (13/45) of the carcinoma samples. The same HPV type was present in only 3/21 pairs of HNC and ESCC samples, suggesting that a clonal expansion from the HNC to a subsequent ESCC, or visa versa, is unlikely. The high prevalence of "low-risk" HPV infections points to the need for studies on possible interactions of these infections with the use of alcohol and tobacco in the pathogenesis of these tumors.

Key words: *papillomavirus; esophageal cancer*

Human papillomavirus (HPV) infections have been associated with a subset of cancers of the head and neck,[1–4] as well as with squamous cell carcinomas of the esophagus.[5] The most prevalent HPV types demonstrated in 20–25% of squamous cell carcinomas of the head and neck have been genital high-risk HPV types.[1,6–9] After the first demonstration of papillomavirus DNA in a tonsillar carcinoma,[10] several studies followed associating about 50% of these carcinomas with papillomavirus infections.[3,11–14] Oropharyngeal carcinomas also frequently harbor HPV DNA, whereas data for the HPV prevalence in laryngeal, hypopharynx and oral carcinomas vary considerably.[1,3,12,14] The data on the prevalence of papillomavirus infection in esophageal squamous cell cancer (ESCC) have ranged from its absence to more than half of the tumors harboring HPV DNA.[5] The majority of studies were designed to test for the presence of the high-risk HPV types only, mainly for HPV 16 and HPV 18.[15–27] A few studies have pointed out that infections with other HPV types were also detectable in these tumors.[28–32]

Esophageal cancer in general has a very poor prognosis, due to the usually advanced disease at the time of diagnosis. Early detection and treatment of asymptomatic second ESCC prolongs survival of patients.[33] In an earlier study, we prospectively screened patients with HNC for asymptomatic ESCC by high-resolution video-esophagoscopy and collected random esophageal biopsies.[34] Low- or high-grade squamous cell dysplasia was detected in 6.8% of the patients and ESCC in 7.4%. Our present study investigated a series of these samples for a possible role for HPV infection in the development of ESCC. In addition, we examined samples from esophageal, as well as the head and neck cancers, from the same patients to determine whether the same HPV type may be involved in both tumors. Three different primer combinations were used for PCR amplification to detect all known as well as putative new HPV types that may be present. We subsequently cloned the amplicons and sequenced at least 10 cloned fragments from each.

MATERIAL AND METHODS

Samples and DNA extraction

All samples were collected at the University Hospital of Berlin.

First group of (asymptomatic) patients who were previously diagnosed for HNC: Biopsies from the esophagus ($n = 60$) were collected randomly during high-resolution video-esophagoscopy over the period May 2000 to August 2001, as described previously.[34] The age of the patients ranged from 45 to 89 years, with a mean age of 60.6 years and included 10 females and 50 males. Samples were frozen and stored at −70°C until use. One sample per patient was analyzed in our present study. Total cellular DNA was extracted from the frozen samples as described previously.[31]

Second group of (symptomatic) patients: Archival samples from 25 patients who had been treated for HNC and ESCC were analyzed. The age of this group ranged from 44 to 90 years with a mean age of 60.0 years. Seventeen males and 4 females were included. Here formalin-fixed, paraffin-embedded samples were sectioned (4–6 sections of 20 μm each) and total DNA extracted from each sample. Great care was taken during sectioning to avoid contamination between samples. The blocks were sectioned in several small random groups by 2 different individuals at different time intervals over a period of 1 week. The microtome was cleaned thoroughly and UV-exposed between samples, in addition to using new blades for each sample. Deparaffinization was performed by rotation overnight in 1 ml xylol, followed by centrifugation and subsequent removal of the supernatant. This step was repeated twice (2 × 1 hr) with fresh xylol. The xylol was in turn removed by rotation in 1 ml 100% ethanol for 1 hr followed by centrifu-

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gation and subsequent replacement of the supernatant with 90% ethanol for 45 min and repeated by steps of 80% ethanol for 45 min and 70% ethanol for 45 min. Samples were then lyophilized and the DNA extracted as described above.

PCR analysis and cloning

DNA (50–100 ng) from each sample was amplified by PCR. The quality of DNA obtained from fixed samples was controlled by amplification with primers detecting the β-actin gene. Both the primer sets PC03/PC04 and RS42/KM29, resulting in amplicons of 110 bp and 536 bp, respectively, were used.[35] The 110 bp fragment could not be amplified in 3 DNA samples, whereas the 536 bp fragment could be amplified in 70% of the samples. The total amount of DNA obtained for 2 samples was not sufficient to perform all PCR reactions. We therefore excluded these 5 samples and their corresponding HNC or ESCC samples (total 4 pairs) for further analyses. Papillomavirus sequences were amplified by using 3 different methods, each targeting highly conserved regions within the L1 open-reading frame of papillomaviruses. These included the GP5+/GP6+ primers,[36] the CP primers using modified conditions as previously described[37,38] and the FAP primers.[39] All amplified products were cloned. At least 10 inserts per amplicon were sequenced. Sequencing was performed by the use of either the Sequenase 2.0 DNA Sequencing Kit (USB, Cleveland, OH) or an ABI Model Sequencer using Big Dye Terminator chemistry (Perkin Elmer Applied Biosystems Division, Darmstadt, Germany).

Sequence analysis

Sequences were compared to all available databanks with the aid of the Husar software package (Deutsches Krebsforschungszentrum).

RESULTS

Patients were screened for asymptomatic ESCC on average 8 months after diagnosis of HNC. Histologic analyses of this first group of esophageal samples revealed 51 biopsies with normal/inflammatory histology, 4 with low-grade dysplasia, 2 with high-grade dysplasia and 3 with carcinoma of the esophagus. The location of the primary carcinomas in this group of patients had included 15 in the oropharynx, 9 in the hypopharynx, 7 in the larynx and 25 in the oral cavity. Four patients suffered from multifocal HNC. The results are summarized in Table I. A total of 34 of 51 (66.7%) esophagus samples with normal/inflammatory histology were HPV DNA positive. Three of 4 (75%) low-grade dysplastic lesions habored HPV DNA, 2/2 (100%) high-grade dysplasias and 1/3 (33.3%) carcinomas, *i.e.*, in total 6/9 (66.7%) pre- and malignant samples. A range of different HPV types was detected in these tissues, including known as well as putative new HPV types. Multiple HPV types within one sample were detected in 21/51 biopsies with normal/inflammatory histology and 2/9 with pre- or malignant histology (Table II). The majority of the HPV-positive normal/inflammatory esophageal biopsies harbored HPV 11 (19/51 samples, 37.3%) followed by HPV 6 (13/51, 25.5%) and HPV 87 (11/51, 21.6%) (Table III). Other HPV types found in the samples with normal/inflammatory histology were HPV 16 in 5 samples, HPV 27 in 3 and HPV 53 in 6 biopsies. In addition, the following HPV types were also detected in the normal/inflammatory epithelium: HPV 20, HPV 21, HPV 25, HPV 32 and 4 putative new HPV types. The 9 pre- and malignant samples harbored HPV 11 (2 biopsies with low-grade and 2 with high-grade dysplasia, 1 carcinoma biopsy), HPV 16 (1 sample of high-grade dysplasia) and HPV 53 (3 biopsies with low-grade dysplasia), as well as HPV 5 and HPV 20 (both in high-grade dysplasia).

We examined a second group of patients of which both the HNC and the ESCC from the same patient were available. HPV DNA was demonstrated in 64.3% (27/42) of these biopsies (Table IV and V). Here the overall rate of HPV detection was very similar to that seen in the normal esophageal tissue from endoscopically screened HNC patients, despite the small numbers of samples for each tumor type (Table I). The spectrum of HPV types detected also overlapped to some extent. Sample pairs from 10 of the 21 (47.6%) patients were both positive for HPV DNA, although the same HPV type was present in only 3 pairs (Table IV). In the latter cases, HPV 11 was present on both the HNC as well as the ESCC from the respective patient. The HNC in 1 case was a hypopharynx carcinoma (harbored HPV 16 DNA in addition) and in the other a carcinoma of the floor of the mouth (also positive for HPV 16 and HPV 20). The ESCC of the first patient harbored HPV 6 as well. HPV 16 and HPV 87 were both present in the 2 tumors from a third patient in which the HNC was a tonsillar carcinoma. In addition, HPV 11 was also found in the esophageal carcinoma of the latter pair. Infections with multiple HPV types were present in 7 of the HNC and in 7 of the ESCC (total 14/42 samples). No HPV DNA could be amplified in any of the samples from 5 patients. Remarkably, little overall variation is seen when comparing HPV prevalences between HNC samples and ESCC biopsies. However, a tendency for a higher prevalence of individual HPV types was noted for HPV 16 present in 10 (47.6%) of the ESCC samples compared to 4 (19.0%) of the HNC samples. HPV 87 was present in 4 (19.0%) HNC biopsies compared to 2 (9.5%) samples from ESCC DNA, although these numbers are small. Combining the results from both HNC and ESCC, HPV 11 (12/42, 28.6%) and HPV 16 (14/42, 33.3%) were the most prevalent HPV types, followed by HPV 87 (6/42, 14.3%), HPV 20 (5/42, 11.9%) and HPV 6 (3/42, 7.1%) (Table V). Other HPV detected were HPV 18 (2 cases), HPV 27 and HPV 32 (1 case each) and the putative new HPV types DL253 and FA24 (1 case each).

DISCUSSION

An etiologic role for papillomavirus infections in malignant tumors of the head and neck as well as in carcinoma of the

TABLE I – PREVALENCE OF HPV IN THE ASYMPTOMATIC ESOPHAGEAL TISSUE FROM 60 SCREENED HEAD AND NECK CANCER (HNC) PATIENTS (FIRST GROUP) AND PAIRS OF TUMOR BIOPSIES FROM 21 PATIENTS WITH BOTH (HNC) AND ESOPHAGEAL SQUAMOUS CELL CANCER (ESCC, SECOND GROUP)

Location of HNC	HPV-positive samples/total					
	First group (from 60 asymptomatic patients)				Second group (from 21 patients each with HNC and ESCC)	
	Esophageal biopsy				ESCC	HNC
	Benign	Low grade	High grade	Carcinoma		
Oropharynx	7/13	—	2/2	—	2/5	2/5
Hypopharynx	5/6	2/2	—	0/1	1/1	1/1
Oral cavity	16/23	0/1	—	0/1	7/11	6/11[1]
Larynx	3/6	1/1	—	—	3/3	3/3
Tonsil	—	—	—	—	1/1	1/1
Multiple	3/3	—	—	1/1	—	—
Total (% positive)	34/51 (66.7)	3/4 (75)	2/2 (100)	1/3 (33.3)	14/21 (66.7)	13/21 (61.9)

[1]Includes tumors of the floor of the mouth, tongue, palate and oral mucosa.

TABLE II – HPV DNA IN ESPOHAGEAL BIOPSIES

Head and neck cancer (HNC)	Esophageal biopsy (HPV+/total)	Results of HPV-positive samples (sets of primers used)[1]		
		GP	CP	FAP
Oropharynx	Normal/inflammatory (7/13)	Neg	Neg	KG463
		Neg	Neg	HPV 11
		HPV 16	Neg	HPV 6
		HPV 11	Neg	HPV 11, HPV 53, HPV 87
		Neg	HPV 6	Neg
		HPV 11, HPV 16	Neg	Neg
		HPV 11, HPV 16	HPV 27	Neg
	High-grade dysplasia (2/2)	HPV 11, HPV 16	HPV 5	HPV 11, HPV 20
		HPV 11	Neg	Neg
Hypopharynx	Normal/inflammatory (5/6)	Neg	IG50	HPV 87, KG306
		Neg	HPV 11	Neg
		Neg	HPV 6, CW760	HPV 53, HPV 87, CW760
		HPV 11	HPV 6, HPV 25	Neg
		HPV 6	HPV 6	HPV 11
	Low-grade dysplasia (2/2)	HPV 11	Neg	HPV 11, HPV 53
	Carcinoma (0/1)	Neg	Neg	HPV 11, HPV 53
Oral cavity	Normal/inflammatory (16/23)	Neg	Neg	HPV 87
		Neg	Neg	KG436
		Neg	HPV 6	Neg
		HPV 6, HPV 11	HPV 6	Neg
		HPV 11, HPV 32	Neg	Neg
		HPV 11	HPV 6	HPV 21
		HPV 11	HPV 6	HPV 53
		Neg	HPV 25	HPV 11, HPV 53, CW760
		HPV 11, HPV 16	Neg	HPV 53
		HPV 11, HPV 27	HPV 6	Neg
		HPV 6, HPV 11	HPV 11, HPV 87	Neg
		Neg	HPV 27	HPV 87
		Neg	Neg	HPV 11
		Neg	Neg	HPV 53, CW760
		Neg	HPV 6	HPV 87
		HPV 11	Neg	Neg
	Low-grade dysplasia (0/1)			
	Carcinoma (0/1)			
Larynx	Normal/inflammatory (3/6)	Neg	HPV 6	Neg
		Neg	Neg	HPV 87
		HPV 16	Neg	Neg
	Low-grade dysplasia (1/1)	Neg	Neg	HPV 53
Multifocal	Normal/inflammatory (3/3)	Neg	Neg	HPV 87
		HPV 11	Neg	HPV 11, HPV 87
		Neg	Neg	HPV 11, HPV 87
	Carcinoma (1/1)	Neg	Neg	HPV 11

[1] References 36–39.

TABLE III – SUMMARY OF RESULTS IN 60 ESOPHAGEAL BIOPSIES (FIRST GROUP)

HPV type	Normal/inflammatory $n = 51$	Pre- and malignant $n = 9$	Total positive (%) $n = 60$
6	13 (25.5%)	—	13 (21.7%)
11	19 (37.3%)	5 (55.6%)	24 (40%)
16	5 (9.8%)	1 (11.1%)	6 (10%)
27	3 (5.9%)	—	3 (5%)
53	6 (11.8%)	3 (33.3%)	9 (15%)
87	11 (21.6%)	—	11 (18%)
5	—	1 (11.1%)	1 (1.7%)
20	—	1 (11.1%)	1 (1.7%)
21	1 (2.0%)	—	1 (1.7%)
25	1 (2.0%)	—	1 (1.7%)
32	1 (2.0%)	—	1 (1.7%)
KG463(HPV50 related)	2 (3.9%)	—	2 (3.3%)
CW760(HPV23 related)	2 (3.9%)	—	2 (3.3%)
IG50 (HPV23 related)	1 (2.0%)	—	1 (1.7%)
KG306(HPV5 related)	1 (2.0%)	—	1 (1.7%)
Total no. of samples positive	34 (66.7%)	6 (66.7%)	40 (66.7%)

esophagus is still not conclusive. The prevalence and type specificity or diversity of HPV DNA in these tumors depend to a large extent on the methodology used. In published data, HPV 16 represents the most prevalent HPV type in HPV-positive biopsies from malignant tumors of the head and neck.[1,3,4,6,7] Many earlier reported studies used restricted methods and probes for HPV DNA

TABLE IV – HPV IN 21 PAIRS OF HEAD AND NECK CANCER (HNC) AND ESOPHAGEAL SQUAMOUS CELL CARCINOMA (ESCC) SAMPLES

Patient no.	HNC (no. patients positive/ patients tested)	Results of HPV-positive samples (sets of primers used)			ESCC (no. patients positive/ patients tested)	Results of HPV-positive samples (sets of primers used)		
		GP	CP	FAP		GP	CP	FAP
1	Oropharynx (2/5)	Neg	Neg	Neg	2/5	HPV 16	Neg	Neg
2		HPV 6, HPV 32	HPV 6	Neg		HPV 11, HPV 16	Neg	Neg
3		Neg	Neg	HPV 11		Neg	Neg	Neg
4	Hypopharynx (1/1)	HPV 11	Neg	HPV 6	1/1	HPV 11, HPV 16	Neg	Neg
5	Oral cavity[1] (6/11)	Neg	Neg	HPV 11, FA24	7/11	Neg	Neg	Neg
6		HPV 11	HPV 16, HPV 20	Neg		Neg	DL253	Neg
7		Neg	Neg	Neg		HPV 16	HPV 20	HPV 87
8		Neg	Neg	HPV 87		HPV 11, HPV 16	Neg	Neg
9		HPV 11	Neg	Neg		HPV 16, HPV 27	Neg	Neg
10		Neg	Neg	Neg		HPV 16	Neg	Neg
11		Neg	Neg	HPV 11		HPV 11, HPV 16	HPV 20	Neg
		HPV 18	Neg	Neg		HPV 16	Neg	Neg
12	Larynx (3/3)	HPV 16	Neg	HPV 87	3/3	Neg	Neg	HPV 6
13		Neg	Neg	HPV 20		HPV 11	Neg	Neg
14		HPV 16	HPV 20	HPV 20, HPV 87		HPV 18	Neg	Neg
15	Tonsil (1/1)	HPV 16	Neg	HPV 87	1/1	HPV 11, HPV 16	Neg	HPV 87

[1] Includes tumors of the floor of the mouth, tongue, palate and oral mucosa.

TABLE V – SUMMARY OF HPV SAMPLES POSITIVE IN 42 PATIENTS WITH HEAD AND NECK CANCER (HNC) AND ESOPHAGEAL SQUAMOUS CELL CARCINOMA (ESCC)

HPV type	HNC $n = 21$	ESCC $n = 21$	Total $n = 42$
6	2 (9.5%)	1 (4.8%)	3 (7.1%)
11	6 (28.6%)	6 (28.6%)	12 (28.6%)
16	4 (19.0%)	10 (47.6%)	14 (33.3%)
18	1 (4.8%)	1 (4.8%)	2 (4.8%)
20	3 (14.3%)	2 (9.5%)	5 (11.9%)
87	4 (19.0%)	2 (9.5%)	6 (14.3%)
27	—	1 (4.8%)	1 (2.4%)
32	1 (4.8%)	—	1 (2.4%)
DL253 (HPV 80 related)	—	1 (4.8%)	1 (2.4%)
FA24 (HPV 43 related)	1 (4.8%)	—	1 (2.4%)
Total no. of samples positive	13 (61.9%)	14 (66.7%)	27 (64.3%)

detection in esophageal cancer,[5] and therefore a joint study between laboratories was performed to determine whether other HPV types might also be involved.[30] A spectrum of different HPV types was demonstrated in samples originating from China, including types that had historically been associated with cutaneous lesions. These data were confirmed in an independent study including samples from other geographic regions.[31]

The aim of our present study was to determine whether HPV DNA is present in the esophageal mucosa of asymptomatic patients, as well as whether the same HPV type was present in both the ESCC and the HNC from the same patient. The papillomaviruses are very diverse in sequence, even in the highly conserved regions of the L1 open-reading frame, and it is difficult to amplify different HPV types with equal sensitivity. Three different PCR primer sets amplifying different regions of the L1 ORF and covering all groups of known HPV types were therefore used in the analyses. In the first group of endoscopic esophageal samples, 85% consisted of only normal/inflammatory epithelium upon histologic analysis. Taken together, HPV DNA was demonstrated in two-thirds of all samples tested. No obvious difference was noted between normal/inflammatory and malignant tissues, and the spectrum of HPV types present was also similar. Although the numbers of the samples tested were small, the tendency was that the low-risk HPV 6 was more prevalent in normal/inflammatory tissue than in malignant tissue and the high-risk HPV 16 higher in carcinomas than in normal/inflammatory samples. It was interesting to note that the low-risk-type HPV 11 was present in almost as many carcinomas (13/45, 29%) as in normal/inflammatory biopsies (19/51, 37.3%) and was present in about the same number of carcinomas as HPV 16 (14/45, 31%). Low-risk HPV types had also been demonstrated in earlier studies of carcinomas of the esophagus.[30,31] The E6 and E7 proteins of the low-risk HPV types do not readily immortalize primary cells, and additional co-factors are probably needed for malignant transformation of infected cells.[40] The use of tobacco and alcohol has been linked to carcinoma development of the upper aerodigestive tract,[42,43] but unfortunately these data were not available for our present study. It should be interesting to study a possible interaction of their carcinogenic metabolites with low-risk HPV infections. HPV 87 was detected in both normal/inflammatory and malignant samples. Available data suggest that its genes stimulate host cell proliferation.[44] HPV 20 found in our study in HNC and in ESCC biopsies may also require additional co-factors for the elimination of cellular proteins controlling functions of its genes in normal cells. HPV 20 belongs to the cutaneous group of HPV types and has been found in several nonmelanoma skin cancers.[45] It has also

been detected in our previous studies on samples from esophageal carcinoma.[30,31] HPV 53 was previously demonstrated in carcinoma of the esophagus[30] but in our present study was only found in normal/inflammatory tissue and low-grade dysplasias. Little is known about the distribution of this HPV type in other tumors. The number of samples from normal/inflammatory esophageal epithelium harboring high-risk-type HPV 16 DNA was relatively low (5/51, 9.8%) but higher in esophageal carcinoma samples (10/24, 41.7%), whereas HPV 18 was detected only in carcinomas. The oncogenic functions of these 2 HPV types have been well established.[41]

Endoscopic surveillance of the esophagus in patients with head and neck cancer has been recommended.[46,47] The detection of identical HPV types in only 3 of 21 pairs of the HNC and ESCC samples suggests that clonal expansion from the HNC to a subsequent ESCC, or visa versa, is unlikely. This has also been analyzed by allelic loss detection by Califano *et al.*[48] On the other hand, the high prevalence of papillomavirus in the normal mucosa of these patients indicates a need for close surveillance. We demonstrated the presence of a spectrum of different HPV types in normal mucosa of the esophagus, as well as in both HNC and ESCC. These include not only high- and low-risk HPV types but also types for which little or no data are available about their capacity to immortalize or transform cells. It appears that the molecular mechanisms differ by which high- and low-risk papillomaviruses contribute to malignant transformation of cells.[40,41] The presence of high-risk HPV types may, to a larger degree, be associated with tumors not linked to tobacco smoking and alcohol consumption,[9] possibly in contrast to those associated with low-risk HPV infections. Our data point to a need to elucidate the mechanisms through which the low-risk HPV types may be involved in the pathogenesis of malignant tumors.

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