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Expression of p53, Cyclin D1 and Ki-67 in Pre-malignant and Malignant Oral Lesions: Association with Clinicopathological Parameters

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Abstract. *In this study, a possible association was examined between the immunoexpressions of p53, cyclin D1, Ki-67 and tobacco exposure and the risk of oral cancer (OC) in pre-malignant and malignant formalin-fixed, paraffin-embedded oral mucosal tissue specimens from patients from Yemen (n=24, all were pre-malignant) and India (n=16, 11 were OCs). Overexpressions of p53, cyclin D1 and Ki-67 were found in 100%, 45.5% and 80% of the OCs, compared to 65.5%, 82.8% and 85.1% of the pre-malignant lesions, respectively. In the pre-malignant lesions, a statistically significant correlation was found between histopathological grading and expressions of cyclin D1 (p=0.001) and Ki-67 (p=0.03), and between anatomical site and expression of Ki-67 (p=0.01). Co-expressions of the three proteins in the cases examined was found to correlate significantly to each other (cyclin D1: p53, r=0.48, p=0.002; p53: Ki-67, r=0.41, p=0.008) except for cyclin D1: Ki-67. These findings suggest that the expressions of p53, cyclin D1 and Ki-67 might contribute to OC susceptibility in oral mucosal lesions examined from Yemen and India. The importance of the three proteins examined as biomarkers in OC and pre-malignant lesions deserves particular attention because it might offer further understanding of the development of these lesions, particularly in populations heavily exposed to tobacco habits. Abnormalities of both cyclin D1 and Ki-67 might play an important role in the development of oral pre-malignant lesions and warrant further studies. Larger studies are, therefore, necessary in the two countries to examine the role of these biomarkers in OCs and pre-malignant oral mucosal lesions.*

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In developing countries, where oral-health-care resources are meagre, oral cancer (OC) is a major health problem (1, 2). The aetiology of this pathology is multifactorial and the most important risk factors include personal habits of tobacco use and alcohol consumption (1-3). Oral leukoplakia is a pre-malignant lesion which usually displays histopathological features of epithelial dysplasia (4). High rates of transformation are found in lesions with epithelial dysplasia, making this a marker of pre-malignancy and, thereby, predictive of the development of OC (5, 6). The number of OC cases in Yemen is reported to be increasing and, during the period 1996-2000, the pattern of distribution of malignant neoplasms in 1491 patients showed that 12% of the cases were head and neck cancers of which 73% were OCs (7). Qat (*Catha edulis* or khat), a plant indigenous to Yemen, Ethiopia and East Africa, was found to be associated with the majority of the OC cases reported from Yemen, with the lesions occurring in areas where qat is the major product (8-11). The high number of OCs observed in Yemen has been attributed to the use of unregulated chemicals for treating qat (8). A study in 1987 reported that some degree of oral keratosis, but not dysplasia or malignancy, was observed among qat users (9). A recent study, involving 1528 cases of qat chewers, demonstrated the development of white oral keratotic lesions

at the site of chewing with a correlation between prevalence and severity of the lesions to duration and frequency of qat use (10). OC is a known major health problem in many parts of the developing world, including India where it accounts for up to 40% of all malignancies (1, 2, 6). The spectrum of OC varies from place to place in India and data from different parts provide various trends and give clues to possible aetiological factors (1, 2, 6). For example, a recent study in patients from the Allahabad region in North India revealed cancer of the tongue to be most prevalent among 759 biopsies from the oral cavity (12). The widespread habit of using tobacco, betel quid (paan) and tobacco-based products plays a major role in the aetiology of the disease in India (12, 13). Although the progress and clinical outcome for patients with OC is largely dependent on the stage of the disease, the biological characteristics of OC are often variable, resulting in divergent clinical disease course despite similar or identical staging (4). In addition, tumour pathology and histological differentiation are also often subjective and are unreliable predictors of disease course (4-6).

D type cyclins, including cyclin D1, are the rate-limiting controllers of the G1-phase progression in mammalian cells and are expressed in the G1- and S-phases of the cell cycle (14). Overexpression of cyclin D1 results in loss of cell cycle control and accelerates progression through the G1-phase (14). The tumour suppressor gene p53, plays a central role in controlling the progression of the cell cycle from the G1-phase to the S-phase (15). Alterations in this gene may provide cancer cells with a growth advantage, leading to uncontrolled proliferation as well as increased p53 protein levels (15). Increased cellular proliferation is associated with more advanced lesions and the distribution of proliferating cells in tissues may tell us more about the regulatory mechanisms that become dysfunctional during carcinogenesis (16). Ki-67 is a nuclear protein expressed in the G2- and M-phases of actively dividing cells (16). This antigen is a proliferation marker that correlates with the presence and severity of epithelial dysplasia (17, 18).

Increased cellular proliferation as a result of changes in proto-oncogene and tumour suppressor genes are believed to drive the development of human cancers (14, 15). These genetic changes are an intimate part of neoplastic development and can serve as markers for the specific changes involved in tumour progression. Moreover, these molecular markers might also allow the classification of a gross tumour into specific stages. The expressions of p53, cyclin D1 and Ki-67 have been examined in pre-malignant and malignant oral mucosal lesions predominantly from the West (17, 19). It was, therefore, the objective of the present study to determine the relative frequency of expression of p53, cyclin D1 and Ki-67 in pre-malignant and malignant oral mucosal lesions from Yemen and India in relation to clinicopathological parameters including qat/tobacco habits.

Materials and Methods

Study subjects. During the period May to November 2001, 24 patients (19 males and 5 females, mean age 39.5±12.36 SE, range 21-60 years) with suspected pre-malignant or malignant oral mucosal lesions presented at the Department of Oral Surgery at the Sana'a Teaching Dental Hospital, University of Sana'a, Yemen. The patients were interviewed on oral habits such as qat use, cigarette smoking and years of usage. The majority of the patients (n=20) were habitual consumers of qat and/or tobacco, while the remaining ones (n=4) did not use qat or any other form of tobacco. Seventeen of the males (89%), with a mean period of 20.5 years of usage, and 3 of the females (60%), with a mean period of 20 years of usage, were found to use qat. Eleven (46%) of the 24 patients were cigarette smokers and 13 (54%) did not smoke. From each patient, a surgical tissue sample was taken, fixed in 10% buffered formalin and embedded in paraffin.

From the period 1996-2002, hospital records of 16 patients (11 males, 5 females, mean age 51.1±12.6 SE, range 30-70 years), previously diagnosed with pre-malignant or malignant oral mucosal lesions in India, were randomly selected from the files of the Department of Pathology, Moti Lal Nehru Medical College, University of Allahabad, India. The corresponding formalin-fixed, paraffin-embedded tissue samples from the 16 cases were obtained and used as control for the cases from Yemen. From the hospital records, 6 of the males (55%) and all the females (100%) were tobacco users (chewers/smokers), with or without further information on the amount consumed daily or weekly. Five of the 16 patients (31%) consumed alcohol. The clinicopathological characteristics of the patients with pre-malignant and malignant oral mucosal tissues obtained from Yemen and India are shown in Table I.

Tissue preparation and evaluation of the haematoxylin and eosin (H&E)-stained sections. From all the formalin-fixed, paraffin-embedded tissue specimens (n=40), serial sections (4-5 μm thick), were prepared and processed for routine histopathological and subsequent immunohistochemical studies. H&E-stained sections were examined under a light microscope and diagnosed according to WHO criteria for histological typing of cancers and pre-cancers of the oral cavity (4). When present, epithelial dysplasias were graded as mild, moderate or severe, while the OCs were graded as well-, moderately- or poorly-differentiated squamous cell carcinomas.

Immunohistochemistry. Forty cases (11 OCs and 29 pre-malignant lesions) were used for the expressions of p53, cyclin D1 and Ki-67 by immunohistochemistry. Sections (on silane-coated slides) were deparaffinized in xylene, rehydrated in graded ethanol and washed in Tris-buffered saline (TBS; pH 7.6). Epitopes were retrieved by heating sections in a microwave oven at high power setting (900W, for a period of 7-9 min) and low power setting (300W for 15 min) using Tris/EDTA (pH 9.0) solution. After cooling (20 min) and washing in TBS (10 min), the sections were incubated with the Dako peroxidase block, 0.03% hydrogen peroxide (H_2O_2) containing sodium azide (Code K4007), for 5 min to eliminate endogenous peroxidase activity. After washing in TBS for 10 min, the sections were incubated with the corresponding primary antibodies for 60 min on the Dako Autostainer Universal Staining System (Dako A/S, Copenhagen, Denmark) using antibodies against p53 (Clone DO-7, Ready-to-use, Autostainer Cat No;

Table I. *Clinicopathological parameters of the patients with pre-malignant and malignant oral mucosal lesions examined from Yemen and India.*

Variable	Yemen samples N (%)	Indian samples N (%)
Total number of samples	24	16
Age mean (yr) (range)	39.5 (21-60)	51.1 (30-70)
Gender		
Male, n (%)	19 (79)	11 (69)
Female, n (%)	5 (21)	5 (31)
Chewing (qat/paan masala)		
Yes, n (%)	20 (83)	4 (25)
No, n (%)	4 (17)	11 (69)
Not known, n (%)		1 (6)
Smoking		
Yes, n (%)	11 (46)	10 (63)
No, n (%)	13 (54)	5 (31)
Not known, n (%)		1 (6)
Anatomical site, n (%)		
Tongue	0	6 (37)
Gingiva	17 (71)	0
Lip/Intraoral	0	3 (19)
Cheek	7 (29)	7 (44)

N158187-2), Ki-67 (MIB-1; dilution 1:100 in Dako Antibody Diluent, Cat No, M724001-2) (both from Dako A/S) and cyclin D1 (NCL-CYCLIN D1, Clone DCS-6, dilution 1:100 in Dako Antibody Diluent; Novocastra Laboratories Ltd., Newcastle-Upon-Tyne, UK). After washing for 10 min in TBS, the sections were incubated with the EnVision Horseradish Peroxidase (DAB) for 30 min, washed twice in TBS for 5 min each, and were further developed twice with the DAB+ chromogen for 5 min each. The sections were counterstained with haematoxylin, rinsed in tap water for 10 min, rehydrated and mounted using the Eukitt mounting medium. Cases in which the primary antibody was omitted and substituted with the diluent TBS served as internal negative controls. Tissue samples of OCs known to show high expression of the proteins, examined from previous studies, were used as positive controls.

Evaluation of the immunohistochemistry and statistical analysis. Whole-tissue sections (including the epithelium adjacent to the non-malignant, pre-malignant and malignant areas when present in the specimens) were examined with a light microscope for p53, cyclin D1 and Ki-67 positive nuclear staining. The positively-stained cells in the pre-malignant and malignant areas of the tissue sections were counted at 400X magnification, in at least 4 randomly selected areas. A minimum of 1000 cells were counted in each section and the percentage was then calculated [labelling index per cent (LI %)]. The staining intensity was graded as negative, mild (nuclear staining of <10% cells), moderate (staining of 10-49% cells) and strong (staining of ≥50% cells). Cells that showed moderate to strong intensity of staining for any of the 3 markers examined were considered to overexpress the corresponding marker. No staining for the 3 markers was observed in the adjacent normal mucosal areas of the tissue samples examined.

SPSS for Windows computer program (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the expression of the proteins examined. The relationship between the clinicopathological parameters and the expression of the 3 markers were analysed with the Kruskal-Wallis analysis of variance (ANOVA). Statistical correlations between the expressions of p53, cyclin D1 and Ki-67 were analysed using Spearman's correlation coefficient.

Results

Immunohistochemical expression of p53, cyclin D1 and Ki-67. Forty cases (29 pre-malignant and 11 OCs), were used for the immunohistochemistry. Among the 11 OCs examined, there were 5 well-, 5 moderately- and 1 poorly-differentiated carcinoma. Of the 29 pre-malignant lesions, there were 2 hyperplasias, 2 Lichen planus, 14 mild, 6 moderate and 5 severe dysplasias. For the immunohistochemistry, cells were considered positive for any of the 3 antigens examined if there was any nuclear staining, regardless of the intensity. In some of the cases, a certain amount of cytoplasmic staining was found. Examples of the immunohistochemical staining of cyclin D1, p53 and Ki-67 found in representative cases of dysplasia (from Yemen and India) and OC (from India) are shown side by side in Figure 1A-I. Among the 40 cases examined, expression of the 3 antigens was limited to the basal and parabasal cell layers in the hyperplasias and the Lichen planus, but it was widely distributed in the mild, moderate and severe dysplasias. The staining with Ki-67 was found to be high (>50% of the cells) and it showed a strong intensity in the oral dysplasias. In the OCs, however, most of the malignant cells were strongly stained for the 3 proteins examined.

p53 nuclear staining was found in all of the OCs (100%) examined. Four of these (36%) showed moderate staining in the infiltrating tumour cells, while 7 (64%) showed strong staining of the infiltrating tumour cells. For the 29 pre-malignant lesions, strong staining for p53 was found in 10 cases (34%), moderate staining in 9 cases (31%) and weak staining in 4 cases (14%), while in 6 of the cases (21%) no staining was found. No statistically significant correlations ($p>0.05$) were found between p53 expression and the patients' clinicopathological parameters in the pre-malignant as well as the OC cases examined.

Expression of cyclin D1 was found in 10 (91%) of the 11 OCs examined. Overall, 5 cases (45%) showed weak nuclear staining, 4 cases (36%) showed moderate nuclear staining and 1 case (9%) showed strong staining of the infiltrating tumour cells. For the 29 pre-malignant lesions examined, positive staining of cyclin D1 was found with different levels of expression, being strong in 4 cases (14%), moderate in 20 cases (69%), mild in 2 cases (7%) and absent in 3 (10%) cases. Overexpression of cyclin D1 was found to be statistically significant ($p=0.001$) when correlated to the histopathological grade of the pre-

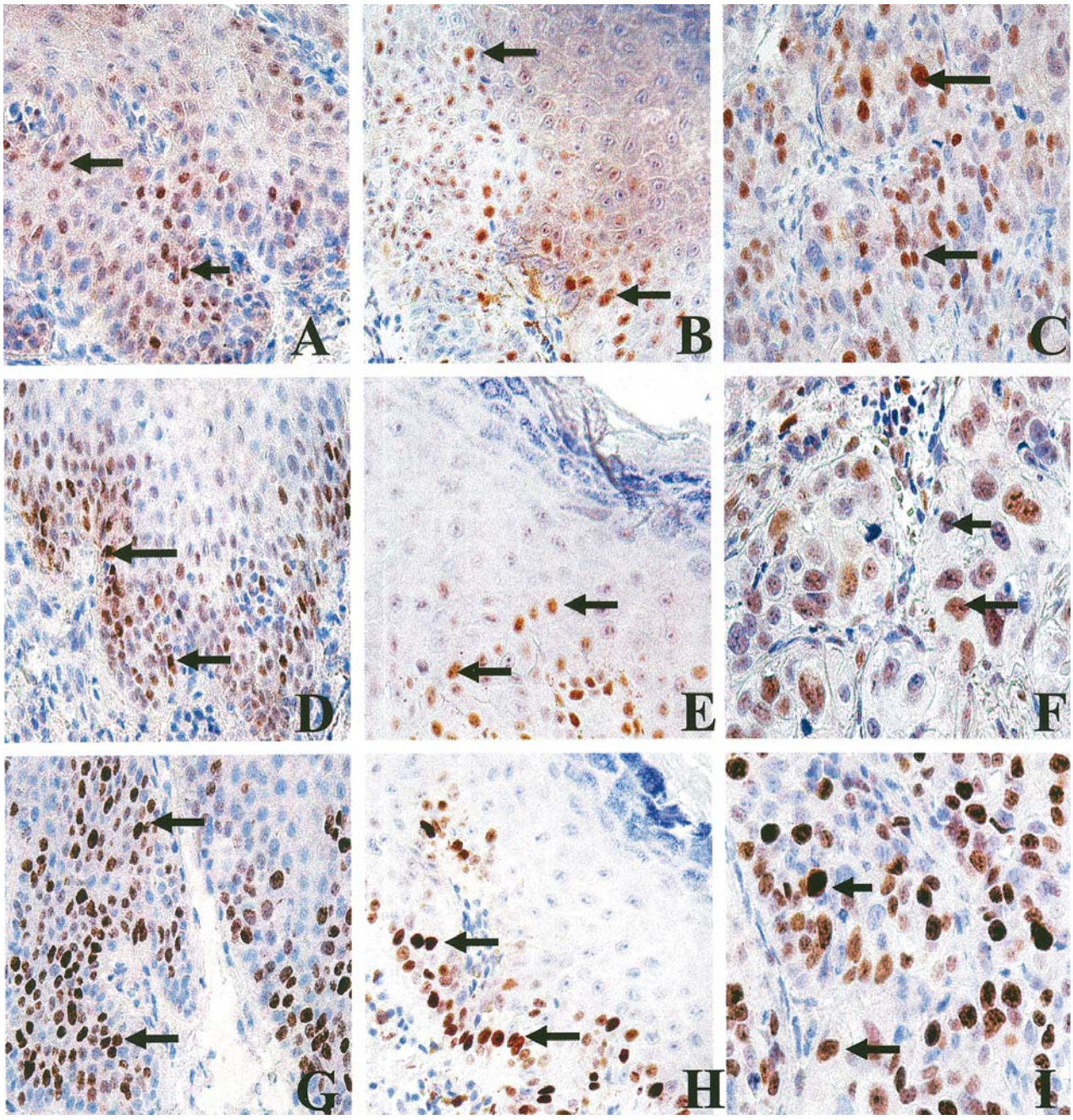

Figure 1. *Immunohistochemical positive staining of cyclin D1 (panels A-C), p53 (panels D-F) and Ki-67 (panels G-I) in a pre-malignant oral mucosal lesion from Yemen (left panel) and India (middle panel) and in an oral squamous cell carcinoma from India (right panel). Arrows indicate positively staining cells.*

malignant lesions (Table II). However, no statistical association was found between the expression of cyclin D1 and other clinicopathological parameters such as gender, tobacco habits, anatomical site/or histological grade of the OCs examined ($p>0.05$). Nevertheless, cyclin D1 was found to be more frequently overexpressed in the pre-malignant cases from qat/tobacco chewers than in those from non-chewers, but the difference was not statistically significant ($p>0.05$).

For Ki-67, expression was found in 9 (90%) of the 10 OCs examined. The staining was found to be strong in 6 cases (60%), moderate in 2 cases (20%) and mild in 1 case

Table II. *Relationship between expression of cyclin D1, p53 and Ki-67 and histopathological differentiation in the pre-malignant and malignant oral mucosal lesions examined from patients from Yemen and India.*

	Cyclin D1 expression			p53 expression			Ki-67 expression		
Variable	-ve	<10%	>10%	-ve	<10%	>10%	-ve	<10%	>10%
Histopathological differentiation									
Pre-malignant lesions									
HP (n=2)	1	1	0	2	0	0	0	1	1
LP (n=2)	1	1	0	2	0	0	1	1	0
Mild dysplasia (n=14)	0	0	14	2	2	10	1	0	13
Mod/ Severe dysplasia (n=11)	1	0	10	0	2	9	0	0	9
P-value			0.001*			NS			0.03*
Site									
Cheek (n=10)	1	1	8	2	2	6	1	1	7
Lip (n=2)	1	1	0	0	1	1	0	1	0
Gingiva (n=17)	1	0	16	4	1	12	1	0	16
P-value			NS			NS			0.01*
Malignant lesions									
Well (n=5)	1	3	1	0	0	5	1	0	4
Mod/ Poor (n=6)	0	2	4	0	0	6	0	1	4
P-value			NS			NS			NS
Site									
Tongue (n=6)	0	3	3	0	0	6	1	1	4
Cheek (n=4)	1	2	1	0	0	4	0	0	3
Oral cavity (n=1)	0	0	1	0	0	1	0	0	1
P-value			NS			NS			NS

LP, hyperplasia; LP, lichen planus; *Statistically significant; NS (not significant).
-ve, no cells stained; <10%, less than 10% cells stained; >10%, more than 10% cells stained.

(10%). For the pre-malignant lesions, expression of Ki-67 was examined in 27 of the 29 cases. The nuclear staining pattern for this marker was found to be strong in 10 cases (37%), moderate in 13 cases (48%), weak in 2 cases (7%) and absent in 2 cases (7%). In 11 (41%) of the 27 cases, mixed cytoplasmic and nuclear staining was found. A statistically significant correlation was found between the expression of Ki-67 and the anatomical site of the lesions ($p=0.01$) as well as with the histopathological grade in the pre-malignant cases examined ($p=0.03$) (Table II).

The results of the immunohistochemical expressions of p53, cyclin D1 and Ki-67 in the pre-malignant oral mucosal lesions and the OCs examined from Yemen and India were graphed as shown in Figure 2. Using Mann-Whitney analysis, the mean LI of p53 expression was found to be significantly higher in the OCs (z=2.199: $p=0.028$), while the mean LIs of cyclin D1 and Ki-67 expressions were not statistically significant in all of the cases examined. The pre-malignant oral mucosal lesions examined from Yemen had a statistically significant high mean of LIs for expression of cyclin D1 (z=2.145: $p=0.028$) and Ki-67 (z=2.330: $p=0.020$), when compared to all lesions examined from India. However, the mean of LIs for p53 expression did not show any statistically significant differences between cases examined from the 2 countries.

Co-expression of p53, cyclin D1 and Ki-67. Co-expression of p53 and cyclin D1 was noted in 78% (n=31) of the 40 cases examined with a significant positive correlation between the 2 proteins (r=0.48; $p=0.002$; Figure 3A). In addition, p53 and Ki-67 were co-expressed in 80% (n=32) of the cases examined, showing a statistically significant correlation for the 2 proteins (r=0.41; $p=0.008$; Figure 3B). Co-expression of cyclin D1 and Ki-67 was noted in 75 % (n=30) of the cases. However, there was no statistically significant correlation between the 2 markers (r=0.29; $p=0.071$; Figure 3C).

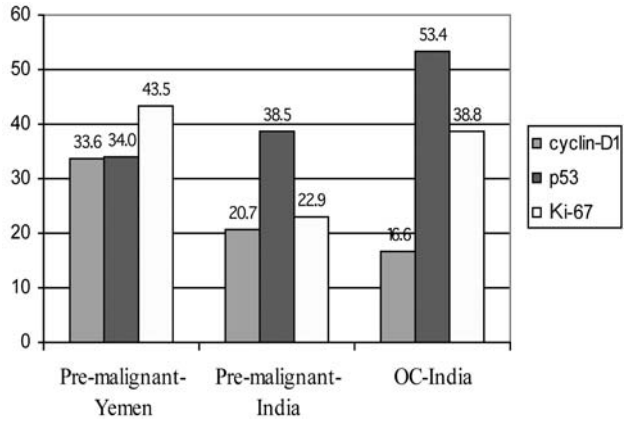

Figure 2. *Histogram for analysis of the mean LI% of the expressions of cyclin D1, p53 and Ki-67 in the pre-malignant and malignant oral mucosal lesions examined from patients from Yemen and India.*

Discussion

Extensive studies related to the expressions and mutations of the cell cycle regulatory genes and cell proliferative activity have been done in pre-malignant and malignant oral mucosal lesions from the West (reviewed in 18, 19). However, only a few studies have been carried out in India (20, 21) and almost none in Yemen. Oral carcinogenesis is a multistep process in which occurrence of a series of genetic events may lead to dysregulation of the cell cycle (19, 22). Usually, expression of p53 protein is very low in normal cells and under normal conditions it prevents the propagation of genetically-damaged cells (15). In many studies including oral lesions, the reported incidence of p53 expression has varied from 11 to over 85% (23) – probably as a result of the substantial differences in detection techniques used as well as the varied oral habits practised in different geographical regions and races (23). In cases examined from patients from India, p53 expression has been shown to be high in oral pre-malignant and malignant lesions (23-25). In our study, all of the OCs and 66% (19/29) of the pre-malignant lesions examined from Yemen and India overexpressed p53. This overexpression was seen in 81% (17/21) of the cases from smokers and in 72% (13/18) of those from non-smokers, albeit with no significant correlations to any of the patients' clinicopathological parameters. Few studies have examined the expression of

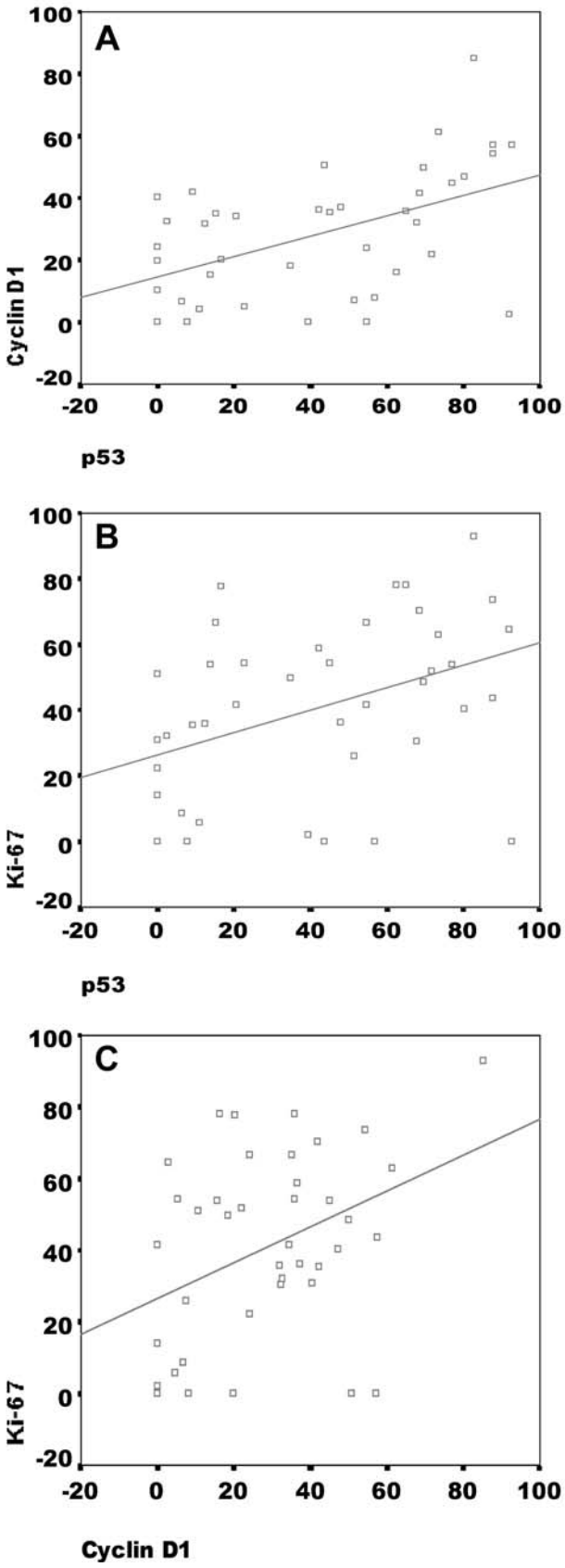

Figure 3. *Analysis for correlations between A) cyclin D1 and p53 expressions, B) Ki-67 and p53 expressions, and C) Ki-67 and cyclin D1 expressions in the pre-malignant and malignant oral mucosal lesions examined from patients from Yemen and India.* →

cyclin D1 in oral lesions and reported prevalence rates ranging from low (27%) to high (71%) (26-28). This discrepancy may be attributed to difficulties in assessing cyclin D1 in formalin-fixed, paraffin-embedded tissue sections by immunohistochemistry (29). We observed overexpression of cyclin D1 in 46% (5/11) of the OCs and in 83% (24/29) of the pre-malignant lesions examined. Several studies have described expression of cyclin D1 in various sites within the oral cavity, with the expression being more often detected in sites like the tongue, retromolar region, palate and gingiva (28). These variations in expression by sites have been proposed to be related to racial differences and varying environmental risk factors (28). We found a positive correlation between cyclin D1 expression and anatomical sites of incidence, with the expression being most common in the gingiva and cheek. Interestingly, most of the gingival lesions overexpressing cyclin D1 were pre-malignant lesions from qat chewers from Yemen, while the cheek lesions were OCs from Indian patients. The findings on OC patients from India might be explained by the habit of keeping oral tobacco in the buccal pouch just before going to sleep – ensuring long contact between the buccal mucosa and the potential carcinogen. In this work, cyclin D1 was found to be overexpressed in 75% (18/24) of the cases from qat/paan masala chewers compared to 80% (12/15) of those from non-chewers, though it is worth noting that some of the non-chewers were smokers and/or consumers of alcohol. These findings might indicate the importance of cyclin D1 as a molecular marker in pre-malignant and malignant oral mucosal lesions associated with tobacco habits. The importance of these findings warrants further studies to understand the importance of cyclin D1 expression in these lesions. In oral mucosal lesions, the expression of Ki-67 has been reported to increase according to the proliferative activity and degree of epithelial dysplasia, suggesting that it is a marker of the presence and severity of epithelial dysplasia (18). Recent studies have reported expression of Ki-67 at the tumour infiltrating front (TIF) of OCs with a strong positive correlation to the histological grading of the carcinoma (30). In our study, staining with Ki-67 was found to be quite high, with a stronger intensity of staining than the other 2 markers examined, especially in the oral dysplasias. It is of great interest to note that Ki-67 overexpression was found in 83% (20/24) of the cases from qat/tobacco chewers and in 67% (10/15) of those from non-chewers.

In OCs, co-expression and correlation between p53 and Ki-67 have been demonstrated, suggesting that alterations in the p53 protein might lead to increased cell proliferation (31). Furthermore, overexpressions of p53 and Ki-67 have been suggested to be reliable indicators for OC development (31). A similar observation was made with cyclin D1, suggesting that its overexpression may be related to local invasion and a more aggressive clinical behaviour of the cancers (32). A positive correlation between the expressions of cyclin D1 and Ki-67 in OCs has been found, suggesting that these 2 proteins were markers of late disease stage and that cyclin D1 was closely associated with high proliferation levels (32). Similar positive correlations between the overexpressions of cyclin D1 and p53 have been shown by other studies (28, 33). In this work, p53 and Ki-67 were found significantly correlated and cyclin D1 and p53 expressions were also found to be positively related to each other in 78% of the cases examined with a significant correlation between the 2 markers. Albeit, there were some cases in which the expression of the 3 markers examined occurred independently of each other.

OC, like other cancers, is a disease where the control of cell growth is dysregulated. Over the past few years, the loss of growth-suppressing activity itself during the pathogenesis of the tumour has been recognised as just as important. The balance however, between the two may be critical in determining the final phenotype of the cell. In summary, the findings of the present work suggest that overexpressions of the p53, cyclin D1 and Ki-67 proteins might contribute to oral cancer susceptibility in oral mucosal lesions examined from patients from Yemen and India. Overexpressions of the 3 protein markers examined were found to positively correlate to each other, but none of them was significantly related to qat/tobacco habits. However, and to validate these findings, larger studies are necessary in oral lesions from the two populations.

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