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Expression of the Histone H3 Gene in Benign, Semi-malignant and Malignant Lesions of the Head and Neck: a Reliable Proliferation Marker

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To search for a reliable proliferation marker in epithelial head and neck lesions, we have analysed the expression of the histone H3 gene by *in situ* hybridisation and compared this with the immunoreactivity of the widely used monoclonal antibody Ki-67. In many lesions, the Ki-67 staining failed to delineate proliferation. In contrast, the H3 hybridisation signals were in accordance with the histopathology of the biopsies: in hyperplastic epithelia, significant H3 mRNA levels were only seen in areas with inflammation. Dysplastic cells showed distinctly elevated H3 expression. Benign and semi-malignant tumours, i.e. basal cell carcinomas, showed moderate H3 signals at the periphery. In squamous cell carcinomas, H3 expression was always high at the expanding zone of the tumour and was most extensive in undifferentiated carcinomas. Thus, the expression of the histone H3 gene closely reflected the dynamics of neoplastic growth within and around head and neck tumours.
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INTRODUCTION

INCREASED, DEREGULATED proliferation is a prime characteristic of a neoplastic tissue. Tumour diagnosis, staging and grading, and treatment modalities, i.e. surgery, chemotherapy or radiotherapy, are all related to the proliferative index of a tumour.

The determination of the proliferation potential *in situ*, i.e. of individual tumour cells or of a subpopulation of tumour cells, is particularly important in studies aiming at the identification and characterisation of tumour cells possessing increased resistance to therapy and advanced metastatic growth potential as compared to their neighbouring cells. The development of such tumour cell heterogeneity within a primary solid tumour may be regarded as the first step in the progression of the primary tumour to a recurring or metastasising tumour.

In order to characterise tumour cell heterogeneities in different types of primary squamous cell carcinomas and in premalignant lesions of the head and neck, we study gene expression at the cellular level by immunohistochemistry (IHC) and *in situ* hybridisation (ISH). We use frozen sections since both IHC and ISH can be performed with higher sensitivity as compared to paraffin embedded material. This requires molecular markers also for the assessment of proliferation, as counting of mitotic figures is unreliable on frozen sections (and tritiated thymidine or bromodeoxyuridine labelling is excluded).

The monoclonal antibody Ki-67 [1] has found wide application as a marker for the proliferation status of normal and neoplastic cells. Various studies using primary and established haematopoietic cells in culture have shown that Ki-67 recognises a nuclear antigen present in cycling cells but not in resting (G_0) cells [2–9]. However, studies by Van Dierendonck and colleagues have disclosed two major pitfalls of Ki-67, at least in epithelial cell lines: (1) expression of the antigen at the onset of DNA synthesis (early S-phase) may be undetectably low, and (2) cells leaving the cell cycle may retain the antigen for a considerable time period [10, 11]. Furthermore, although part of the gene coding for the antigen recognised by Ki-67 has been isolated and sequenced [3], its identity, biochemical characteristics and mechanism of regulation is still unknown. In human biopsy specimens, Ki-67 has turned out to be a good marker of proliferation in lymphoid tissues derived from tumours [12, 13] and other lymphoid diseases [14]. However, in epithelial tumours as well as in soft tissue lesions [15–17], the staining patterns observed have not been unambiguous. Rather, most published studies report on a very pronounced variation of Ki-67 staining of tumour cells and a lack of correlation of Ki-67 staining with clinical and histopathological criteria of different epithelial tumours [18–27].

PCNA (proliferating cell nuclear antigen), an auxilliary protein to DNA polymerase delta [28], whose expression seems to be restricted to the S-phase of the cell cycle [5], has similar pitfalls. The cell cycle-specific regulation of PCNA has not yet been worked out and the antibody to PCNA has been reported to produce higher background staining than Ki-67 [26].

In contrast, the regulation of expression of the histone H3 gene which, like the other histone genes, is tightly coupled to DNA synthesis, is well characterised. Structural features of its mRNA involved in its regulation have been elucidated in regions both upstream and downstream from the coding sequences (e.g. lack of a poly A-tail). The regulation of histone expression within the cell cycle occurs at both transcriptional and post-transcriptional levels, resulting in low mRNA levels during G_2, M (in this phase mRNA levels should be lowest) and early G_1, and high levels in late G_1 and the S-phase. In resting (G_0) cells no expression should be detectable [29–33].

A heterologous H3 antisense RNA probe derived from a rat

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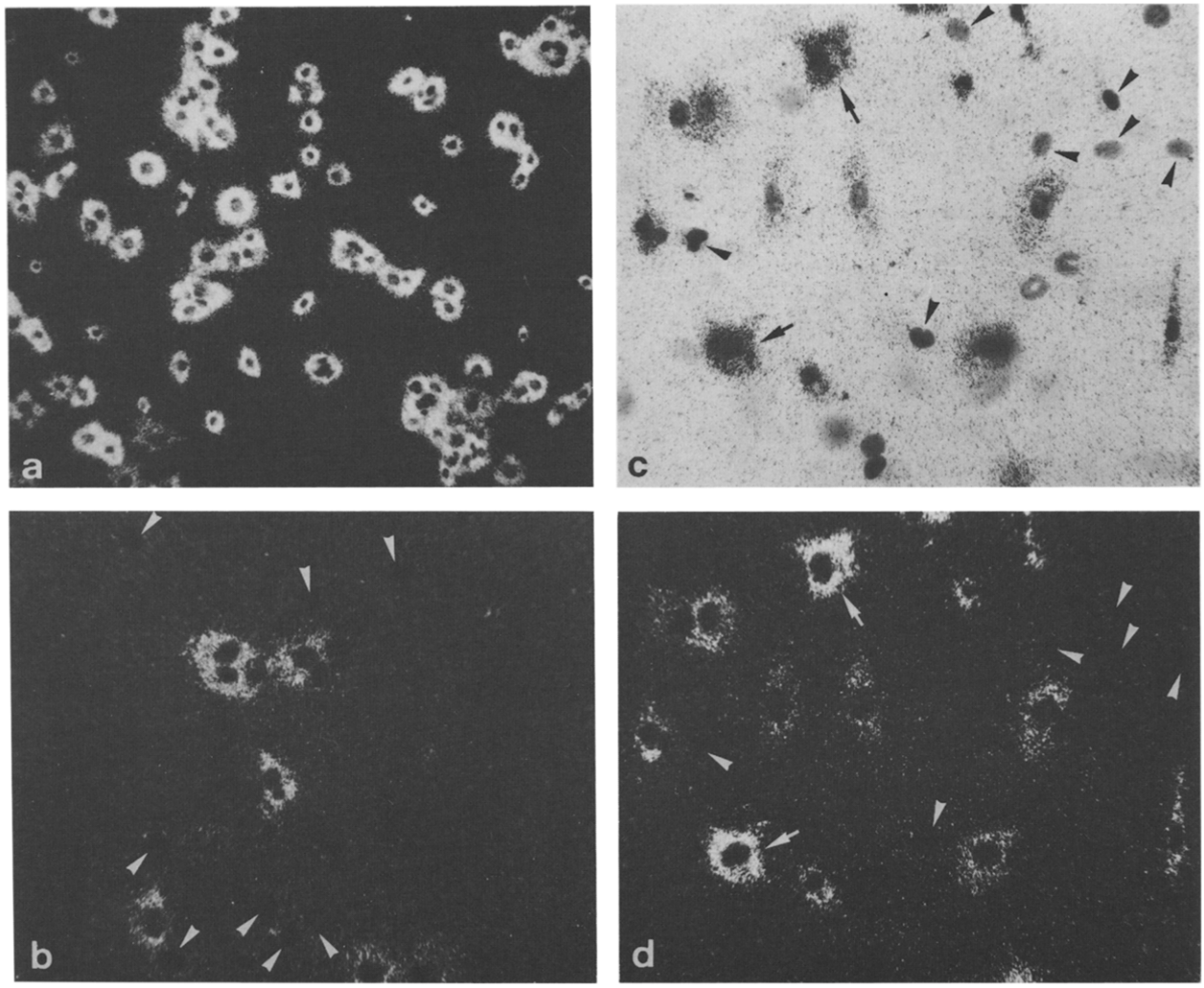

Fig. 1. Histone H3 and β-actin mRNA levels in cultured human glioblastoma cells analysed by *in situ* hybridisation (ISH). The cells were grown on microscopic slides to semiconfluency, washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. Hybridisation to the ^{32}P-labelled probes was for 5 h at 50°C. (a) Homogeneous β-actin mRNA in all cells. (b) Histone H3 mRNA without and (c), (d), with fresh serum stimulation prior to ISH. (d) Dark field photomicrograph to (c). For orientation, two strongly H3 positive cells are marked by arrows, negative or weakly labelled cells in (b), (c), (d) are marked by arrow heads. Note increase of positive cells from (b) to (c), (d). Magnifications: × 380 in (a), × 800 in (b) (c) (d).

H3 cDNA clone has indeed been applied in ISH studies to label proliferating cells in human oral cancer specimens and in experimentally induced tumours in hamster cheek pouch [34, 35]. However, in these studies, neither a direct comparison to other established proliferation markers nor a correlation to established differentiation markers has been included. Also, no ISH to mRNA of intraspecies house keeping genes have been performed as internal controls for the efficacy of ISH.

For the analysis of the differentiation status of the tissues under study, we employ molecular probes specific for individual members of the multigene family of cytokeratins (cks), which in human epithelia comprises at least 20 polypeptides and has proven to provide specific and sensitive markers for the differentiation status of normal as well as neoplastic epithelia [36–38]. Noteworthy in the context of this study is the fact that cells within the suprabasal, differentiated layers of stratified squamous epithelia express cks 4 and 13 in non-keratinised regions and cks 1 and 10/11 in ortho-keratinised regions. The basal keratinocytes express cks 5 and 14 [38, 39]. The immunoreactivity (reflecting antigen expression) or the signals generated from ISH (reflecting expression of the corresponding mRNA) to the corresponding probes is a very useful guideline along which the expression of different markers of proliferation can be assessed.

We have collected a large number of tissue specimens which could be classified as histologically inconspicuous, hyperplastic and dysplastic squamous and respiratory epithelia, benign and malignant primary, secondary and recurrent tumours and lymph nodes containing regional metastases, respectively. After staining many of these tissue specimens with Ki-67, with unsatisfactory results, we have set out, as described in the present communication, to compare in a representative series of these specimens the immunohistochemical staining patterns of Ki-67 with the mRNA expression patterns of histone H3 and have assessed them in correlation to the expression of differentiation related cytokeratins and to the histopathological staging and grading. The results demonstrate that histone H3 but not Ki-67 fulfils the requirements of a reliable proliferation marker in head and neck carcinomas.

MATERIALS AND METHODS

Cultured cells

The cells of a glioblastoma cell line, RO p94, kindly provided by Dr G. Schackert (Institute of Neurosurgery, University of Heidelberg) were propagated in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. They were allowed to attach to microscope slides at semiconfluency by overnight incubation. They were then either processed further or were restimulated to grow by starvation from and readdition of serum, as described [44]. The cells were washed with phosphate buffered saline, fixed in 4% formaldehyde (made from paraformaldehyde) for 15 min at room temperature and hybridised for 5 h at 50°C to ^{32}P-labelled histone H3 and β-actin cRNA probes.

Tissue specimens

Specimens from different types of head and neck tumours were collected in the operation room immediately after operation. All specimens of squamous cell carcinomas showed, at least in some areas, signs of dedifferentiation, histological grading varying between G2 and G4. When large tumour areas were to be resected, hyperplastic and dysplastic squamous and respiratory epithelia in close vicinity to the tumour were also obtained from the operation and they were processed separately.

The tissues were snap-frozen in isopentane/liquid nitrogen and stored at −80°C. Serial cryostat sections (5–6 μm thick) were mounted on 3-aminopropyl-triethoxysilane-coated slides.

Immunohistochemical staining

The frozen sections were fixed for 10 min in acetone at −20°C and air dried. Primary monoclonal antibodies were applied to the sections in phosphate buffered saline (pH 7.4). Antibody staining, performed on at least two sections per specimen, was detected by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method, using either the protocol provided by Dakopatts (Kopenhagen, Denmark) or a modified, shortened protocol ("SIMIT"-APAAP) developed by Progen Biotechnics (Heidelberg, Germany).

Antibodies

The murine monoclonal antibody (Mab) Ki-67, staining a nuclear antigen expressed in proliferating cells [1, 3], was obtained from Dianova (Hamburg, F.R.G.). Some cks-specific Mabs were used for comparison. These included K_s 8.60 reacting

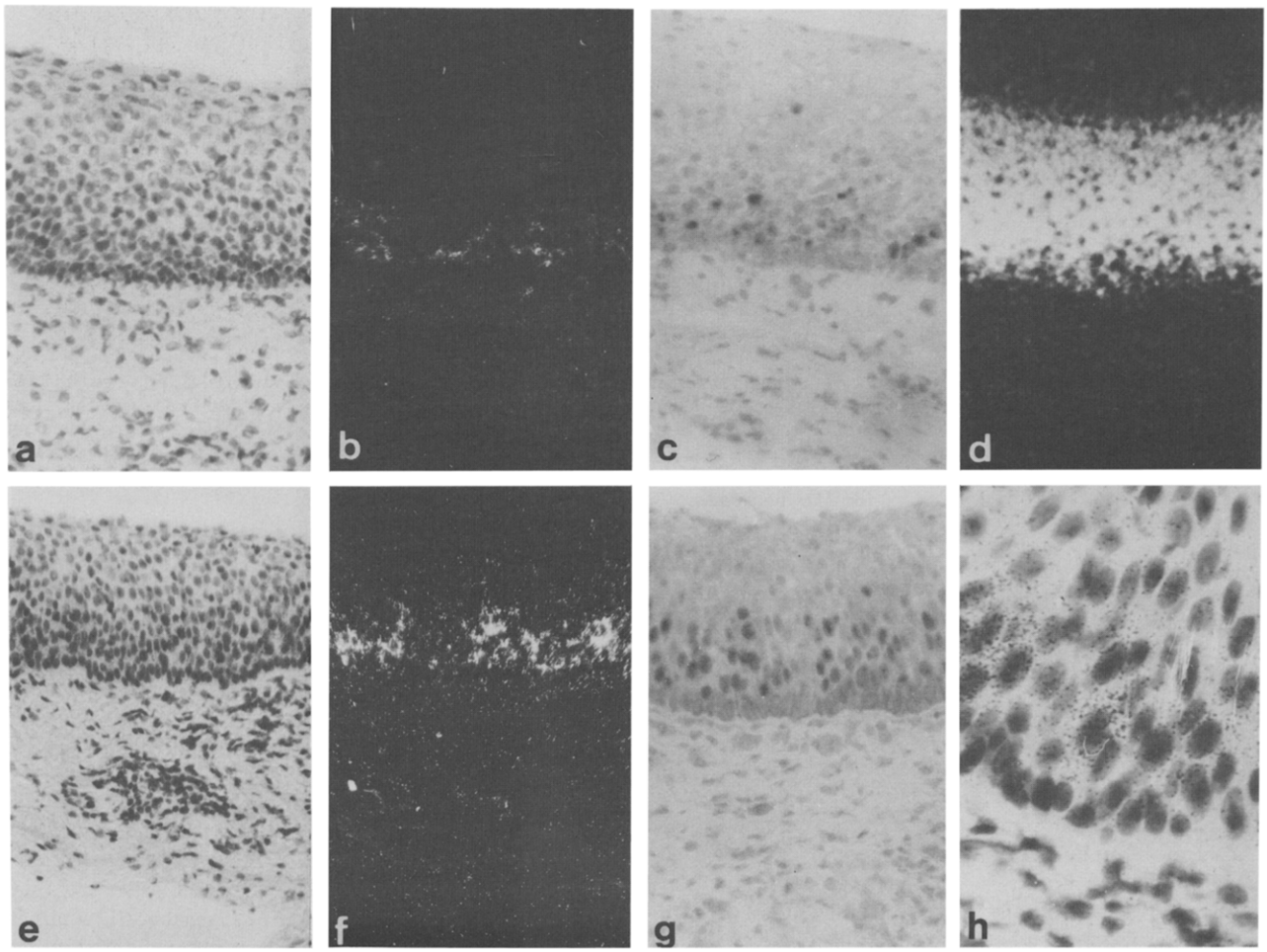

Fig. 2. Comparison of histone H3 mRNA expression (a, e, h: bright field; b, f: dark field illumination) with Ki-67 staining (c, g) in hyperplastic squamous epithelium of laryngeal ventricle. The upper row shows a region with little inflammation; the lower row (from the same tissue sections) shows a region with visible inflammation of the underlying connective tissue. There is little H3 and Ki-67 expression in the hyperplastic region (a–c), but significantly elevated expression in the inflamed region (e–h). H3 and Ki-67 (compare a, c with e, g) are concordant except that some Ki-67 immunoreactive cells are located in suprabasal layers. For comparison, expression of cytokeratin 4 is shown in (d). (h) High magnification of part of (e) showing H3 mRNA in parabasal cells rather than in the basalmost cells. Magnification: × 300 in (a–g), × 800 in (h).

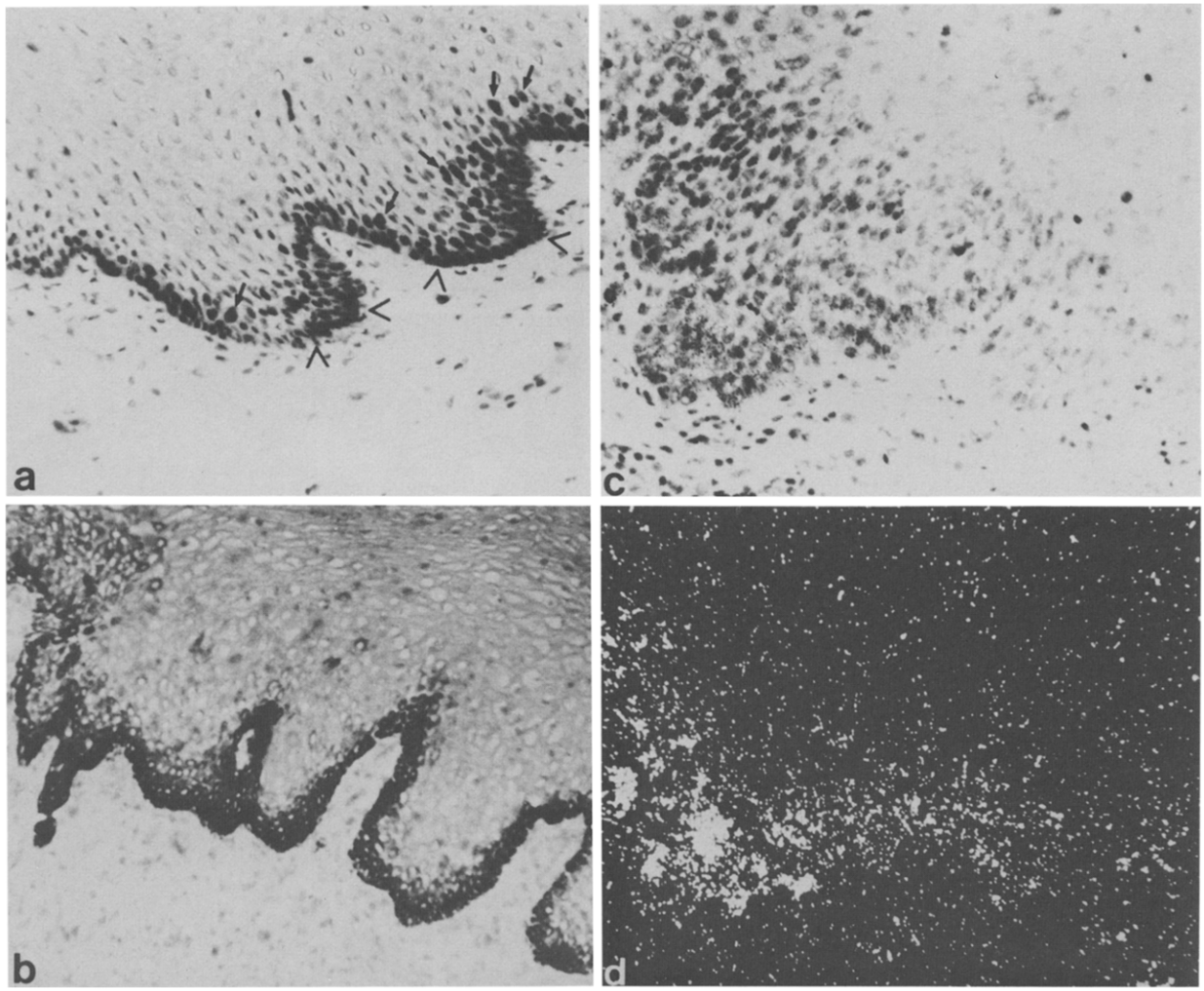

Fig. 3. Comparison of Ki-67 and H3 expression in hyperplasia vs. dysplasia. (a) Homogeneous but mostly cytoplasmic Ki-67 staining of the basal layer (arrow heads), plus nuclear staining of parabasal cells in a hyperplastic squamous epithelium from the base of tongue (arrows). (b) Hyperplastic region (at the right) and dysplastic focus (at the left) from the floor of the mouth. There is homogeneous, mostly cytoplasmic staining of the basal layer, without discrimination between hyperplasia and dysplasia. (c) Bright field and (d) dark field photographs of H3 mRNA in another region of transition from hyperplasia to dysplasia from the same tissue block. Hyperplasia with very little H3 expression is seen at the right, and moderate and then severe dysplasia with strongly increasing H3 expression is visible at the left.

with cks 10/11; 6B10 and 1C7, specific for cks 4 and 13, respectively (for references and sources [45]).

In situ *hybridisation*

The procedure, based on the studies of Cox *et al.* [46], was carried out essentially as described previously [41, 45] with some minor modifications. For instance, both ^{32}P-labelled as well as ^{35}S-labelled cRNA probes were employed. In the latter cases, the hybridisation solution contained 50 mmol/l DTT (dithiothreitol) and the sections were covered with parafilm in order to avoid evaporation of DTT. The probes were prepared by incorporation of either alpha-^{32}P or ^{35}S-CTP or UTP into transcripts synthesised *in vitro* from cDNA cloned into Bluescribe or Bluescript vectors (Stratagene). Hybridisations were also carried out on sections which, after paraformaldehyde fixation and washing, had been refrozen at −80°C. Here, the concentration of the proteinase K was lowered from 0.5 to 0.2 μg/ml. The concentration range of RNase A which is used after hybridisation to efficiently remove unbound probes from the sections was lower than described previously [41, 45], being 1–5 μg/ml for ^{32}P-labelled probes and 10 μg/ml for ^{35}S-labelled probes. At least two sections were hybridised with each probe. The Bluescript-based human histone H3 cDNA, containing a 330 base pair insert, was the kind gift of Drs H. Smola and N.E. Fusenig (German Cancer Research Center, Heidelberg, F.R.G.). The cDNA probes used to analyse mRNA expression patterns of stratification related cks, were kind gifts of Drs B. Bader, R. Leube and W. W. Franke, also of the German Cancer Center.

RESULTS

Histone H3 mRNA expression in cell culture

A non-synchronised human glioblastoma cell line, RO p94, was used to test whether *in situ* hybridisation with the histone H3 cRNA probe yielded the expected signals over a certain fraction of these cells [40], in comparison to an expected homogeneous hybridisation signal generated by a probe specific for the cytoskeletal gene β-actin. After a 1-day growth period, which in some cases was followed by serum restimulation, the cells were washed with phosphate buffered saline and subjected to ISH with ^{32}P-labelled human histone H3 and β-actin probes as described [41, 42]. As outlined in the Introduction, the

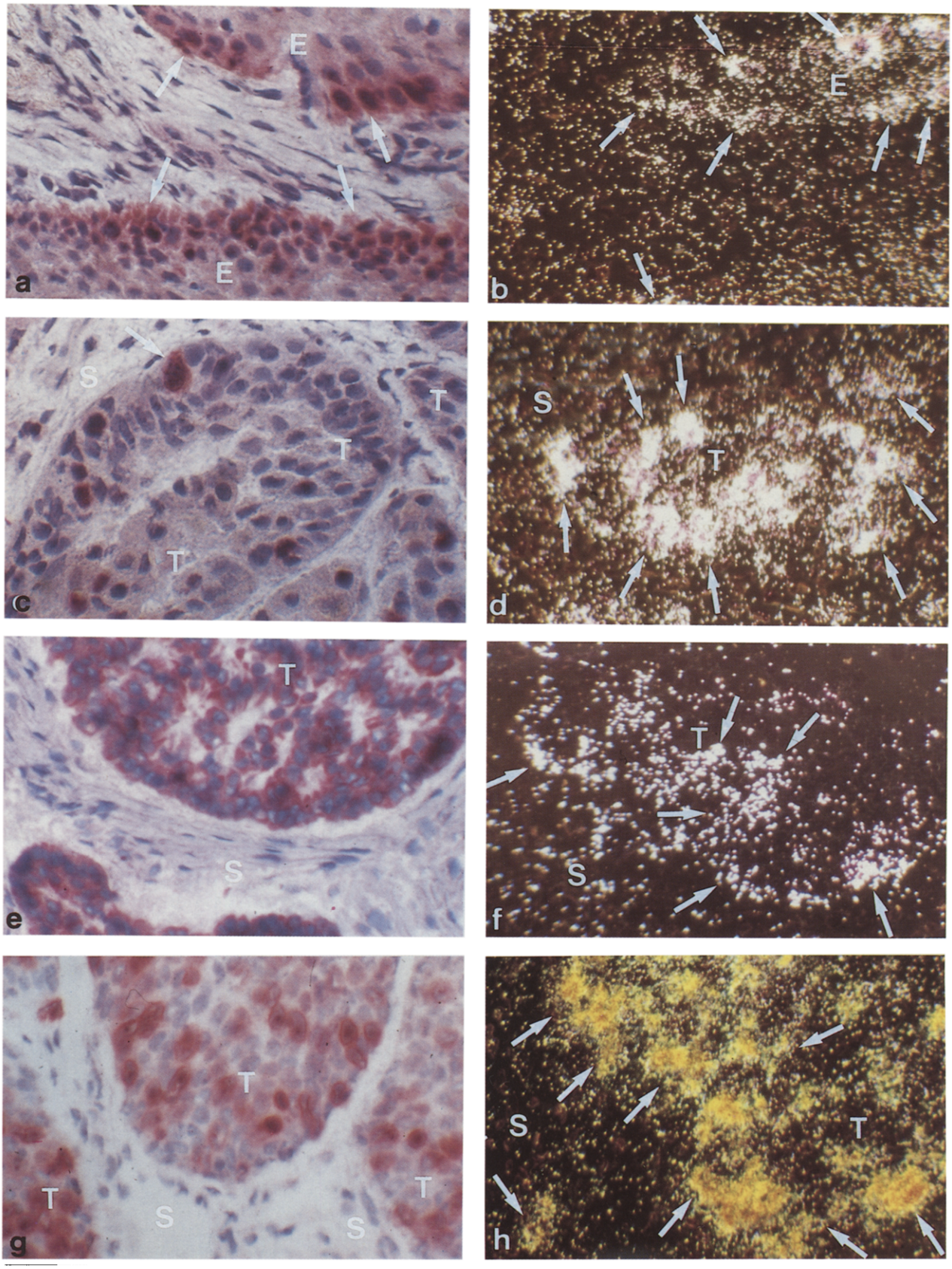

Fig. 4. Comparison of Ki-67 and H3 mRNA expression in akanthotic epithelium vs. invasive, poorly differentiated squamous cell carcinoma, in semi-malignant basal cell carcinoma, and in anaplastic squamous cell carcinoma with small cell morphology—colour illustration. The left column shows Ki-67 staining, the right column shows dark field photomicrographs from ISH with the ^{35}S-labelled H3-probe. (a), (b) Akanthotic epithelium. Note confinement of H3 expression to a few basal cells but homogeneous basal staining with Ki-67 (arrows). (c), (d) Adjacent invasive tumour nodules on the same section. Very few tumour cells are nuclearly stained with Ki-67; many tumour cells are labelled with H3 (see arrows in c, d). (e), (f) Semi-malignant basal cell carcinoma. Uniform, perinuclear Ki-67 staining (e) but clustered H3 mRNA expression in some peripheral tumour cells (f, arrows). (g), (h) Undifferentiated squamous cell carcinoma, $T_4N_{2b}M_1/G_4$. Strong, but dispersed nuclear Ki-67 staining (g) in contrast to extremely strong H3 expression in all peripheral tumour cells, in many central tumour cells and in individual tumour cells in the stroma (h, see arrows). E: epithelium, T: tumour, S: stroma. Magnification: × 800.

histone H3 mRNA should display a very heterogeneous pattern of expression, depending on the cell cycle status of the cells, whereas the mRNA of the β-actin gene should be expressed constitutively in all cells in a cell cycle-independent manner. Fig. 1 confirmed these differential expression patterns. As visualised by dark field microscopy, the hybridisation signals specific for the β-actin mRNA were fairly homogeneous in intensity and all cells were labelled (Fig. 1a), whereas a strong H3 hybridisation signal could be seen only in a subpopulation of cells (approximately 25%), presumably those in late G_1 and S-phase (Fig. 1b). Other cells (indicated by arrow heads) showed a much lower but detectable accumulation of silver grains over their cytoplasm, consistent with their being in other phases of the cell cycle. The percentage of cells with strong H3 expression could be significantly increased to about 40–50% by addition of fresh serum prior to ISH, as demonstrated in Fig. 1c (bright field) and Fig. 1d (dark field photomicrograph to Fig. 1c), consistent with serum stimulation of growth.

Comparison of histone H3 mRNA expression patterns with Ki-67 immunostaining patterns

Non-tumorous squamous epithelia. We examined frozen sections of approximately 25 different non-tumorous, squamous or respiratory epithelia obtained from the vicinity of surgically removed head and neck tumours. As a typical example, Fig. 2 shows staining of a hyperplastic, non-keratinising epithelium of the laryngeal ventricle in the vicinity of a squamous cell carcinoma of the larynx/hypopharynx. Both histone H3-ISH as well as Ki-67-IHC yielded similar signals in cell layers close to the basal layer (the parabasal compartment), varying from very little to significant (compare Fig. 2b, c with f, g). This difference in the proliferative activity might be due to the presence of increased inflammation in the underlying connective tissue in the region shown in Fig. 2e–h. In these areas, the expression of H3 as well as of the Ki-67 antigen was elevated (Fig. 2e–g). Such variation was not observed when expression of cks 4 (Fig. 2d) and 13 (not shown), which are typical suprabasal components of this type of epithelium, was analysed. It was interesting to note that the cells positive for Ki-67 and H3 were mainly located in the parabasal compartment, as shown in Fig. 2c, g (Ki-67) and h (H3, bright field high magnification photomicrograph), suggestive of a reserve cell character of the basal-most cell layer. Although a few cells in suprabasal layers were still positive for Ki-67 but not for H3 (compare Fig. 2c, g with f, g; this phenomenon due to the longer half life of the Ki-67 antigen), in the majority of non-tumorous epithelia the H3 and Ki-67 expression patterns were concordant. It may also be mentioned that in uninvolved lymph nodes which were occasionally included in tumour resections, the H3 probe reproducibly hybridised to the germinal (i.e. proliferative) centres of these lymph nodes. These also reacted positively with the Ki-67 antibody (data not shown).

Hyperplasia vs. dysplasia (grade II). In some biopsy specimens from the oral cavity displaying hyperplasia, the Ki-67 antibody yielded a mixture of nuclear staining of individual parabasal cells and a homogeneous, but mostly cytoplasmic staining of the basal layer (Fig. 3a, see arrows and arrow heads, respectively). In most hyperplastic specimens containing dysplastic regions (n = 10), there was a homogeneous, but mostly cytoplasmic staining of the basal layer both in the hyperplastic areas as well as in the dysplastic regions (Fig. 3b, the dysplasia being at the left margin). Since the Ki-67 antigen is generally accepted to be nuclearly localised, the authenticity of these staining patterns is difficult to evaluate. Indeed, the presence of proliferation in the regions showing cytoplasmic staining with Ki-67 was not confirmed by ISH with the H3 probe. On the contrary, as shown in Fig. 3c (bright field) and Fig. 3d (dark field photomicrographs), the number of H3 mRNA-expressing cells in the area of moderate hyperplasia was low (see right margin of Fig. 3c) but increased strongly a small distance further along the same section where the hyperplasia turned into moderate and severe dysplasia (grade II dysplasia at left margin of Fig. 3c, d).

Akanthotic squamous epithelium vs. areas of invasive tumour (T4N3Mx; G3 grade). In four different tumour specimens which displayed akanthotic/dysplastic squamous epithelium and poorly differentiated or undifferentiated tumour nodules close by, the failure of Ki-67 to correlate with these features was clearly evident. In Fig. 4a–d, the Ki-67 staining in akanthotic squamous epithelium (Fig. 4a) is compared with that in invasive areas with poorly differentiated tumour cells (Fig. 4c) and is contrasted to the H3 mRNA expression patterns. A largely homogeneous, nuclear as well as cytoplasmic basal Ki-67 staining demarcated the akanthotic epithelium, whereas in the small invasive tumour islands only very few cells were stained, the staining pattern being of the expected nuclear nature (see arrows in Fig. 4a and c, respectively). ISH with the H3 probe resulted in the opposite labelling pattern, as demonstrated in Fig. 4b and d by dark field illumination. It can be seen that only individual basal keratinocytes in the akanthotic epithelium (arrows in Fig. 4b) showed distinct labelling, whereas an invasive tumour island of poorly differentiated cells showed very extensive H3 expression indicating aggressive proliferation (Fig. 4d, arrows). It must be stressed that these differential expression patterns shown were in both cases derived from the same tissue section and similar regions from a consecutive series of sections were compared.

Semi-malignant basal cell carcinoma vs. highly malignant, undifferentiated squamous cell carcinoma (T4N2bM1; G4 grade). A positive immunoreactivity to the Ki-67 antibody in a basal cell carcinoma tumour specimen was observed in virtually all tumour cells. Again, however, this staining pattern was mostly perinuclear and/or cytoplasmatic (Fig. 4e) and, therefore, difficult to assess. In contrast, the results of ISH with the histone H3 probe revealed that in large tumour islands, only a small fraction of evenly distributed tumour cells was moderately labelled. Clusters of tumour cells expressing significant H3 mRNA levels were to be seen only in small tumour islands expanding into the connective tissue (Fig. 4f; the arrows demarcate the tumour front).

The comparison presented in Fig. 4g and h revealed another deficiency of Ki-67 in the head and neck tissues under study, i.e. its failure to specifically demarcate the expanding zones of tumours or premalignant lesions. Here we have examined the case of a highly aggressive, undifferentiated squamous cell carcinoma of the hypopharynx displaying the morphology of a small cell carcinoma. In Fig. 4g, the Ki-67 staining of this tumour is contrasted to the histone H3 mRNA expression pattern (Fig. 4h, dark field photomicrograph). The Ki-67 positive tumour cells are relatively dispersed. However, the periphery of the tumour islands is not demarcated by an accumulation of Ki-67 positive cells. In contrast, the periphery of such tumour islands is very strongly and almost homogeneously labelled after ISH with the H3 probe. Clearly, many centrally located tumour cells also proliferate and even individual invasive tumour cells

can be seen in the stroma (at the bottom left in Fig. 4h, see arrows).

DISCUSSION

We have compared the expression of the histone H3 gene analysed at the mRNA level with the expression of the Ki-67 antigen as detected by immunohistochemical staining in hyperplastic and neoplastic human epithelial tissues from the head and neck region. This comparison has been performed because Ki-67, whose high value as proliferation marker in other cell types, in particular in haematopoietic cells is undisputed, in many cases gave results which were in conflict with histological and histopathological assessments. The data presented show that Ki-67 failed to reliably measure proliferation in the neoplastic epithelial tissues analysed. Only in sections lacking neoplastic features, i.e. in hyperplastic epithelia, including areas of inflammation, were the Ki-67 staining patterns accordant with histological criteria, at least with some consistency. In these sections both Ki-67 as well as the histone H3 cRNA probe labelled similarly low numbers of cells in the basal and parabasal cell layers (Fig. 2). Therefore, when assessing tumour biopsies, in should be kept in mind that inflammation of the connective tissue may add significantly to the proliferation index of the epithelial lesion.

In hyperplastic epithelia containing dysplastic areas, the Ki-67 antibody staining failed to correlate reliably with proliferation as assessed by other criteria and often differed drastically from the histone H3 labelling patterns. In addition, the Ki-67 positive reactivity frequently involved cytoplasmic staining, making interpretation difficult. In line with the findings of Van Dierendonck *et al.* [10, 11], one could speculate that in these cells, the down-regulation of expression and degradation of the Ki-67 antigen is very slow and immunoreactive degradation products appear in the cytoplasm, yielding the observed Ki-67 staining. In contrast, the number of H3-expressing cells was low in hyperplasia without dysplasia, but increased strongly in the epithelial tips of dysplastic foci (Fig. 3). Whether this surprisingly low H3 mRNA expression in hyperplastic regions devoid of visible dysplastic features (Fig. 3) is a diagnostic finding awaits the analysis of a larger number of hyperplasias with and without dysplastic foci.

As exemplified in Fig. 4a–d, the Ki-67 staining was often inverse to the H3 mRNA expression patterns. There was too much and partially cytoplasmic Ki-67 staining in akanthotic squamous epithelium, too little in the invasive tumour.

Not considering the cytoplasmic staining, the uniform Ki-67 staining pattern of the basal cell carcinoma (Fig. 4e, f) which appeared to consist of a single cell type, could be regarded as being compatible with the concept that all tumour cells were cycling. However, it is incompatible with the slow growth behaviour of this tumour. The slow growth of this tumour was reflected much better by the observed H3 mRNA labelling pattern which was low in the majority of tumour cells and relatively high only in small ("young") tumour islands at the tumour front.

The lack of a specific demarcation of the expanding zones of various types of head and neck tumours by Ki-67 was another observation in this study. Although in some biopsies of squamous cell carcinomas with regions of moderate to well differentiated morphology and significant keratinisation, the Ki-67 staining was nuclear and confined to peripheral tumour cells (data not shown), in many others a growth cone with highest proliferative potential was not evident from the Ki-67 staining pattern. In contrast, the H3 mRNA invariably showed a topographical distribution consistent with increased proliferation in the labelled region (e.g. see Fig. 4g, h). Again, the H3 expression pattern demarcated the expanding zone and reflected the aggressive nature of this tumour much better than the Ki-67 staining.

The two peculiar features of Ki-67 already mentioned, i.e. the very low level of expression at entry into S-phase and the persistence of immunoreactive antigen when the cells leave the cell cycle [10, 11], may be relevant to the observed unreliable Ki-67 immunoreactivity. We, therefore, propose that the assessment of proliferation in epithelial diseases should not be addressed by Ki-67 immunohistochemistry alone.

A realistic measure of the proliferation status of the head and neck tumours was provided by the analysis of the histone H3 gene expression. Both tumour stage and differentiation grade were closely reflected by the expression profile of the histone H3 gene. This conclusion was further substantiated by the analysis of the differentiation status. The expression of the keratinisation-related cks showed an inverse relationship to the expression of histone H3 (Fig. 2 and unpublished data).

Our choice of employing ISH to histone H3 mRNA as a reliable proliferation marker was based on the fact that H3, like the other histone genes, is actively expressed only in cycling cells, its mRNA synthesis is completely shut down when cells leave the cell cycle (while the protein will persist as is probably also the case with the Ki-67 antigen) and some of the mechanisms governing regulation of H3 mRNA expression within the cell cycle have been elucidated [33]. Besides the regulation occurring at the transcriptional level, the very selective modulation of the stability of the H3 mRNA adds substantially to the efficacy of regulation of H3 gene expression [43]. Consequently, analysing mRNA levels rather than antigen levels (a suitable antibody or antiserum for H3 is not available) should, in fact, reflect much closer the dynamics of growth. In all specimens analysed, starting from the arbitrarily chosen tumour cell line to the most aggressive carcinomas, the dynamics of cell proliferation were apparently correctly reflected by the gene expression profile of histone H3. In several instances the ISH results with the H3 probe actually assisted in identifying small areas of increased growth (Figs 2, 3 and unpublished observations), particularly so in biopsies taken in close vicinity to some of these tumours. Thus, the data presented indicate that the expression of the histone H3 gene can be used as a highly sensitive and reliable marker of proliferation in head and neck tumours and presumably in other tumours as well.

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