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Original Paper

Detection of Human Papillomavirus (HPV) in Laryngeal Carcinoma Cell Lines Provides Evidence for a Heterogeneic Cell Population

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The role of human papillomavirus (HPV) has been studied in laryngeal carcinomas with contradictory results. To evaluate the causal relationship between HPV infection and epithelial malignancies of the larynx, 27 laryngeal carcinoma cell lines from 22 patients were studied. Also, paraffin-embedded biopsy samples of the original tumours were available from 12 patients. First, Southern blot hybridisation (SBH) was used for the analysis of 18 cell lines and 12 original tumour sections were studied by *in situ* hybridisation (ISH) to detect HPV. Further, cell lines and tumour biopsy samples were investigated with polymerase chain reaction (PCR) using three sets of consensus primers directed to L1 and E1 ORFs (open reading frames) and type-specific primers to HPV 16 E6 region. The adjacent apparently normal epithelium of one original biopsy sample showed positive signals for HPV by ISH. All other samples were HPV negative with these methods. The study was then extended to 27 laryngeal carcinoma cell lines, including the 18 cell lines studied earlier. A new nested PCR method was used with MY as external and general primers (GP) as internal primers for the cell lines and original tumour samples to achieve a maximal sensitivity. Subsequent SBH was performed to confirm the specificity of PCR products with both low- and high-risk HPV oligonucleotide probe mixtures and also with the HPV 16 oligoprobe. With this method, seven of 27 (26%) cell lines and seven of 12 (58%) tumour samples were found to harbour high-risk HPV. In two cases both the original tumour sample and the derived cell line showed HPV positivity. These results indicate that HPV copy numbers are low and only a minority of tumour cells harbour HPV DNA, explaining partly the controversial results reported earlier.

Key words: HPV, nested PCR, laryngeal cancer cell line
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INTRODUCTION

THE CAUSATIVE role of the low-risk human papillomavirus (HPV) types in the development of laryngeal papillomas is well established: HPV infection, predominantly by types 6 and/or 11, has been found in the majority of laryngeal papillomas derived from adults and from younger patients [1]. Malignant transformation, which may follow a severe papillomatosis of long duration, is possibly associated with a prior low-risk HPV infection [2]. Alternatively, the low-risk types may just be bystanders during tumorigenesis [3].

Numerous studies on the role of high-risk HPV types, predominantly HPV 16, in laryngeal carcinomas have been published with contradictory results. Using *in situ* hybridisation (ISH), 5% of laryngeal carcinoma samples have been found to contain HPV 16 DNA [4]. With Southern blot hybridisation (SBH), Scheurlen and colleagues [5] detected 3% and Watts and associates [6] 11% HPV 16 positivity. The HPV 16 detection rate has ranged from 4 to 54% with polymerase chain reaction (PCR) [7, 8].

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The variation in the results may partly be derived from the tissue material used: in the paraffin-embedded blocks the quantity of intact DNA is minimal and impairs the amplification of longer sequences [9]. All biopsy samples, either fixed or fresh, always contain cells other than cancer cells, which may also have an effect on the results. In addition, there are remarkable differences in the sensitivity of methods used for HPV analysis: from 20 to 50 viral copies per cell are required for signal in ISH, whereas SBH finds one HPV copy among 10 and PCR among 100 000 HPV-negative cells. The conflicting findings may also result from the low HPV copy number [10, 11] and the scattered distribution of viral DNA in only some tumour cells, as has been seen with ISH in head and neck cancers [12]. If only few tumour cells harbour more than 20 copies of HPV DNA per cell, ISH is the only method that can detect them. If several cells contain one viral copy or less, all other DNA methods but ISH will give positive results if enough cells are collected for analysis.

According to the 'hit and run' theory, the papillomavirus genome is considered necessary in the initiation of malignant transformation. HPV is thought to exert its oncogenic capacity through maintaining continuous cell proliferation and preventing the function of *p53* and *Rb* tumour-suppressor genes by its E6 and E7 proteins. Subsequently, if new, irreversible malignant changes develop that have the same effect as E6 and E7, the viral genome could be lost from the cell [13]. This theory cannot be excluded as a reason for the low HPV detection rate in the upper respiratory tract malignancies either [10].

To elucidate the role of HPV in laryngeal carcinogenesis and to avoid contamination problems from viral infection in adjacent normal cells, we analysed 27 cell lines established from laryngeal carcinomas or their metastases from 22 patients by PCR using two sets of consensus primers and HPV 16 E6 primers. Also, paraffin-embedded biopsy samples from the original tumours of 12 of the cell line donors were available for ISH and PCR analyses. Further, in order to find even minimal amounts of HPV DNA, a nested PCR method was applied, known to detect only 2.5 copies of HPV 16 DNA in a PCR reaction mixture [14]. After nested PCR, SBH was performed both with low- and high-risk HPV oligonucleotide probe mixtures and separately with the HPV 16 oligonucleotide probe to confirm the results.

MATERIALS AND METHODS

Patients and samples

The permanent cell lines were established from human laryngeal cancers or metastases as described by Carey [15]. Six UM-SCC cell lines were obtained as a gift from Dr T. Carey (Ann Arbor, Michigan, U.S.A.). Twenty-one UT-SCC cell lines were established by Grenman and colleagues [16]. Thus, 27 cell lines established from 22 patients were used. The paraffin-embedded biopsy samples from the original laryngeal carcinomas of 12 patients of the UT-SCC series were available for this study. Table 1 gives the patient data and the characteristics of the original tumours and metastases.

Cell lines

The cells were cultured in Eagle's complete minimal essential medium (DMEM) containing 2 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin supplemented with 10% fetal bovine serum. Subcultures were performed weekly and bi-weekly. The cells were grown in 75 cm^2 flasks and harvested at mid-logarithmic growth with trypsin/ethylene diamine tetraacetic acid (EDTA) for DNA extraction. The cell cultivation and DNA

Table 1. *Characterisation of the cell lines used*

	Cell line	Sex	Primary carcinoma location	TN	Type of lesion	Grade
1.	UT-SCC-4	F	Supraglottic larynx	T4N0	Recurrent	G2
2.	UT-SCC-6A	F	Supraglottic larynx	T2N1	Recurrent	G1
3.	UT-SCC-6B	F	Supraglottic larynx	T2N1	Neck metastases	G1
4.	UT-SCC-8	M	Supraglottic larynx	T2N0	Primary	G1
5.	UT-SCC-9	M	Glottic larynx	T2N0	Neck metastases	G1
6.	UT-SCC-11	M	Glottic larynx	T1N0	Recurrent	G2
7.	UT-SCC-13	M	Glottic larynx	T3N0	Persistent primary	G2
8.	UT-SCC-17	M	Supraglottic larynx	T2N0	Neck metastases	G3
9.	UT-SCC-19A	M	Glottic larynx	T4N0	Primary	G2
10.	UT-SCC-19B	M	Glottic larynx	T4N0	Persistent primary	G2
11.	UT-SCC-22	M	Glottic larynx	T1N0	Recurrent	G2
12.	UT-SCC-23	M	Glottic larynx	T3N0	Persistent primary	G1
13.	UT-SCC-29	M	Glottic larynx	T2N0	Primary	G1
14.	UT-SCC-34	M	Supraglottic larynx	T4N0	Primary	G1
15.	UT-SCC-35	M	Glottic larynx	T2N0	Recurrent	G2
16.	UT-SCC-38	M	Glottic larynx	T2N0	Primary	G2
17.	UT-SCC-39	M	Supraglottic larynx	T2N0	Primary	G2
18.	UT-SCC-42A	M	Supraglottic larynx	T4N3	Primary	G3
19.	UT-SCC-42B	M	Supraglottic larynx	T4N3	Neck metastases	G3
20.	UT-SCC-49	M	Glottic larynx	T2N0	Primary	G2
21.	UT-SCC-50	M	Glottic larynx	T2N0	Recurrent	G3
22.	UM-SCC-5	M	Supraglottic larynx	T2N1	Persistent primary	G3
23.	UM-SCC-11A	M	Supraglottic larynx	T2N2	Primary	Unknown
24.	UM-SCC-11B	M	Supraglottic larynx	T2N2	Persistent primary	Unknown
25.	UM-SCC-12	M	Supraglottic larynx	T2N1	Persistent primary	G2
26.	UM-SCC-17A	F	Glottic larynx	T2N1	Persistent primary	G2
27.	UM-SCC-17B	F	Glottic larynx	T2N1	Neck metastases	G2

extraction of a series of 18, and later a larger series of 27, laryngeal carcinoma cell lines, including the 18 cell lines, were carried out in separate laboratories to exclude cross-contamination between the series. The passages of the series of 27 cell lines were selected on the basis of the lowest passage available. In all but one sample the passage was the same as or earlier than in the corresponding sample in the series of 18 cell lines.

DNA extraction from cell lines and paraffin-embedded blocks

Cells (approximately 10^6) were lysed with 0.3 mg proteinase K in a buffer containing 10 mM Tris, pH 8.3, 400 mM NaCl, 2 mM Na_2 EDTA and 1% sodium dodecyl sulphate (SDS) at 37°C overnight. After digestion, proteins were first precipitated with saturated 6 M NaCl and then centrifuged at 2500 rpm for 15 min. Thereafter DNA was precipitated with absolute ethanol, washed with 70% ethanol and dissolved in 20 µl of sterile H_2O.

The histology of the original tumour samples was confirmed in re-cut, haematoxylin and eosin-stained sections. Several 5 µm thick sections were cut to achieve a total surface area of 1 cm^2. DNA was extracted through xylene, graded ethanol and acetone treatment. Protein digestion was carried out with proteinase K (0.2 mg/ml) in 50 µl of sterile water at 37°C overnight.

SBH

SBH was used for the analysis of 18 cell lines under low stringency conditions. The DNA was digested with restriction endonuclease *Pst I* at 37°C overnight, run on a 1% agarose gel (Bio-Rad, Richmond, California, U.S.A.) and transferred to a Nylon filter (Gene Screen Plus, NEN Research Products, DuPont, Boston, Massachusetts, U.S.A.) according to the routine protocol. The prehybridisation was performed in a solution containing 30% formamide, 1% SDS, 5×SSC, 0.05 M sodium phosphate, 1×Denhardt and 0.5 mg/ml t-RNA at 42°C for 3 h. The heat-denatured whole-genomic ^{32}P-labelled HPV probe mixture of HPV 6, 16 and 18, was added to the prehybrisation solution at a concentration of 10^6 cpm/ml at 42°C overnight. After washing the filters the results were visualised with autoradiography (Kodak X-Omat AR, intensifying screens) exposed at − 70°C for 1, 3 and 7 days. DNA samples isolated from HPV 11 DNA and human fibroblasts were used as positive and negative controls, respectively.

ISH

ISH was used for the evaluation of 12 original laryngeal carcinoma samples. Two sequential 6 µm sections from the formalin-fixed, paraffin-embedded carcinomas were cut and mounted on 1% organosilane slides. The slides were deparaffinised and hybridised with the biotinylated whole-genomic DNA probe mixture of HPV 6, 16 and 18 under low stringency (Tm − 30°C). Hybridisation was carried out at 37°C overnight. Posthybridisation washes were as follows: three times in 2×SSC at room temperature (RT) for 5 min, twice in 0.2×SSC at 37°C for 5 min and once in 2×SSC at RT for 5 min. The biotinylated hybrids were detected by streptavidin–alkaline phosphatase complex using 5-bromo-4-chloro-3-indolylphosphate (BCIP) as the substrate and nitroblue tetrazolium (NBT) as the chromogene. The sections were then dehydrated and mounted. The slides were reviewed for the presence of a purple nuclear staining reaction. Tissue sections fixed in a similar manner and known to harbour HPV 6, 11, 16, 18, 31 and 33 were run simultaneously as positive controls. Negative controls were run by omitting the probe and replacing it with plasmid DNA (pBR 322).

PCR

PCR was carried out first from the same 18 cell lines as SBH and also from 12 paraffin-embedded biopsy samples. To increase the accuracy of PCR analysis, two sets of oligonucleotide consensus primers, MY09/MY11 and GP05/GP06, directed to the L1 open reading frame (ORF) of the HPV genome were used [17, 18]. Additionally, two sets of oligonucleotide primers targeting HPV E1 [19] and HPV 16 E6 ORFs were included. The amplification of the human β-globin gene was performed using PCO3B/4B primers to check the adequacy of the DNA from each specimen. Table 2 shows the sequences of oligonucleotide primers and the sizes of PCR products. PCR was performed in a 50 µl reaction volume. From cell lines, 5 µl and from tumour biopsy samples, 10 µl of the template DNA solution were used, approximating 300 ng of DNA. The PCR mixture contained PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100) (Finnzymes, Espoo, Finland), 20 pmol of each primer, 200 µM of each deoxynucleotide triphosphate and 1 U of DynaZyme DNA polymerase (Finnzymes). PCR was performed with a DNA thermal cycler (Perkin–Elmer Cetus, Norwalk, Connecticut, U.S.A.). Table 3 depicts the PCR cycles and the number of cycles for

Table 2. Sequences and lengths of the oligonucleotide primers used

Region and primers	Sequence	Size (bp)
HPV LI-MY09/11	5′-CGT CCM ARR GGA WAC TGA TC-3′ 5′-GCM CAG GGW CAT AAY AAT GG-3′	448–454
HPV L1-GP05/06	5′-TTT GTT ACT GTG GTA GAT AC-3′ 5′-GAA AAA TAA ACT GTA AAT CA-3′	140–150
HPV L1-GP05+/06+	5′-TTT GTT ACT GTG GTA GAT ACT AC-3′ 5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′	140–150
HPV E1-p1E1/p2E2	5′-TATGGCTATTCTGAAGTGGAA-3′ 5′-TTGATATACCTGTTCTAAACCA-3′	526–583
HPV 16 E6-A1B/A2	5′-GGA TCC ACA GGA GCG ACC CAG AAA G-3′ 5′-CTG CAG ATG GGG CAC ACA ATT CTT A-3′	447
β-globin-PCO3B/4B	5′-ACA CAA CTG TGT TCA CTA GC-3′ 5′-CAA CTT CAT CCA CGT TCA CC-3′	110

M = A + C; R = A + G; W = A + T; Y = C + T.

Table 3. Polymerase chain reaction (PCR) protocol steps for each primer

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
HPV-MY09/11	95°C, 4.5 min	95°C, 30 sec	55°C, 50 sec	72°C, 60 sec	72°C, 7 min	30
HPV-GP05/06	95°C, 5 min	95°C, 60 sec	40°C, 60 sec	72°C, 90 sec	72°C, 7 min	40
HPV-p1E1/p2E2	94°C, 3 min	94°C, 60 sec	42°C, 2 min	72°C, 90 sec	72°C, 7 min	34
HPV-16 E6	95°C, 6.5 min	95°C, 60 sec	62°C, 60 sec	72°C, 60 sec	72°C, 7 min	30
β-globin-PCO3B/4B	95°C, 10 min	95°C, 30 sec	55°C, 50 sec	72°C, 60 sec	72°C, 7 min	30

each primer set. The amplified products were screened by agarose gel electrophoresis (Nusieve, FMC Corp Bio-Products, Rockland, Maine, U.S.A.) under ultraviolet light. CaSki cell line, containing 600 copies of HPV 16 DNA per cell, served as a positive control. The PCR reaction mixture without the template was used as a negative control. Due to the negativity of the first 18 cell lines in PCR with all HPV primers, the study was repeated with 27 cell lines, including nine new laryngeal carcinoma cell lines. PCR was carried out with MY, GP05 +/06 + and HPV 16 E6 primers (Table 2). β-globin amplification was also repeated. PCR was performed using the DNA amplification kit (Perkin–Elmer, New Jersey, U.S.A.) and DNA thermal cycler (Perkin–Elmer Cetus). The 50 µl reaction volume contained 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.4 µmol of each primer, 200 µM of each deoxynucleotide triphosphate and 1.25 U AmpliTag Gold DNA polymerase (Perkin–Elmer). The PCR programme with longer GP05 +/06 +-primers started with an initial denaturation of 10 min instead of 5 min, otherwise the programmes for each primer as well as the control samples were identical to the protocols described above.

Nested PCR and SBH using digoxigenin detection

Nested PCR was performed first for 14 of 18 cell lines using MY09/MY11 primers as external primers and GP05 +/GP06 + primers as internal ones. The oligonucleotides were synthesised by Kebo Lab (Huddinge, Sweden). The specificity of the amplimer was confirmed by subsequent SBH using low- and high-risk HPV oligonucleotide probe mixtures and the HPV 16 oligonucleotide probe. After the primary positive results, 13 additional cell lines from laryngeal carcinomas and 12 original biopsy samples were included for the study. First, PCR was performed as described above using MY09/11 primers. Second, the re-amplification was performed, taking 2 µl of the product from the first PCR reaction as a template. In the re-amplification general primers (GPs), amplifying the sequence inside the L1 ORF targeted by MY primers, were used. CaSki DNA amplified with both conventional and nested PCR were used as positive controls. One or two negative controls, which were obtained by omitting the template from the PCR mixture, were used in every reaction. After re-amplification, the results were confirmed by viewing the samples run on agarose gels under ultraviolet light. DNA was then transferred to a Nylon membrane (GeneScreen Plus, Biotechnology Systems, Boston, Massachusetts, U.S.A.). The prehybridisation was performed in a solution containing 25% formamide, 3×SSC, 1% SDS, 5% Denhardt and 10% Dextran sulphate at 55°C for 2 h, the background was blocked with salmon sperm DNA (2 mg/ml). Thereafter, the membranes were hybridised with both low- and high-risk HPV oligoprobe mixtures, containing six and 12 different HPV oligonucleotides, respectively. Subsequently, the type-specific HPV 16 oligonucleotide probe was used. Oligonucleotides were labelled with digoxigenin according to the manufacturer's instructions (DIG Oligonucleotide 3′-end Labeling Kit, Boehringer Mannheim, Germany). After denaturation of the probes at 95°C, 150 pmol of probe mixtures or probe were added to 10 ml of prehybridisation solution. The hybridisation was carried out at 55°C overnight. After the hybridisation the filters were washed three times in 2×SSC/1% SDS at 55°C and twice in 100 mM Tris/150 mM NaCl (pH 7.0) at RT. After incubation in the blocking reagent (Boehringer Mannheim) at 55°C for 30–60 min, positive hybridisation was immunodetected at RT for 40 min using antidigoxigenin Fab fragments conjugated to alkaline phosphatase. The filters were rinsed and the positive signals were visualised using the chemiluminescence substrate CSPD (DIG Luminescent Detection Kit, Boehringer Mannheim). The filters were incubated in 100 µl CSPD/10 ml buffer for 5 min in the dark. Following the washes with 100 mM Tris/150 mM NaCl and 100 mM Tris/100 mM NaCl/50 mM $MgCl_2$ (pH 9.5), chemiluminescence was recorded on X-ray films (DuPont, Cronex 4) with the exposure time from 5 to 60 min. Amplified DNA extracted from SiHa cells, containing one HPV 16 copy per cell, was used as a semiquantitative assay to estimate the HPV copy number. A SiHa dilution series, ranging from 1000 to 1 SiHa cell against 1 200 000 background fibroblasts, was amplified with nested PCR and hybridised with the HPV 16 probe. The positive results were compared with the intensity of signals found in the SiHa dilution series. Nested PCR was repeated twice with subsequent hybridisation with the HPV 16 oligoprobe from the series of 27 cell lines to confirm the results.

RESULTS

SBH and ISH

None of the 18 cell lines studied were HPV positive using SBH under low stringency conditions. There was also no positive signal found in the original carcinomas studied by ISH. However, HPV DNA-positive signals were seen in a few normal epithelial cells lining the carcinoma tissue in one sample. The same sample, UT-SCC-13, proved to be HPV 16 positive in nested PCR, but the cell line established from it did not contain HPV DNA.

PCR

None of the DNAs extracted from the 18 cell lines could be amplified with the four sets of HPV primers used. The series of 27 cell lines was also HPV DNA negative with three different primers. Additionally, the original tumours were also HPV DNA negative after repeated amplification. Amplification of the 27 cell lines and the original tumour samples with β-globin PCO3B/4B primers resulted in a PCR product 110 bp in size visible under ultraviolet light, indicating a sufficient amount of DNA in all extracts.

Nested PCR

Table 4 presents the results obtained from the cell lines and original tumour samples with nested PCR and subsequent SBH with the high-risk oligoprobe mixture and HPV 16 probe. From 14 cell lines analysed initially, five cell lines (36%) were found to contain HPV DNA with the high-risk HPV probe mixture; the finding was identical with the HPV 16 oligoprobe. To confirm the first result with nested PCR, those 14 cell lines were recultured and DNA was isolated again in a separate laboratory to avoid the possibility of contamination. Moreover, 13 new cell lines were included for the analyses.

Nested PCR of 27 cell lines with subsequent SBH with the high-risk HPV probe mixture revealed that five cell lines (19%) from 5 patients harboured HPV. In the first screening with the HPV 16 oligoprobe three of those samples proved to be positive (Figure 1). The analysis was repeated twice with the HPV 16 probe to confirm the results. One cell line, UT-SCC-50, was confirmed to be HPV DNA positive through all these analyses. Four other cell lines showed fluctuating results in the repeated analyses: UT-SCC-22 gave positive signals twice with the HPV 16 oligoprobe hybridisation whilst three other cell lines showed HPV 16 DNA positivity once during three hybridisations. A total of seven cell lines (26%) in both series of 14 and 27 cell lines together were found to harbour HPV 16 in nested PCR. Only one cell line gave a positive signal with the low-risk HPV probe cocktail; it was not positive with the high-risk HPV probe.

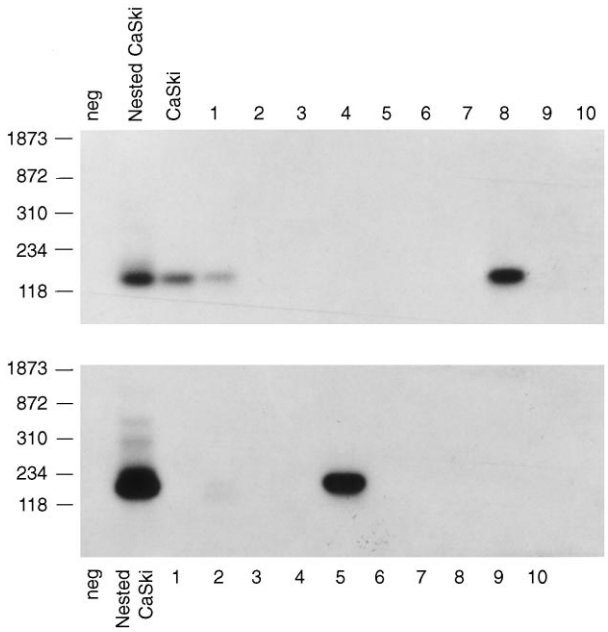

Figure 1. Southern blot hybridisation (SBH) of the nested polymerase chain reaction (PCR) product with a high-risk human papillomavirus (HPV) oligoprobe mixture resulted in three positive signals in the cell lines as shown in lanes 1 and 8 on the upper part of the filter and in lane 5 on the lower part of the filter. Nested and conventional PCRs of the CaSki cell line as positive controls and negative controls are marked separately.

Table 4. *Human papillomavirus (HPV) positivity as detected with nested polymerase chain reaction (PCR) confirmed by Southern blot hybridisation*

	Tumour biopsy sample		Cell line samples			Cell line samples, second DNA isolation				
Cell line	High-risk probe	HPV 16 probe	Passage	High-risk HPV probe cocktail	HPV 16 probe	Passage	High-risk HPV probe cocktail	HPV 16 probe/I	HPV 16 probe/II	HPV 16 probe/III
1. UT-SCC-4	+	–	32	–	–	32	–	–	–	–
2. UT-SCC-6A	+	+	23	+	+	3	+	–	–	+
3. UT-SCC-6B	BNA	BNA	24	–	–	24	–	–	–	–
4. UT-SCC-8	BNA	BNA	33	–	–	33	–	–	–	–
5. UT-SCC-9	–	–	12	+	+	3	–	–	–	–
6. UT-SCC-11	–	–	32	+	+	2	+	–	–	+
7. UT-SCC-17	BNA	BNA	22	–	–	22	–	–	–	–
8. UT-SCC-19A	BNA	BNA	17	–	–	17	–	–	–	–
9. UM-SCC-5	BNA	BNA	16	+	+	18	–	–	–	–
10. UM-SCC-11A	BNA	BNA	19	–	–	19	–	–	–	–
11. UM-SCC-11B	BNA	BNA	13	+	+	12	+	+	–	–
12. UM-SCC-12	BNA	BNA	14	–	–	14	–	–	–	–
13. UM-SCC-17A	BNA	BNA	28	–	–	28	–	–	–	–
14. UM-SCC-17B	BNA	BNA	36	–	–	36	–	–	–	–
15. UT-SCC-13	+	+		ND	ND	16	–	–	–	–
16. UT-SCC-19B	BNA	BNA		ND	ND	34	–	–	–	–
17. UT-SCC-22	+	+		ND	ND	19	+	+	+	–
18. UT-SCC-23	+	+		ND	ND	18	–	–	–	–
19. UT-SCC-29	–	–		ND	ND	20	–	–	–	–
20. UT-SCC-34	+	+		ND	ND	18	–	–	–	–
21. UT-SCC-35	+	+		ND	ND	34	–	–	–	–
22. UT-SCC-38	–	–		ND	ND	20	–	–	–	–
23. UT-SCC-39	BNA	BNA		ND	ND	4	–	–	–	–
24. UT-SCC-42A	BNA	BNA		ND	ND	30	–	–	–	–
25. UT-SCC-42B*	BNA	BNA		ND	ND	12	–	–	–	–
26. UT-SCC-49	BNA	BNA		ND	ND	15	–	–	–	–
27. UT-SCC-50	–	–		ND	ND	13	+	+	+	+

BNA, block not available; ND, not done. High risk oligoprobe contained 12 different HPV oligonucleotides. *Low-risk HPV DNA-positive sample.

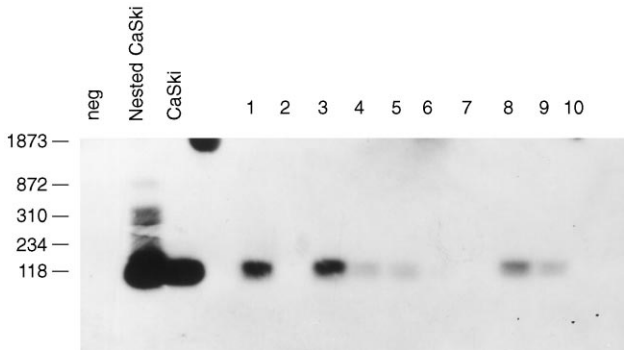

Figure 2. Original tumour samples gave positive signals in nested polymerase chain reaction (PCR) with subsequent Southern blot hybridisation (SBH) with the high-risk human papillomavirus (HPV) oligoprobe mixture in lanes 1, 3, 4, 5, 6, 8 and 9. Positive and negative controls are marked.

Two different passages were analysed in five cell lines. In three of them, HPV DNA was found in both the earlier and the later passages. UT-SCC-9 turned HPV DNA positive at a later passage. HPV DNA positivity disappeared in UM-SCC-5 at passage 18.

From the 12 original tumours investigated, seven (58%) showed HPV DNA positivity after nested PCR with subsequent SBH with the high-risk HPV oligoprobe (Figure 2). Thus, HPV was a more frequent finding in the original tumours than in the cell lines derived from them. In two cases, HPV DNA was found both in the tumour biopsy sample and in the cell line derived from it. All the tumour samples were then rehybridised with the HPV 16 oligoprobe. One sample did not hybridise with it, implicating the presence of some other high-risk HPV type. The sample containing signals with ISH, indicating the presence of HPV DNA, was also positive with nested PCR. No signals for the presence of HPV were achieved when using the low-risk HPV oligoprobe mixture.

In the SiHa dilution series, the last positive signal was seen in nested PCR with 20 SiHa cells in the reaction, equal to 20 HPV 16 copies. Semiquantitative assessment of the results obtained from the cell line samples showed the positive signals in four samples to be faint and identical to that found in a PCR with 20 SiHa cells, i.e. 20 HPV copies in the sample of 1 200 000 cells. In the other three cell line samples signals were stronger.

DISCUSSION

The causal relationship between laryngeal cancer and high-risk HPV types is complex. Wide variation has been found in the detection rate of HPV DNA in laryngeal carcinomas, even when the same method is applied, and the findings concerning the correlation of HPV with clinical parameters and survival have been contradictory [7, 20, 21]. Further, high-risk types have also been detected in benign and premalignant lesions of the larynx [22]. The application of sensitive PCR has increased the detection of HPV infections in laryngeal carcinomas, but the role of HPV in laryngeal carcinogenesis is still under discussion.

In the present study, HPV DNA could not be amplified with PCR either from DNA extracted from the cell lines or from paraffin samples with the primers targeting L1 ORF. The disruption of L1 ORF is possible in laryngeal carcinomas [5]. Also, the L1 region may be fragmentary even in the episomal form in oral keratinocytes [23]. The fragmentation of the HPV genome may prevent the annealing of general primers like MY and GP primers to target DNA. However, the total negativity even with primers specific for E1 and HPV 16 E6 ORFs indicates an absence of HPV DNA in the samples. Bradford and colleagues [24] analysed five UM cell lines, which were also included in our series, by SBH and were unable to find HPV. When investigating cell line material, it is easy to grow cells to the quantity which is sufficient for DNA extraction and PCR analysis. Thus, the inadequacy of DNA was probably not the reason for the failure to show HPV DNA in the samples. The negative results with the paraffin-embedded biopsy material may partly be due to their storage for many years, impairing the quality of the DNA. The size of the tumour biopsy samples was also small. Nevertheless, the DNA extraction method used here should not cause a considerable loss of DNA from formalin-fixed samples. Also, PCR with β-globin primers was positive, indicating a sufficient amount of proper DNA in the original biopsy samples and the absence of non-specific PCR-inhibiting factors.

Nested PCR resulted in positivity: 26% of all cell lines were found to contain HPV 16 DNA. Considering the established importance of HPV 16 in cervical carcinogenesis, its predominance in laryngeal carcinomas may indicate its potential role in this site. As normal SBH did not detect any positive signals, the results suggest that some cells in the cell line population do not contain the HPV genome or that the HPV genome is in a form that cannot be amplified under the conditions used. In fact, in half the positive cell line samples, the portion of cells harbouring HPV was minimal, as estimated from the SiHa dilution series, corresponding to approximately 20 cells among 10^6 tumour cells. Tumour tissue is heterogeneous in many respects; our findings also suggest heterogeneity in carcinoma cell lines in the light of a subpopulation of HPV DNA-containing cells. The inconsistent detection of HPV 16 in four samples in three identical analyses from the same sample tube also supports this. The relative 'oversensitivity' of nested PCR, when using in total 70 cycles in two successive PCRs, may also cause inappropriate primer annealing. The risk of contamination with this highly sensitive method must also be taken into account. However, negative controls used remained appropriately negative, indicative of the absence of contamination. In addition, 20 of 27 cell lines were consistently HPV DNA negative. These findings point to the non-clonal persistence of HPV in laryngeal carcinomas, also proposed by Snijders and associates [11], when observing less than one HPV 16 copy per 1000 cells in head and neck cancers.

The integration of HPV 16 into the human genome and the expression of its oncoproteins E6/E7 have been reported to provide cervical epithelial cells with a growth advantage in cell culture [25]. After the integration, HPV-infected cell clones could occupy and transform a cervical cell line during several passages [26]. One of our cell lines was HPV DNA negative in the earlier passage but showed HPV 16 positivity subsequently, after nine passages, suggesting possible selective growth of the HPV-containing clone. Also, two of our cell lines were investigated at passages 2 and 3 and at passages 32 and 23, respectively, and they maintained their HPV 16 positivity. We need further studies to determine the optimal growth conditions for these cell clones to achieve a homogeneus HPV-positive cell line.

In line with the concept of a growth advantage associated with HPV integration, Williams and colleagues [27] found a steep decline in the episomal HPV copy number from passage 0 to passages 1–3 in oral wart and anal mucosal keratinocytes. Moreover, the cells floating freely in the culture flask were observed to contain higher copy numbers than the adhered ones. Paralleling that, diLorenzo and associates [28], using laryngeal papilloma cell lines, found that the plating of HPV-infected cells to culture flasks was impaired compared with uninfected cells or cells with low HPV copy number. The loss of HPV during multiple passages has also been observed in a dysplastic genital lesion by our group [29]. A selection pressure towards the eradication of episomal HPV from cell lines is possible and reasonable as a cellular defence mechanism against the infecting virus. As the integration is not a frequent finding in head and neck cancers [23, 30], it might even explain the minor contribution of HPV to laryngeal carcinogenesis compared with the involvement in genital carcinomas.

When analysed with nested PCR, 58% of the original biopsies were found to harbour high-risk HPV DNA. A recent study found HPV DNA in 50% of oral cancer samples with nested PCR, supporting our observation [31]. However, the samples harboured HPV at low copy numbers, since there were no positive signals with ISH in the carcinoma tissues. Very low HPV copy numbers have also been observed in studies of oral cancers [12, 32]. In our material, 11 of 22 patients had a T3-T4 tumour and/or a recurrent disease. Considering the advanced stage of tumours in half the patients, HPV might have been involved in the initiation of cancer and subsequently lost during the course of the disease. Then, only minimal amounts of HPV genome would have been left at the time of the biopsy, which would support the 'hit and run' theory. The higher HPV detection rate in original tumours compared with cell lines indicates the eradication of the HPV genome in early passages of cell cultivation. However, the higher HPV detection rate may speak for the presence of adjacent, HPV-infected normal cells in biopsy samples, as indicated by an ISH-positive sample in the present series. The majority of the samples were HPV 16 positive, a result in line with the predominance of HPV 16 in carcinomas of the larynx found in other reports [8, 11, 33].

A considerable amount of interest has focused on the involvement of HPV in epithelial malignancies of the upper aerodigestive tract, but the results have been contradictory. The study materials and methods vary in the reports. Further, the copy number and the total amount of HPV in laryngeal carcinomas, as shown by the present study, are usually low. HPV persistence appears to be non-clonal, arguing for heterogeneous laryngeal cancer cell populations. These findings may partly explain the discrepancy in the results of the role of high-risk HPV in carcinomas of the larynx. Moreover, taking the more infrequent HPV detection rate in laryngeal carcinomas compared with that in the genital cancers, the mechanism of HPV-associated carcinogenesis may be different in these two anatomical sites. When HPV is present, it probably co-operates with, for example, tobacco carcinogens, to promote malignant transformation, as reported with experiments on oral keratinocyte cell lines [34].

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