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Original

Translational approach utilizing COX-2, p53, and MDM2 expressions in malignant transformation of oral submucous fibrosis

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Abstract: About 20% of the world's population uses some form of betel nut, which suggests that the incidence of oral submucous fibrosis (OSF) is higher than current estimates. OSF has the potential to undergo malignant transformation; thus, there is a need to identify relevant markers to assess its aggressiveness. We evaluated changes in COX-2, p53, and MDM2 expressions in progressive OSF. Expressions of COX-2, p53, and MDM2 increased with OSF progression. There was a strong association between COX-2 overexpression and recurrence of oral squamous cell carcinoma ($P < 0.001$) and a positive relation between increased MDM2 expression and failure of radiotherapy ($P = 0.007$). These findings suggest that COX-2 is an important marker of disease progression and that MDM2 expression is useful for treatment planning. (J Oral Sci 57, 169-176, 2015)

Keywords: oral submucous fibrosis; COX-2; p53; MDM2.

Introduction

Oral submucous fibrosis (OSF) is a chronic disorder characterized by fibrosis of the mucosa lining the upper digestive tract and has the potential for malignant transformation (1). It is a progressive, scarring, debilitating disorder and is not limited to Southeast Asian countries, as previously believed. In reality, it has been observed in many countries and poses a serious public health problem. OSF was believed to be an idiopathic condition but is in fact multifactorial in origin. Although capsaicin and micronutrient deficiencies are important, areca nut consumption is the main factor (2). Oral squamous cell carcinoma (OSCC) is believed to be the most common malignant neoplasm of the oral cavity, and is associated with OSF in Southeast Asian countries (3). The poor overall survival rates for oral cancer patients in the last two decades highlight the need to elucidate the molecular interplay of relevant markers, as increased understanding would enable early identification of malignant transformation and timely intervention for successful management of OSCC (4).

The *COX-2* gene is an immediate early-response gene that is triggered by growth factors, oncogenes, carcinogens, and tumor-promoting phorbol esters. p53 is encoded by *TP53* and is a key transcription factor that controls cellular pathways and plays a vital role as a tumor suppressor protein (5). p53 is negatively regulated through interaction with the oncoprotein mouse double minute 2 (MDM2) (5). In cancer, the *TP53* gene is often mutated or deleted, or wild-type p53 is inhibited by MDM2, resulting in downregulation of tumor-suppressive p53 pathways. Inhibition of MDM2-p53 interaction is an interesting therapeutic strategy in cancer treatment (5,6). In this study, we determined expressions of COX-2, p53, and MDM2 during various stages of OSF and in OSCC associated with OSF (OSCC+OSF). The roles of p53 and MDM2 as early prognostic indicators in malignant transformation of OSF were also examined. To our knowledge, this is the first attempt to evaluate COX-2,

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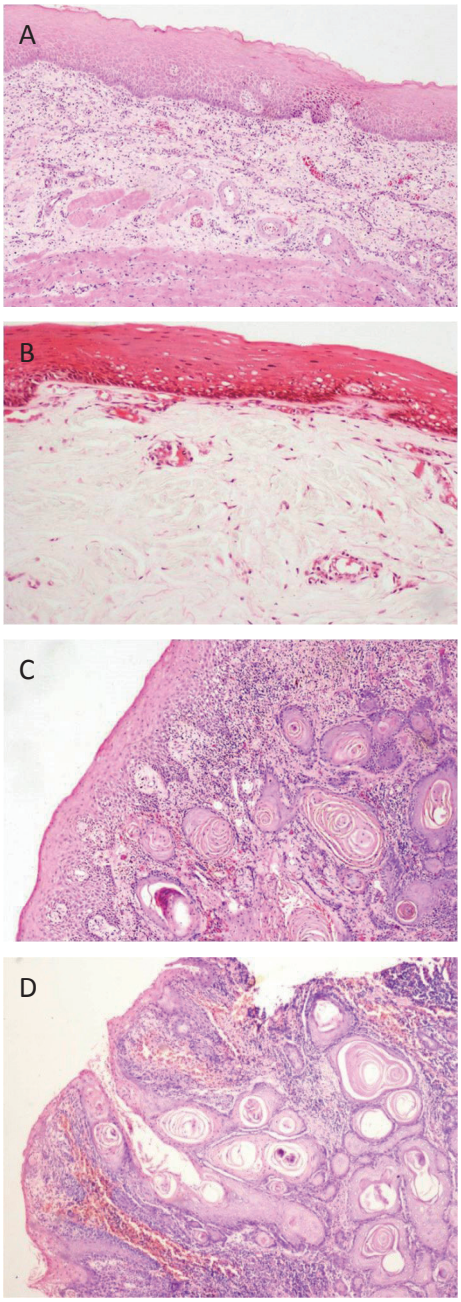

Fig. 1 Representative H&E staining of EOSF (A), AOSF (B), OSCC+OSF (C), and OSCC-OSF (D) specimens (original magnification ×4).

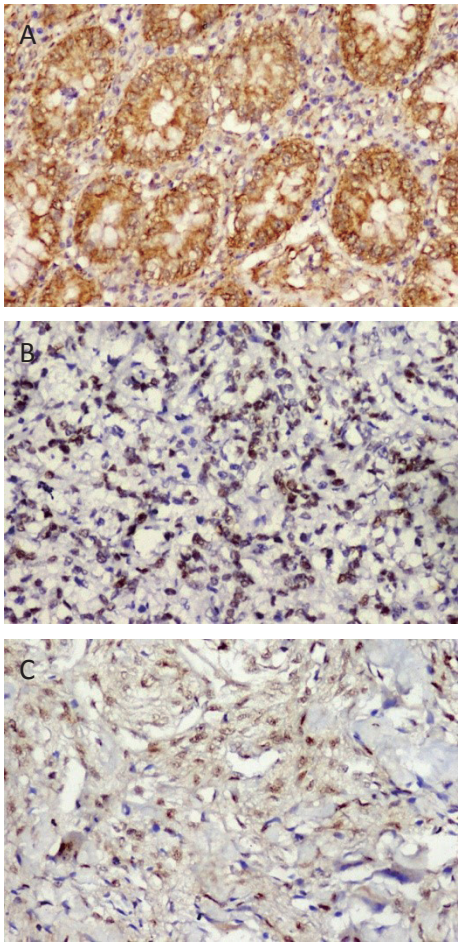

Fig. 2 A, Image of ulcerative colitis (COX-2 stain; original magnification ×20); B, image of carcinoma of the colon (p53 stain; original magnification ×20); C, MDM2 immunopositivity in osteosarcoma (original magnification ×20).

p53, and MDM2 as a means to develop an algorithm to assess malignant transformation of OSF and shed light on treatment planning.

Materials and Methods

This retrospective chart analysis used formalin-fixed, paraffin-embedded tissue specimens obtained from departmental archives, after obtaining approval from the Institutional Ethics Committee of Kasturba Hospital, Manipal (IEC 422/2013). The study group comprised histologically confirmed specimens (n = 10 each) of early oral submucous fibrosis (EOSF), advanced oral submucous fibrosis (AOSF) (7), well-differentiated OSCC associated with OSF (OSCC+OSF), and well-differentiated OSCC in patients with no history of OSF (OSCC-OSF).

Specimens with juxta-epithelial areas of early hyalinization, collagen in separate thick bundles, moderate numbers of fibroblasts, congested blood vessels, and an inflammatory component composed of lymphocytes, eosinophils, and occasional plasma cells were classified as EOSF (Fig. 1A). Specimens with completely hyalinized collagen, no or few fibroblasts, completely obliterated blood vessels, and an inflammatory component predominantly composed of lymphocytes and plasma cells were classified as AOSF (Fig. 1B). Specimens in which the connective tissue stroma contained dysplastic epithelial cells arranged in nests, cords, and islands and with

features of cellular pleomorphism, vesicular nuclei with prominent nucleoli, individual cell keratinization, and keratin pearl formation were classified as OSCC+OSF (Fig. 1C) or OSCC-OSF (Fig. 1D). All 20 of the patients from which OSCC specimens had been collected in this study had been followed up for a minimum of 5 years. Specimens from patients in poor general health and those from patients with unhealthful oral habits other than areca nut chewing were excluded.

Immunohistochemistry was performed with an avidin-biotin technique and 4-μm sections placed on slides coated with 3-aminopropyltriethoxysilane (APES) (8,9), utilizing commercially prepared Leica Novocastra (Newcastle upon Tyne, UK) antibodies for COX-2, p53, and MDM-2 (NCL-COX-2, RTU-p53-DO7, and NCL-MDM2, respectively). The clone and Ig class of COX-2, p53, and MDM2 were 4H12 and IgG1 (kappa), DO7 and IgG2b, and 1B10 and IgM, respectively. Briefly, the sections were deparaffinized, rehydrated, and quenched for endogenous peroxidase activity. Epitope retrieval in sodium citrate buffer (pH 6.0) under pressure, nonspecific antigen blocking, and incubation with primary antibody was done using monoclonal antibody raised in mouse. COX-2 and MDM2 were diluted at 1:100, whereas p53 was ready to use. The sections were then covered with a polymer penetration enhancer (ie, post-primary block), which was followed by incubation with secondary antibody. Antigen-antibody binding was detected with a DAB chromogen system, and sections were counterstained with Mayer's hematoxylin. The recommended positive controls—including ulcerative colitis for COX-2 (Fig. 2A), colon carcinoma for p53 (Fig. 2B), and osteosarcoma for MDM2 (Fig. 2C)—were used, and expressions of these markers were also evaluated in morphologically normal oral epithelium.

Positive COX-2 exhibited cytoplasmic staining, while p53 and MDM2 stained the nucleus. COX-2-positive cells were evaluated semi-quantitatively in five representative fields at 20× magnification (10). The scoring criteria were as follows: 0, no stained cells; 1, less than 25% of cells stained positively; 2, 25% to 50% of cells stained positively; 3, 50% to 75% of cells stained positively; and 4, greater than 75% of cells stained positively (10). Staining was also assessed in the basal, suprabasal, and keratin layers (11). For antibodies staining nuclei (p53 and MDM2), the labeling index (LI) was calculated by using image analysis software (Image J), as follows:

LI = (No. of positive cells / No. of cells) × 100

Statistical analysis was carried out using SPSS version 16.0 for Windows. One-way ANOVA was used to assess statistical differences in the percentage of positive cells between the EOSF, AOSF, OSCC+OSF, and OSCC-OSF specimens. The independent sample *t* test was used to compare groups. A *P* value of less than 0.05 was considered to indicate statistical significance in all analyses. Bivariate correlation analysis was used to assess COX-2, p53, and MDM2, and Pearson's correlation coefficients (r) were calculated.

Table 1 Clinical characteristics of patients

Group	Age	Sex	Site	Treatment
EOSF	30	Male	BM	M
EOSF	58	Female	BM	M
EOSF	49	Male	BM	M
EOSF	40	Female	BM	M
EOSF	35	Female	BM	M
EOSF	48	Male	BM	M
EOSF	30	Male	BM	M
EOSF	51	Male	BM	M
EOSF	55	Female	BM	M
EOSF	30	Male	BM	M
AOSF	30	Male	BM+LM+T	M
AOSF	26	Male	BM+LM+T	M+S
AOSF	21	Male	BM+LM+T+SP	M
AOSF	56	Male	BM+LM+T	M+S
AOSF	53	Male	BM+LM+T	M
AOSF	34	Female	BM+LM+T	M+S
AOSF	24	Male	BM+LM+T	M
AOSF	33	Female	BM+LM+T	M
AOSF	45	Male	BM+LM+T	M+S
AOSF	41	Female	BM+LM+T+SP	M
OSCC+OSF	40	Male	BM	S+RT
OSCC+OSF	30	Female	T	S
OSCC+OSF	58	Male	BM	RT
OSCC+OSF	45	Male	BM	S
OSCC+OSF	63	Male	BM	S
OSCC+OSF	35	Female	BM	RT
OSCC+OSF	51	Male	BM	RT
OSCC+OSF	47	Female	T	S+RT
OSCC+OSF	57	Female	BM	RT
OSCC+OSF	66	Male	BM	RT
OSCC-OSF	24	Male	BM	S
OSCC-OSF	46	Female	BM	S
OSCC-OSF	49	Male	T	S+RT
OSCC-OSF	59	Male	T	RT
OSCC-OSF	73	Female	BM	S
OSCC-OSF	51	Male	BM	RT
OSCC-OSF	54	Male	BM	RT
OSCC-OSF	66	Female	RMP	RT
OSCC-OSF	78	Male	T	RT
OSCC-OSF	63	Female	T	RT

Site: BM, buccal mucosa; LM, labial mucosa; T, tongue; SP, soft palate, RMP, retromolar pad

Treatment: M, medical treatment of OSF, including antioxidants, nutritional supplements, and mouth opening exercises; S, surgery; RT, radiotherapy.

Results

The specimens were collected from 25 men and 15 women (mean age, 46.1 ± 14.3 years) who had chewed areca nut habitually for 18.1 ± 4.8 years. The characteristics of the patients (group, age, sex, site of involvement, and treatment) are shown in Table 1. All EOSF cases had

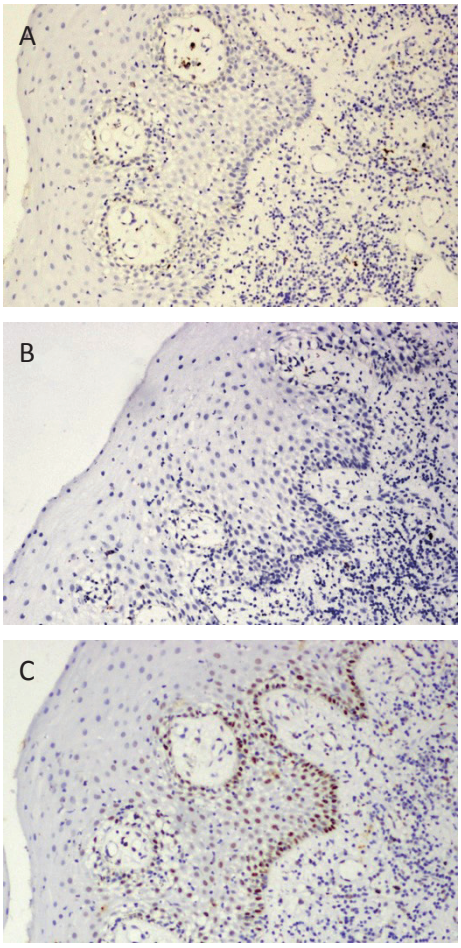

Fig. 3 A, Absence of COX-2 staining in normal oral mucosa (original magnification ×20); B, absence of immunoreactivity to p53 (original magnification ×20); C, immunopositive basal cells in normal oral mucosa (original magnification ×20).

bilateral involvement of the buccal mucosa and clinically evident blanching. Among the AOSF cases, in addition to buccal mucosa, eight patients had involvement of the labial mucosa and tongue and two had involvement of the labial mucosa, tongue, and soft palate. In the OSCC+OSF group, six patients had a unilateral ulcer on the buccal mucosa, two had an ulcer on the tongue, and two had an ulceroproliferative lesion on the buccal mucosa. In the OSCC-OSF group, five patients had an ulcer on the buccal mucosa, four had an ulceroproliferative lesion on the tongue, and one had an ulcer on the retromolar pad.

Neither COX-2 nor p53 was expressed in morphologically normal oral epithelium, whereas immunoreactivity to MDM2 was detected in basal region (Figs. 3A-C).

COX-2 staining was predominantly cytoplasmic and was restricted to basal cells in EOSF samples (Fig. 4A) but extended suprabasally in AOSF cases (Fig. 4B). The

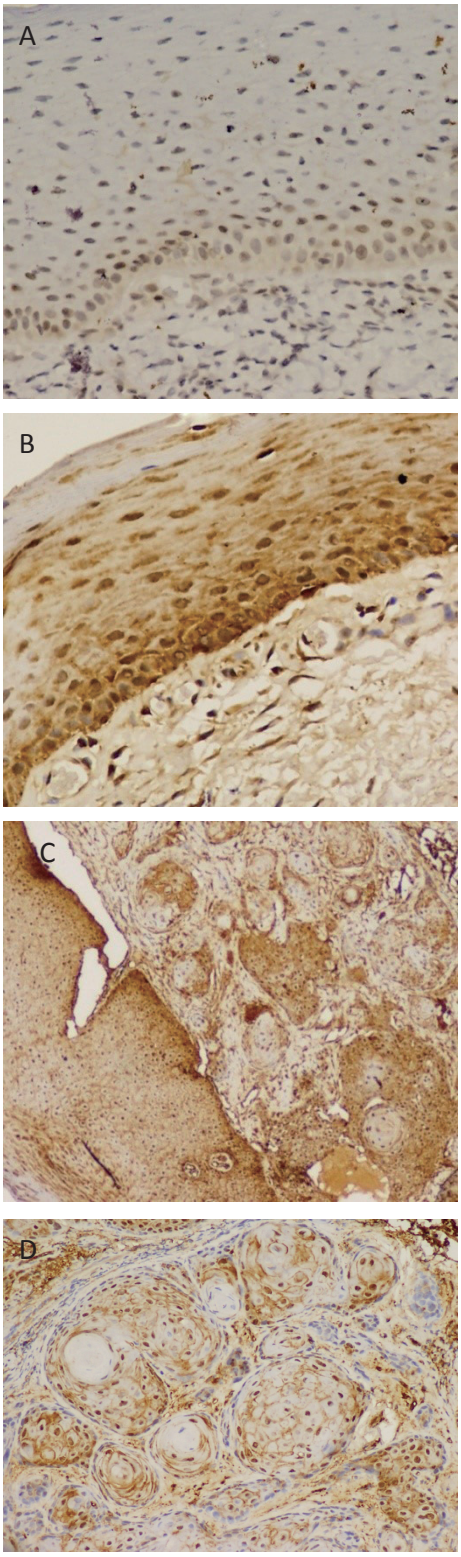

Fig. 4 A, COX-2 staining chiefly restricted to basal cells in an EOSF specimen (original magnification ×20); B, COX-2 staining extending suprabasally in an AOSF specimen (original magnification ×20); C, immunopositivity throughout the epithelium and invading islands in an OSCC+OSF specimen (original magnification ×10); D, epithelial islands showing intense COX-2 staining in an OSCC-OSF specimen (original magnification ×20).

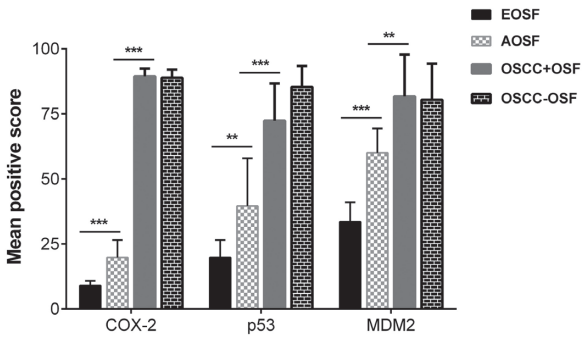

Fig. 5 Mean positive scores for COX-2 and LIs for p53 and MDM2 in EOSF, AOSF, OSCC+OSF, and OSCC-OSF specimens. ** $P < 0.01$, *** $P < 0.001$

Table 2 Intensity of COX-2, p53, and MDM2 immunopositivity in study groups

Marker	Group	Staining intensity				*P* value
		–	+	++	+++	
COX-2	EOSF	2	8	0	0	<0.001
	AOSF	0	0	8	2	
	OSCC+OSF	0	0	0	10	
	OSCC-OSF	0	0	1	9	
p53	EOSF	10	0	0	0	<0.001
	AOSF	0	1	8	1	
	OSCC+OSF	0	0	1	9	
	OSCC-OSF	0	0	2	8	
MDM2	EOSF	0	9	1	0	<0.001
	AOSF	0	8	2	1	
	OSCC+OSF	0	0	1	9	
	OSCC-OSF	0	0	1	9	

mean positive score was 1 (<25% cells positive) for eight EOSF cases and 0 (no cells stained) for two EOSF cases. The mean positive score was 2 (25-50% positive) for nine AOSF cases and 1 for the remaining case. There was a significant difference in COX-2 score between EOSF and AOSF cases. The difference in staining pattern for COX-2 expression between EOSF and AOSF was statistically significant ($t = 9.0$, $P < 0.001$). All OSCC cases (OSCC+OSF and OSCC-OSF) showed strong COX-2 immunoreactivity throughout the epithelium; the mean positive score was 4 (>75% cells positive) (Figs. 4C, 4D). There was a significant difference between OSF and OSCC cases ($t = 11.13$, $P < 0.001$), but no significant difference was noted between the scores for OSCC+OSF and OSCC-OSF cases (Fig. 5).

Cytoplasmic staining was also assessed in terms of the intensity of the immunopositive reaction. A statistically significant difference was observed among groups, and intensity was higher in OSCC cases (+/−OSF) than in EOSF cases and extended to suprabasal cells (Table 2).

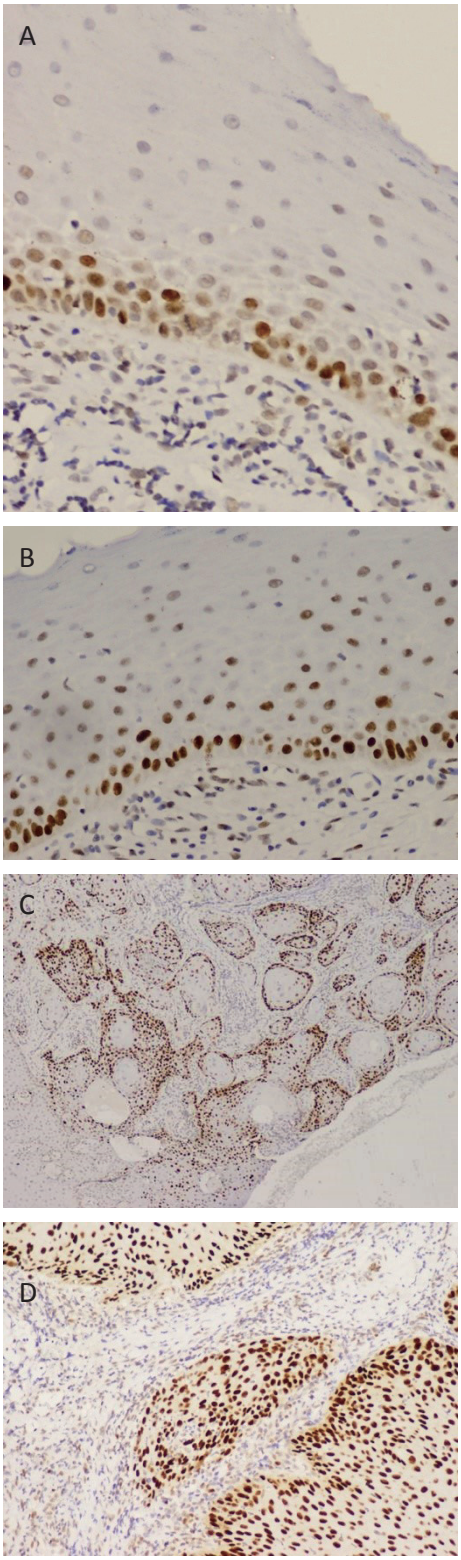

Fig. 6 A, An EOSF specimen with p53 expressed mainly in basal epithelial cells (original magnification ×20); B, p53 expression extends suprabasally in an AOSF specimen (original magnification ×20); C, p53 immunoreactivity in the epithelium and infiltrating islands in an OSCC+OSF specimen (original magnification ×10); D, epithelial islands revealing p53 staining in an OSCC-OSF specimen (original magnification ×20).

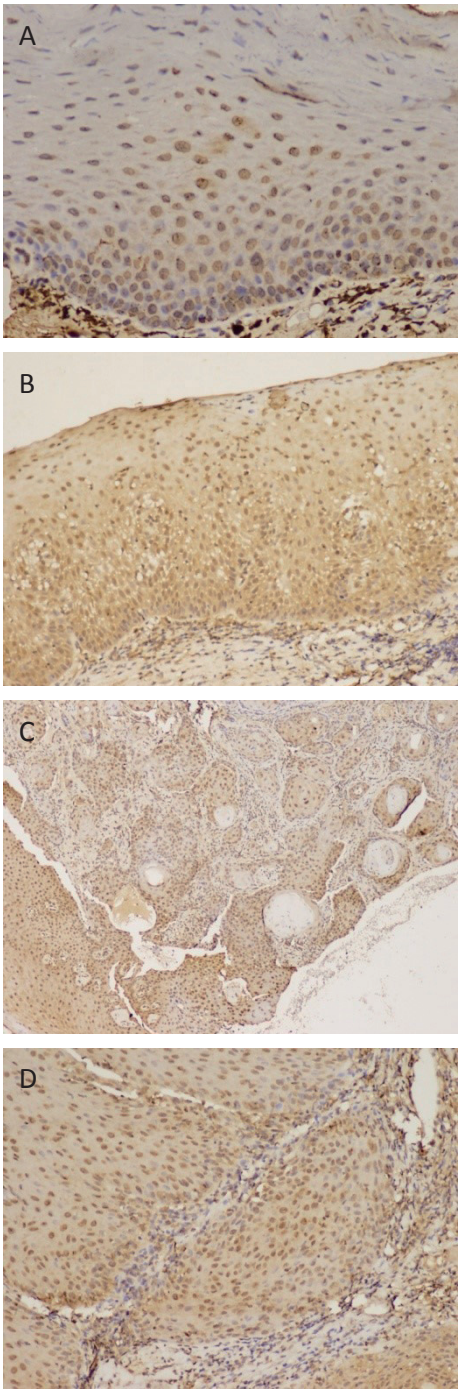

Fig. 7 A, MDM2 is taken up by basal and suprabasal cells in an EOSF specimen (original magnification ×20); B, MDM2 is expressed throughout the epithelium in an AOSF specimen (original magnification ×20); C, MDM2 positivity in the epithelium and advancing islands in an OSCC+OSF specimen (original magnification ×10); D, epithelial islands showing MDM2 staining in an OSCC-OSF specimen (original magnification ×20).

All EOSF samples showed p53-positivity restricted to basal cells. However, in AOSF cases immunoreactivity extended into the suprabasal layer in 7/10 samples. In the remaining cases, only basal cells showed positivity (Figs. 6A, 6B). The difference in mean score was statistically significant (t = 3.3, P = 0.004) between the two OSF groups. Both OSCC groups (Figs. 6C, 6D) had positively stained cells in all epithelial layers. There was a statistically significant difference in mean LI score between the AOSF and OSCC+OSF cases (t = 7.5, $P < 0.001$). Mean LI score did not significantly differ between OSCC+OSF and OSCC-OSF cases (Fig. 5).

Table 3 Mean positive score for COX-2 and LI scores for p53 and MDM2 in OSCC cases

Marker	Recurrence		*P* value
	No	Yes	
COX-2	70.43 ± 3.51	88.46 ± 2.63	<0.001
p53	71.14 ± 19.68	79.92 ± 10.97	0.17
MDM2	67.14 ± 20.68	88.54 ± 7.52	0.007

Immunoreactivity to MDM2 extended into the suprabasal layer in 4/10 EOSF cases and 6/10 AOSF cases (Figs. 7A, 7B); in the remainder positivity was restricted to the basal cell layer. All OSCC samples showed positivity in all cell layers of the epithelium (Figs. 7C, 7D). There was a statistically significant difference in mean LI score between OSF groups (t = 7.0, $P < 0.001$) and in LI score between AOSF and OSCC+OSF cases (t = 3.7, P = 0.002). Mean LI did not significantly differ between the OSCC+OSF and OSCC-OSF groups (t = 0.2, P = 0.8) (Fig. 5).

There were 13 cases of recurrent OSCC (six OSCC+OSF cases and seven OSCC-OSF cases). The recurrent cases had a higher mean score for COX-2 positivity and greater LIs for p53 and MDM2 as compared with non-recurrent cases. This association was statistically significant with regard to COX-2 score and the LI for MDM2 (Table 3). Among recurrent cases, 3 were treated with surgery and postoperative radiotherapy and 10 were treated with radiotherapy only. Among non-recurrent cases, 6 / 7 were treated with surgery and only 1 was treated with radiotherapy.

One-way ANOVA revealed that expressions of COX-2, p53, and MDM2 increased as OSF progressed ($P < 0.001$ for all three markers). The bivariate correlation test showed a strong positive correlation between COX-2 and the LI for p53 (r = 0.93, $P < 0.001$) and MDM2 (r = 0.79, $P < 0.001$) (Fig. 8).

Discussion

Early detection and prompt intervention can prevent malignant transformation of OSF (7). Examination of OSF pathogenesis reveals that localized mucosal

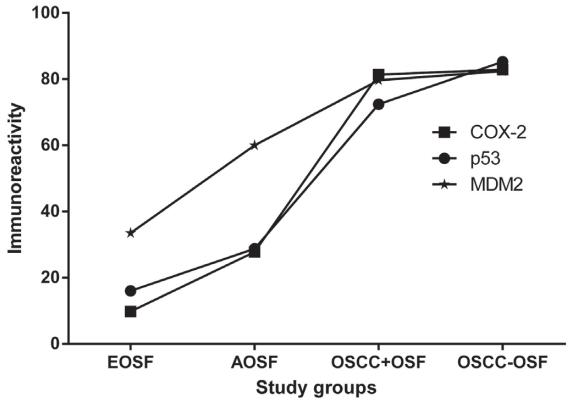

Fig. 8 Mean immunoreactivity against IHC markers in the study groups.

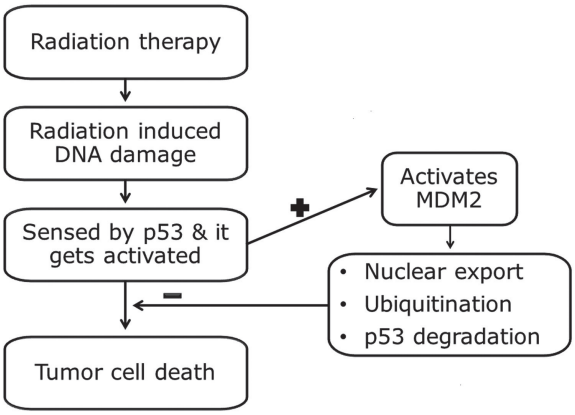

Fig. 9 Positive induction of MDM2, which leads to suppression of tumor cell death by p53 and radiotherapy failure.

irritation and inflammation leads to recruitment of T lymphocytes and macrophages, which brings cytokines and signaling molecules into play. This culminates in abnormal collagen metabolism, through various pathways (4).

COX-2 overexpression is closely associated with tumor recurrence in OSCC (12). Enhanced synthesis of prostaglandins due to increased COX-2 activity increases proliferation of neoplastic cells, promotes angiogenesis, and inhibits immune surveillance (9,12). Additionally, COX-2 overexpression inhibits apoptosis and increases invasiveness (10).

In vitro studies indicate that buccal mucosal fibroblasts do not constitutively express COX-2 (9). However, when fibroblasts are challenged with agents causing OSF, upregulation of COX-2 has been observed as early as 30 minutes later (9). Our findings regarding COX-2 expression suggest that as OSF progresses the population of epithelial cells immunoreactive for COX-2 also increases. This indicates that COX-2 may be an important marker of disease progression. Our finding regarding increased COX-2 expression in OSCC is similar to the results of Itoh et al. (12), who found that disease-free and overall survival were significantly worse in patients with COX-2 overexpression. Taken together, these findings indicate that COX-2 immunoreactivity might be modulated by the interaction of stromal cells and cancer cells during progression to advanced disease or invasion. COX-2 expression in AOSF and OSCC with or without associated OSF might be a reliable prognostic indicator.

Degradation of p53 has been implicated in OSCC pathogenesis. Cellular stresses, such as DNA damage and oncogenic signals, activate the appropriate p53 pathways, leading to cell cycle arrest (8,11). Overexpression of p53 in premalignant conditions suggests malignant transformation, and this has been observed in OSF cases, in which expression is believed to be as high as 70% (13). Moreover, p53 staining in the parabasal region is strongly associated with cancer progression (11,14).

In healthy individuals, p53 levels are strictly controlled by the p53 negative regulator, MDM2. Binding of MDM2 protein to the p53 protein leads to exportation of p53 out of the nucleus into the cytoplasm, where it is degraded through the ubiquitin pathway (8). Thus, in healthy persons, p53 is not detected, but MDM2, a protein activated by p53, can be traced. With DNA damage, the ATM kinase enzyme is activated, which leads to phosphorylation of p53 at Ser15, Thr18, or Ser20. This obstructs formation of the p53-MDM2 complex. With increased p53 activity, there is a burst of MDM2 (8).

A study of the relation between expression of wild-type p53 and genetic aberrations found that p53-positive tumors had a higher allelic imbalance (15). Even limited exposure to tobacco carcinogens is thought to have a mutagenic effect on *TP53* (6). In dysplastic lesions, the positive predictive value of suprabasal staining in developing carcinomas was 86%, which suggests that it may be a marker for early detection (14).

The MDM2 gene is a proto-oncogene and is amplified in 25% to 40% of all human cancers and 40% to 80% of all OSCC cases (16). Although there was no significant difference in MDM2 expression between the two OSCC groups in our study, Girod et al. (8) found that MDM2 expression was prevalent in OSCC associated with betel nut chewing and was associated with worse outcomes. Our study did agree with theirs in finding that combined overexpression of p53 and MDM2 correlated with disease progression.

The p53 molecule is a known fundamental factor in cancer and is thus central in the development of anti-

cancer therapies (17). Understanding the p53-MDM2 pathway might hasten development of a tailored treatment algorithm. p53-induced suppression is important in cancer treatment, as radiotherapy and postoperative radiation damage rapidly proliferating cells. This is sensed by elements in the p53 pathway and eventually results in tumor cell death (18). In the MDM2-controlled cascade, p53 is exported to the cytoplasm and undergoes degradation, which leads to poor radiotherapy outcomes and failure of locoregional control (Fig. 9). A study of patients receiving postoperative irradiation found that a single-nucleotide polymorphism of MDM2 was linked to poor prognosis if patients were treated with radiotherapy. This might be due to MDM2-related p53 degradation that results in treatment failure (19).

Despite the social burden of OSCC, reports indicate that translational research on OSCC is inadequate (13,20). The present preliminary findings and prudent application of molecular markers may provide a critical breakthrough in the development of appropriate treatment protocols.

The intensity of COX-2 expression is associated with OSF progression, induction of p53 indicates malignant transformation, and greater MDM2 activity suggests a worse prognosis. Therefore, we propose the use of targeted drug therapy instead of postoperative radiotherapy in cases of MDM2 overexpression. Use of these three immunohistochemical markers and confirmation of the present results in a larger study may assist in treatment planning and improve clinical outcomes. To our knowledge, this is the first study to use COX-2, p53, and MDM2 for the development of an algorithm that can predict malignant transformation of OSF and shed light on possible therapeutic interventions.

Conflict of interest

The authors have no conflicts of interest to declare.

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