J.Cell.Mol.Med. Vol 5, No 1, 2001 pp. 49-59

Detection of human papillomavirus gene sequences in cell lines derived from laryngeal tumors

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Received: January 15, 2001; Accepted: March 19, 2001

Abstract

The role of Human Papillomaviruses (HPV) in laryngeal carcinomas has been studied with conflicting results. To evaluate the etiologic relationship between HPV infection and epithelial malignancy of the larynx we studied five laryngeal carcinoma cell lines obtained from patients undergoing surgery for laryngeal tumors. The paraffin embedded biopsy samples of the original tumor and different passages of the new established cell lines were investigated by PCR with consensus primers specific for HPV DNA. The findings indicate that HPV infection is associated with some larynx carcinomas. The positive association has been enhanced when a method of enrichment of epithelial cells from fresh tumor samples was used. All tumor cells enriched smears were positive for HPV DNA not only by PCR but also by *in situ* hybridization (ISH). Investigated by PCR, different passages of larynx tumor cell lines maintained expression of HPV DNA. At subsequent passages ISH gives constantly no signals suggesting a minimal amount of viral harbored sequences. In one cell line propagated more than 60 population doublings, the chromosomal frequency distribution shifted from modal number 46 at the 5th passage to 63 at the 60th passage. The mechanisms by which persistent HPV infection maintains continuous cell proliferation were discussed.

Keywords: laryngeal carcinomas - enrichment of epithelial cells - tumor derived cell lines - HPV DNA detection

Introduction

The association between Human Papillomaviruses (HPV) infections and a subset of head and neck cancers was established by molecular genetic assays which signaled the presence of fragments of virus genome in tumors [1]. The assessment was done using direct methods: polymerase chain reaction (PCR), *in situ* hybridisation (ISH), genomic Southern blot or, indirectly, by analysis for p53 tumor-suppressor gene and protein [2]. HPV positivity was found especially in oropharynx, larynx and tonsils and in basaloid tumors (over 80% tumors being positive). HPV infection seems to have a predilection for the border between the external and internal epithelia where frequent metaplasic proces-

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ses can take place. Moreover, because of their invaginating crypts, such anatomical sites have an extensive epithelial surface, facilitating the viral access to basal cells and intensive antigenic stimulation. In addition, the presence of lymphoid tissue may affect viral transcription and cellular transformation.

Well-differentiated epidermoid carcinomas with small and dark crowded cells, scant cytoplasm and hiperchromatic nuclei are histological features associated with persistent HPV infection. Persistent infections with HPV genotypes with high carcinogenic risk show also striking mitotic activity and absence of normal keratinisation pattern. The HPV infection is not the only missing link between laryngeal cancers and histological features. Other lifetime carcinogen exposure needs careful assessment: smoking, consumption of different alcoholic beverages, chewing different products. In view of the need of more effective and less mutilating treatments the diagnostic of infection with biologically relevant HPV genotypes would be useful.

High-risk human papillomavirus types have been found in patients with respiratory papillomas and squamous cell carcinoma using PCR, immunochemistry (IHC) and ISH. Virological studies of juvenile laryngeal papillomatosis also involved the persistent HPV infection. Detection and typing of human papillomavirus DNA in benign and malignant tumors of laryngeal epithelium was proposed as a tool of therapeutic management [3].

Clinical and molecular biology studies of respiratory papillomatosis provide conflicting results. Some authors noted low detection rate of HPV in oral and laryngeal carcinomas [4, 5]. On the other side, demonstration of multiple HPV types in laryngeal premalignant lesions using PCR and ISH further supports the etiological role of HPV infection in the transformation of laryngeal epithelium [6]. We tried to solve the inconstant reports by using a rapid and simple method of epithelial cell enrichment proposed by Maitra *et al.* [7].

A new perspective was brought on the possible mechanisms by which papillomaviruses contribute to cancer. HPV is thought to exert its oncogenic capacity through maintaining continous cell proliferation and preventing function of two suppressor genes. Two HPV early proteins, E6 and E7 were shown to interact with two cellular tumor-suppressing proteins: p53 and retinoblastoma, respectively [8]. P53 is one of the most important guardians in DNA repairing processes and in maintaining chromosomal stability. That explains why mutational changes observed in HPV related tumors are present in immortalized cell lines derived from the original samples.

The involvement of genetic factors in HPV-linked carcinogenesis has been postulated in the past and recently a high mutator phenotype responsible for increased degradation of p53 has been signaled. There is likely to be a gradual accumulation of specific cellular changes during the malignant progression that can explain the long latency after the primary infection and the failure to detect viral sequences in some cases.

Materials and methods

Patients

A multidisciplinary team including a head and neck surgeon, a radiation oncologist and a medical oncologist carefully examined five patients who complained of hoarseness for longer than one month. The clinical diagnostic of cancer of the larynx was confirmed and evaluated for the extent of the tumor by computed tomography scanning. All patients were informed of the treatment alternative and risks. The patients gave informed consent to participate in the study in accord with a protocol that had received ethical approval from the internal ethical board.

Histopathology

During surgical intervention (total laryngectomy), tissue samples were removed. One fragment including part of the primary tumor and uni- or bilateral neck lymph nodes was fixed in 10% buffered formaldehyde and embedded in paraffin, then sectioned for staining with hematoxylin and eosin. The remaining fragment obtained by removal of the primary tumor was collected in tissue culture medium and immediately transported on ice to the Institute of Virology. The smears were obtained from this last fragment and tissue cultures were initiated using standard protocols.

The same pathologist semi-quantitatively appreciated the level of inflammation on microscopic sections on a scale from 0 to 4. Histological parameters, including subpopulations of malignant cells, keratosis, lymphocytic infiltration, non-neoplasic epithelium were all assigned scores on a scale: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; 4, severe extension of tumor or of lymphohistiocytic infiltration.

Cell cultures

Tissue samples from the original tumors were treated by trypsin digestion. Cells were plated in Dulbecco's modified Eagle medium with 10% fetal bovine serum (Sigma). Regular passages were done at 5 – 7 days interval with a 1:2 split ratio. At fifth passage representative samples were cryopreserved. A human diploid cell line and a HeLa clone positive for HPV-18 were maintained in the same medium and used as negative and positive controls, respectively.

Enrichment of normal and tumor cells

Accurate molecular analysis of tumors and their precursor lesions requires extraction of specific subpopulations of cells (normal, preneoplasic and tumoral) from a composite background of multiple cell types. Attempts to obtain pure tumor cell samples have resulted in the initiation of several methods of cell enrichment, including the use of tumor cell lines, xenografted tumors, and microdissection of frozen or paraffin-embedded tissues. All methods used in the present have advantages and limitations. To enhance the potential of diagnostic methods, we used a rapid, simple procedure for enrichment of normal and tumor cells developed by Maitra *et al.* [7] called epithelial aggregate separation and isolation (EASI). EASI is based on two general principles: the innate property of desmosome-rich epithelial cells to separate from surrounding stromal tissues as tightly adherent clusters, and the use of rapid, alcohol-based fixation of thin layers of cells resulting in improved preservation of DNA, RNA and proteins. EASI is applicable only to fresh tissues that have not been frozen or formalin-fixed. This method routinely dislodges many aggregates of epithelial cells. Multiple slides (for example, 5–50) can be prepared from scraped normal or neoplasic epithelium within a few minutes. Epithelial aggregates are precisely identified by microscopic examination and appear as tight cellular aggregates or tissue fragments. Each fragment usually contains an estimated number of 10,000–20,000 cells.

DNA isolation and PCR assay

Total cell DNA from tumor smears or from initiated cell cultures was isolated with proteinase K and SDS by standard procedure [8].

The viral DNA amplification was performed according to the protocol of Manos *et al* [8] slightly modified by Anton *et al* [9]. The degenerated consensus primers for HPV/L1 protein were produced by Biometra GmbH. The primers sequences were as follow: MY11 (+5'-GCMCAGGWCATAAYAATGG-3') and MY09 (-5'-CGTCCMARRGGAWACTGAT-3'). Samples were defined as positive for DNA/HPV if they showed an ethidium bromide stained band of ~450 bp in the electrophoresis of amplicons. The positive control was represented by plasmidial HPV DNA, (HPV 16), kindly provided by Ethel-Marie de Villiers, Deutsches Krebsforschungszentrum – Heidelberg, Germany.

This PCR assay was used for the identification of DNA from the mucosal HPVs types -6, -11, -16, -18, -31 and -33 with consensus primers, for detecting DNA from more than 25 characterized mucosal HPVs.

Viral genome sequence localization

Separate tissue sections and smears from the original tumors transported in cold MEM medium were studied by ISH to detect HPV. Further, the original new initiated "TV" cell lines and tumor biopsy samples were investigated with PCR using a set of consensus primers directed to HPV L1 gene.

Cytogenetic analysis

Two hours prior to cell cultures harvest cholcemid was added to a final concentration of 0.2 mg/ml. After harvesting cells by trypsinization, KCl hypotonic solution (0.075 M) was added to the pelleted cells at room temperature, gently resuspended and incubated at 37ºC for 30 minutes. The cells were fixed by adding 3:1 methanol: acetic acid mixture. The mitotic chromosomes obtained were used to identify numerical and structural chromosomal abnormalities by various classical staining techniques. The G banding technique, which shows more details about microdeletions and rearrangements, was adapted for karyotype analysis. For this, the slides were dipped in trypsin solution at 37ºC for 30 - 90 seconds, adjusting the time according to banding quality and stained 10 minutes in Giemsa 10% in Sörensen phosphate buffer.

Results

Evaluation of histological samples

The clinico-pathological assessment of five cases of larynx cancer resumed in table 1 was in accordance with UICC TNM System [10]. The T classification indicating the extent of the primary tumor is in line with anatomic considerations for glottic larynx involving the vocal cords (main symptom: hoarseness and vocal cords fixation) or supraglottic larynx (main symptoms: dysphagia, throat pain, hoarseness). T0 indicates no evidence of primary tumor, Tis - carcinoma *in situ*, T1 - tumor 2 cm or less, T2

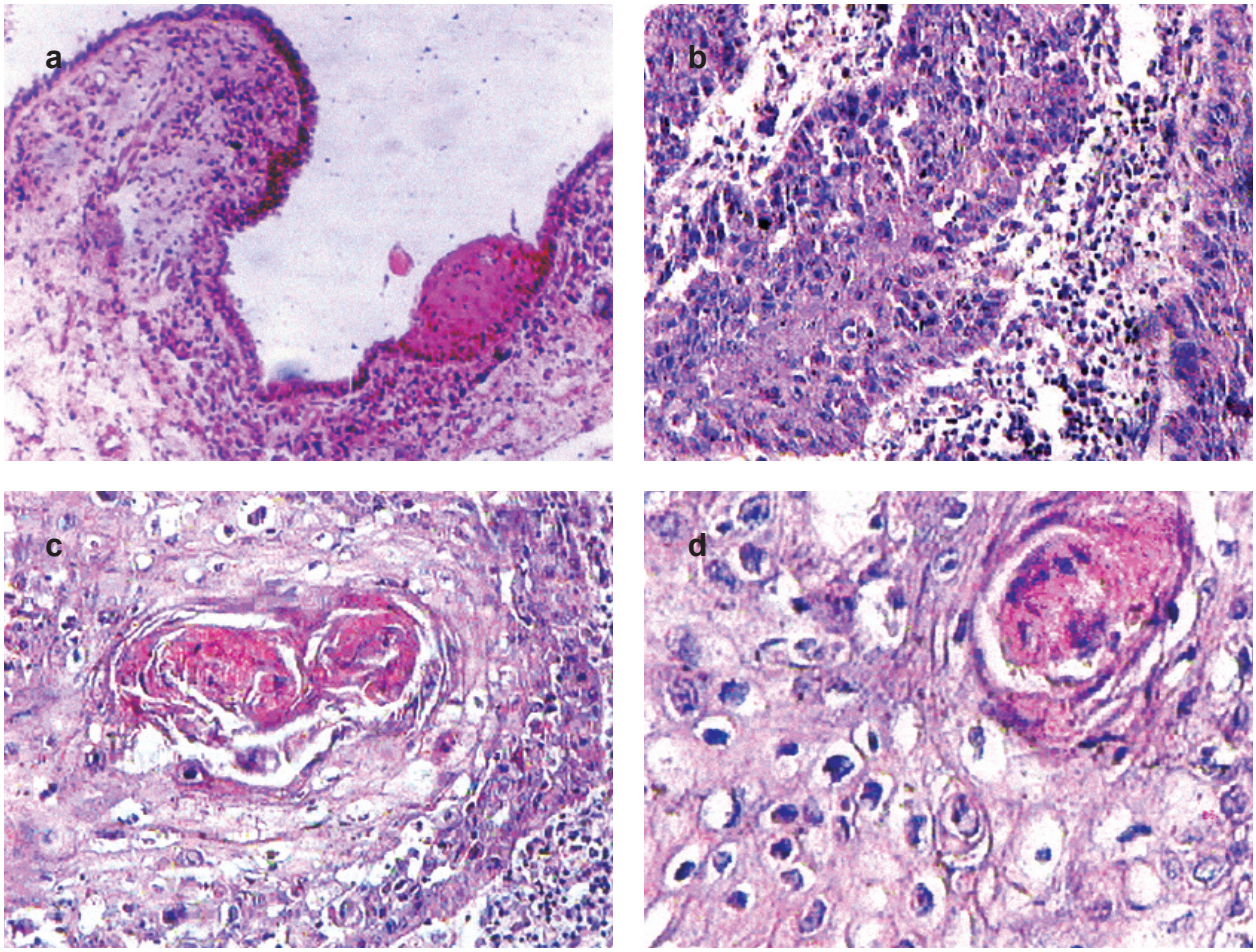

Fig. 1 Sections from squamous carcinoma (TV patient). Morphological alterations suggestive for HPV infection: **(a)** the tumors usually arise from the surface epithelium of the larynx, HE, x40; **(b)** the thickened of the epithelial cover penetrates into the underline chorion and induces digitizations of various shapes and sizes, HE, x100; **(c)** the orthokeratozic pearls are ovoid circular formations made up of elongated cells arranged in a foliate bulb shape HE, x250; **(d)** adjacent to the orthokeratotic pearls, cells with picnotic nuclei, HE, x400. Perinuclear cytoplasmic cavitation with thickened plasma membrane and nuclear atypia are characteristic alterations for an HPV infection.

- tumor 2-4 cm and T3/4 - tumor more than 4 cm. The N classification refers to regional lymph node involvement: N1 – metastasis in a single ipsilateral lymph node 3 cm or less, N2 – metastasis in multiple ipsilateral lymph nodes, N3 - metastasis in multiple lymph nodes more than 6 cm in the greatest dimension. All patients were in poor general health and surgical interventions (total laryngectomy) were done without preoperative irradiation. Postoperative radiotherapy to the tumor bed and bilateral nodes was applied to all patients.

The patients were 4 males and 1 female with the mean age of 63.8±8.4. Tumors were histologically characterized by lobules and nests of basaloid cells with scanty cytoplasm, comedonecrosis and adenoid features, and by concomitant presence of squamous cell carcinoma. The features of squamous cell carcinoma are presented in Fig. 1. HPV DNA was detected in all but one of the original tumor sample. This study reaffirms that larynx carcinoma may have a peculiar topographic distribution and distinct pathologic features. Koilocytosis referring to productive viral infection and the signs of abnormal keratinisation were present in each primary tumor sample (Fig. 1c and 1d). HPV are thought to infect the basal or parabasal cells of the squamous epithelium and to give rise either to non-productive (latent) or a productive infection. The latent infection is defined as one in which the virus replication is synchronized with the cell cycle but, in our study,

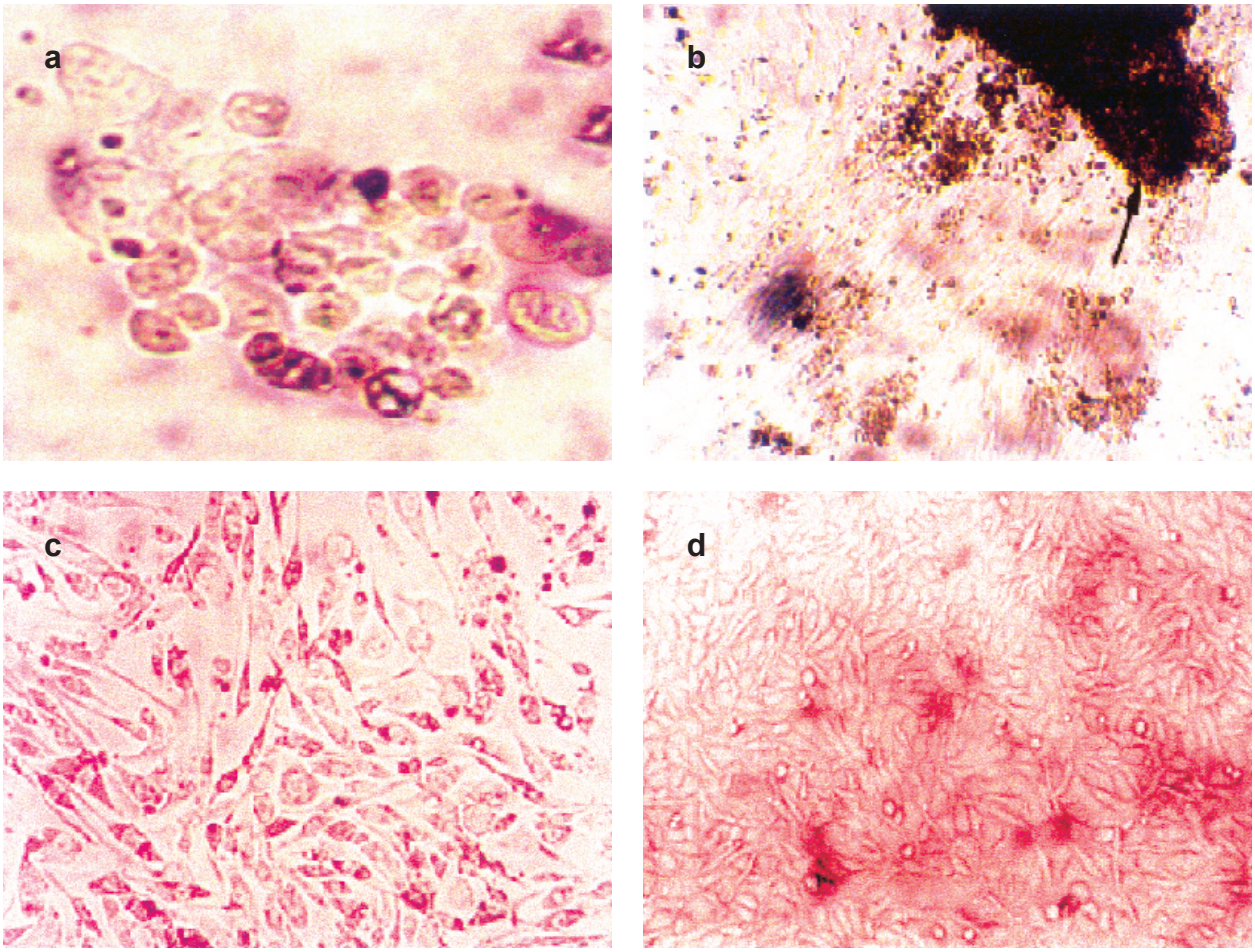

Fig. 2 Morphological pattern observed at different passages of TV cell line: (a) epithelial aggregate obtained by cell enrichment method, magnification: x600; **(b)** tumor explants from TV patient, x40; **(c)** early, fifth passage, presenting mixed population of fibroblasts and epitheloid cells, x200; **(d)** late passage (>40th) with uniform epithelial cell population, x100; The b, c, d observations were made with a Zeiss inversed microscope.

none of the cytopathogenic effect could be detected. The productive viral replication began in the upper parabasal and lower intermediate layers of the epithelium where characteristic cytological alterations become evident. We observed effects that are most specific including perinuclear cytoplasmic cavitation with thickened cytoplasmic membrane and, most importantly, nuclear atypia. The combination of nuclear atypia and perinuclear halo formation is referred to as koilocytosis or koilocytotic atypia (Fig. 1d). These koilocytotic cells are the principal hallmark of a productive HPV infection.

Culture of human cancer cell

There is a misapprehension that primary culture cells from tumoral tissue almost always spontaneously become immortal [11]. Cancer human cells (in contrast to rodent cells) can only be used a limited period until their senescence. The lifespan can be extended by transfection with viral genes. We obtained primary adherent cultures from each tumor processed. By passaging cells in standard culture condition (with the MEM enriched with 10% fetal calf serum) we succeeded to make a stock of cells that were cryopreserved at passage five. Only two lines have been further propagated *in vitro*. At an early passage, the cultures still senesce (the crisis period) but occasional cells acquire some mutations that make them immortal. Important morphological changes accompanied the immortalization process (Fig. 2a to 2d). The nuclear architecture was particularly affected with the chromatin structure positioned randomly but frequently with a

Table 1 The clinico-pathologic characteristics of five cases of larynx cancer

Patient, Sex & age	Clinical Classification[1]	Histology	Post-surgical evolution	Other risk factors
TV, male, 54	T3N3	Spinocellular carcinoma[2]	Latero-cervical metastasis	TB, tobacco
UC, male, 66	T2N2	Spinocellular carcinoma [2]	Latero-cervical metastasis	Prostatectomy
GA, male, 73	RRA [3]	Basaloid squamous carcinoma	Good	Diabetes, tobacco
GI, male, 70	T1aN1	Pavimentous carcinoma	Good	Tobacco
PF, female, 56	T2N2	Spinocellular carcinoma [2]	Latero-cervical metastasis	

[1]TNM clinical classification for the head and neck area corresponds to the UICC general rules. [2]Spinocellular carcinoma with hiperkeratosis; [3]Recurrent respiratory papillomatosis

tendency to cluster in a heterochromatin fraction that stay compact through the cell cycle (Fig. 2c). The shape of the cytoplasm was predominantly fibroblastic-like at early passages and mostly epitheloid above the 30th passage (Fig. 2d). In Fig. 3 the natural history of one of these new cell lines, named TV, developed from a larynx tumor biopsy is depicted. The TV cell line, initiated from an old male patient with spino-cellular carcinoma, was maintained over 600 days *in vitro*. Three phases of cell cultures can be described. Phase I with predominant cultured normal fibroblasts was specific for the first six weeks, after which the luxuriant growth ceased and large, senescent spindle cells accumulated. In phase II a mixed population of cells with increasing morphologic changes heralded the approach of Hayflick limit (cellular aging) [12] was present together with islets of epitheloid cells that had a good proliferative potential. Finally, in phase III, after supplementary 18 – 20 weeks *in vitro*, the epitheloid cells demonstrate an intrinsic capacity for replacement and for normal growth with biweekly doublings. These populations represent the expression of maximum potential longevity, even immortality. For control, we traced the well-known three-phase evolution of a human diploid cell (HDC) strain initiated from embryonic skin and the passage history of a HeLa clone. The succession of three phases in HDC strain is different: (*i*) – the primary culture dependent of density cell seed, (*ii*) – exponential replication period: 40 – 50 doublings at three days interval and (*iii*) senescence and arrested cell division.

Chromosomal abnormality

It is well known that tumoral tissues, as well as immortalized cell lines, involve disruption of stable patterns of chromosomal positioning. The detection of numerical changes was reported both in established tumors and in derived cell lines. In Fig. 4 the histograms of chromosomes frequency distribution in TV cell line at different passage level were compared. At the early passages the mean value and the standard deviation of chromosome number was 47.5± 9.4 (modal no 46) slightly different from passage 60, mean value 55.8±21.4 (modal no 63). Chromosomes rearrange naturally during *in vitro* cultivation: they undergo duplication, translocations, inversion and so on. It remains to be established if the chromosomal alteration is non-random and reveals a possible loss of tumor suppressor gene.

Aneuplody is characterized by an abnormal number of sets of chromosomes and influence gene expression not only in the duplicated chromosome

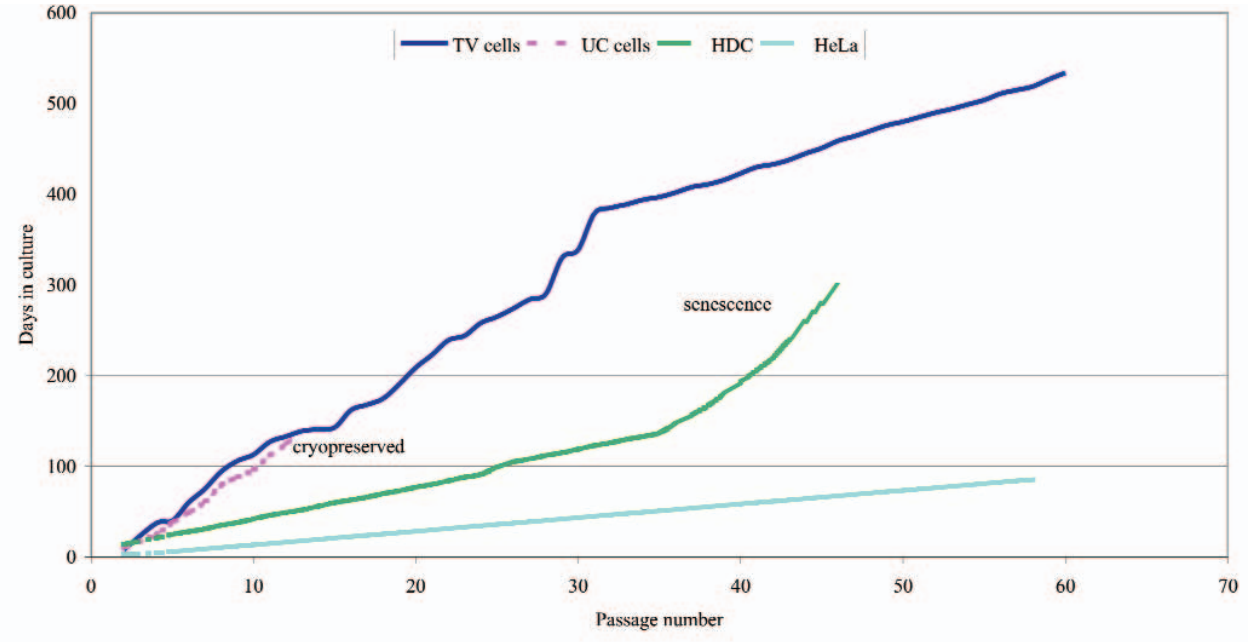

Fig. 3 The accumulated cell population doublings during days in culture. TV and UC are cell lines initiated from larynx carcinoma. HDC - human diploid cell line - and HeLa cells were propagated in the same conditions.

but also in nearby chromosomal regions. Investigations into links between structural and functional aspects of metaphase chromosomes promise to reveal other insights into development of phenotypic changes during *in vitro* cell differentiation.

Expression of HPV DNA in laryngeal carcinoma

To clarify the role of viral DNA in cellular transformation we examined by PCR amplification original tumor samples: methanol fixed smears obtained from tumors as well as different passages of cell lines initiated from tumors. Only the sample from one patient has been constantly negative. The abundance of inflammatory infiltrate can explain the negative result in the original tumor. Smears of tumor tissues were prepared on glass slides (Fig. 2a) and processed either fresh or fixed. The rapidly fixed (in methanol) and stained samples showed improved preservation of viral DNA. Possible virus integration into the cellular genome was consistent with the continuous expression of viral DNA at different passage levels in cell lines (Table 2). Noteworthy, all tumor cells enriched smears were positive and the absence of keratosis in the original tumor was associated with negative viral DNA detection. Different passages of larynx tumor cell lines with positive viral DNA signal in the original tumor maintained expression of HPV DNA. Combining the high sensitivity of PCR with the cell localizing ability of *in situ* hybridization (ISH) allows for the reproducible detection of low copy targets in nucleus of intact cells. Fig. 5 shows the strong positive signal with ISH in a TV cell at an early passage. At subsequent passages ISH gives constantly no signals suggesting a selection pressure towards the eradication of episomal HPV DNA. These results indicate that only a minority of cells harbors HPV DNA, explaining partly the controversial results reported earlier [16, 22].

Discussion

The frequency of HPV in benign and precancerous head and neck lesions ranged from 18.5% to 35.9%, depending on the detection methodology [13]. Based upon the most sensitive method of detection, PCR, the overall prevalence of HPV in head and neck tumors was 34.5% (416 of 1205 tumors) in the review of Mc Kaig *et al* [14]. The majority of HPV-

Table 2 Expression of HPV DNA in larynx carcinoma and in derived cell lines

ID	Tumor size	Infiltrate	Keratosis[1]	HPV DNA tumor §	HPV DNA smears §¶	Cell passage§[2]
TV	T4	Massive	Nodular	Positive	Positive	45
UC	T4	Moderate	Absent	Negative	Positive	15
GA	T2	Massive	Discrete	Negative	Positive	5
GI	T4	Moderate	Absent	Positive	Positive	15
PF	T4	Discrete	Nodular	Negative	ND	5, negative

Expression of HPV DNA was tested by PCR (§) or/and ISH (¶); [1] for histological parameters see figures 1 to 4; [2] last cell line passage tested with presence of HPV DNA; ND - not done.

positive tumors contained the "high risk" HPV types 16 (40.0%) and 18 (11.9%). The frequency of HPV positivity in oral samples from healthy individuals ranged from 1% to 60% [15]. A limited number of descriptive and analytic epidemiological studies in region with high incidence of head and neck cancer have indicated that age (<60 years) and sex (male) were associated with the presence of HPV in the tumor, whereas tobacco and alcohol use were not [16, 17]. The occurrence of squamous cell carcinomas (SCC), in patients previously treated for papillomas, underlines the need for repeated virological studies with sensitive detection methods [18, 19].

Most cases of laryngeal papillomatosis are cytologically benign and do not undergo malignant transformation. However, SCC can arise in recurrent respiratory papillomatosis in the absence of known risk factors such as radiation and smoking [20].

To evaluate the causal relationship between HPV infection and epithelial malignancies of the larynx, 27 laryngeal carcinoma cell lines from 22 patients were studied by Atula and colleagues [21]. Also, paraffin-embedded biopsy samples of the original tumors were available from 12 patients. Southern blot hybridization (SBH) and *in situ* hybridization (ISH) were used for the analysis of 18 cell lines and 12 original tumor sections to detect HPV. The disappointing results forced the application of more sensitive techniques in order to found even minimal amount of HPV DNA. Cell lines and tumor biopsy samples were investigated with PCR using three sets of consensus primers directed to L1 and E1 open reading frames (orf) and type-specific primers to HPV 16 E6 region in order to achieve a maximal sensitivity. Also, a new nested PCR method was used with MY as external and general primers (GP) as internal primers, for the cell lines and original tumor samples. 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. Only with this last method, seven of 27 (26%) cell lines and seven of 12 (58%) tumor samples were found to harbor high-risk HPV. In laryngeal verucous carcinoma, a variant of well-differentiated SCC that clinically resembles laryngeal papilloma, HPV DNA was detected in 31 out of 34 cases. HPV 16 was by far the most frequent type detected in these tumors [21]. Scarce detection of human papillomavirus (HPV) in laryngeal carcinoma cell lines provides evidence for a heterogeneic cell population or cells harbored HPV at a very low number of copies.

Tumors are composite structures consisting of host inflammatory and desmoplastic responses, angiogenic elements, non-neoplasic epithelium and stroma. Subpopulations of viable premalignant or malignant cells may occupy less than 5% of the tissue volume.

The explants of tumor tissue sample give more frequent positive results for the presence of HPV genome sequences and allow a semi-quantitative estimation of viral copies number. Analytical methods for evaluation the sensitivity of various techniques for the detection of HPV infection used as standards cell lines containing different copy number of HPV DNA [23]. These cell lines are derived from cervix carcinoma *e.g.*: Ca Ski with 600 copies of HPV 16 DNA, SiHa with 1-2 copies of HPV 16 DNA or HeLa with 10 –50 copies of HPV 18 DNA.

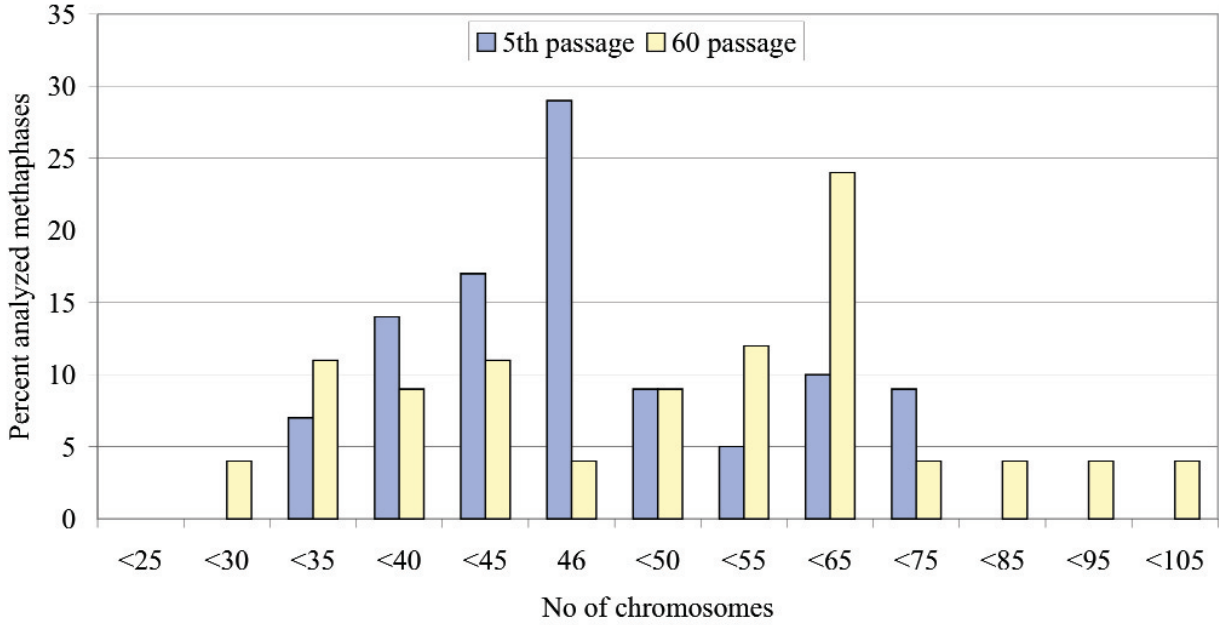

Fig. 4 Chromosome frequency distribution in TV cell line derived from a male patient with larynx carcinoma. The chromosome frequency, distribution and morphology in at least 50 representative metaphases were determined. The observations were made on cells from the second passage, following recovery from frozen preserved stock ampoules.

The TV cell line presented in this paper has the same low level of copies as HeLa, but expressed in cells originating from a male patient and not from a female one like HeLa. The pattern of oncogenesis has not been frequently documented amongst most of the common adult epithelial cancers. The experimental model offered by our new cell line is interesting because Y chromosome is less variable than X and non-sex chromosomes.

Non random alterations, frequently involving chromosomes 1 and 3 were reported in cell lines derived from cervix carcinoma. It is interesting that this is correlated with earlier observation that the tumorigenicity of HPV 18 expressing HeLa cells and of HPV 16 expressing SiHa cells can be suppressed by the addition of a normal human chromosome 11 in somatic cell hybrids [24]. The detection of numerical and structural changes in chromosome 1 may provide a method of studying premalignant lesions.

Chromosomal deletions have been identified in both premalignant and malignant lesions, as have mutations in tumor suppressor genes, commonly the p53 gene [25]. Amplifications of oncogenes are less common but overexpression of the epidermal growth factor receptor has been described [26]. The latest finding correlates positively with tumor size and poor outcome. Attempts have been made to use this information on therapy. A tumor specific p53 mutation can be detected on some phenotypically "normal" surgical margins indicating residual disease. The use of molecular techniques for the early diagnosis or screening in high risk populations (smokers and heavy drinkers) seems a promising research way.

HPV types 16 or 18 related sequences were identified only in a SCC specimen. p53 was detected in 64% of the cases. All p53+ specimens showed no HPV related sequences; the only HPV positive case was p53 negative. The increased p53 expression in the process from dysplastic to invasive SCC indicates that p53 overexpression is an early event in laryngeal carcinogenesis [25].

The occurrence of SCC in patients previously treated for papillomas underlines the need for repeated histological studies. The surgical treatment remains the mainstay in the management of laryngeal papillomatosis. The laser surgical technique is superior to conventional removal. Using the present most sensitive and specific methods HPV DNA can be detected in a large percentage of laryngeal papillomas. A part of the patients with larynx cancer developed recurrence in spite of normal lymph nodes biopsy and clear surgical margins around the

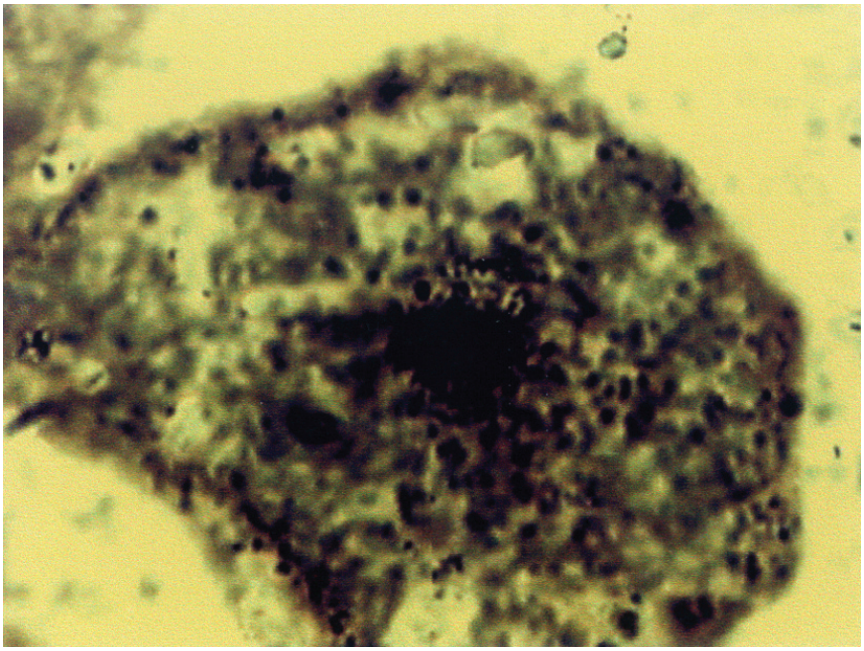

Fig. 5 ***In situ*** **hybridization assay for HPV DNA.** Strong positive signals revealed by a digoxigenin (dig) labeled probe (5 ng ADN in 20 ml solution) in TV cell line at passage 5th. The molecular probe was obtained with dig-11-dUTP by PCR. Detection was performed by alkaline phosphatase labeled anti-dig antibody, using as substrate nitroblue-tetrazolium and bromo-chloro-indolyl-phosphate. Magnification: x1000.

total laryngectomy. Histologically undetectable micrometastases in the lymphatic system can be discovered by molecular technology methods. The long lasting presence of HPV DNA sequences in some cell lines derived from larynx tumours suggests the need to analyze lymphatic tissues for the presence of HPV DNA.

Conclusion

The prevalence of HPV, particularly the high-risk types, suggests a potential etiologic role of the virus in head and neck cancer. Molecular biology has provided important data on the interaction of the HPV oncoproteins with genes important in cell cycle control.

Cell lines derived from epithelial cells enriched samples give more frequent positive results for the presence of HPV genome sequences in comparison with commonly used fresh or fixed tumor tissue samples. This system, representing self-replicating tumor cell populations, yield high quality DNA, RNA and protein for subsequent studies. However, the explant method is a relatively cumbersome, and expensive technique and is not always successful. Nonetheless, more basic research is needed to describe the physical state of the virus in a variety of cell types and the interaction with other genes. In addition, epidemiological research is required for a further understanding of the association between HPV and demographic and other risk factors, as well as possible routes of transmission. Finally, further studies are needed much work is warranted to provide a definitive assessment of the prognostic significance of HPV in premalignant larynx lesions.

Acknowledgements

The authors thank Dr. Daniela Rojanski for technical assistance in karyotyping studies. We are grateful to Dr. Simona Ruta for helpful discussions. The work was supported by a grant from National Agency for Science, Technology and Innovations.

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