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Characterization of human papillomavirus type 16 activity in separate biopsies from a carcinoma of the cervix uteri

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Abstract Human papillomavirus (HPV)16-specific nucleic acid sequences were analysed in separate biopsies taken from a patient with a poorly differentiated squamous cell carcinoma of the uterine cervix. Biopsies were obtained from histopathologically normal epithelium adjacent to the carcinomatous epithelium, the primary carcinoma and a metastatic lymph node. Signals characterizing viral DNA and oncogene transcription were obviously differentiation dependent as shown by in situ hybridization of viral nucleic acids and immunofluorescence of epithelial differentiation specific proteins. In histologically normal parts of the epithelium viral DNA was amplified at the transition from basal to maturing cells, whereas E6/E7 genes were actively transcribed mainly in maturing epithelial cells following the basal cell layer. Some of the cells in the primary carcinoma and in the metastatic lymph node expressed involucrin at increased levels. Signals for viral DNA and HPV16-specific E6/E7 transcripts decreased in intensity during differentiation in an inverse relationship to the observed involucrin increase in those cells. The absence of Ki67 in cells expressing large amounts of involucrin as revealed by immunostaining, support the inverse correlation between differentiation of cancer cells, HPV16 replication and E6/E7 transcription. The changes in cytokine expression may indicate an HPV16 associated disruption of normal cytokine expression pattern in the carcinoma.

Key words Human papillomavirus 16 differentiation dependence · Separate biopsies

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Introduction

Cervical cancer is one of the most common malignant diseases in women worldwide. Human papillomaviruses (HPV) are strictly epitheliotropic viruses and are presumed to be an important risk factor for the development of this type of cancer [15, 27]. The presence of the viral DNA in up to 90% of all anogenital carcinomas and the necessity of expression of viral genes E6 and E7 of the "high risk" HPV types 16/18 for immortalization of cells of epithelial origin in vitro and in vivo has led to the current understanding of the role that HPV play in the pathogenesis of cervical neoplasia [2, 6, 25, 33].

It is evident that HPV DNA is mostly maintained episomally in benign productive infections and in premalignant lesions [8, 20]. In contrast, in high grade dysplasias and invasive cervical cancers viral DNA is characterized as rarely episomal, as coexisting episomal and integrated copies and as predominantly integrated into the host genome [8]. How viral gene expression is deregulated during tumour initiation and progression is uncertain; initiation and maintenance of the transformed phenotype of epithelial cells are presumed to be associated with an efficient expression of E6/E7 open reading frames (ORF) [5, 19, 32].

Histological grading of dysplastic cervical epithelia is based on the proportion of the epithelial thickness occupied by dedifferentiated basal-layer-like cells and on single cell criteria such as nuclear atypia. HPV activity was correlated with morphologically defined changes, characterized by epithelial hyperplasia and cells exhibiting a number of abnormalities, such as bi- and multinucleated cells, dyskeratosis and changes in the nuclear-to-cytoplasmic ratio [12, 16].

In productive infections, amplification of viral genomes takes place in terminally differentiated epithelial cells and early transcripts are expressed before the onset of vegetative DNA replication. They continue to be expressed in increasing amounts in the maturing epithelium [1, 34, 35]. In cervical intraepithelial neoplasia (CIN) replication of HPV is restricted to the superficial cell layer, whereas E6/E7 ORF were found to be expressed in the upper third of the epithelium [4, 7, 10, 35].

A major problem is the correlation of HPV activity with the initiation and progression of cancer in histologically normal epithelia or fully transformed epithelial cells and it is difficult to analyse the dynamics of cervical carcinogenesis in vivo. Previous data were obtained analysing tumour biopsies at different stages of progression from independent patients [11, 13, 34, 35]. In each case, results were representative only for a single point in a multistep process; a chain of events, however, which is greatly influenced in the individual.

We analysed HPV 16-specific nucleic acid sequences in separate biopsies of one patient with a cervical carcinoma. In nonmalignant parts of the epithelium surrounding the tumour ORF E6/E7 were found to be transcribed. HPV replication and transcription in separate biopsies of the same patient were shown to be affected by differentiation. The changes in cytokine expression may indicate HPV16 associated disruption of normal cytokine expression pattern in the carcinoma.

Although the results described in this study do not permit final conclusions, it seems to be relevant to verify subjective histological findings by molecular-biological methods.

Materials and methods

Serial 5 µm cryostat sections were analysed by indirect immunofluorescence. Primary antibodies were rabbit antihuman involucrin and monoclonal antihuman-cytokeratins (K) 10 (LH2) and 14 (LH8), kindly provided by Drs. Fionna Watt (Imperial Cancer Research Fund, London) and Irene Leigh (The Royal London Hospital, London), respectively [22, 28]. Mouse monoclonal antibodies directed against human Ki67 (DAKO), human K13 (Sigma) and epidermal growth factor (EGF)-receptor (Oncogene Science) were assayed according to the manufacturer's instructions. Cryostat sections were incubated with primary antibodies diluted in phosphate buffered saline containing 0,5% Triton X100 for 60 min at room temperature. Secondary fluorescein isothiocyanate-conjugated antimouse and rhodamine anti-rabbit immunoglobulins were used at dilutions recommended by the manufacturer (Sigma). Incubation was performed for 60 min at room temperature. The sections were embedded in Citifluor AF1, and fluorescence photographs were obtained with a Zeiss Aksioskop microscope by using Agfachrome 1000RS films.

For *RNA-RNA in situ hybridization (ISH)* serial cryostat sections mounted on 3-aminopropyl-triethoxysilane-coated slides were fixed in 4% paraformaldehyde in 2x standard sodium phosphate EdTA (SSPE) for 10–15 min at room temperature, digested with proteinase K (5 µg/ml) for 10 min at 37 °C, and hybridized with strand-specific RNA probes spanning the E6–E7 ORF of HPV 16 (nucleotide positions 99–880) [30] or the human cytokeratin 1 gene (provided by F. Bosch and M. Dürst, DKFZ, Heidelberg, Germany) as described [10]. Briefly, radioactively labelled RNA probes were generated from the transcription vector Bluescribe in a solution containing either T3 or T7 RNA polymerase, 100 µCi of α-^{32}P-uridine triphosphate (800 Ci/mmol; Amersham), and 0.25 mM each of the remaining precursors as a cold substrate. This procedure yields RNA with a specific activity of 10^9 cpm/µg. After DNase treatment, probes were subjected to limited alkaline hydrolysis. The sense orientation of each probe served as negative control. Hybridization was performed overnight at 42 °C in a solution containing 50% formamide, 2x SSPE, 10% (w/v) dextran sulphate, 10 mM TRIS (pH 7.5), 1x Denhardt's solution (0,02% each of bovine serum albumin, ficoll, and polyvinylpyrrolidone), 500 µg of tRNA per ml, 100 µg of salmon sperm DNA (sonicated and denatured) per ml, 0.1% (sodium dodecyl sulphate) SDS, and 10^5 cpm of probe per µl. Sections were washed in 50% formamide-2x SSPE-0.1% SDS for 30 min at 50 °C, treated with RNase A (50 µg/ml) for 30 min at 37 °C and subsequently washed in 50% formamide-0.5× standard saline citrate 0.1% SDS for 30 min at the same temperature. Slides were dehydrated in graded alcohols containing 300 mM ammonium acetate, dried, and dipped in Kodak NTB 2 emulsion diluted 1:1 in 600 mM ammonium acetate. After storage for 1–14 days the slides were developed in Kodak D-19 developer, fixed and counterstained with haematoxylin and eosin. The sections were examined and photographed with a Zeiss Aksioskop microscope equipped with rotatable bright-and darkfield condensors to reveal tissue histology and to enable good signal visualization, respectively.

DNA-RNA in situ hybridization was performed with E6/E7 sense probes at conditions modified from RNA-RNA ISH as follows: after proteinase treatment the tissue sections were treated either with RNAse or 0.2 N sodium hydroxide for 30 min at room temperature. The hybridization mixture was added to the tissue sections and incubated for 10 min at 95 °C.

Results

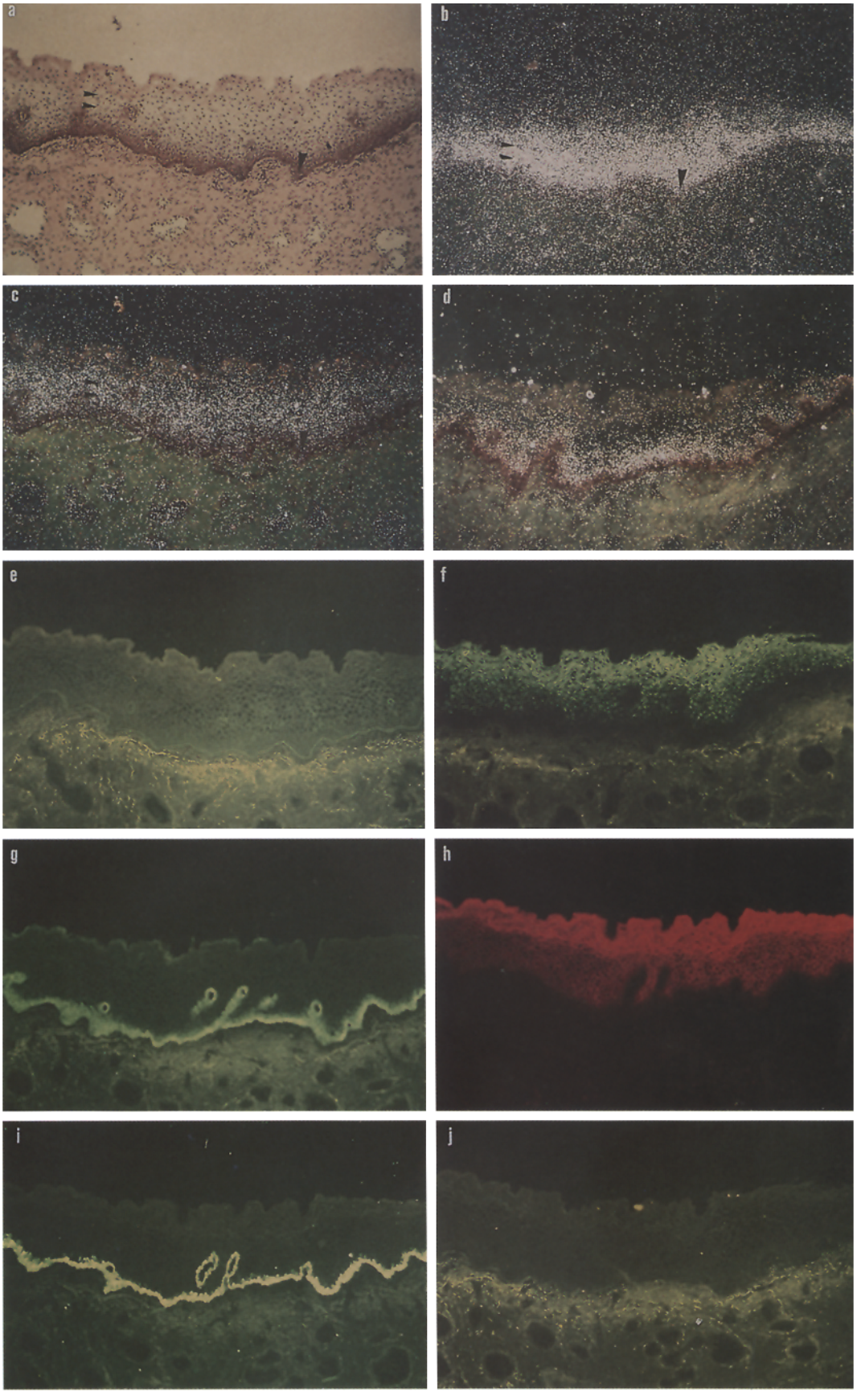
The cervical epithelium consisted of nonkeratinized squamous cells without any dysplastic changes. No koilocytes were observed (Fig. 1a). The tumour was characterized as a poorly differentiated squamous cell carcinoma of the cervix at a stage G3 pT2a pN1 in the TNM system (International Federation of Obstetricians and Gynacologists IIa). Solid nests of invasive squamous carcinoma cells were visible (Fig. 2a). Peripheral cell layers showed a moderate eosinophilic cytoplasm, whereas cells inside the tumour nests contained pleomorphic large vesicular nuclei. Stromal fibroblasts were admixed with lymphocytes and macrophages.

In one lymph node metastases were found (Fig. 3a). Metastatic cell nests therein had pleomorphic nuclei and showed regressive prenecrotic changes of tumour cells.

HPV16 DNA and viral transcripts were analysed by in situ hybridization in cryostat tissue sections (Figs. 1–3). In histopathologically normal epithelium signals for viral DNA were detected at low but significant intensity in the basal cell layer. A marked increase of DNA signals was seen in suprabasal, maturing epithelial cells. In the upper part of the epithelium, consisting of terminally differentiated cells, no further increase of viral DNA was seen (Fig. 1a, b).

HPV 16 E6/E7 gene expression was low and restricted to the suprabasal part of the epithelium (Fig. 1c). In basal layer cells no transcriptional activity was obtained.

Fig. 1a–j In situ hybridization (ISH) and immunofluorescence analysis of tissue sections from histological normal epithelium adjacent to the carcinoma Haematoxylin and eosin (H&E) staining (**a**), darkfield illuminations of HPV16 DNA-RNA ISH (**b**), HPV16 E6/E7 antisense RNA-RNA ISH (**c**), cytokeratin (k) 1 antisense RNA-RNA ISH (**d**), immunostains of K10 (**e**), K13 (**f**), K14 (**g**), involucrin (**h**), Ki67 (**i**), epidermal growth factor (EGF)-receptor (**j**), using tetra methylrhodamine isothiacyanate (TRITC)-conjugated antirabbit (**h**) or fluorescein isothiocyanate (FITC)-conjugated antimouse (**e, f, g, i, j**) secondary antibodies (magnification ×25). △ basal epithelial cells, △△ – maturing epithelial cells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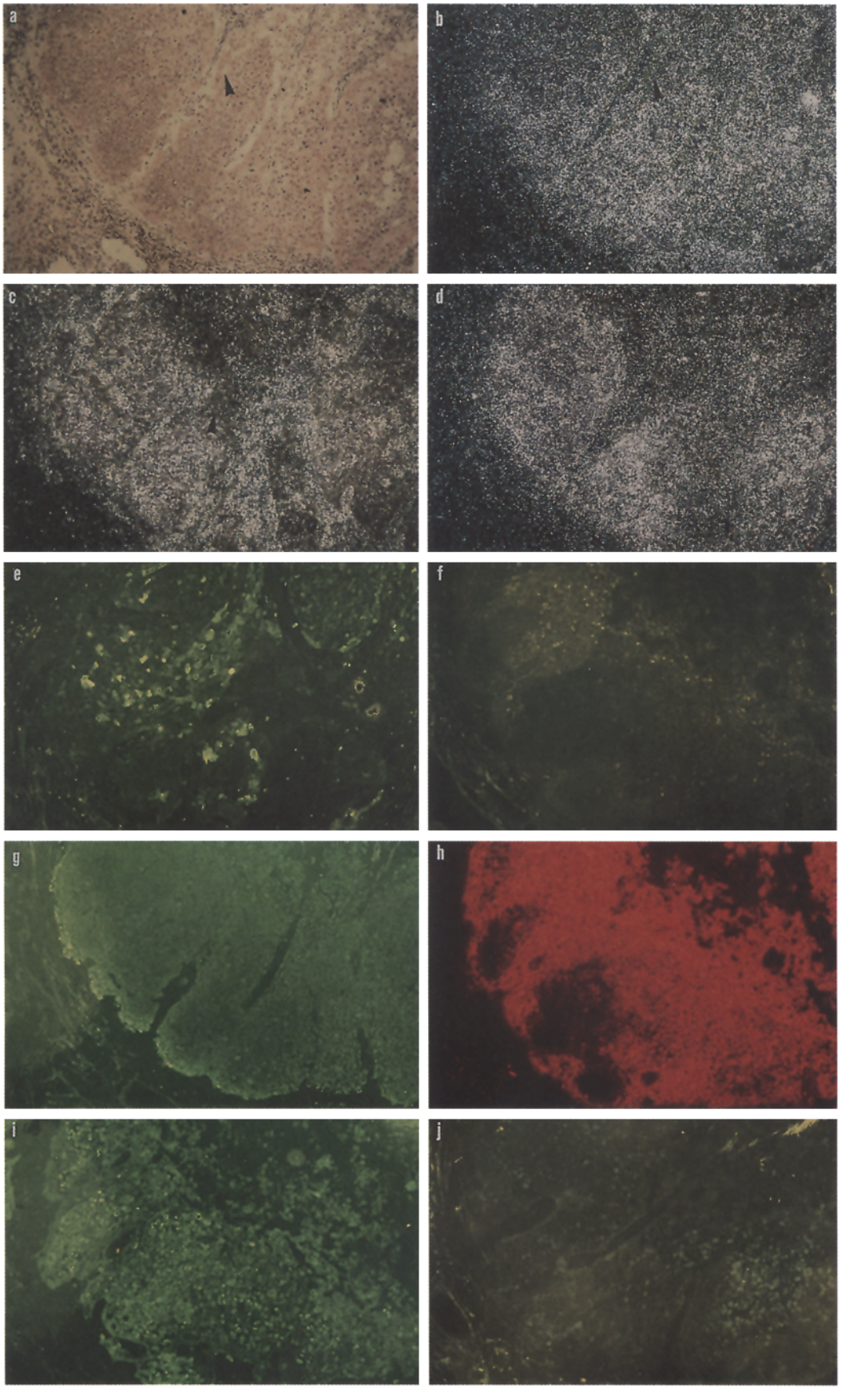
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The primary carcinoma consists of solid nests of invasive squamous carcinoma cells. Dedifferentiated cells inside the tumour, characterized by pleomorphic large vesicular nuclei, were shown to contain the highest level of viral DNA and transcripts of ORF E6 and E7 (Fig. 2a–c). Signals for both viral DNA and RNA transcripts were reduced in peripheral tumour cells with moderately eosinophilic cytoplasma (Fig. 2a–c).

Metastatic cells were detected by in situ hybridization in one lymph node analysed (Fig. 3a–c). In the metastatic lymph node as in the primary carcinoma viral DNA and oncogene transcripts were detected in dedifferentiated squamous carcinoma cells.

Serial sections of all biopsies were hybridized with specific probes to visualize viral DNA and transcripts of viral E6/E7 as well as cytokeratin 1. Immunostaining was performed with antibodies directed against involucrin, Ki67, proliferating cell nuclear antigen, urokinase, EGF-receptor and the K10, 13, 14 (Figs. 1–3). K10 and involucrin are normaly expressed in suprabasal cells, which are commited to terminal differentiation. K13 is found in differentiated squamous epithelial cells. K14 and Ki67 are expressed in proliferative active basal cells of the normal epithelium [38]. K1 mRNA was found to be expressed in the normal epithelium (Fig. 3d), in agreement with previous findings [10]. In only very few epithelial cells was in the maturing part K10 detected (Fig. 3e). K13 was found in differentiated squamous epithelial cells and increasing amounts of involucrin were seen in maturing epithelial cells (Fig. 3f, h). Only basal epithelial cells were positive for K14 and Ki67, indicating their proliferative activity (Fig. 3g, k). EGF-receptor expression was found to be at background levels (Fig. 3l).

In maturing epithelial cells viral DNA and E6/E7 gene expression reached the maximum level. Viral oncogenes were transcribed in differentiating, nonproliferating cells, where both involucrin and K13 were expressed (Fig. 3).

In both the biopsy and the metastatic lymph node viral DNA and E6/E7 gene expression were always shown in cells morphologically classified as poorly differentiated (Figs. 2, 3). K14 was found in most cancer cells at very low levels (Figs. 2g, 3h). At the boundary between tumour and connective tissue, cells were identified which synthesized higher amounts of K14. Ki67 was detected in tumour cells with pleomorphic nuclei, demonstrating their proliferative activity (Figs. 2k, 3l) [9]. It has previously been shown in cell culture that Ki67 is present only in the nuclei of cycling cells [14]. No correlation between Ki67 and K14 immunostaining was observed as found in the normal epithelium. Because of the increase of involucrin expression from central to peripheral cells in the primary carcinoma (Fig. 2h), we assume that cells in the carcinoma and the lymph node are still able to differentiate. Ki67 expression was greatly enhanced in that part of the carcinoma, where involucrin staining was absent. Cells at the periphery of the tumour nests contained reduced amounts of viral DNA and HPV16-specific E6/E7-transcripts. Both invasive carcinoma and lymph node biopsies were negative for K13 (Figs. 2f, 3g). Inside the undifferentiated tumour cell rests, EGF-receptor expression was found in some cells.

Fig. 2a–j ISH and immunofluorescence analysis of tissue sections of the primary carcinoma. H&E-staining (**a**), darkfield illuminations of HPV16 DNA-RNA ISH (**b**), HPV16 E6/E7 antisense RNA-RNA ISH (**c**), K1 antisense RNA-RNA ISH (**d**), immunostains of K10 (**e**), K13 (**f**), K14 (**g**), involucrin (**h**), Ki67 (**i**), EGF-receptor (**j**), using TRITC-conjugated antirabbit (**h**) or FITC-conjugated antimouse (**e, f, g, i, j**) secondary antibodies (magnification ×25). △ cells with eosinophilic cytoplasma

Discussion

We have compared viral DNA and oncogene transcription of HPV16 in separate biopsies of a poorly differentiated squamous cell carcinoma from one patient. The biopsies were from different locations with regard to the tumour, from normal epithelium adjacent to the carcinoma, the primary carcinoma and a metastatic lymph node.

Unlike other comparative studies, these biopsies were obtained from one patient. The immunological and humoral status, which are presumed to influence tumour development [31, 35] were, therefore, identical.

Biological and clinical evidence suggest that carcinoma of the uterine cervix is preceded by a series of precancerous intraepithelial lesions exhibiting varying degrees of morphological alterations, although the process of carcinogenesis may be continuous or discontinuous with respect to the histologically defined cancer stages. It is assumed that HPV activity is subjected to different regulation during the process of epithelial cell transformation of the female genital tract to carcinoma [6, 15, 23, 31]. The correlation between replication and transcription of "high risk" types HPV 16/18 and the normal and abnormal epithelial morphology has not been investigated systematically. Viral DNA in this case was restricted to the upper part of the epithelium, where cells terminally differentiate, whereas the early genes of the virus were transcribed in the maturing epithelium. In differentiating squamous epithelial cells in benign lesions and low grade CIN's viral DNA and eleveated levels of E4 expression were described in the same type of cells [6, 17]. In carcinomas however, viral DNA was found focally [4, 11, 27, 35].

We have demonstrated the topographic distribution of HPV16 DNA and E6/E7 transcription pattern in separate biopsies of a cervical carcinoma by in situ hybridization, in their relationship to the differentiation potential of the epithelial cells. The topography seems to be different with respect to differentiation when compared the pattern of biopsies obtained from various patients at different tumour progression stages.

In normal epithelium, adjacent to the carcinoma, the K1 gene was transcribed in suprabasal epithelial cell layers, typical of differentiation of non-neoplastic squa-

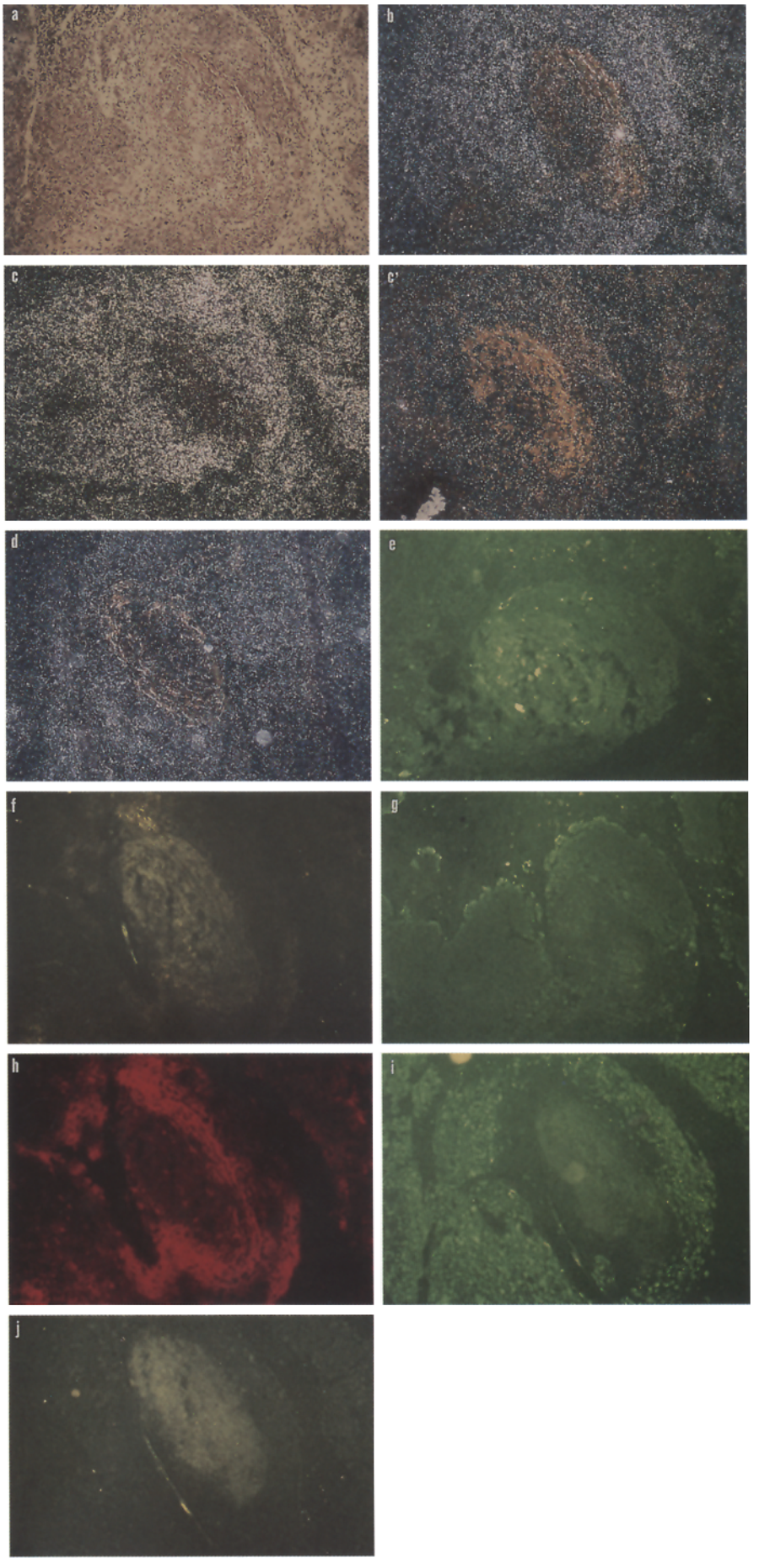

Fig. 3a–j. ISH and immunofluorescence analysis of tissue sections from a metastatic lymph node. H&E-staining (**a**), darkfield illuminations of HPV16 DNA-RNA ISH (**b**), HPV16 E6/E7 antisense RNA-RNA ISH (**c**), HPV E6/E7 sense RNA-RNA ISH (**c'**), K1 antisense RNA-RNA ISH (**d**), immunostains of K10 (**e**), K13 (**f**), K14 (**g**), involucrin (**h**), Ki67 (**i**), EGF-receptor (**j**) using TRITC-conjugated antirabbit (**h**) or FITC-conjugated antimouse (**e, f, g, i, j**) secondary antibodies (magnification ×25)

mous epithelia. Involucrin expression was found at a low level revealing little differentiation in upward layers of the epithelium [37]. K10 and 13 characterize cells of the superficial layer in squamous epithelia [29, 36] whereas only low levels of K10 were observed in our study. In contrast to previous findings, viral DNA was amplified at the transition from the basal cell layer to the upper quarter of the epithelium. In the basal cell layer viral DNA persists at a low copy number, and is transcribed at a comparably low rate [15, 27]. Elevated levels of HPV16 E6/E7-specific transcripts as well as viral DNA amplification were shown to coincide locally, revealing a cooperative effect of replication and E6/E7 transcription. The increase of E6/E7 gene expression might be the consequence of an increased number of viral DNA copies rather than the result of deregulated viral transcription. The upper part of the epithelium was shown to contain much less viral DNA in contrast to the situation in productive infections or benign lesions, where upward amplification of DNA in the epithelium takes place [6, 34]. Though biopsies were morphological nearly normal, we found changes at the molecular level clearly distinguishing them from nonmalignant tissue. The absence of koilocytic cells, dysplasia and nuclear abnormalities in the morphological picture on the one hand and the transcription of viral oncogenes E6/E7 in the same tissues on the other, do not match the scheme of HPV infection [15, 16]. It still remains unclear why there is no vegetative replication in this part of epithelium, but a detectable level of E6/E7 gene Sexpression was found [3, 10, 11, 35]. Cells established from a HPV31b-containing condyloma showed amplified viral DNA in raft cultures only in some differentiated koilocytic cells. Moreover, production of viral structural proteins was evident in these cells [24]. The absence of cytokeratin 10 may also indicate that the epithelial differentiation programme has already undergone some changes. We therefore conclude that during HPV16 associated carcinogenesis in morphological normal epithelia pathological processes take place which are not manifest in an alteration of cellular morphology.

However, the distribution of viral DNA and HPV16-E6/E7 transcripts and K1 in the primary carcinoma and the metastatic lymph node was rather diffuse. Cells appeared morphologically dedifferentiated. However, in eosinophilic cells at the periphery of the carcinoma, involucrin, a marker of epithelial maturation, was detected. In these cells viral transcriptional activity was found to be reduced.

Our results demonstrate the capability of epithelial cells within the invasive part of the carcinoma to express proteins typical for maturing epithelial cells (involucrin) [38]. The absence of K13 immunostaining, reduced levels of K14, but increased K10 expression could be explained by a change in the differentiation programme of HPV-associated carcinoma cells. Differentially disrupted cytokine expression patterns in stratified squamous epithelia associated with HPV have been described for carcinoma derived cell lines in our group [18], as well as for reconstructed epithelia from HPV immortalized cell lines [36]. Expression of EGF-receptors was very low in undifferentiated cells but might be connected to their uncoupled growth behaviour in the carcinoma.

The data described in this paper clearly show differences between separate biopsies obtained from one particular patient, which in part, reflect those described previously comparing independent samples at various progression stages.

Furthermore, our results again support the usefulness of viral nucleic acid analysis in clinical biopsies to verify histological data and to minimize the probability of incorrect or inappropriate diagnosis in this area of pathology [13, 21].

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