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The Presence of p53 Protein in Relation to Ki-67 as Cellular Proliferation Marker in Head and Neck Squamous Cell Carcinoma and Adjacent Dysplastic Mucosa

P.J. Slootweg, R. Koole and G.J. Hordijk

Paraffin embedded material from 15 patients suffering from head and neck squamous cell carcinoma (HNSCC) bordered by dysplastic mucosal areas was immunohistochemically investigated for the presence of p53 protein and Ki-67 proliferation marker. p53 protein was present in 9 cases (60%), invariably in invasive cancer areas as well as in adjacent non-invasive dysplastic mucosa. Only cells exhibiting atypia contained p53 protein. Ki-67 proliferation marker was present in the basal cells of the normal epithelium and more extensive in dysplasias and HNSCC. The presence of Ki-67 closely coincided with p53 protein in the 9 cases exhibiting this. No differences in Ki-67 expression were found between p53 positive and negative cases. It is concluded that the appearance of p53 protein occurs early in carcinogenesis but that cells also may show increased proliferation without involving immunohistochemically detectable alterations in the p53 gene.

Key words: Oral squamous cell cancer; p53 oncoprotein; Ki-67; immunohistochemistry.

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INTRODUCTION

THE p53 gene, located on the short arm of chromosome 17 normally behaves as a tumour suppressor gene but alteration or inactivation of p53 by mutation can lead to cancer. The normal p53 protein has a very short half life which prevents its immunohistochemical detection but mutation of the p53 gene commonly leads to a more stable protein that can be detected using immunohistochemical methods and therefore, it is assumed that the detection of the p53 protein is synonymous with mutation of the p53 gene [1, 2].

As p53 protein has been detected in a variety of malignancies, this mutation might be considered to be associated with tumour development. The occurrence of p53 protein has been extensively investigated in head and neck squamous cell cancer (HNSCC) by immunohistochemical methods [3–10] and the presence of the protein has been shown to be related with mutations of the p53 gene thus substantiating the assumption that occurrence of immunohistochemically detectable p53 protein is due to mutation of the corresponding gene [11–13]. Other studies have investigated the occurrence of p53 gene alterations without concomitant immunohistochemical investigation of the presence of p53 protein [14–17].

Although it appears that p53 alterations are correlated with the development of HNSCC, its exact role is not yet clear. As quite a substantial number of HNSCC-cases lack immunohistochemically demonstrable p53 protein, it is suggested that p53 mutations are neither sufficient nor necessary for the development of HNSCC [5]. Moreover, there is uncertainty as to whether it is an early [5, 6, 9] or a late [12] event in tumorigenesis.

A way to investigate whether p53 mutations do occur early or late in tumorigenesis is to look for the presence of p53 protein in premalignant lesions of the mucous membranes. This has been done by Ogden *et al.* [7] who report absence of this protein in premalignant lesions, whereas the opposite was found by Langdon and Partridge [4] and Nakanishi *et al.* [18]. Other authors have looked for the presence of p53 protein in dysplastic mucosal areas adjacent to invasive carcinomas where it was observed to occur although to a varying extent [5, 6, 9, 11]. In the present paper, we report the results of our investigation on the occurrence of p53 protein in HNSCC with adjacent carcinoma *in situ* (CIS) or various degrees of dysplasia. In addition, we investigated whether occurrence of demonstrable p53 protein in an individual case coincided with an increase of the proportion of cells exhibiting proliferation.

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MATERIALS AND METHODS

The material consisted of tissue specimens from 15 patients that were surgically treated for HNSCC.

Four of them were females and 11 were males. The tumours were located at the following sites: palate ($N=10$), floor of mouth ($N=2$), piriform sinus ($N=2$), tongue ($N=1$). The mean age of the patients was 59.3 years with a range varying from 43 to 76. No patients had undergone any previous treatment other than biopsies as part of the diagnostic procedures.

Paraffin blocks were selected in which invasive tumour was present together with adjacent CIS or dysplasia and 4 μ slides were cut for immunohistochemical demonstration of p53 protein and the proliferation marker Ki-67.

Immunohistochemistry

Immunohistochemistry was performed on deparaffinised 4 μ sections using a murine monoclonal antibody, BP53-12-1, directed against both wild-type and mutant p53 protein (Biogenex, San Ramon, California, U.S.A.) and a murine monoclonal antibody MIB-1 which is an antibody that reacts with the Ki-67 nuclear antigen designed for use on formalin-fixed, paraffin-embedded material (Immunotech. S.A., Marseille, France) [19].

The procedure for the detection of p53 protein was as follows. Sections were deparaffinised and incubated with anti p53 antibody BP53-12-1 in a dilution of 1:100. Sections were washed in phosphate buffered saline (PBS) and incubated with biotinilated horse antimouse antibodies (1/500; Vector, Burlingham, California, U.S.A.) for 30 min, followed by washing in phosphate buffered saline (PBS) and incubation with alkaline-phosphatase labelled streptavidin (1/200; Dakopatts) for 30 min. The alkaline phosphatase activity was developed by naphthol-AS-BI-phosphate and hexazotised new fuchsin resulting in a bright red reaction product. Sections were counterstained with haematoxylin and mounted.

The Ki-67 antigen was detected conforming to a technique more extensively described elsewhere [20]. Briefly, sections are treated with a boiling solution of freshly prepared citrate/HCL buffer 10 mmol, pH 6.0. After cooling down to room temperature, sections are rinsed three times in PBS, pre-incubated for 15–30 min in PBS with 10% horse serum and incubated for 60 min with MIB-1 (1:200 dilution). The following steps were the same as for the p53 protein detection.

RESULTS

Overexpression of p53 protein in HNSCC as detected by the BP 53-12-1 antibody was found in 9 out of the 15 cases (60%). All immunostaining for p53 protein was confined to the cell nuclei. In all cases in which the invasive tumour component exhibited p53 protein expression, the adjacent CIS or dysplasia areas did also (Figs 1 and 2). Expression of p53 protein was closely correlated with cellular atypia. No expression of p53 protein was observed in adjacent epithelium composed of normal-looking cells. When the HNSCC lacked positivity, the adjacent non-invasive abnormal epithelium also failed to exhibit detectable p53 protein.

Nuclear expression of the Ki-67 antigen as detected by the MIB-1 antibody was found in all cases. In normal epithelium, it remained confined to isolated cells adjacent to the basal lamina. In the dysplastic or CIS areas, proliferative cells were present at abnormal sites within the epithelium, sometimes throughout the entire epithelial thickness (Fig. 3). In the invasive parts, Ki-67 positivity was observed in the peripheral tumour parts and in the cells in the periphery of the tumour

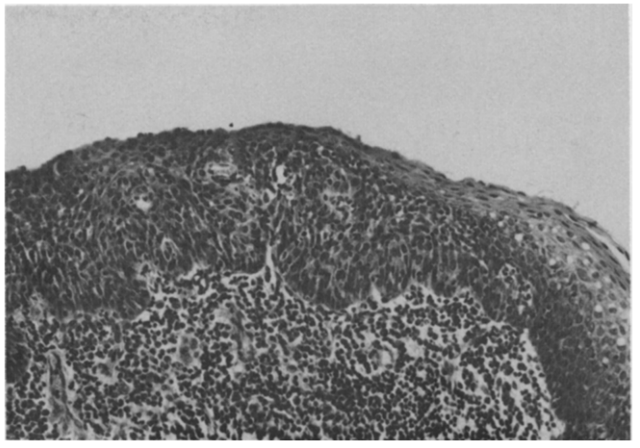

Fig. 1. Photomicrograph showing border between dysplasia and normal mucous membrane. Haematoxylin and eosin × 100.

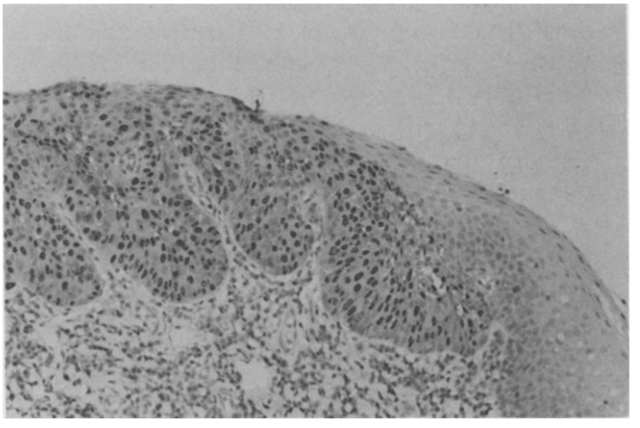

Fig. 2. Same area as shown in Fig. 1. Dark dots indicate presence of p53 oncoprotein. Immuno-alkaline phosphatase haematoxylin × 100.

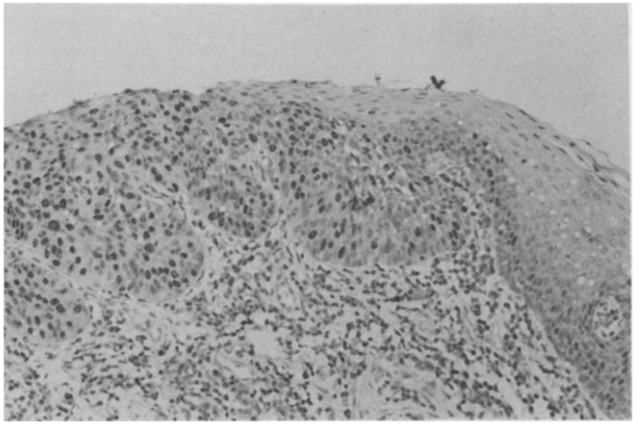

Fig. 3. Same area as shown in Fig. 1. Dark dots indicate presence of Ki-67 proliferation marker. Immuno-alkaline phosphatase haematoxylin × 100

nests. When the tumour cells matured into squamous pearls, positivity of Ki-67 as well as of p53 protein tumour disappeared (Fig. 4). In the 9 cases that exhibited positivity for Ki-67 as well as p53 protein, both markers were present at the same areas, the only difference being found in the normal epithelium that did contain occasionally Ki-67 positive cells but lacked any positivity for p53 protein.

DISCUSSION

Our data on immunohistochemically detectable p53 protein in HNSCC agree very well with those obtained previously. A percentage of 60% lies in a range that varies from 35% [5] to 90% [6]. As there is also quite a substantial amount of cases that lack presence of p53 protein, the assumption that p53 mutation is not obligatory in HNSCC [5] seems at first substantiated. However, lack of p53 protein expression by the tumour cells does not always mean that the p53 is uninvolved. Tumours with no p53 oncoprotein staining may have lost the expression of both alleles or may contain a level of p53 mutant that cannot be detected by the anti-p53 protein antibodies used in the various studies [3]. To exclude unequivocally the presence of p53 gene alterations in HNSCC, one has to study the gene itself. When this was done by several molecular biological methods one study indicated a 100% mutation frequency [13] but other reports mention p53 alterations ranging from 50% to 90% [12, 15]. Development of HNSCC without participation of p53 gene alterations seems to occur by a very low level of p53 oncoprotein positive cases in oral carcinoma associated with betel and tobacco chewing [10]. p53 gene alterations are probably related to some aetiological factors, e.g. smoking [3], whereas other aetiological agents may induce HNSCC without involvement of the p53 gene.

The next point for discussion is how p53 gene alterations are related to cell proliferation. One previous study indicated an increase of Ki-67 cells not only in p53 protein positive tumours but also in the p53 negative cases [4] and the same was found in a study that combined detection of p53 protein with assessment of the proliferative cells by PCNA [8]. We also obtained these results; an increase in proliferating cells not confined to the invasive tumour areas but also in the adjacent CIS or epithelial dysplasia. Assuming that p53 negativity means that at least in some tumours the p53 gene is normal indicates that the tumour cells are in some way able to bypass the suppressive action of the normal gene.

When returning to the question if p53 gene alterations occur early or late in tumour development, we feel that the presence of p53 protein found in tumours bordering atypical cells in previous studies [5, 6, 9, 11] supports the idea that the p53 gene is involved in the first transformations of a cell to the cancer cell stage. The idea that p53 gene alterations occur late in tumour development was based on the observation that the most intense p53 protein staining was found in the more advanced cases [12] but other studies [3, 6] have failed to find a correlation between tumour stage and p53 protein expression and the presence of p53 oncoprotein in epithelial cells exhibiting atypia but no invasive growth is difficult to reconcile with p53 gene alterations as a late event. As p53 expression only occurs in cells already exhibiting morphological aberrations, it cannot be used as a marker for cellular events leading to cancer but not yet showing visible changes. The fact that these not yet visible precancerous stages do occur became apparent from our studies on the activity of tyrosine-phosphokinase (TPK) in HNSCC and adjacent mucosa in which we found that TPK activity in microscopically normal mucous membranes obtained from HNSCC patients was much higher than in mucosa from healthy control patients but not as high as in tumour tissue itself [21].

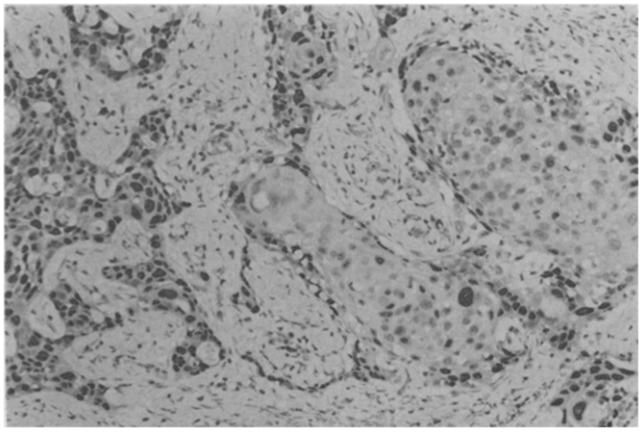

Fig. 4. Invasive carcinoma. Dark dots indicate presence of p53 protein. Ki-67 showed the same distribution: occurring peripherally in the tumour nests while being absent in more centrally located cells. Immuno-alkaline phosphatase haematoxylin × 100.

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