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ORIGINAL ARTICLE

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p16 Alterations and retinoblastoma protein expression in squamous cell carcinoma and neighboring dysplasia from the upper aerodigestive tract

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Abstract The progression potential of preinvasive epithelial lesions is usually evaluated by assessing the degree of histologic dysplasia. We examined p16, retinoblastoma protein (pRb), and proliferating cell nuclear antigen (PCNA) immunophenotypes in 57 cases of previously untreated squamous cell carcinoma (SCC) of the upper digestive tract and in the neighboring normal and dysplastic epithelia. Tissue samples were examined for homozygous deletion of exon 2 of the *p16* gene using polymerase chain reaction (PCR) analysis. The PCNA index increased with increasing grade of dysplasia. The pRb protein was expressed in 89% of the samples of SCCs and in the neighboring dysplasias and carcinoma in situ (CIS). In cases with a lack of pRb expression, corresponding preinvasive lesions were also negative. Lack of p16 expression was found in 82% of SCCs. The prevalence of p16 expression decreased with increasing grade of dysplasia. Molecular analysis of the *p16* gene showed homozygous deletion in 37% of SCCs, 33% of CIS, and 15% of the samples of normal epithelia. Our data indicate that inactivation of *p16* may play an important role in early head and neck carcinogenesis, whereas the mutation of *Rb* may be an infrequent event. The p16 immunophenotype might be a biomarker for an increased risk of progression in squamous dysplasia.

Keywords Tumor suppressor · p16 · pRb · Carcinogenesis · Head and neck squamous cell carcinoma

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Introduction

Epithelial carcinogenesis in the head and neck area is believed to be a multistep process. Mutations that develop may be silent if no vital genes are affected, but mutations significant for the development of carcinoma will affect genes important in determining cell behavior. These implicate a number of genes involved in the control of the G1 phase of the cell cycle. The G1 phase is controlled by phosphorylation of the retinoblastoma protein (pRb) and mediated by cyclin D cyclin-dependent kinase (cdk) complexes. It was recently shown that clonal events in the form of loss of heterozygosity at the 3p14 and 9p21 chromosomal regions are detectable early in head and neck carcinogenesis [4]. The search for a tumor suppressor gene on chromosome 9p21 has identified CDKN2/MTS1/INK4 A coding for the cdk4 inhibitor, p16 [11, 33]. The result of p16 binding is the release of cyclin D1 from association with cdk4/cdk6, subsequently targeting cyclin D1 for destruction. The fact that these molecules are all part of the same signal transduction pathway, indicates that inactivation of *p16* and/or *pRb* has the potential to deregulate the G1 phase of the cell cycle and lead to a selective growth advantage for tumor cells. Mutations within the *p16* gene inhibit the cdk inhibitory activity of p16 and thus contribute to carcinogenesis. The presumed role of *p16* as a tumor suppressor in the genesis of head and neck squamous cell carcinoma (SCC) was recently supported by transfecting the gene in *p16* negative squamous carcinoma cell lines, resulting in markedly reduced proliferative activity [16, 24].

The identification of markers relevant to an increased risk of progression toward carcinoma would be invaluable to the clinician for the classification of precancerous lesions and assessing the individual risk of progression. We describe here an analysis of the expression pattern of the tumor suppressor proteins, pRb and p16, in normal tissue, squamous dysplasia, carcinoma in situ, and carcinoma, and further demonstrate frequent homozygous deletion of *p16* in these lesions. The proliferative activity was assessed by immunostaining for proliferating cell nuclear antigen (PCNA).

Materials and methods

Tissue specimens

Paraffin-embedded tissue specimens from 57 previously untreated patients with SCC of the head and neck were available for this study. The patient population comprised 38 males (67%) and 19 females (33%), ranging in age from 38 years to 91 years, with a median age of 57 years. Specimens included three different sites of the head and neck (13 oral cavity, 30 oropharynx, and 14 hypopharynx). Staging was performed according to the 1992 International Union Against Cancer (UICC) criteria. The distribution of pT categories was as follows: 23 pT1, 22 pT2, 8 pT3, and 4 pT4. Only 25 patients were in UICC stage I or II, and 32 patients had lymph node metastases in the neck at diagnosis. All specimens were selected because they contained, besides carcinoma, adjacent normal tissue, squamous dysplasia, and/or preinvasive carcinoma. Samples were serially sectioned. One section was stained with hematoxylin and eosin (HE) and reviewed by a pathologist (IR) for histologic features. Dysplasia grading was done according to the criteria for the definition and classification of squamous dysplasia from the upper aerodigestive tract given by the World Health Organization (WHO) in 1991 [34]. The adjacent sections were immunostained or processed for DNA extraction.

Immunohistochemistry

Dewaxed 3-µm sections were immersed in a citrate buffer solution (0.01 M sodium citrate, pH 6.0) and boiled in a microwave oven at 700 W (three times, 5 min each). Immunostaining for pRb and PCNA was performed using the monoclonal mouse antibodies 1F8 (Novocastra Laboratories Ltd., Newcastle, G.B.) and 5A10 (Immunotech, Marseille, France), respectively. A rabbit polyclonal p16 antibody was obtained from PharMingen (San Diego, Calif.). Using well-defined p16 positive and p16 negative cell lines, this antibody was shown to have adequate sensitivity and specificity and can be used on paraffin sections [7, 12]. The sections were incubated with primary antibody (1:250 dilution for PCNA, 1:30 for pRb, and 1:250 for p16 in stock serum). For the PCNA and pRb antibodies, slides were incubated for 120 min at room temperature. For the p16 antibody, slides were incubated overnight at 4°C.

Slides were then sequentially incubated at room temperature with the second antibody [anti-mouse or anti-rabbit immunoglobulin (Ig)G, 1:200 dilution] for 60 min and alkaline phosphatase–antialkaline phosphatase (APAAP) complex for 60 min. Neufuchsin was used as a chromogen. Slides were washed with tris-buffered saline (TBS) between incubations and counterstained with Meyers Hämalaun. Negative controls were established by replacing the primary antibody with TBS and normal mouse or rabbit serum. Known immunostaining positive slides were used as positive controls. One of each (negative and positive control) was included in each staining procedure along with the patient slides.

All slides were reviewed by two independent observers (PA, IR) under light microscopy. A cell was considered positive if there was a visibly detectable signal within the nucleus (PCNA, pRB) or within the nucleus and cytoplasm (p16). In p16, only nuclear staining was regarded as positive staining; inflammatory cells and reactive stromal cells served as positive internal controls. If the latter were negative as well, the stain was considered uninterpretable.

Immunostaining was graded and scored based on positive cell count in more than ten fields under 250× magnification. PCNA expression was measured semiquantitatively with an ocular micrometer. For this purpose 598–838 epithelial nuclei (697 nuclei on average) were counted in each lesion. The labeling index of PCNA was calculated as the total number of positively stained cells divided by the total number of cells counted. The PCNA labeling index was determined in invasive carcinoma and in preinvasive lesions. The expression of pRb protein was graded as: – 0% cells positively stained; + 1–33% cells positively stained; ++ 34–66% cells positively stained; +++ 67–100% cells positively stained. The expression of p16 protein was not quantified.

Microdissection and DNA extraction

The epithelial layer of each lesion was microdissected from five to seven serial sections (5 µm) of each specimen using a 25.5-gauge steel needle. The microdissected specimens of squamous epithelium contained 100% morphologically normal cells, the specimens of carcinoma in situ (CIS) contained greater than 75%, and the specimens of carcinoma contained greater than 90% neoplastic cells. The samples were dewaxed with xylol and digested in 100 µl 50 mM Tris-HCl (pH 8.0) containing 1% dodecyl sulfate-proteinase K (1.8 mg/ml) and incubated at 55°C for 12 h. DNA was isolated with the QIAamp Tissue Kit (Qiagen, Hilden, Germany). The DNA concentrations were assessed using spectrophotometry.

PCR analysis for homozygous deletion of the *p16* gene

PCR analysis was performed to detect a homozygous deletion, because it has been suggested that a homozygous deletion is the predominant mechanism for inactivation of *p16* in head and neck cancer [3]. Of all coding regions of the *p16* locus (exon 1β, 1α, 2, and 3) on chromosome 9p21, most deletions and mutations were found in exon 2 (total 307 bp) [1, 14, 36]. The first 240 bp of this exon were amplified, using the sense primer (5′-TCATGATGATGGGCAGCGCC) and the antisense primer (5′-ACCGTGCGACATCGCGATGG).

Each fragment of exon 2 was coamplified with a fragment of the *mdm2* gene, which is present in each cell, as an internal positive control in the same reaction. Control primers were: the sense primer (5′-GTGTGATTTGTCAAGGTCGA) and the antisense primer (5′-ATTGGTTGTCTACATACTGG). PCR amplification yielded a *p16* product of 234 bp and a *mdm2* product of 135 bp. All PCR reactions were carried out in 50-µl reactions using 200 ng genomic DNA. The reaction mixture further contained PCR buffer [50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (Pharmacia, Freiburg, Germany)], 0.6 µM each of sense and antisense primer of both primer pairs, 400 µM each of four deoxynucleoside-triphosphates (Pharmacia, Freiburg, Germany), and 5 U *Taq* DNA polymerase (Pharmacia, Freiburg, Germany). The PCR parameters were 94°C for 5 min (initial denaturation), followed by 40 cycles of denaturation (94°C for 45 s), annealing (48°C for 60 s), and elongation (72°C for 60 s), with a final elongation at 72°C for 5 min. PCR products were run on 2.5% agarose gels, stained with ethidium bromide, and visualized on a ultra violet (UV) lightbox. The intensity of the bands was not quantified. As a DNA standard, pUC19 DNA, following incubation with the restriction endonucle-

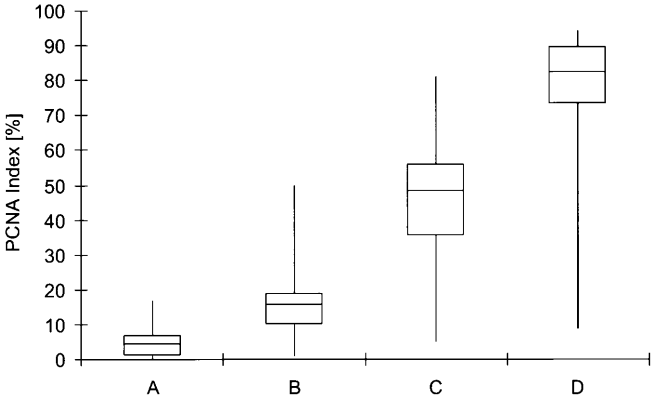

Fig. 1 The proliferating cell nuclear antigen (PCNA) index in squamous cell carcinoma, and the PCNA index in preinvasive lesions in normal epithelium and squamous hyperplasia (*A*), mild and moderate dysplasia (*B*), severe dysplasia and carcinoma in situ (*C*), and carcinoma (*D*). Data are shown as box plots, including minimum (25% quantile), maximum (50% quantile), and median (50% quantile)

ases *Msp*I and *Hpa*II, was used. All PCR reactions were repeated at least three times to ensure accuracy of data. A homozygous deletion of the *p16* gene was only assumed if *mdm2* was successfully amplified but *p16* amplification products could not be detected.

Results

Histologic features

Among the 57 cases of surgically resected tumors analyzed, there were 49 cases with moderately and 8 cases with poorly differentiated SCC. The surrounding mucosa showed mild or moderate dysplasia in 49 cases and severe dysplasia or CIS in 43 cases. Normal mucosa was present in each case.

PCNA immunohistochemistry

The median value of the PCNA index was 4% in normal epithelium and squamous hyperplasia, 16% in mild and moderate dysplasia, 48% in severe dysplasia and carcinoma in situ, and 82% in carcinoma (Fig. 1). The proliferative activity differed from case to case but, in the same case, the higher degree of dysplasia possessed higher proliferative activity. Whereas the PCNA positive cells in normal epithelia were mostly located in the

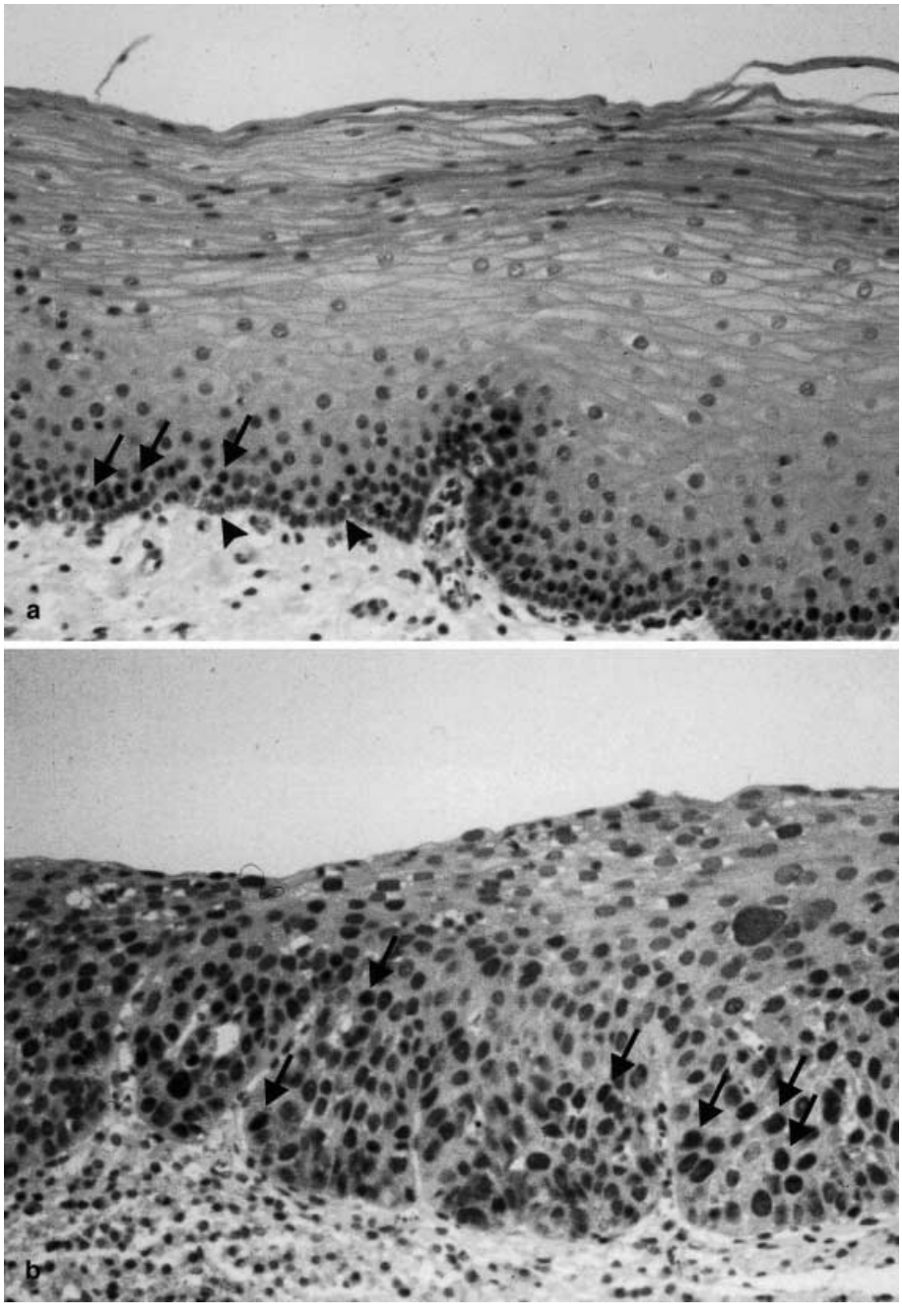

Fig. 2 **a** Expression of retinoblastoma protein (pRb) protein in normal squamous epithelium. Suprabasal nuclei stained positive (*arrows*), and basal nuclei stained negative (*arrowheads*). **b** Expression of pRb protein in a severe squamous dysplasia. pRb protein positive nuclei (*arrows*)

basal cell layer, a greater number of positive cells were seen in the upper cell layers when the degree of dysplasia increased. In squamous hyperplasia and in mild dysplasia, positive cells were found in the lower third of the epithelium. In moderate dysplasia, positive cells were found in the lower two thirds and, in severe dysplasia and CIS, positive cells were found in all epithelial layers. Positive cells were randomly distributed in carcinoma and, in all cases, nearly all cells of the invading front were positive.

pRb immunohistochemistry

In normal squamous epithelium and squamous hyperplasia, the parabasal and superficial cells were pRb positive. The basal cells, however, showed no detectable pRb protein (Fig. 2a). In squamous dysplasia, positive nuclei were present in basal and superficial cell layers (Fig. 2b).

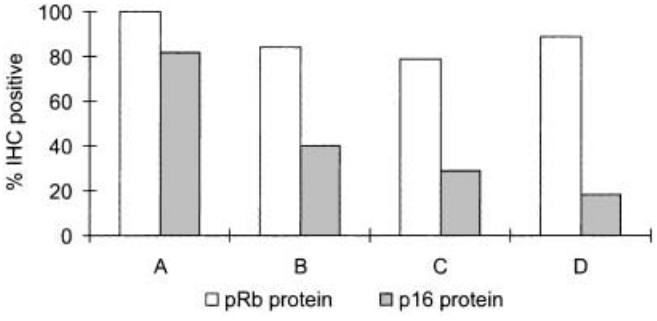

Fig. 3 Expression of retinoblastoma protein (pRb) and p16 protein in squamous cell carcinoma and preinvasive lesions. Normal epithelium and squamous hyperplasia (*A*), mild and moderate dysplasia (*B*), severe dysplasia and carcinoma in situ (*C*), and carcinoma (*D*)

Preinvasive and invasive carcinoma showed randomly distributed positive nuclei. As could be expected, staining was strongest in actively proliferating cells, as indicated by parallel staining for the proliferation marker PCNA. The normal mucosa adjacent to the carcinoma stained positive in all 57 cases. In six (11%) of the SCCs analyzed, no pRb protein could be detected. In these cases, the surrounding dysplasia and preinvasive carcinoma also stained negative. In the group of pRb-positive carcinoma all squamous hyperplasia, dysplasia, and CIS stained positive. Protein expression was lacking in the precancerous stages only when the carcinoma was also negative. Therefore, loss of expression was not dependent on the stage of progression (Fig. 3).

p16 Immunohistochemistry

Expression of the p16 protein was studied in sections of 50 SCC; the stains of seven cases were considered uninterpretable. The protein could be identified in 9 (18%) of 50 invasive lesions. Loss of p16 protein expression correlated with histologic progression: normal mucosa and squamous hyperplasias were positive in 41 of 50 (82%) cases, mild or moderate dysplasias were positive in 6 of 15 (40%) cases, and severe dysplasias or CIS were positive in 10 of 34 (29%) cases (Fig. 3 and Fig. 4).

Comparison of p16 and pRb expression

In 50 SCC, expression of the p16 and the pRb protein was determined. We found an inverse expression of both proteins in 43 (86%) carcinomas. Proteins were coexpressed in seven (14%) cases, and no tumor had lost both proteins.

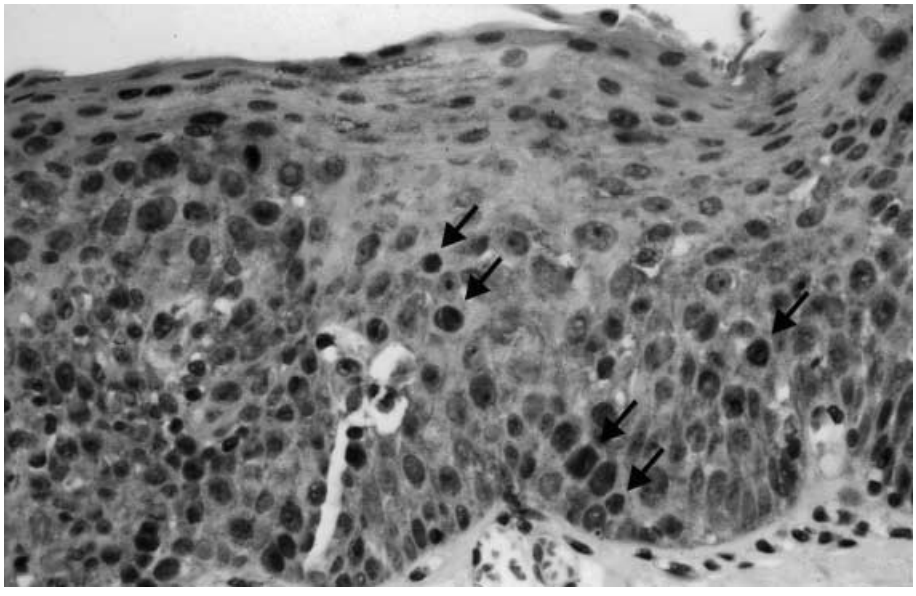

Fig. 4 Expression of p16 protein in a moderate squamous dysplasia p16 protein positive nuclei (*arrows*)

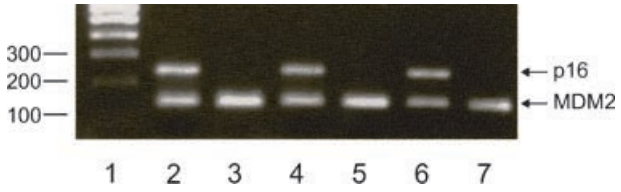

Fig. 5 Representative results of polymerase chain reaction (PCR) analysis of the *p16* gene, exon 2. The *p16* gene, exon 2 was amplified from DNA derived from normal squamous epithelium (*lanes 2* and *3*), carcinoma in situ (*lanes 4* and *5*), and carcinoma (*lanes 6* and *7*). The *p16* amplification products were detectable in lanes 2, 4, and 6. Corresponding tissue samples (*lanes 3*, *5*, and *7*) showed homozygous deletion of the *p16* gene. PCR products of the coamplified *mdm2* gene were detectable in each specimen

p16 PCR analysis

Homozygous deletions were found in 17 of 46 (37%) carcinomas, in 6 of 18 (33%) CISs, and in 2 of 13 (15%) samples of normal epithelium (Fig. 5). The comparison between immunohistochemistry and genetic analysis revealed that in 14 of 35 (40%) carcinomas, in 4 of 8 (50%) CISs, and in 2 of 5 (40%) samples of normal epithelium without expression of the p16 protein, a homozygous deletion had occurred. A deletion was never present when protein was expressed.

Discussion

Head and neck SCCs arise from a clonal population of cells that accumulate genetic alterations. These alterations preferentially appear in different stages of malignant transformation. The identification of these changes may provide molecular diagnostic markers for clinical applications. In the present study, we attempted to define the role of *Rb* and *p16* inactivation in head and neck carcinogenesis. We used normal mucosa dysplasia and CIS adjacent to carcinoma as a progression model. This model assumes that the surrounding dysplasia represents a precursor to the invasive tumor.

In addition to histomorphologic classification of preinvasive and invasive lesions, proliferative activity was evaluated using immunostaining for PCNA. To date, the monoclonal antibody Ki-67 (MIB-1) has been mostly used for assessment of proliferation. It was shown, however, that both PCNA and Ki-67 expression correlate well with increasing grade of dysplasia in oral carcinogenesis as indicated through DNA measurements [37]. No significant difference between Ki-67 and PCNA scores could be demonstrated in head and neck SCC, showing that PCNA immunostaining can be alternatively used for assessing proliferation [17].

It is well known that PCNA expression in premalignant and malignant lesions from the mucosa of the upper aerodigestive tract correlates with the grade of dysplasia and the loss of differentiation in tumors [35, 41]. In accordance with others, we found that the expression of PCNA in squamous epithelia increased with progression toward carcinoma. With increasing grade of dysplasia, PCNA expression increased in the suprabasal cell layers, reflecting an abnormal proliferation pattern in these lesions.

pRb immunohistochemistry showed strong nuclear staining in both tumor and normal cells. We have found that the expression of pRb protein is restricted to the suprabasal epithelial layers in normal epithelium and squamous hyperplasia. In dysplasia and CIS, however, pRb is also expressed in the basal cells. The expression pattern may be explained by considering the observation that pRb expression was found to be largely or entirely confined to the differentiating cells in fetal mouse tissues with a growing and differentiating compartment. A differentiation-dependent pRb expression was found in squamous epithelia with no detectable pRb staining in the basal cell layers but positive staining in the more differentiated superficial layers, suggesting that the protein plays a role in differentiation of dividing precursor cells into mature resting cells [38]. The expression pattern of the normal epithelia examined by us corresponds well to the pattern observed in normal murine squamous epithelia. The appearance of the protein in the basal cell layer in dysplasia and CIS might reflect an altered differentiation pattern in these lesions.

Immunohistochemical analysis has proven reliable for the assessment of the *Rb* genetic status despite multiple mechanisms of inactivation; the presence of *Rb* mutations correlates with a lack of immunostaining [7, 39, 40]. In our series, *Rb* inactivation with no detectable protein expression was observed in 11% of SCCs. The incidence of *Rb* inactivation was consistent with previous studies on primary head and neck SCC [2, 20, 39]. The protein was not expressed in the precancerous stages only when the carcinoma was also negative. Therefore, loss of expression was not dependent on the stage of progression. Our observations show that inactivation of the *Rb* gene might occur early but seems to be a rare event and therefore plays a less important role in head and neck carcinogenesis.

A prevalence of *p16* alterations, ranging from 25% to 83%, has been reported in head and neck SCC [5, 6, 8, 10, 21, 27, 30]. The variable findings may be attributable to different patient populations and varying methodologies used for analysis. The *p16* gene can be inactivated by a variety of mechanisms, including deletion, point mutation, and silencing by means of methylation [3, 6, 8, 9, 14, 22, 23, 32]. A comprehensive genetic analysis, including a search for homozygous deletion, point mutation, and methylation status would require amounts of DNA not extractable from foci of preinvasive carcinoma. Thus, we focused our genetic analysis on a screening for homozygous deletion, in the knowledge that methylation might be another important mechanism of *p16* inactivation in head and neck SCC [6, 31].

We found a homozygous deletion in 37% of the carcinomas. This figure is in accordance with other reports, describing a prevalence of homozygous deletion of

10–55% [3, 5, 6, 8, 18, 21, 27, 29, 30]. Furthermore, we could show that the homozygous deletion of the *p16* gene was detectable in 33% of preinvasive carcinoma and even in 15% of normal epithelia of cancer patients. This is in agreement with findings of Califano et al. [4], who examined hyperplastic and dysplastic epithelia surrounding invasive head and neck cancer and found loss of heterozygosity (LOH) of chromosome 9p21 in 29% of hyperplasia, 57% of dysplasias, and 80% of CIS, with no further increase in invasive cancers. A recent study of LOH in mucosa specimens from the upper aerodigestive tract has shown that deletions of 9p21 occurred in 8 of 13 dysplasia and in 6 of 13 mucosa specimens in the absence of histologic changes [19]. These findings further support the assumption that deletion of 9p21 and loss of *p16* function is an early event in head and neck carcinogenesis.

The two most common inactivation mechanisms of the *p16* gene result in a loss of protein expression, detectable in formalin-fixed, paraffin-embedded tissue. We confirm that the p16 protein is predominantly nuclear localized, with a variable nonspecific cytoplasmic reactivity, as previously noted [7, 26, 30]. We could demonstrate that the loss of p16 protein expression correlates with histologic progression. Specimens were p16-negative in 18% of normal mucosa and squamous hyperplasia, 60% of mild or moderate dysplasia, 71% of severe dysplasia or CIS, and 82% of SCC. To our knowledge, only one previous report determined immunohistochemically the prevalence of p16 protein expression in oral preinvasive lesions [28]. These authors described a loss of p16 protein expression in 43% (10 of 23) of oral leucoplakias with mainly mild dysplasia – only two cases exhibited moderate to severe dysplasia – and a loss of p16 protein expression in 35% (18 of 51) of leucoplakia without dysplasia. Undetectable p16 protein in 83% of head and neck SCC using immunohistochemistry was reported by others [30].

p16 Appears to function in a feedback loop that involves pRb. In cell lines lacking functional pRb, high levels of p16 were observed [15, 26]. *p16* And *Rb* inactivation have been reported to be inversely correlated in some tumor types, including head and neck SCC [2, 13, 25, 32]. In our series, we found an inverse expression of p16 and pRb in 86% of SCC. This inverse relationship indicates that a mutation in either *Rb* or *p16* is sufficient to disrupt the G1 checkpoint pathway. In head and neck SCC, *p16* is the most frequent target of inactivation.

Our observations further confirm the importance of the *p16/Rb* pathway in the initiation of head and neck SCC. The inactivation of *p16* appears to be critical to early head and neck cancer development, because we could demonstrate mutation and loss of protein expression in the absence of histologic changes. The immunohistochemically detectable loss of p16 protein expression might become a clinically valuable biomarker in the classification and assessment of the prognosis of precancerous lesions of the upper aerodigestive tract.

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