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p53 and PCNA Expression in Carcinogenesis of the Oropharyngeal Mucosa

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Hyperplastic lesions of the oral mucosa such as leukoplakia and oral lichen planus can eventually develop into squamous cell carcinomas (SCC) and provide an excellent model for multistage carcinogenesis. The development of carcinomas is assumed to be the result of interaction of genetic factors, locally applied carcinogens and immununological unresponsiveness. The purpose of this study was, therefore, to determine the role of alterations of the tumour suppressor gene p53, and the proliferation status of the lesions determined by PCNA expression. We investigated p53 and PCNA expression in 265 tissue sections of normal mucosa, premalignant, malignant and metastatic lesions of the oral mucosa by immunohistology. Quantitative analysis showed a gradual increase in PCNA expression from normal mucosa to moderately differentiated SCC. p53 expression was detectable in benign premalignant lesions. The increase in the number of p53-positive biopsies was correlated with the dysplasia and loss of differentiation in the premalignant and malignant lesions.

Keywords: Tumour suppressor genes, PCNA, oral mucosa, carcinogenesis, leukoplakia, lichen planus, squamous cell carcinoma

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INTRODUCTION

FOR HYPERPLASTIC lesions of the oral mucosa with an increased risk of malignant development the term "preneoplastic" lesions is widely used [1]. Examples of such lesions are oral leukoplakia and oral lichen planus. Around 4% of oral leukoplakia can develop into squamous cell carcinoma [2]. In highly dysplastic lesions the rate of malignant development can be as high as 43% [3–6]. In oral lichen planus the frequency of carcinomatous changes varies between 1 and 10% [7–10]. It is impossible though to assess in an individual patient the biological behaviour of the premalignant lesions. Histological evaluation of the lesions for presence or absence of dysplasia still seems to be the most reliable indicator for future carcinomatous development [6]. It is, therefore, necessary to understand the molecular changes in the carcinogenesis of the oral mucosa.

Oncogenes and tumour suppressor genes are known to play an important role in the development of the malignant phenotype. Mutations in the p53 tumour suppressor gene are the most common genetic alterations in human cancer [11]. Increased levels of p53 protein are often found in malignant tumours, but rarely in benign tumours and normal tissue [12, 13]. The purpose of this study was to determine whether p53 plays a role in the carcinogenesis of the oral mucosa.

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Proliferating cell nuclear antigen (PCNA) is a nuclear protein synthesised in late G1 and S phase of the cell cycle. Immunohistological detection of the protein represents a useful marker of the proliferation status of the investigated lesions [14].

MATERIAL AND METHODS

Paraffin-embedded tissue sections of preneoplastic lesions and normal mucosa of the oropharynx from 121 patients and paraffin-embedded tissue sections of squamous cell carcinomas (SCC) of the oropharyngeal mucosa from 144 patients were immunohistochemically stained with monoclonal antibodies for the presence of p53 (MAb Do 7; Dako) and PCNA (PCNA PC 10; Dako) expression.

Immunohistochemistry was performed using the APAAP technique. The tissue sections were first dewaxed with xylene (30 min), dehydrated with ethanol and rehydrated gradually with ethanol and water. The tissue sections were then incubated with TBS (pH 7.4) in the microwave (650 W) twice for 5 min to resolve the protein fixation. The TBS-treated sections were subsequently incubated with goat serum (1:10; Dako X 907) for 10 min and then incubated with the specific antibody (MAb Do 7; Dako, 1:25 and MAb PC 10; Dako, 1:100). The p53 antibody was incubated for 12 h at 4°C and the PCNA MAb was incubated for 60 min at room temperature. After washing twice with TBS the sections were incubated with pig serum (1:20; Dako X 901) for 10 min followed by incubation with the bridging antibody (Dako Z 259) for 60 min. After further washing with TBS the sections were incubated with goat serum (1:10, 10 min) and the

APAAP complex (1:50, 60 min; Dako D 651). The steps starting with the pig serum, incubation with the bridging antibody, washing and incubation with goat serum and the APAAP-complex were repeated once. After further washing with TBS the reaction product was stained with Fast Red (Sigma F-1500) and counterstained with haematoxylin. Levamisole 1 mM was added to block endogenous alkaline phosphatase activity. All incubations were carried out at room temperature if not indicated otherwise. Each set of experiments included positive and negative controls.

The preneoplastic lesions ($n=104$) were classified histopathologically. The squamous cell carcinomas ($n=144$) were classified according to the loss of differentiation (UICC classification; G1-G3 for differentiated, moderately differentiated and poorly differentiated SCC). Tissue samples were counted as positive for p53 expression when a single positive cell of presumably epithelial origin could be detected in the specimen. In the PCNA-positive tissue samples, cell counts of the positive cells in 10 different areas of the slide, in defined size and standardised location, were performed to assess the PCNA expression semiquantitatively.

RESULTS

As in mutant p53, the protein is stabilised, the half-life is extended and it becomes detectable by immunohistological staining [11]. Detection of p53 by immunohistology can, therefore, either be caused by stabilisation of the protein due to the presence of mutation or result from promotion-driven mechanisms that lead to increased p53 at the steady state in a cell cycle checkpoint response mechanism [15].

Of 104 tissue samples of benign lesions of the oropharyngeal mucosa (leukoplakia and lichen planus), 44 samples did not show any dysplasia (G0) (Figs 1 and 2). Thirty-nine samples showed epithelial dysplasia (Fig. 3). Twenty-one specimens were classified as lichen planus of the oral mucosa and in 17 tissue samples the epithelium was normal. These samples served as normal controls.

The SCC of the oral mucosa ($n=144$) were classified according to the loss of differentiation (G1-G3). Sixty-seven carcinomas were well differentiated (G1). Sixty-four carcinomas were classified as moderately differentiated (G2). Seven tumours were poorly differentiated (G3). Six tissue samples were metastases of oropharyngeal SCC.

None of the normal controls showed any p53 expression. In lichen planus, four samples (19%) showed p53 expression at a detectable level (Fig. 2). In the group of hyperplastic lesions without dysplasia ($n=44$), 16 lesions (36%) were p53 positive. A detectable level of p53 was also found in 14 (36%) benign lesions that showed epithelial dysplasia (Fig. 3).

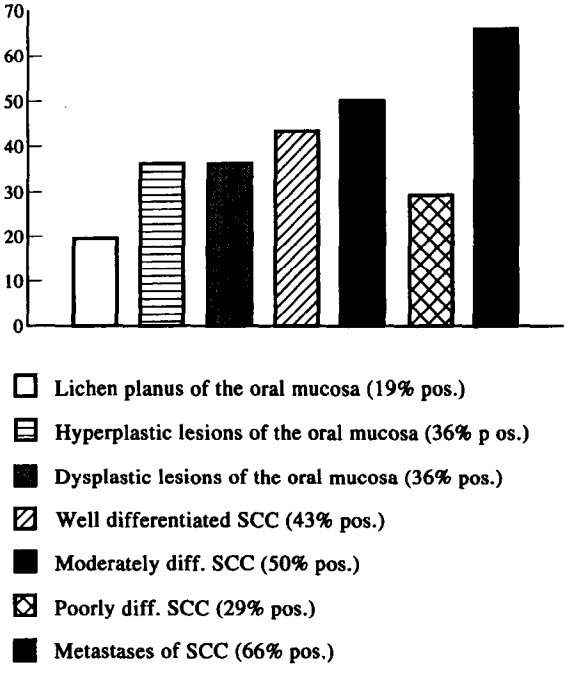

Fig. 1. Positive p53 expression in the lesions in the oral mucosa.

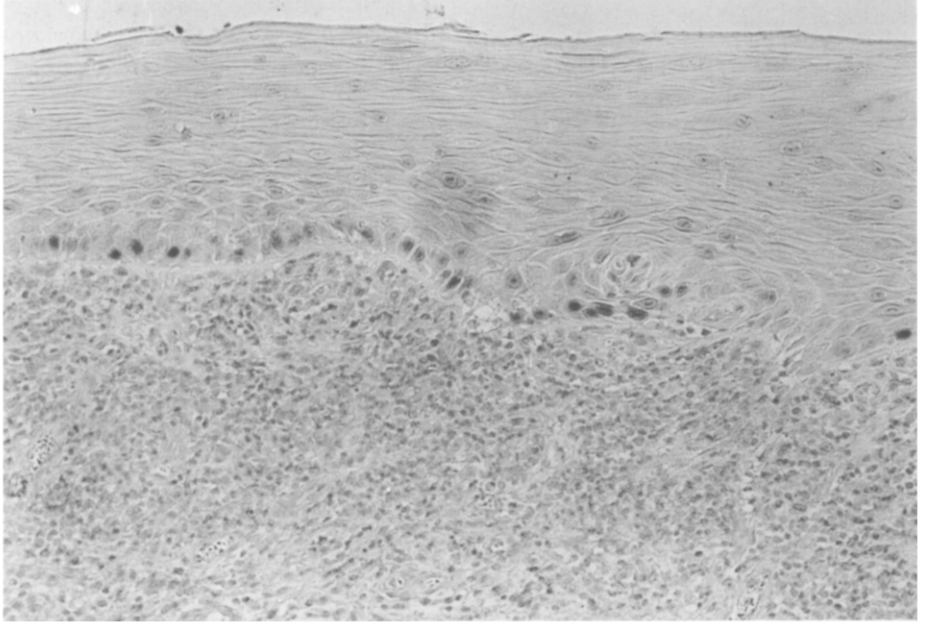

Fig. 2. p53 expression in hyperplasia of the oral mucosa without dysplasia (lichen planus) (100 ×).

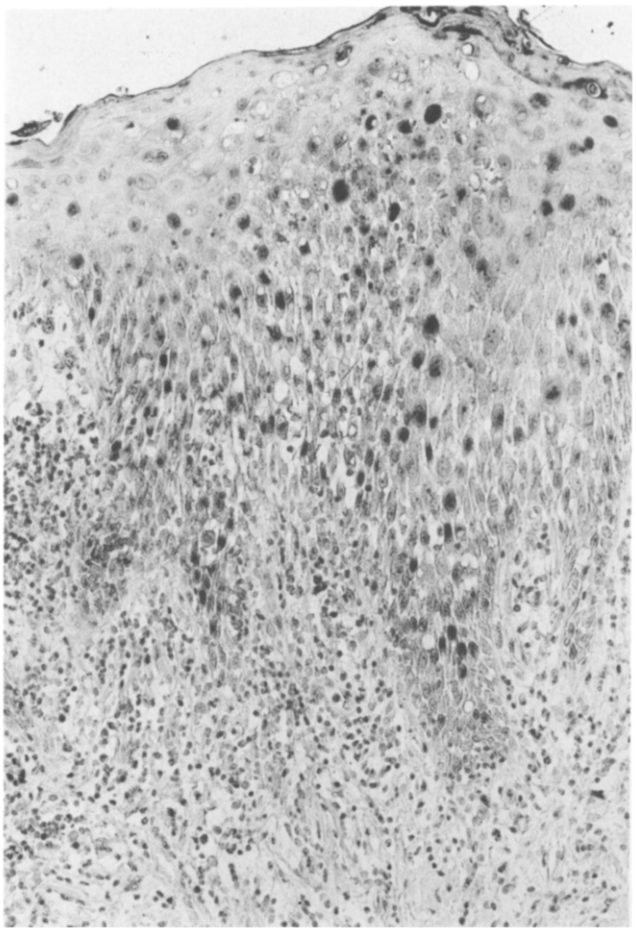

Fig. 3. p53 expression in hyperplasia of the oral mucosa with dysplasia (leukoplakia) (100 ×).

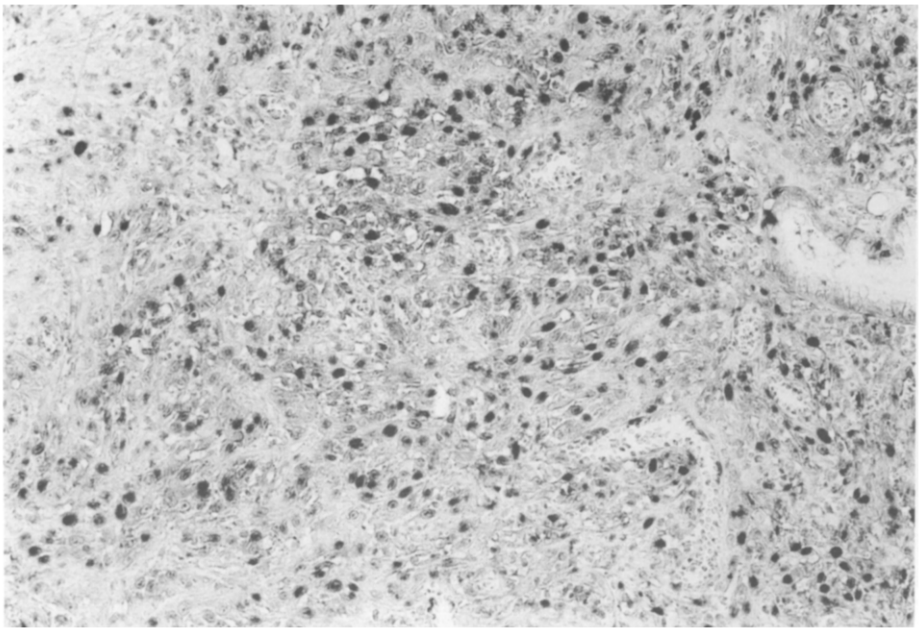

Fig. 4. p53 expression in SCC of the oral mucosa (G2) (100 ×).

In the G1 group of the SCC (well differentiated) ($n=67$), 29 tumours (43%) were p53 positive. Among the SCC that showed only moderate differentiation (G2; $n=64$), 32 tumours (50%) were positive for p53 (Fig. 4). Only two (29%) specimens of the G3 tumours ($n=7$) were p53 positive, whereas four (66%) tissue samples from lymph node metastases of oropharyngeal SCC ($n=6$) were p53 positive.

The quantification of p53 expression in the positive tissue

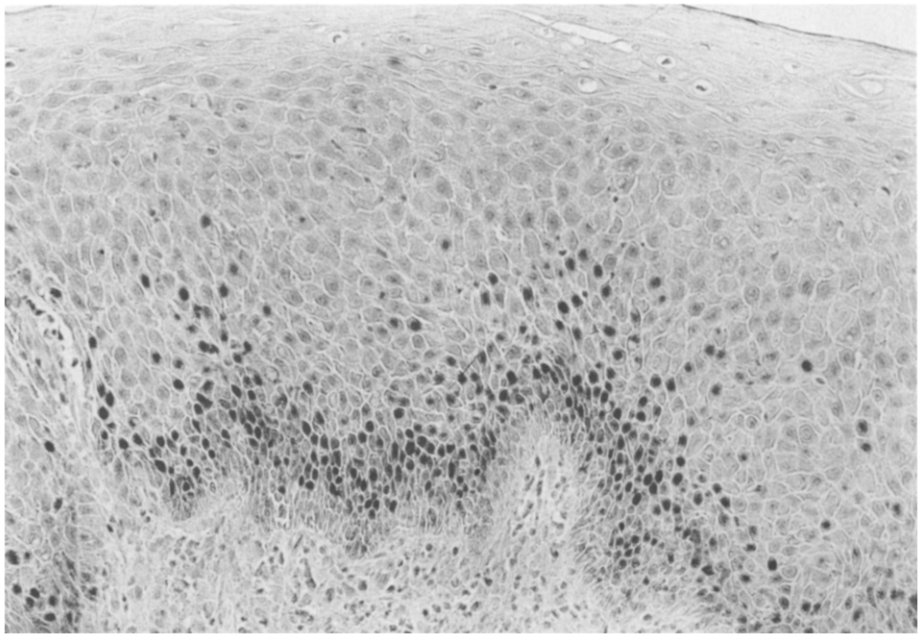

Fig. 5. PCNA expression in hyperplasia of the oral mucosa with dysplasia (leukoplakia) (100 ×).

samples did not show any correlation with histological parameters, such as dysplasia and loss of differentiation. p53-positive cells were commonly found in the basal cell layer (Figs 2 and 3) or randomly distributed in the poorly differentiated tumours (Fig. 4).

For semiquantitative analysis of PCNA expression, cell counts of the positive cells in 10 different areas of the slide, in defined size and standardised location, were performed. All specimens were PCNA positive. PCNA-positive cells were usually located in the basal cell layer or randomly distributed in tumours, thus showing a pattern similar to p53 expression (Fig. 5). In the normal controls an average of 27 cells were PCNA positive per field (Fig. 6). In lichen planus 56 cells were positive per field. In hyperplastic epithelium an average of 31 cells were positive and in dysplastic lesions 47 per field showed PCNA expression (Fig. 5). The number of PCNA-positive cells increased in well differentiated tumours (74 cells positive per field) and with loss of differentiation. In moderately differentiated SCC (G2) 80 cells were positive per field. In poorly differentiated SCC and metastases the number of PCNA-positive cells decreased again (60 cells positive in G3; 46 cells positive in metastases) (Fig. 6).

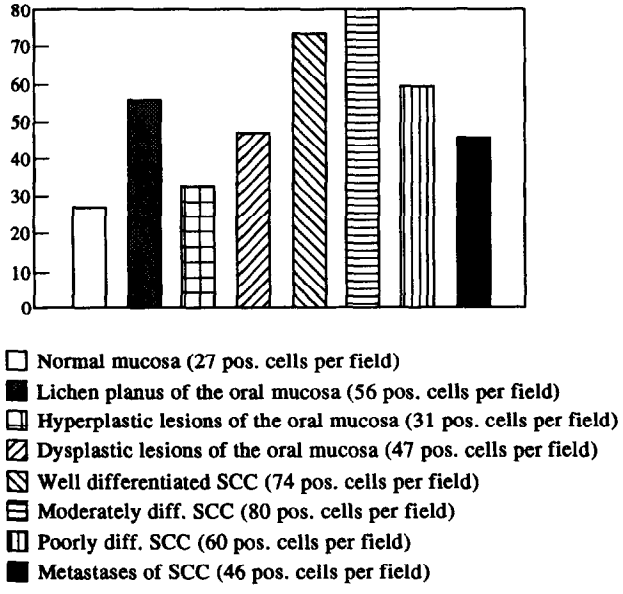

Fig. 6. PCNA expression in different stages of the carcinogenesis in the oropharyngeal mucosa.

DISCUSSION

Clinically, it is well known that oral leukoplakia and lichen planus can develop into squamous cell carcinoma [1–10]. Burkhardt [16] found a correlation between the development of carcinomas and the grade of dysplasia of the primary lesions. Other investigators found that grade of dysplasia seems to be unreliable as the only diagnostic parameter in predicting cancer development [6]. PCNA expression seems to be a good marker to assess the proliferation activity of premalignant and malignant lesions in the oral mucosa [17].

p53 mutation is a common genetic change in human oral cancer. Most cell lines of SCC of the oropharynx show mutations of the p53 tumour suppression gene [12, 18, 19]. Using different monoclonal antibodies, Langdon and Partridge [13] found detectable levels of p53 in up to 80% of 15 SCC lesions they studied, whereas no detectable level of p53 could be found in normal mucosa. Field found that the expression of p53 in carcinomas of the head and neck was correlated with a history of heavy smoking and drinking [20]. So far, elevated p53 expression had been detected in premalignant lesions in the oral mucosa [21]. Thus, p53 mutation seemed to be an interesting gene to investigate in carcinogenesis in the oral mucosa. The presented data suggests that p53 gene mutation is an early event in the carcinogenesis in the oropharyngeal mucosa and positively correlated to increasing dysplasia, loss of differentiation and proliferation status of premalignant and malignant lesions. One third of preneoplastic lesions showed positive p53 expression in this study. Interestingly, a smaller percentage of oral lichen planus was

also p53 positive. This is consistent with the clinical finding that, in comparison with leukoplakia, lichen planus only rarely shows malignant transformation [7–10]. It was also attempted to quantify p53 expression among the lesions by counting the positive cells. No correlation could be found between the number of positive cells and the grade of dysplasia of the lesions. If a cell appears negative in an otherwise positive sample this might be again due to the fact that this special cell either belongs to a non-mutated clone or is currently in a state of the cell cycle where it contains undetectable levels of p53 protein which cannot be measured by immunohistology. In addition, other mechanisms are known that impair p53 function without causing an increase in protein expression that may then become detectable by immunohistochemistry [22].

PCNA expression in premalignant and malignant lesions of the oropharyngeal mucosa correlated well with the dysplasia grade and loss of differentiation in the benign lesions and tumours. Lichen planus of the oral mucosa showed a very active proliferation status as determined by PCNA expression (Fig. 6) though malignant changes are rare [7–10]. Mitosis could be induced by inflammation in lichen planus, e.g. in psoriasis and may not be an indicator of malignancy. The p53 expression could, therefore, be a better indicator of malignancy versus benign proliferative activity as determined by PCNA expression.

Cells with positive p53 expression as well as PCNA-positive cells were located in the basal cell layer of the epithelium or randomly distributed in the tumours, indicating areas of different proliferation activity. This could be due to the heterogeneity of the polyclonal tumours and hyperplastic lesions with areas of different proliferation status. p53 expression can also probably be better detected in proliferating cells by immunohistology, as p53 expression is cell cycle-dependant [23–25]. Thus, p53- and PCNA-positive cells show a similar distribution.

The results of this immunohistological investigation imply that mutation of the p53 tumour suppressor gene may play an important role in carcinogenesis in the oral mucosa. With morphology being the least sensitive technique, dysplasia in benign lesions may indicate an already advanced state of atypia. Demonstration of p53 mutation may suggest atypia is not yet overt by light microscopy. Further investigations on the molecular level are needed though to determine whether detection of p53 is caused by the presence of mutation or due to promotion-driven mechanisms that lead to increased p53 at the steady state in a cell cycle checkpoint response mechanism.

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