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Establishment and characterisation of human papillomavirus type 16 DNA immortalised human tonsillar epithelial cell lines

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Abstract

Oncogenic human papillomavirus (HPV) plays a possible aetiological role in a subset of head and neck cancers, particularly in tonsillar carcinomas. For establishing a model to study mechanisms involved in HPV-associated tonsillar carcinogenesis, normal human tonsillar epithelial (HTE) cells were transfected with full-length HPV-16 DNA. The transfections produced four immortalised cell lines, designated HTE-114/K1, HTE-114/K2, HTE-114/K3 and HTE-114/B. All transfected HTE cell lines were cytogenetically abnormal. They exhibited altered morphology and impaired expression of cytokeratins in organotypic cultures. They failed to form colonies in soft agarose and formed no tumours in nude mice within 6 months. Each of them contained integrated viral DNA in a distinctive pattern as shown by Southern blot hybridisation. Early viral transcripts containing the E7 gene were detected by northern blot hybridisation. In conclusion, primary HTE cells can be immortalised following transfection with full-length HPV-16 DNA; the immortalised cell lines had partially retained epithelial characteristics in their morphology and function. They seem to represent early stages of premalignant epithelial cells and thus provide a useful model for studying further the multistep molecular events of HPV-16-associated tonsillar carcinogenesis.

Keywords: Human papillomavirus; Human tonsillar epithelial cell; Immortalisation; Transformation

1. Introduction

Human papillomavirus (HPV) is a small double-stranded DNA virus epitheliotropically infecting the skin and mucosa. An increasing number of studies have suggested that high-risk types of HPV play an aetiological role in the pathogenesis of squamous-cell carcinoma of the head and neck (HNSCC), particular in tonsillar malignancies [1]. Oncogenic HPV DNA is more frequently found in tonsillar squamous-cell carcinoma (SCC) than in other sites of HNSCC [2]. Approximately 20% of oropharyngeal cancers contain the same HPV types as anogenital malignancies [3]. Patients with a history of HPV-associated anogenital cancers have a significantly increased risk for tonsillar SCC [4]. The reason for the association of HPV with tonsillar SCC remains unclear. It may be because of the tonsils' extensive epithelial surface area, they are uniquely sensitive to the transforming effects of the virus; alternatively, the surrounding hormonal and paracrine milieu may affect viral transcription, resulting in cellular transformation, as reviewed by Gillison and colleagues [5].

High-risk types of HPV are a causative agent in uterine cervical cancer, and molecular biological approaches have provided evidence for specific mechanisms [6]. However, the role of HPV infection in tonsillar carcinogenesis is less well established. Study of HPV in cell culture has been hindered because a complete viral life cycle cannot be duplicated *in vitro*. Transfection of primary human epithelial cells with high-risk types of HPV DNA often results in cell transformation and immortalisation

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[7]. The immortalised cell lines such as human cervical epithelial cells, foreskin keratinocytes, and oral epithelial cells have served as an *in vitro* model for studying the role of HPV in human carcinogenesis [8,9]. In the present study, normal HTE cells were transfected with full-length HPV-16 DNA, and this transfection resulted in cell immortalisation. These cell lines may provide a useful model to study further the multistep molecular events of HPV-associated tonsillar carcinogenesis, the interaction of HPV-transformed HTE cells and stroma, as well as the effects of antivirus and antitumour agents.

2. Materials and methods

2.1. Cell culture and transfection

Primary HTE cells were derived from children's bilateral tonsillectomies. A small piece of tissue from each tonsil was examined by human papilloma virus-polymerase chain reaction (HPV-PCR) in order to exclude pre-existing HPV infection. The tissues had normal histological morphology as judged from haematoxylin- and eosin-stained tissue sections. The methods used in cell cultures and electroporation have been previously described in Ref. [8]. Two clones of HPV-16 DNA, 114/K and 114/B [10], were used for the transfection.

2.2. Organotypic culture

The organotypic culture previously described in Ref. [11] was used with minor modifications. Briefly, for each raft, a mixture of 4×10^5 human embryonic skin (HES) fibroblasts and 1.75 mg of rat tail collagen type I (Upstate Biotechnology, Lake Placid, NY, USA) in Modified Eagle Medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics was placed on top of 0.8% agarose (w/v) in a 12-well plate. The next day, 2×10^5 epithelial cells were seeded on the top of the collagen gel, submerged in culture medium. Three days later, when the epithelial cells had grown to confluence, the collagen raft was lifted onto a metal grid and further cultured at the air-medium interface for 12 days. The cell lines cultured in rafts were HPV-16-transfected cells (between the 15th and 21st passages), primary HTE cells, and SiHa carcinoma cells. The rafts were fixed with 4% paraformaldehyde and embedded in paraffin.

2.3. Immunocytochemistry

5-μm cross-sections of rafts were deparaffinised and rehydrated. Immunostaining for cytokeratins was performed with the Ventana Gen II *in situ* hybridisation/immunohistochemistry slide stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) using the alkaline phosphatase Fast Red Detection Kit (Ventana) (positive signal appears in red) and counterstained with haematoxylin. The primary mouse IgG monoclonal antibody (MAb) to human cytokeratins 1, 5, 10 and 14 (1:50) (DAKO-Cytokeratin, 34BE12, Carpinteria, CA, USA) was used after antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) in a microwave oven. An unrelated murine IgG MAb (1:200) (Clone X63, American Type Culture Collection, Manassas, VA, USA) served as a negative control.

2.4. Electron microscopic examination

The rafts from HPV-16-transfected cell lines were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Ultra-thin sections were stained with uranyl acetate and analysed at 80 kV with a Jeol 100 CX-II electron microscope.

2.5. Anchorage-independent growth in soft agarose

The assay was previously described in Ref. [8]. Briefly, 0.4% top agarose (SeaPlaque agarose; FMC BioProducts, Rockland, ME, USA) containing 10^4 cells was layered over prehardened 0.64% bottom agarose in a 12-well plate. On the next day, 200 μl of culture medium was pipetted on top of the agarose to prevent dehydration. The cultures were monitored for colony formation for 4 weeks.

2.6. Tumorigenicity assay

Five million cells in 100 μl of medium were injected subcutaneously (s.c.) into the right flank of 4-week-old nude mice (Balb/c nu/nu, Harlan, Blackthorn, Bicester, UK). Cell lines used in the assay were HPV-16-transfected HTE cell lines (between the 30th and 40th passage), SiHa (positive control), and HaCaT cells (negative control), as well as medium alone. The tumorigenicity of each cell line was performed in quintuplicate. After injection, the animals were followed-up for 6 months, and their weight, injection site, and overall health condition was recorded weekly (0–2.5 months postinjection) or biweekly (2.5–6 months postinjection).

2.7. Cytogenetic analysis

Monolayer cultures in the logarithmic phase were incubated with 0.1 μg/ml of colcemide overnight. Subsequently, the metaphase cells were trypsinised and swollen in 0.075 M of KCl, fixed in a mixture of ice-cold methanol and acetic acid at 3:1 (v/v), and spread on alcohol-treated slides. The metaphase spreads were stained with Giemsa.

2.8. Fluorescence in situ hybridisation (FISH)

Full-length HPV-16 DNA was labelled with biotin-16-deoxyuridine triphosphate (dUTP) by nick translation

(Roche Diagnostics GmbH, Mannheim, Germany), co-precipitated with human *Cot-1* DNA (Life Technologies, Inc., Gaithersburg, MD, USA), and dissolved in a hybridisation solution containing 50% formamide, 20% dextran sulphate, and 2×SSC (saline-sodium citrate). The metaphase spreads were denatured at 70 °C in 2×SSC containing 70% formamide for 2 min and hybridised with the heat-denatured probe at 37 °C for 48 h. The hybridised probe was detected by fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories Inc., Burlingame, CA, USA). The signals were then amplified with anti-avidin D/avidin-FITC (Vector). Chromosomes were counterstained with diamidino-2-phenylindole. Signals were captured by an Olympus fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) connected to a charge-coupled devise (CCD) camera and the *in situ* imaging system (ISIS) digital image analysis system (Meta Systems GmbH, Altlussheim, Germany). For each cell line, a minimum of 30 metaphases was analysed.

2.9. Southern blot hybridisation

Total cellular DNA was isolated by proteinase K lysis followed by phenol extraction. Ten micrograms of DNA was digested with the indicated restriction endonuclease and electrophoresed in a 0.8% agarose gel (SeaKem, BioWhittaker Molecular Applications, Rockland, ME, USA). The DNA fragments were blotted onto an uncharged nylon membrane (Hybond, Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK) by a capillary transfer system and fixed by heating at 80 °C for 2 h. The membrane was prehybridised at 60 °C for 1 h and hybridised with a ^{32}P-labelled full-length HPV-16 DNA probe at the same temperature for 1 h in ExpressHyb hybridisation solution (Clontech Laboratories, Inc. Palo Alto, CA, USA) in a cylinder. After low- and high-stringency washes, the membrane was exposed to X-ray film (Fuji Photo Film GmbH, Düsseldorf, Germany) for 3–10 days at −70 °C with an intensifying screen.

2.10. Northern blot hybridisation

Total RNA was isolated with TRIZOL Reagent (Life Technologies, Inc.). 20 μg of total RNA was fractionated by electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde. The fractionated RNA was transferred onto an uncharged nylon membrane (Hybond, Amersham Pharmacia Biotech) as described above. The membrane was prehybridised at 68 °C for 30 min and then hybridised with a ^{32}P-labelled probe at the same temperature for 1 h in ExpressHyb hybridisation solution. Three probes were used: full-length HPV-16 DNA, PCR-derived fragments corresponding to the E7 sequence [12], and the L1 sequence [13]. Hybridising transcripts were visualised by autoradiography as described above.

3. Results

3.1. Growth properties of HPV-16-transfected cells

Normal HTE cells had a limited lifespan of approximately 6–8 subcultivations at a 1:2 split ratio for 3 months. They formed clusters of small polygonal cells with pale nuclei in monolayer culture. In contrast to the primary HTE cells, all HPV-16-transfected cell lines acquired extended lifespans; currently, they are beyond 80 subcultures. The primary HTE cells transfected with clone 114/K (HTE-114/K) underwent a 3-week crisis at the 9th passage. Addition of 40 ng/ml of dexamethasone was used at passage 9 to assist the cells to escape senescence. After this crisis, three individual clones were cultured separately, designated HTE-114/K1, HTE-14/K2 and HTE-114/K3. The primary HTE cells from another donor, transfected with clone 114/B (HTE-114/B), had no intervening crisis period. All HPV-16-transfected cells contained large or multiple nuclei and each nucleus contained 3–8 prominent nucleoli. HTE-114/K1 and HTE-114/K3 had a morphology similar to that of primary HTE cells, whereas HTE-114/K2 and HTE-114/B were larger and had loose cell-to-cell contacts (Fig. 1).

3.2. Cell differentiation

In vitro, the differentiated phenotype of epithelial cells can be induced by growing the cells at the air–liquid interface on collagenous substrata. If HPV-immortalised cells represent a tissue culture counterpart to naturally occurring premalignant cells, they should share many characteristics of dysplastic cells [14]. We observed that the primary HTE cells in raft cultures had a well-differentiated morphology similar to the normal tonsillar epithelial architecture. Though the number of cell layers was rather small, the layers were well polarised. Cubic epithelial cells were present in the basal layer, and from the middle to superficial layers, the cells were gradually flattened, indicating terminal differentiation of normal HTE cells in the raft cultures. During normal differentiation, epithelial cells are characterised by the expression of intermediate filaments, notably keratins. Cytokeratins were expressed strongly throughout all epithelial layers in the normal HTE raft (Fig. 2a). In contrast, all transfected cells in organotypic cultures showed considerable variability in size and shape. They had a disturbed cellular polarity, vacuolated cytoplasm, and an increased nucleus-to-cytoplasm ratio. Furthermore, the cells infiltrated into the collagen matrix (Fig. 2d). The appearance of the transfected HTE cells in rafts resembled epithelial dysplasia *in vivo*. A weak-to-intermediate expression level of cytokeratins was detected in the HPV-transfected cells in the rafts (Fig. 2b–e). SiHa carcinoma cells in the raft formed a non-stratified epithelium, and exhibited a

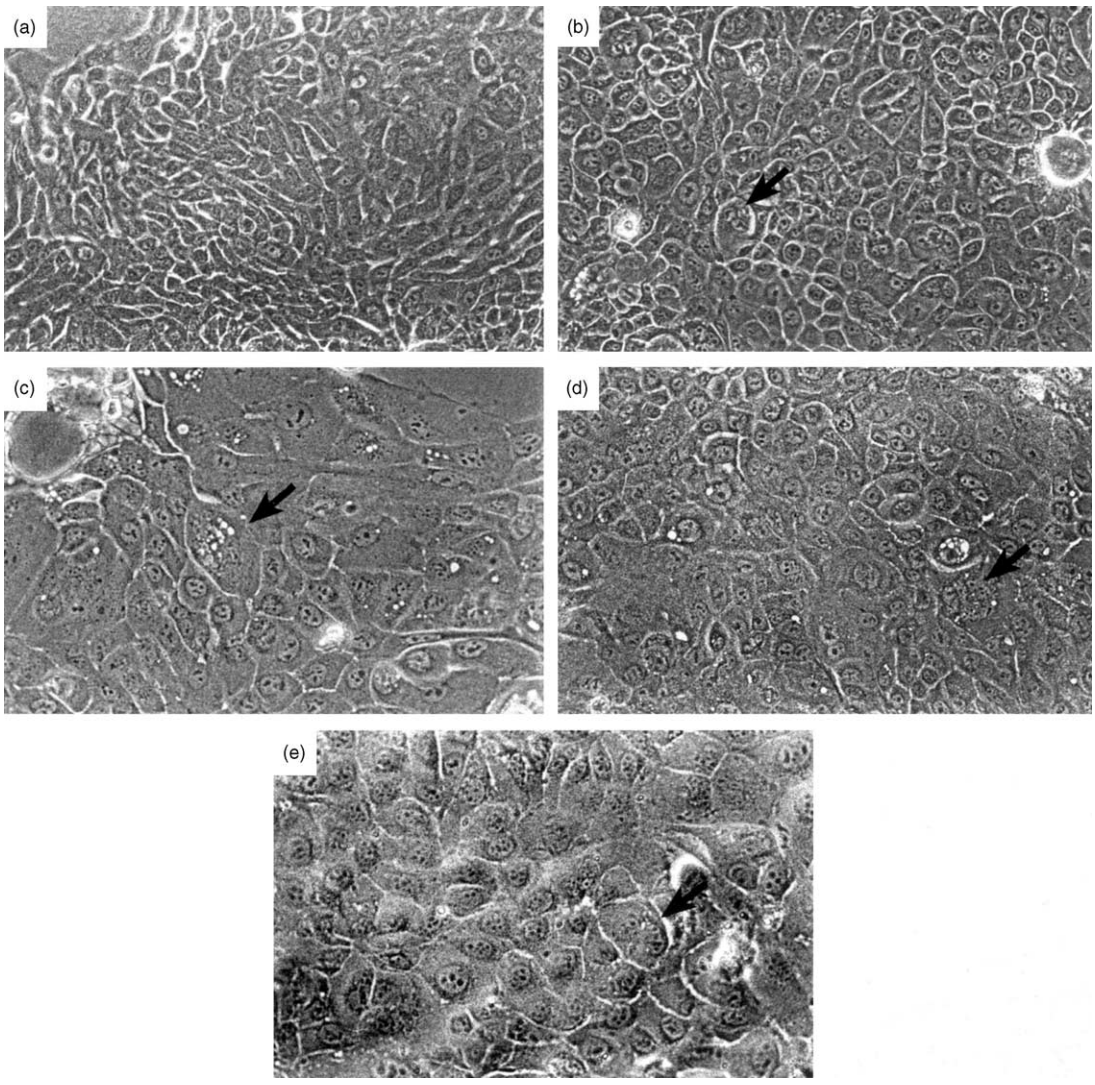

Fig. 1. Phase-contrast photomicrographs of monolayer cultured cells. (a) Primary human tonsillar epithelial (HTE) cells at passage (p) 4, (b) HTE-114/K1 at p29, (c) HTE-114/K2 at p28, (d) HTE-114/K3 at p28, (e) HTE-114/B at p37. Microscopic magnifications are ×200. Arrows in (b)–(e) indicate multi-nuclear or abnormal mitotic cells.

reversed nucleus-to-cytoplasm ratio. The expression of cytokeratins was absent (Fig. 2f).

3.3. Ultrastructure of transfected cells

All HPV-16-transfected HTE cell lines presented numerous desmosomes and microvilli (Fig. 3), which are characteristic components of epithelial cells. No virus-like particles (VLPs) were detectable.

3.4. Colony formation in soft agarose

When HTE-114/K1, HTE-114/K2, HTE-114/K3 and HTE-114/B were cultured in soft agarose for 4 weeks, none of them formed colonies. In contrast, SiHa carcinoma cells grew to large colonies within 9 days.

3.5. Tumorigenicity in nude mice

Tumorigenicity of the transfected HTE cells in nude mice was assayed in parallel with control SiHa and HaCaT cells. During the first month after the injection, we observed the formation of small nodules with a diameter of up to 4 mm in all of the injected animals, with the exception of mice injected with medium alone. The mice injected with HTE-114/K1, HTE-114/K2, HTE-114/K3, HTE-114/B or HaCaT cells did not differ from each other in response to the treatment: after the initial nodule formation during the first month, a gradual decrease occurred in the size and number of nodules, with no nodules larger than 1 mm present 3.5 months after the injection. In SiHa-injected mice, these nodules persisted and started to grow rapidly during the second month after the injection, with the tumour diameter

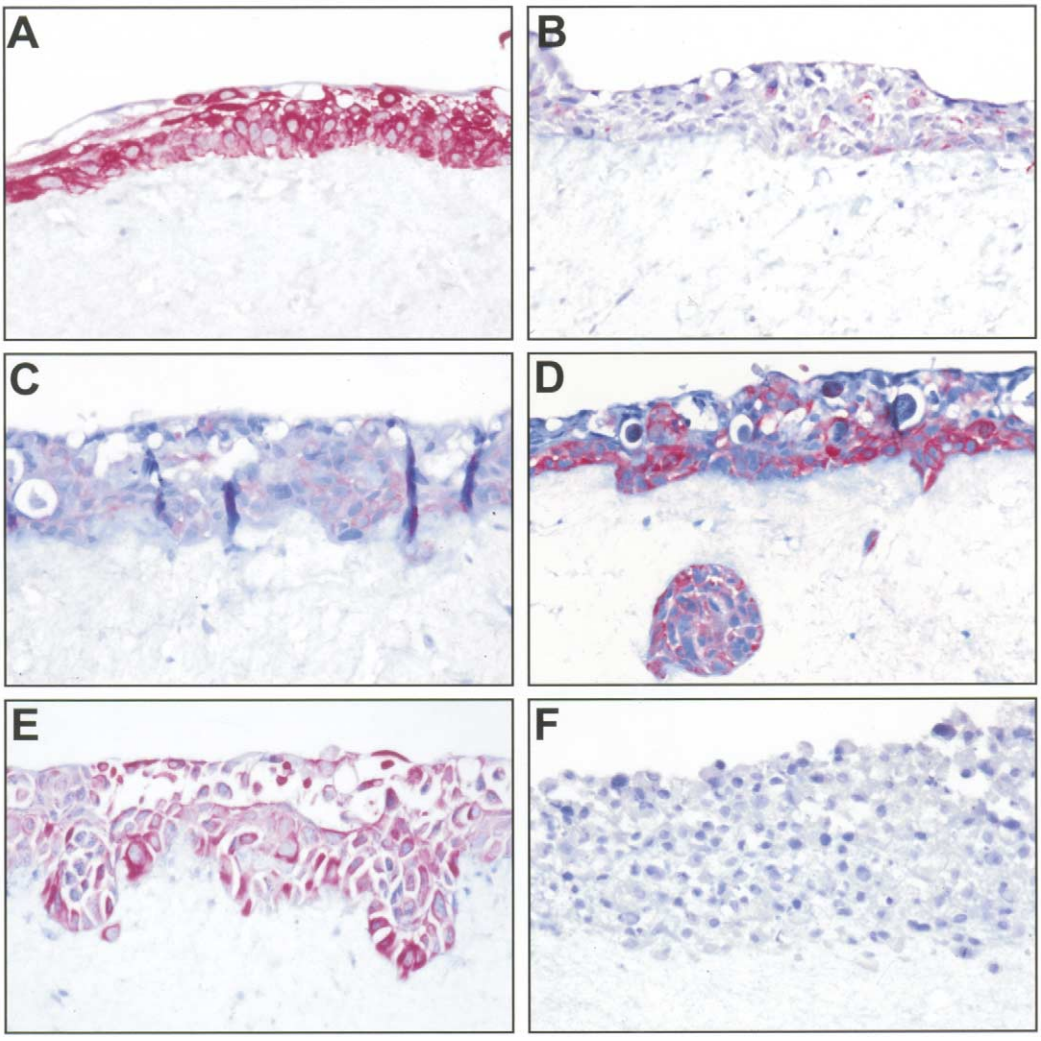

Fig. 2. Immunocytochemical staining of cytokeratins in collagen rafts. Normal human tonsillar epithelial (HTE) cells in organotypic culture had well-differentiated morphology and cytokeratins were expressed strongly throughout all epithelial layers (a). In contrast, HTE-114/K1 (b), HTE-114/K2 (c), HTE-114/K3 (d) and HTE-114/B (e) had considerable variability in size and shape. They showed disturbed cellular polarity, vacuolated cytoplasm, and an increased nucleus-to-cytoplasm ratio. They exhibited impaired expression of cytokeratins. Colonies of HTE-114/K3 cells infiltrated into the collagen matrix (d). SiHa carcinoma cells in the raft formed a non-stratified epithelium. The expression of the cytokeratins was absent (f). Microscopic magnifications are ×200 in (a)–(f).

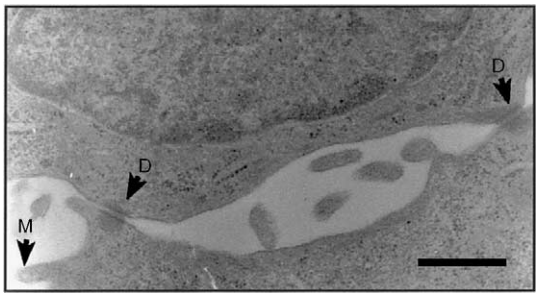

Fig. 3. Ultrastructure of an human tonsillar epithelial (HTE)-114/B in organotypic culture. D, desmosome; M, microvillus. The bar is 500 nm.

reaching 20 mm. SiHa-treated animals were killed due to wasting and poor overall health condition 8 weeks after the injection, and during necropsy the tumour mass was found to range from 0.9 to 1.9 g.

3.6. Cytogenetic aberration

Unbanded metaphase spreads from all HPV-16-transfected HTE cell lines showed chromosome abnormalities, such as chromosomal breakage, chromatid elongation (premature chromosome condensation), dicentric and acentric chromosomes, and numerical chromosomal changes ($n = 80-100$) (Fig. 4).

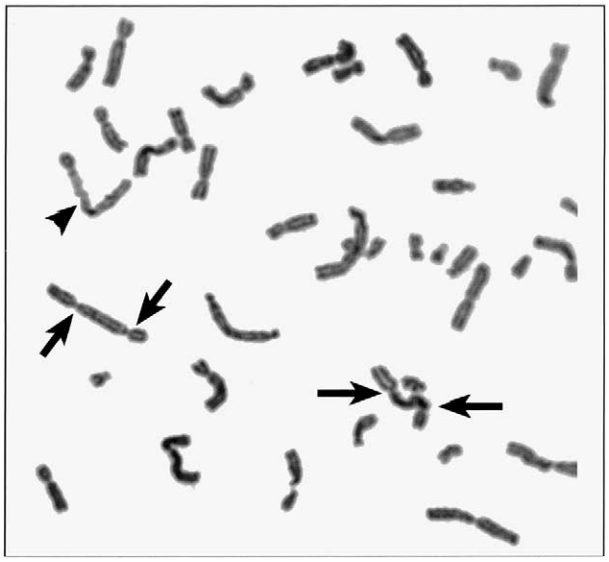

Fig. 4. Unbanded metaphase of an human tonsillar epithelial (HTE)-114/K2 cell. Arrows indicate dicentric chromosomes, and the arrowhead indicates chromatid elongation (premature chromosome condensation).

Our observations are in line with those by others [15,16].

3.7. Presence of viral DNA in transfected cells

Southern blot analysis of undigested DNA of HPV-16-transfected cells demonstrated the presence of high-molecular-weight bands, indicating no episomal viral DNA. *Bam*HI digestion of cellular DNA of all the transfected cell lines yielded fragments larger than 7.9 kb, suggesting that in all cases the viral DNA was integrated into the cellular genome. Digestion of cellular DNA with *Hind*III, which does not cleave the transfecting DNA, resulted in one or two major fragments between 9 and 23 kb in all of the HPV-16-transfected cell lines, indicating that the viral DNA had integrated in monomeric, dimeric or a combined form. However, each cell line showed a distinctive integration pattern (Fig. 5). Digestion with *Pst*I, a multicut enzyme for HPV-16 DNA, resulted in an identical and characteristic pattern [15] in HTE-114/K1, HTE-114/K2, and HTE-114/K2, indicating that no viral rearrangement had occurred; whereas in HTE-114/B, only three fragments were detected, indicating deletion or rearrangement of viral sequences upon integration (data not shown).

3.8. Chromosomal localisation of HPV-16 DNA in transfected cells

FISH assay revealed that viral DNA had integrated exclusively at chromosomal 7q31 in the HTE-114/K1, HTE-114/K2 and HTE-114/K3 cell lines, and at chromosomal 9q34 in the HTE-114/B cell line (Fig. 6).

3.9. Viral gene expression in transfected cells

The viral DNA was transcriptionally active in all of the transfected cells. Northern blot hybridisation with a full-length HPV-16 DNA probe showed the presence of two major (1.7 and 3.7 kb) and one minor (2.4 kb) transcripts (Fig. 7). A similar pattern of northern analysis was observed with a PCR-derived probe corresponding to the E7 region. In contrast, no viral transcriptional signal was detectable when the filter was hybridised with a fragment corresponding to the L1 region.

4. Discussion

Four human tonsillar epithelial cell lines were established by introducing full-length HPV-16 DNA into primary HTE cells. Normal human epithelial cells in culture have a limited lifespan. Spontaneous immortalised cells have rarely been obtained [17,18]. All of our transfected cell lines acquired extended lifespans of more than 80 subcultures, and they may be considered immortalised. The immortalising property has been mapped to the viral oncogenes E6 and E7 [19]. The parental HTE-114/K underwent a 3-week crisis whereas the other transfectant did not. Dexamethasone is known to stimulate glucocorticoid-responsive elements in the upstream regulatory region of HPV-16 [20]. Consequently, it upregulates the transcription of the viral early genes [21] and increases viral DNA replication [22]. Intrinsic and external factors involved in HPV-mediated cell immortalisation have been characterised elsewhere in Ref. [23]. Treatment with dexamethasone in this cell line led to the cells overcoming senescence.

Although the HPV-16-transfected HTE cells acquired immortalisation, they essentially maintained normal growth characteristics, since they required all the supplements in culture medium for growth and propagation that the primary cells did. Unlike SiHa carcinoma cells, they did not form colonies in soft agarose or tumour nodules in nude mice. Thus, they appear to represent premalignant epithelial cells.

Subculturing HPV-transfected HTE cells on plastic dishes revealed no significant changes in cellular morphology. Therefore, we used an organotypic culture system, which reproduces *in vitro* the differentiation patterns of epithelial cells *in vivo*, to search for the differences between normal and HPV-transfected HTE cells. In contrast to the primary HTE cells, all transfected cells exhibited abnormal cytomorphology and impaired cytokeratin expression in the rafts. Possibly, the expression of viral oncogenes E6 and E7 stimulates the proliferation of cells in organotypic culture, and leads to the formation of a disorganised epithelial layer

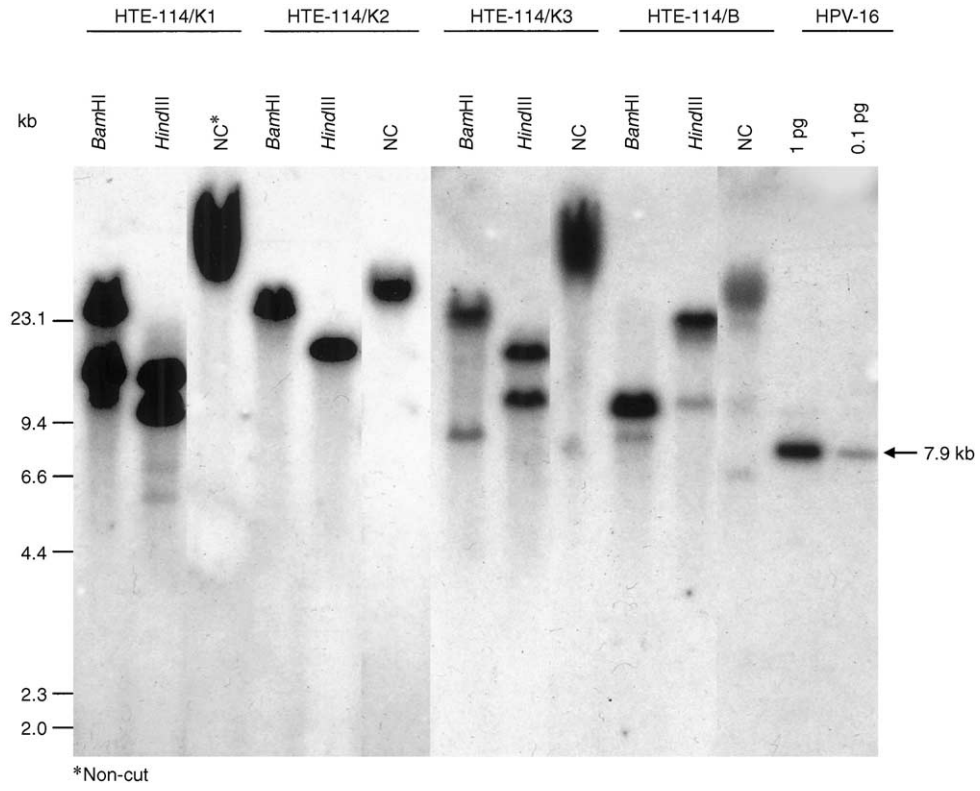

Fig. 5. Southern blot hybridisation analysis of HPV-16-transfected human tonsillar epithelial (HTE) cells. Fragments derived from *Bam*HI digestion in all of the transfected cell lines were larger than 7.9 kb, suggesting that the viral DNA was integrated into the cellular genome. *Hind*III digestion resulted in one or two major fragments between 9 and 23 kb in all of the human papillomavirus epithelial (HPV)-transfected cell lines. This pattern is compatible with monomeric, dimeric or a combined form of integrated viral DNA. All the HPV-transfected cell lines exhibited a single fragment larger than 23 kb with no restriction digestion, indicating no episomal viral DNA. Two lanes on the right indicated 1 and 0.1 pg of HPV-16 genomic DNA, respectively. Numbers on the left indicate the size of molecular weight marker fragments derived from λDNA by a *Hind*III digestion.

[24]. In HPV-associated tonsillar SCC cells, the viral oncoproteins have been found to reduce the expression of pRb and p53, resulting in a non-keratinised morphology in the tumours [25].

VLPs have been identified in HPV-immortalised keratinocytes containing episomal viral DNA, as well as in a cell line derived from a cervical intraepithelial neoplasia type I lesion when the cells were grown in an organotypic culture [26,27]. However, we were unable to demonstrate VLPs in the rafts because these cells contained no episomal viral DNA; the L1 open reading frame, which encodes for the major capsid protein of HPV, had possibly been disrupted upon viral integration [28].

Integration of HPV DNA is considered an important step in tumour progression [29]. There seems to be no specific preferred chromosomal sites for viral DNA integration in HPV-immortalised cell lines [30,31]. Interestingly, we observed that the viral DNA had integrated exclusively at chromosomal 7q31 in HTE-114/K1, HTE-114/K2 and HTE-114/K3, which all originated from the parental HTE-114/K transfectant. The integration event may have occurred in the parental HTE-114/K cells, and subsequently the three clonal cell lines each had possibly a different genomic instability which resulted in distinctive southern patterns. In HTE-114/B, a non-clonal cell line from another transfectant of a different donor, the viral DNA was integrated at a single locus near the telomere of the long arm of chromosome 9. It is unclear whether the host cells, HPV variants, transfection conditions, or any combination of these may favour specific loci for viral integration. The HPV-16 E2 gene is often disrupted upon viral DNA integration *in vivo* [32]. We used a real-time PCR with primers specific for the HPV-16 E2 and E6 regions to quantitate the copy numbers of the E2 and E6 DNA and found that these cell lines contained equal amount of E2 and E6 DNA copies suggesting that the integration site was not within the E2 region. It would be of interest to determine at which region the HPV-16 genome was integrated into cellular genome.

Evidently, the presence of HPV alone does not necessarily result in malignancy. Other co-factors may be necessary for full oncogenic expression, of which fibroblasts are perhaps one of the most important [33,34]. The viral integration loci have often been mapped near proto-oncogenes [35,36]. We found that the viral DNA integrated at chromosomal 7q31 in HTE-114/K1, HTE-114/K2 and HTE-114/K3, which is near the *met* proto-oncogene [37]. The *met* gene is known to encode for the

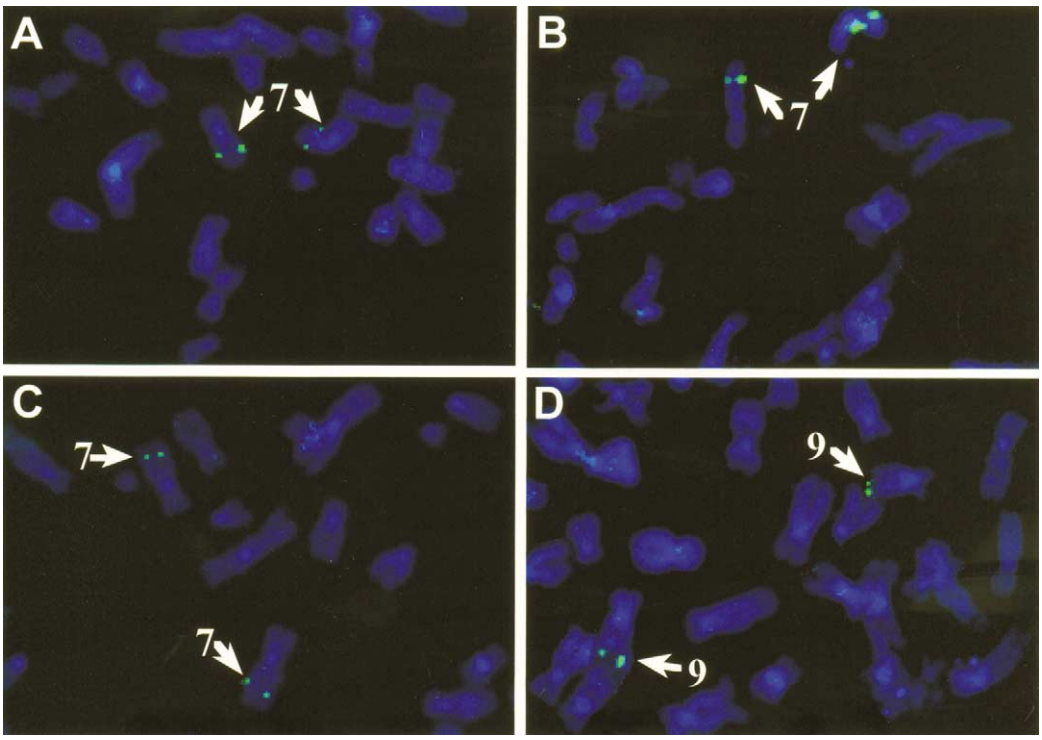

Fig. 6. Chromosomal localisation of human papillomavirus (HPV)-16 DNA in the transfected human tonsillar epithelial (HTE) cells by fluorescence *in situ* hybridisation (FISH). The signals in green are located at chromosomal 7q31 in HTE-114/K1 (a), HTE-114/K2 (b), HTE-114/K3 (c), and at chromosomal 9q34 in HTE-114/B (d). Microscopic magnifications in (a)–(d) are ×1000.

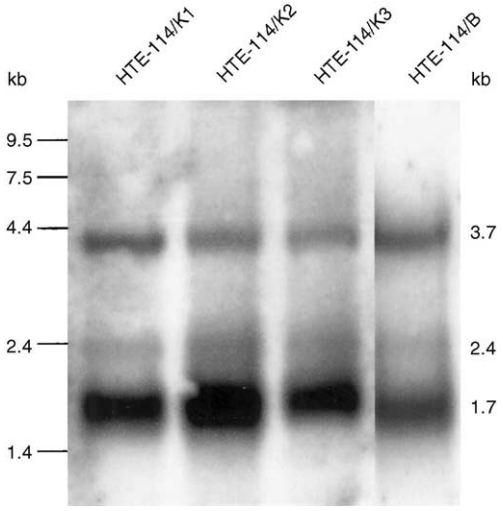

Fig. 7. Northern blot hybridisation analysis of human papillomavirus (HPV)-16-transfected cell lines. All the transfected human tonsillar epithelial (HTE) cell lines presented two major (1.7 and 3.7 kb) and one minor (2.4 kb) transcripts. Numbers on the left are for the RNA ladder (Life Technologies).

hepatocyte growth factor (HGF) receptor, a specific cell-surface tyrosine kinase receptor for HGF [38]. Several growth factors are known to modulate cascades of signalling pathways in epithelial cell lines. Dysregulation of such cascades may lead to cell transformation and tumorigenesis [39–41]. For example, upregulated keratinocyte growth factor (KGF) receptor in HPV-immortalised cells may subsequently result in KGF-induced anchorage-independent growth of the cells [42]. KGF is a member of the fibroblast growth factor family and has unique target-cell specificity restricted to the epithelial cell type [43,44]. Our studies aim to define which factors are involved in the malignant transformation of HPV-16 immortalised tonsillar epithelial cells.

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