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Detection of human papillomavirus type 16 DNA in carcinomas of the palatine tonsil

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Abstract

Twenty eight tonsillar carcinomas of various histological types were investigated for the presence of Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human papillomavirus (HPV) types 6, 11, and 16 by in situ hybridisation using highly stringent procedures. In six cases an autoradiographic signal was obtained in the tumour cell nuclei with the HPV type 16 specific probe. No signal was obtained with any of the other probes. Immunohistochemical investigations with mouse monoclonal antibodies directed against the L1 protein of HPV type and a rabbit antiserum that detects common protein determinants of HPV gave negative results, thus indicating latent infection. Furthermore, a series of tonsils from controls with comparable age distribution was negative by both in situ hybridisation and immunohistology. These results indicate a possible role for HPV 16 in the aetiology of a proportion of tonsillar carcinomas.

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A possible aetiological role for DNA tumour viruses has been postulated for several carcinomas. In particular, human papillomaviruses (HPV) have been associated with a variety of squamous cell carcinomas, such as cervical, anal, and laryngeal carcinomas.[1–5] The Epstein-Barr virus (EBV) has been implicated in the pathogenesis of undifferentiated nasopharyngeal carcinoma.[6] Tumours from other sites with similar morphological features (so-called lymphoepithelioma-like carcinomas) have also been shown to harbour EBV-DNA occasionally.[7–9]

Carcinoma of the palatine tonsil is the second most common malignant tumour of the upper respiratory tract, with tobacco and alcohol abuse being the most common risk factors.[10 11] An association between undifferentiated tonsillar carcinomas and EBV has been reported in a study using in situ hybridisation and, more recently, serological anti-EBV antibody patterns, characteristic of undifferentiated nasopharyngeal carcinoma, have also been described in patients with lymphoepithelioma-like tonsillar carcinomas.[12 13] In an in situ hybridisation study investigating epithelial malignancies from various sites for the presence of EBV DNA, we therefore included 28 tonsillar carcinomas. As all of these cases were negative with EBV-specific probes (G Niedobitek, unpublished observations) we extended our study to other DNA tumour viruses and report here the results obtained with HPV specific DNA probes.

Methods

Formalin fixed, paraffin wax embedded biopsy specimens of 28 tonsillar carcinomas were studied. Sixteen (57%) patients were male and 12 (43%) were female. The age of the patients ranged from 43 to 85 years, with a median of 66 years (table). The women were older than the men, the median ages being 70 years and 59 years, respectively. Tobacco smoking or alcohol abuse, or both, were risk factors for most of the patients (table). The tumours were graded according to WHO criteria.[14] Two carcinomas were well differentiated, 12 moderately differentiated, and 11 poorly differentiated squamous cell carcinomas. One case was an in situ carcinoma and two were lymphoepithelioma-like carcinomas with morphological similarities with undifferentiated carcinomas of nasopharyngeal type (table). Frozen tissue for DNA extraction was available from one case.

A series of 30 tonsils surgically removed because of chronic inflammation served as controls. The male to female ratio of this group was one, the age ranged from 47 years to 80 years (median 60 years). The median age of male and female patients was 55 years and 61 years, respectively.

PLASMIDS AND PROBES

Plasmids harbouring HPV6b, HPV11, and HPV16 DNA were a gift from Dr H zur Hausen, Heidelberg.[15–17] The plasmids *pBa-W*, containing the 3·1 kilobase pair *BamHI-W* internal repetitive fragment of EBV strain M-ABA, and *pM961-20*, harbouring the 6·4 kilobase pair *HindIII/SalI* fragment of the terminal region of the EBV genome, were kindly provided by Dr GW Bornkamm, Freiburg.[18] The plasmid *pCM5018* (human cytomegalovirus, CMV, strain AD169 *EcoRI-J* fragment subcloned into *pACYC184)*, was a kind gift from Dr B Fleckenstein, Erlangen, and served as a probe in negative control experiments.[19] Total plasmid DNA was labelled with ^{35}S-dCTP (New England Nuclear, Dreieich, West Germany; specific activity 1200 Ci/mmol) by nick translation to a specific activity of 3–5 $\times$ 10^8 dpm/μg.[20]

Age, sex, and risk factors of patients with tonsillar carcinoma, and in situ hybridisation results

Case No	Age	Sex	Risk factors	Diagnosis	In situ hybridisation
1	43	M	Tobacco/alcohol	SCC, well differentiated	Negative
2	45	M	Tobacco/alcohol	SCC, moderately differentiated	Negative
3	46	M	Tobacco	In situ carcinoma	Negative
4	47	M	Alcohol	SCC, moderately differentiated	Negative
5	49	M	Alcohol	SCC, moderately differentiated	Negative
6	51	M	None	SCC, moderately differentiated	Negative
7	52	M	Tobacco	SCC, poorly differentiated	Negative
8	59	M	Tobacco/alcohol	SCC, well differentiated	Negative
9	60	M	Tobacco	SCC, poorly differentiated	Negative
10	64	M	Tobacco/alcohol	SCC, moderately differentiated	Negative
11	66	M	Alcohol	SCC, poorly differentiated	Negative
12	67	M	Tobacco	Lymphoepithelioma-like carcinoma	Negative
13	68	M	Tobacco/alcohol	SCC, poorly differentiated	HPV16
14	71	M	Alcohol	SCC, poorly differentiated	Negative
15	75	M	Tobacco/alcohol	SCC, moderately differentiated	Negative
16	83	M	None	SCC, poorly differentiated	Negative
17	51	F	Tobacco	SCC, poorly differentiated	HPV16
18	64	F	Tobacco/alcohol	SCC, moderately differentiated	Negative
19	65	F	None	SCC, moderately differentiated	Negative
20	66	F	None	SCC, moderately differentiated	Negative
21	67	F	Unknown	SCC, moderately differentiated	Negative
22	68	F	Tobacco	SCC, moderately differentiated	Negative
23	72	F	Unknown	SCC, poorly differentiated	HPV16
24	73	F	Tobacco	SCC, poorly differentiated	HPV16
25	77	F	None	SCC, poorly differentiated	Negative
26	77	F	None	Lymphoepithelioma-like carcinoma	Negative
27	83	F	Tobacco	SCC, poorly differentiated	HPV16
28	85	F	None	SCC, moderately differentiated	HPV16

SCC: squamous cell carcinoma.

IN SITU HYBRIDISATION

In situ hybridisation was performed as described previously.[21 22] In brief, rehydrated paraffin wax sections were pretreated with 0·2 N HCl for 10 minutes at room temperature, 0·1% Triton X-100 for one and a half minutes at room temperature, and 0·1 mg/ml pronase (Boehringer, Mannheim, West Germany) for 30 minutes at 37°C, followed by acetylation with 0·1 M triethanolamine (pH 8·0)/0·25% acetic anhydride for 10 minutes at room temperature. Slides were washed in 2 × SSC (0·3 M sodium chloride/0·03 M sodium citrate, pH 7·6) between the incubation steps. Slides were then dehydrated through graded ethanols and 25 μl hybridisation mixture (50% deinonised formamide/2 × SSC/10% dextran sulphate/0·1 mg/ml herring sperm DNA/20 to 40 ng/ml of labelled probe) were applied. Probe and tissue DNA were denatured simultaneously by heating the slides to 90°C for three minutes. Subsequently, slides were incubated at 37°C for 12 hours (21°C below melting point), followed by washing in several changes of 50% formamide/0·1 × SSC at 37°C (about melting point) for four hours and rinsing in 0·1 × SSC for 30 minutes at room temperature. The hybridisation mixture and all washing solutions contained 10 mM dithiothreitol. The slides were then dehydrated through graded ethanols and dipped into Ilford-G5 emulsion. After exposure times of three to 10 days slides were developed with Kodak Rapid Fixer, followed by counterstaining with haematoxylin and eosin.

SOUTHERN BLOT HYBRIDISATION

DNA was extracted from frozen tissue, digested with *BamHI* or *EcoRI* restriction enzymes, separated by electrophoresis in a 0·8% agarose gel, and transferred to nylon membrane filters. Total HPV 16 plasmid DNA was labelled with ^{32}p-dCTP (New England Nuclear, specific activity 3000 Ci/mmol) by random primer extension. Hybridisation and washing was performed as previously described.[23] Placenta DNA provided the negative control.

IMMUNOHISTOLOGY

Four monoclonal antibodies specific for the L1 capsid protein of HPV 16, 8C4, 3D1, 5A4, and 1D6 were used in this study.[24] Undiluted cell supernatants were applied to sections. A polyclonal anti-bovine papillomavirus antiserum (Dakopatts, Glostrup, Denmark) was also used in a 1 in 16000 dilution. Paraffin wax sections were dewaxed and rehydrated. The immunoalkaline phosphatase anti-alkaline phosphatase (APAAP) method was used as described previously.[25 26]

Results

In situ hybridisation to the HPV 16 specific probe resulted in an accumulation of grains over tumour cell nuclei in six tonsillar carcinomas while hybridisation to the HPV 6, HPV 11, EBV, or CMV specific probes gave negative results (fig 1A–B) (table). Five of the positive cases were poorly differentiated carcinomas and one was of intermediate differentiation. The labelling intensity of tumour cells was fairly homogenous. Adjacent non-neoplastic squamous epithelium was available in five cases. This was negative in all instances on hybridisation to the HPV specific probes. In one case epithelial cells of an in situ carcinoma in the vicinity of the invasive carcinoma were also positive for HPV 16. Thirty tonsils with chronic inflammatory disease investigated for control purposes were negative with all probes.

Frozen tissue from one carcinoma positive

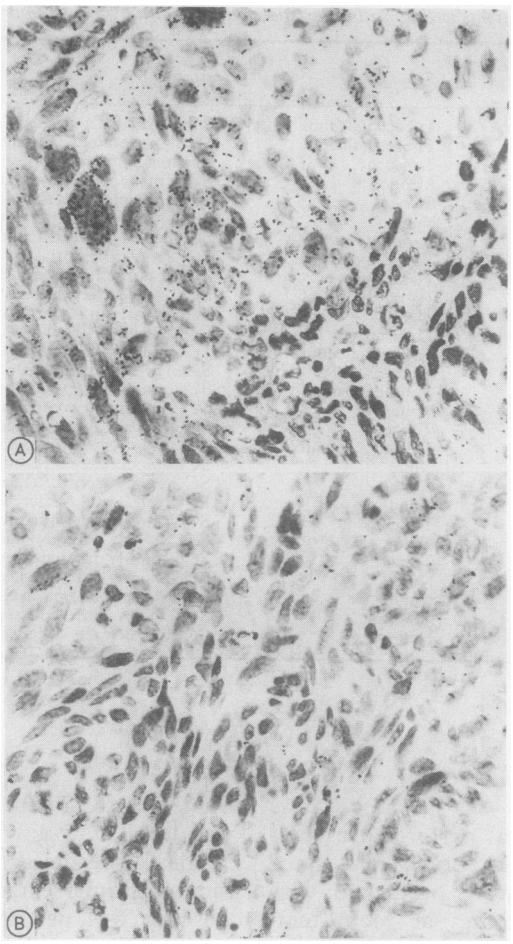

Figure 1A Demonstration of HPV type 16 DNA in a tonsillar carcinoma (case 24). Accumulation of grains over tumour cell nuclei; inflammatory cells are negative. Exposure time seven days (haematoxylin and eosin).

Figure 1B Same case as fig 1A hybridised to a CMV specific probe displays background distribution of grains. Exposure time seven days (haematoxylin and eosin).

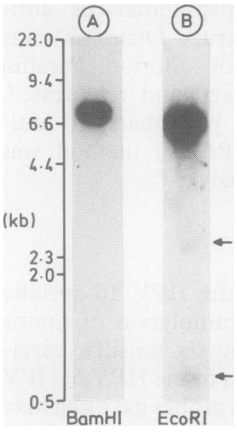

Figure 2 Southern blot autoradiograms of (A) BamHI and (B) EcoRI digested DNA from a tonsillar carcinoma (case 28) hybridised to a ^{32}p-labelled HPV16 specific probe. Hybridisation of BamHI digested DNA results in a single band of 7·2 kilobase pairs; hybridisation of EcoRI-digested DNA displays a major band of 7 kilobase pairs and two weak bands of 0·8 and 2·5 kilobase pairs, respectively.

for HPV 16 by in situ hybridisation was available for DNA extraction and Southern blotting. Hybridisation of *BamHI* digested DNA to the HPV 16 specific probe resulted in one band of 7·2 kilobase pairs while hybridisation to *EcoRI* digested DNA produced a major band of about 7 kilobase pairs and two additional bands of 0·8 and 2·5 kilobase pairs, respectively (fig 2).

Immunohistological staining with four HPV type 16 specific monoclonal antibodies and a polyclonal antiserum directed against viral capsid proteins did not result in any specific staining, while in simultaneous control experiments all four monoclonal antibodies strongly stained nuclei of a cervical intraepithelial neoplasia harbouring HPV 16 DNA, as determined by in situ hybridisation. The monoclonal antibodies did not react with condylomas harbouring HPV type 6 (data not shown). The antiserum produced a weak staining of superficial cells in condylomata acuminata (data not shown). Furthermore, 30 non-neoplastic tonsils were investigated for the expression of common determinants of HPV capsid proteins using a polyclonal antiserum, and of HPV 16 L1 protein group-specific viral capsid antigens, with negative results in all cases.

Discussion

Carcinoma of the palatine tonsil is a common neoplasia usually associated with risk factors such as alcohol or tobacco abuse.[10 11] Some DNA viruses have also been implicated as cofactors in the pathogenesis of this malignancy. Some reports have linked undifferentiated and lymphoepithelioma-like carcinomas of this site with EBV infection.[12 13] In a study investigating a total of 115 epithelial tumours from various sites by in situ hybridisation, however (G Niedobitek, unpublished observations) we have shown that 28 tonsillar carcinomas of all histological types were negative with two different EBV specific probes.

HPV 16 DNA was found in the tumour cell populations of six of these 28 carcinomas: five were poorly and one was moderately differentiated, while all of two highly differentiated carcinomas and one in situ carcinoma did not show any autoradiographic signals with any of the probes. Our results confirm the findings of a report showing the presence of HPV 16 DNA in two of seven tonsillar carcinomas.[27] These results, however, were obtained by dot blot hybridisation only and the number of tonsillar carcinomas investigated was comparatively small.[27] Therefore, our results are, to the best of our knowledge, the first demonstration of HPV type 16 DNA in the tumour cell population of tonsillar carcinomas.

The specificity of our in situ hybridisation results was confirmed by Southern blot hybridisation of DNA from one carcinoma. The enzymes chosen for digestion of DNA were those used in the study describing the cloning of HPV 16 DNA, and the pattern of bands obtained is characteristic for this virus.[17] Though we could not assert the presence of HPV 16 in the other five cases by Southern blot hybridisation due to the lack of frozen tissues, in view of the highly stringent hybridisation and washing conditions of our protocol it seems unlikely that the autoradiographic signal obtained in these cases with the HPV 16-specific probe is due to cross hybridisation with an HPV 16-related DNA. Cross hybridisation with any other viral nucleic acid can also be regraded as unlikely, as hybridisation to HPV 6, HPV 11, EBV, and CMV specific probes yielded negative results.

Immunohistochemical investigations with four monoclonal antibodies directed against the L1 capsid protein of HPV 16 and an antiserum recognising a common determinant of HPV capsid proteins did not produce any staining of tumour cell nuclei in any of the carcinomas harbouring HPV 16 DNA. As these proteins are expressed only in replicative infection, these results do not contradict our hybridisation data but rather point to a latent infection of tumor cells.[24]

Interestingly, five of the patients with HPV 16 positive carcinomas were female and only one was male. The number of positive carcinomas, however, is certainly too small to draw any conclusions from this observation. This finding may also only reflect the slightly different age distribution as the male patients were, on average, younger than the female patients (table).

In four out of the six patients with HPV 16 positive tonsillar carcinomas alcohol and tobacco abuse were anamnestically identified risk factors; in one case alcohol and tobacco abuse was denied, and in another case no information was available. Thus there was one case with no other known risk factor than HPV 16 infection. It has been suggested that HPV 16 is involved in the pathogenesis of squamous cell carcinomas from various sites.[1] In particular, the presence of HPV 16 DNA has been confirmed in most carcinomas from the uterine cervix.[3 17] The clinical importance of this finding, however, has been questioned recently by epidemiological studies.[28] To preclude the possibility that the presence of HPV 16 DNA in the tonsillar carcinomas is merely circumstantial, a series of non-neoplastic tonsils from patients with similar age distribution was investigated for the presence of viral DNA and viral proteins. The fact that none of these cases displayed evidence of HPV infection by any of the methods used points to a possible co-carcinogenic role for this virus for a proportion of tonsillar carcinomas, possibly acting synergistically with other risk factors such as excessive alcohol and tobacco consumption.

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