Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: correlation with expression of Ki67

S Kannan, G Jagadeesh Chandran, K Raveendran Pillai, Babu Mathew, K Sujathan, K R Nalinakumary, M Krishnan Nair

Abstract

Aim—To study p53 expression in relation to proliferative status in normal and non-dysplastic, dysplastic and malignant lesions of the oral mucosa.

Method—The standard avidin-biotin complex (ABC) immunohistochemical staining method was used to study the expression of p53 and Ki67 on frozen sections of oral leukoplakias and carcinomas.

Results—Of the leukoplakia and carcinoma samples, 70% expressed p53 in over 5% of cells. In normal mucosa less than 5% of cells expressed p53. The proliferation index, as assessed by expression of Ki67, was highest in the malignant lesions (43%) and lowest in normal mucosa (11%). Statistical analysis revealed that expression of both p53 and Ki67 was correlated significantly with the histopathological stage of the tumour. However, expression of p53 was not correlated with that of Ki67. In leukoplakias lesions with proliferative features p53 immunostaining was less intense than in non-proliferative lesions; this difference was statistically significant.

Conclusions—These results emphasise the potential of Ki67 and p53 as biomarkers of carcinogenesis in oral cancer and may also serve as intermediate points for cancer prevention programmes, such as the oral chemopreventive trials. Factors other than p53 may have a more important role in the deregulation of proliferation in pre-malignant oral lesions.

Keywords: p53, Ki67, oral leukoplakia, oral squamous cell carcinoma, tumour progression, immunohistochemistry.

In recent years more emphasis has been placed on cancer prevention programmes and related studies. In these programmes, success has relied on the development of efficient, early detection methods to identify patients at high risk of developing cancer. Several clinical and aetiological factors have been identified as markers for the detection of these high risk groups for various malignancies. However, these factors are not efficient and studies are currently underway to identify biological markers for this purpose. In this regard, unravelling of biological processes during the pre-neoplastic stages is of paramount importance.

Of the pre-malignant lesions, oral leukoplakia has greatest relevance in the study of the biology of carcinogenesis because the oral cavity is easily accessible for clinical examination and also because it has a multistage carcinogenesis pathway and of the concept of field cancerisation. The oral carcinogenesis pathway can be broadly classified histopathologically into normal, non-dysplastic, dysplastic, carcinoma-in-situ, and invasive carcinoma. Molecular biological studies on oral cancer have identified several genetic aberrations in the cells during various tumour stages.

Mutations in the p53 tumour suppressor gene are the most common genetic changes in humans cancers and are regarded as early events in carcinogenesis. In pre-malignant and malignant oral lesions, alterations in p53 expression have been reported both at protein and DNA levels. As cancer is characterised by uncontrolled cell proliferation, markers of proliferation, such as Ki67 and proliferating cell nuclear antigen, have been studied extensively in neoplastic lesions. Ki67 is a nuclear antigen which is present in the perichromosomal region during mitosis and seems to be a non-histone protein, representing a new class of cell cycle maintaining proteins. Studies on proliferation markers in lesions of the oral mucosa have shown that expression of Ki67 is correlated with the severity of the lesion.

Studies on the relation between p53 and the proliferation index in various malignancies, including lesions of the oral mucosa, have been reported previously. Our aim was to elucidate the relationship, if any, between expression of p53 and Ki67 in normal, non-dysplastic, dysplastic, and neoplastic lesions of the oral mucosa and to determine whether these markers have potential as early indicators of malignancy.

Methods

Fifty tissue samples, five of normal oral mucosa, 30 of leukoplakia and 15 of invasive carcinoma, were studied. Demographic details were recorded for all patients and included age, sex and whether they used tobacco products. Only those patients with oral leukoplakia and carcinoma at non-keratinising sites of the oral cavity were included. A 4 mm punch biopsy specimen was collected from each patient, immediately snap frozen and stored in liquid nitrogen. Cryostat sections, 5 μm thick, were cut from each biopsy specimen and fixed in cold acetone. One section from each biopsy...
The specimen was stained with haematoxylin and eosin for histopathological examination. Dysplasia in leukoplakia samples and differentiation in carcinoma specimens were graded as recommended by the World Health Organisation (WHO). The standard avidin-biotin complex (ABC) (Unitec immunohistochemistry detection system, Oncogene Science Inc. USA) immunohistochemical staining method was used to detect expression of p53 and Ki67, as described previously, using primary monoclonal antibodies directed against p53 (Pab 1801 (Oncogene Science, USA) and DO7 (Dako, Glostrup, Denmark)) and Ki67 (Ki67; Dako). Pab1801 and Ki67 were used at a dilution of 1 in 10 and DO7 at a dilution of 1 in 20. Negative controls (lacking primary antibody) were also included in each assay to assess the specificity of staining. Aminothiolcarbazole (AEC) was used as the chromogen and the slides were counterstained with Mayer’s haematoxylin.

The intensity of immunohistochemical staining was graded as follows: negative, mild, moderate, or intense. The pattern of expression was also analysed semiquantitatively by counting the number of positive cells per 100 basal/basaloid cells and was recorded as a percentage. The area with the maximum number of positive cells was considered in each section. The slides were coded and examined blindly.

The relation between the pattern of expression of both antigens and tumour progression was evaluated statistically. The grade of staining was converted into numerical scores as follows: negative = 0; mild = 2; moderate = 4; and intense = 6. The percentage of positive cells was also scored: 0 = 0–5%; 2 = 6–25%; 4 = 26–60%; 6 = 61–99%. Tumour progression was classified as follows: normal non-keratinising mucosa, stage 0; non-dysplastic mucosa, stage 1; dysplastic mucosa, stage 2; all invasive cancer lesions were grouped together and classified as stage 3. As the scores were not continuous variables, statistical analysis was done using non-parametric methods. The pattern of expression of each protein was compared between histopathological groups using the Mann–Whitney U and Wilcoxon rank sum W tests. Kruskal–Wallis one way analysis of variance was used to test the significance of alterations in expression of p53 and Ki67 during the various stages of tumour progression. The association between the staining pattern and stage of tumour progression was assessed by Spearman’s rank correlation analysis.

Results with confidence intervals above 95% (p < 0.05) were considered significant.

**Results**

**HISTOPATHOLOGY**

Seven (23%) of the 30 patients with leukoplakia had low grade dysplastic features. Excluding dysplastic features, the leukoplakias were classified into proliferative (hyperplastic) and non-proliferative (hyperkeratosis) subtypes based on the presence of basal cell hyperplasia, proliferation and elongation of rete pegs, and thickening of the entire epithelium. Thus, the proliferative group comprised seven leukoplakias, three of which exhibited dysplastic features. Of the carcinomas, nine were well differentiated, four were moderately differentiated and remaining two were poorly differentiated. Comparative analysis of the patients’ clinical details and histopathological features of the lesions did not reveal any significant relations.

**IMMUNOHISTOCHEMISTRY**

Table 1 shows the pattern of expression of Ki67 and p53 in the various oral lesions studied. Both antigens were immunolocalised to the nucleus of basal and parabasal cells of the normal and leukoplakic epithelium (figs 1A and 1B). The two p53 antibodies produced almost identical staining patterns in all tissues evaluated. Interestingly, Ki67 positivity was more pronounced in parabasal than in basal cells. In carcinomas immature basaloid cell nuclei were positive for both antigens (figs 1C and 1D). The staining intensity of both antigens was correlated with the percentage of positive cells—that is, staining was more intense in lesions with larger numbers of positive cells and less so in lesions with fewer positive cells.

In normal mucosa less than 5% of cells expressed p53 in all cases, while in two cases more than 20% of cells expressed Ki67. For convenience, 25% immunopositivity was taken as the cut off value to differentiate low from high antigen expression. Ki67 and p53 were highly expressed in 10 (45%) of the 23 non-dysplastic lesions. Ki67 was highly expressed in four (58%) of the seven dysplastic lesions, whereas p53 was highly expressed in one (13%) only (figs 1A and 1B). Ten (67%) of 15 carcinomas were strongly positive for both Ki67 and p53 (figs 2A and 2B). With regard to the grade of differentiation in carcinomas, poorly differentiated lesions showed more

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**Table 1** Ki67 and p53 nuclear positivity in normal, non-dysplastic, dysplastic, and neoplastic lesions of the oral mucosa

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Percentage nuclear positivity</th>
<th>Ki67</th>
<th>p53</th>
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<tr>
<td></td>
<td></td>
<td>0-5</td>
<td>6-25</td>
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<tr>
<td>Normal (n = 5)</td>
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<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Non-dysplastic (n = 23)</td>
<td></td>
<td>7</td>
<td>6</td>
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<tr>
<td>Dysplastic (n = 7)</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Well differentiated (n = 9)</td>
<td></td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Moderately differentiated (n = 4)</td>
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<td>2</td>
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<td>Poorly differentiated (n = 2)</td>
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prominent alterations in both Ki67 and p53 expression than well differentiated ones. With respect to the proliferation status of the leukoplakias on a histopathological level, Ki67 and p53 exhibited some conspicuous differences in their expression (figs 2A–F). Of the 23 non-proliferative lesions, over 25% of cells were Ki67 and p53 positive in eight (35%) and 11 (48%) cases, respectively. Of the seven proliferative lesions, six (85%) were strongly positive for Ki67; none of the lesions exhibited high expression of p53. Thus, Ki67 and p53 were found to have an inverse relation with respect to the proliferative status of the leukoplakia lesions. This relation was not observed in carcinomas.

**STATISTICAL ANALYSIS**

The mean percentage of Ki67 and p53 positivity in the various histopathological groups is presented graphically in fig 3. Univariate analysis showed a significant difference between the expression pattern of the normal and other histopathological groups for both Ki67 and p53. No significant differences were observed between non-dysplastic, dysplastic and carcinoma groups (table 2). When the patterns of expression of p53 and Ki67 were compared in the different leukoplakias with respect to proliferative status, p53 showed a significant difference (p = 0.0169), but for Ki67 the difference was statistically insignificant (p = 0.0758). Kruskal–Wallis one way analysis of variance and Spearman’s rank correlation analyses showed significance in the expression pattern for both the Ki67 and p53 in relation to tumour progression (table 3). Correlation analysis of the proliferative status of leukoplakias and staining showed a significant negative correlation coefficient for p53 (r_s = -0.4436; p = 0.014), but not for Ki67 (r_s = 0.3297; p = 0.075). Correlation analysis of the percentage of positivity and intensity of staining also showed a significant positive correlation for both the Ki67 (r_s = 0.5143; p = 0.0001) and p53 (r_s = 0.7482; p = 0.0001). The association between the percentage Ki67 and p53 positivity was insignificant (r_s =

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**Figure 1** Immunohistochemical pattern of expression of (A) Ki67 and (B) p53 in dysplastic oral leukoplakia and of (C) Ki67 and (D) p53 in well differentiated squamous cell carcinoma of the mouth (original magnification, x100).
Expression of p53 in leukoplakia and SCC of the oral mucosa

Discussion

Mutations in the p53 gene are the most common genetic changes observed in human carcinomas. Mutant p53 protein is more stable than its wild type counterpart and thus can be visualised by immunohistochemistry. Accumulation of p53 protein in the cell is generally considered to be the result of mutation of the p53 gene. These mutations lead to uncontrolled cell proliferation, resulting in further genetic abnormalities and finally in malignancy. Therefore, the nature of the p53 gene and the proliferative status of a cell are

Figure 2 Immunohistochemical pattern of expression of Ki67 and p53 in non-proliferative (A, haematoxylin and eosin; C, p53; E, Ki67) and proliferative (B, haematoxylin and eosin; D, p53; F, Ki67) oral leukoplakia lesions (original magnification, x250).

Figure 3 Mean (SD) percentage of Ki67 and p53 nuclear positivity in various oral lesions. A = normal mucosa; B = non-dysplastic leukoplakia; C = dysplastic leukoplakia; D = squamous cell carcinoma; E = non-proliferative leukoplakia; F = proliferative leukoplakia.
closely linked and the loss of this linkage is one of the main causes of tumour formation and is considered to be an early event in this process. To analyse the proliferative status of a cell or tissue various markers are available, of which Ki67 is the most widely used and reliable. It recognises a proliferation related nuclear antigen present at all phases of cell cycle except G0.

In the present study 70% of the samples showed greater than 5% positivity for p53 while normal mucosa expressed p53 in less than 5% of cells. Of the leukoplakias 78% of the non-dysplastic and 86% of the dysplastic lesions expressed p53 in more than 5% of cells. In carcinomas most of the lesions (73%) exhibited high expression of p53. The proliferation index, as evaluated by expression of Ki67, was highest in carcinomas (43%) and lowest in normal mucosa (11%). In leukoplakias expression of Ki67 was intermediate and was almost identical in non-dysplastic (24%) and dysplastic (23%) lesions. Previous studies of p53 expression in oral pre-malignant and malignant lesions showed a range of positivity of 35 to 90% and also reported a correlation between p53 positivity and the severity of the lesion. However, a study from Sri Lanka found a low prevalence (11%) of p53 expression in oral carcinoma. The reason for this notable variation in p53 positivity from that observed in the present study, as well as in other reports, is difficult to explain. Both studies were carried out on frozen sections using the same antibody. Interestingly, population differences in ras mutation have been reported previously in oral carcinoma for Indian and UK samples.

A significant relation between the staining pattern and tumour progression has been observed for both Ki67 and p53. Earlier studies using various proliferation markers also reported a similar association between the proliferative index and tumour progression in oral mucosa. However, no correlation was found between the pattern of expression of p53 and Ki67 in oral pre-malignant and malignant lesions. This suggests that p53 expression have a direct effect on the proliferative status of oral lesions, supporting the conclusions of earlier reports. Of the two markers, Ki67 exhibited a more pronounced association with tumour progression, although its expression was more heterogeneous in oral leukoplakias. This variation in expression may be of prognostic importance for the early detection of pre-malignant lesions. These results emphasise the potential use of Ki67 and p53 as biomarkers of carcinogenesis in oral cancer and also may serve as intermediate points for cancer prevention programmes, such as the oral chemopreventive trials.

At the histopathological level a higher percentage of p53 positivity was observed in in non-proliferative hyperkeratotic lesions (mean 33.22 ± 25.8) than in their proliferative counterparts (mean 11.43 ± 8.58). This observation is not compatible with current thinking on the stability of wild type and mutated p53 protein—that is, immunohistochemical positivity for p53 protein in a cell is suggestive of a mutation in the p53 gene with the subsequent induction of proliferation by the mutated protein. In the present study fewer cells expressed p53 in the proliferative than in non-proliferative leukoplakia lesions. The proliferative status of the lesions was confirmed by Ki67 expression; the proliferative lesions have a mean percentage Ki67 positivity of 32.57 ± 21.72 whereas that of the non-proliferative lesions was 21.48 ± 21.72. The results of some recent studies have also raised doubts about the concept of the stability of p53 proteins and inferences of immunohistochemical results (reviewed by Soussi et al). Some mutations lead to transcription or translation errors in the p53 gene, which result in the arrest of p53 protein synthesis and negative staining on immunohistochemistry. Tumours with nonsense or frameshift mutations also result in the production of unstable, truncated proteins, which are also negative on immunohistochemistry. These types of mutations are estimated to account for less than 15% of human tumour mutations, depending on the cancer. Overexpression without p53 gene mutation has also been observed in breast carcinoma. The study on the oral cancers from Sri Lanka also found that of the few immunohistochemically p53 positive cases, none had mutations in exons 5–8, which are considered to be a “hot spot” for mutation. Accumulation of p53 proteins in normal cells followed by certain stresses, such as ultraviolet irradiation and exposure to carcinogens, has also been reported. Hence, it is not possible to determine whether p53 positivity observed in the present study was a result of mutation or the accumulation of normal p53 induced by carcinogens present in tobacco, as all of the patients were chronic betel quid chewers. However, previous studies, including those on head and neck tumours, have compared the results of p53 immunexpression with those of molecular methods and most of them found a significant association between these two techniques. On the basis of the present report, the present result can be interpreted as indicating that factors other than p53, or which act prior to the appearance of p53 aberrations, may be responsible for alterations in the proliferation index of these pre-malignant oral lesions. Molecular analysis of the p53 gene in these tissues to confirm the present findings is essential, and is currently underway. Although the relation between the p53 and Ki67 expression is not clear, especially in pre-malignant oral lesions, the significant correlation between tumour progression and expression of these antigens suggests that they may be useful biomarkers of oral carcinogenesis.

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