RESEARCH ARTICLE

Editorial Process: Submission:12/29/2023 Acceptance:12/20/2024

P53 and *Ki67* Biomarkers are Predictors for Malignant Transformation in Oral Submucous Fibrosis: A Prospective Study

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Abstract

Objectives: Oral submucous fibrosis (OSMF) is a potentially malignant disorder (PMD) characterized by a high rate of malignant transformation (MT). OSMF exhibits atrophic epithelium yet has a high proliferation rate. Both *p53* and *Ki67* are nuclear proteins associated with cell proliferation, detectable in the early stages of oral cancer (OC). This study aimed to analyze the efficacy of *p53* and *Ki67* immuno-expression as tools for predicting malignant transformation in OSMF cases. The objective was to correlate the expression of *p53* and *Ki67* with demographic and chewing habits data. **Materials and Methods:** The study group consisted of 60 histopathologically diagnosed cases of OSMF, 60 cases of OC as positive controls, and 60 cases of NOM as negative controls. Immunohistochemistry was performed on neutral-buffered formalin-fixed, paraffin-embedded tissue sections of 3 µm thickness, using ready-to-use anti-human *p53* protein (clone DO-7) and monoclonal antibody for *Ki67* antigen (clone MIB-1). Statistical analysis was conducted using SPSS software version 21, employing the chi-square test ($p < 0.05$). **Results:** The expression of *p53* and *Ki67* was significantly higher in OSMF samples compared to NOM samples, but lower than in OC samples. When the expression levels of both *p53* and *Ki67* were correlated with demographic and chewing habits data, the results were statistically significant. **Conclusion:** The overexpression of *p53* and *Ki67* may contribute to the progression of MT in OSM. Early detection of these biomarkers is crucial for preventing MT, which also helps reduce the morbidity and mortality of OC. Therefore, both *p53* and *Ki67* can serve as predictive biomarkers for the early detection of MT in high-risk OSMF patients.

Keywords: Oral cancer- malignant transformation- immunohistochemistry- early detection- correlation

Asian Pac J Cancer Prev, **25 (12)**, 4129-4136

Introduction

Oral Submucous Fibrosis (OSMF) is a potentially malignant disorder (PMD) that significantly affects a large population in India and other South East Asian countries [1]. In India, oral cancers (OC) account for 30 to 40% of all reported cancer cases [2], and approximately 90% of these oral malignancies are believed to develop from pre-existing potentially malignant disorders (PMDs) [3].

Recent epidemiological studies indicate a significant rise in the prevalence of OSMF [4] and in India, the number of OSMF cases has surged rapidly, exceeding two million in the last decade [5]. The rate of malignant transformation (MT) in the Indian subcontinent has been reported to be as high as 7.6% over a span of 17 years (Murti et al., 1985).

Previous data and intervention studies indicate that areca nut (AN) is the primary etiological factor for OSMF. It is also the fourth most commonly used psychoactive substance in the world, following nicotine, ethanol, and caffeine [2, 1, 6]. The activation of oncogenes, inactivation of tumor suppressor genes (TSG), and increased cellular proliferation are critical events in the multistep process of carcinogenesis. One of the key hallmarks of MT is uncontrolled growth, which is commonly evidenced by increased cell proliferation [7]. The high proliferative activity, basal cell hyperplasia, rapid exfoliation of superficial cells, and epithelial atrophy all indicate a high epithelial turnover rate in OSMF [8].

It is suggested that genetic alterations in specific signaling pathways are believed to play critical roles in OC tumorigenesis and progression [9]. Both *p53* and *Ki67* are nuclear proteins associated with cell proliferation. Evidence indicates that *p53* is the most frequently mutated gene in human cancers, with up to 80% of OCs harboring mutated *p53* tumor suppressor genes (TSGs). This high mutation frequency underscores the significant role of this gene in carcinogenesis [10]. The accumulation of

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p53 protein within cells is generally considered to result from mutations in the *p53* gene [7]. *Ki67* is a proliferative marker present in all phases of the cell cycle except the G0 phase and the early G1 phase. Its expression increases during the S phase and reflects the cell's proliferative capacity. Due to its relative resistance to internal and external influences, *Ki67* is considered an excellent marker for assessing cell proliferation [11].

OSMF exhibits atrophic epithelium but has a high proliferation rate that cannot be detected under hematoxylin and eosin (H&E) staining by assessing dysplasia [5]. The proliferative activity of any tissue or neoplasm can be determined by its growth rate using antibodies directed against specific antigens expressed by proliferating neoplastic cells [12].

The rising incidence of OC is a major concern for public health, as it is one of the most prevalent types of cancer in India. Due to late-stage detection, the chances of a cure are very low, with five-year survival rates around 20%. The primary reason for late detection is the lack of awareness about early symptoms of OC [13]. Early diagnosis of PMDs is crucial to prevent their progression into OC. However, there is limited data on biomarkers such as *p53* and *Ki67* in OSMF and its MT at the molecular level. This study aims to analyze the efficacy of *p53* and *Ki67* expression as tools for MT in OSMF. It also seeks to correlate the expression of these biomarkers with different types of habits, duration, and frequency in OSMF patients.

Materials and Methods

Study Design, and Participants

This prospective cross-sectional observational study was conducted in the Department of Oral Medicine and Radiology at the School of Dental Sciences, KIMSDU, Karad, Satara, Maharashtra, from April 2018 to April 2021. Patients were selected from the outpatient department (OPD) of Oral Medicine and Radiology using a purposive/ subjective sampling technique. Ethical clearance from the institutional ethics committee and written informed consent (in the local language Marathi) from all patients were obtained prior to the start of the study.

Samples Size

A total of 180 cases were considered for this study, including 60 cases of OSMF and OC in individuals with habits of chewing areca nut (AN) in processed forms, tobacco, and other products, along with 60 cases of normal oral mucosa (NOM) without any habits. Normal tissues were obtained from the buccal mucosa of 60 patients during the surgical removal of their third molars, serving as negative controls, while OC patients served as positive controls.

The inclusion criteria for the study encompassed individuals clinically diagnosed with OSMF and OC who had these habits. Exclusion criteria included medically compromised patients and those who did not provide written informed consent. Demographic details were recorded for all patients. The diagnosis of OSMF and OC was based on characteristic clinical features as described by Shih et al. [14] and Carreras-Torras et al. [15]. All patients underwent incisional biopsy, and clinical diagnoses were confirmed histopathologically.

Biopsied tissue specimens were fixed in 10% neutral buffered formalin for 24 to 48 hours and embedded in paraffin wax using standard procedures. The tissue sections, cut to a thickness of 3 μm, were stained with Hematoxylin and Eosin (Loba Chemie Pvt. Ltd, Mumbai, India) for histopathological diagnosis. Additional sequential sections were prepared for immunohistochemical (IHC) studies.

Immunohistochemistry protocol

All 180 tissue sections were available for high-quality immunohistochemical (IHC) staining. The sections on frosted slides were deparaffinized in xylene for three cycles of 5 minutes each, and then rehydrated in different concentrations of ethanol (Dako Denmark).

Antigen retrieval was performed in Envision FLEX target retrieval solution, high pH (Dako; K8004), containing Tris-EDTA buffer pH 9, for 30 minutes in an autoclave. After washing with distilled water at 25ºC, the slides were incubated with Envision FLEX wash buffer containing Tris-buffered saline solution with Tween 20, pH 7.6 (Dako; K8007), for 20 minutes, followed by blocking with Envision FLEX peroxidase blocking reagent (Dako Denmark), which contains phosphate buffer with 15 mmol/L hydrogen peroxide, sodium azide, and detergent (Dako; SM801). Following 20 minutes of H_2O_2 blocking, the sections were incubated directly with primary antibodies, anti-human *p53* protein (clone DO-7) and *Ki67* antigen clone MIB-1, ready to use (Dako Autostrainer/ Autostrainer Plus, Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark), for 1 hour at room temperature in a humidity chamber.

Subsequently, the sections were washed with wash buffer for 5 minutes, followed by treatment with Envision FLEX/HRP goat secondary antibody against rabbit and mouse immunoglobulins coupled with peroxidase molecules (Dako; SM802). After completing the 1-hour incubation with the secondary antibody, sections were washed with distilled water for 5 minutes. The sections were then stained with Envision FLEX DAB + Chromogen (3,3' diaminobenzidine tetrahydrochloride) (Dako; DM827) in Envision FLEX substrate buffer containing hydrogen peroxide and preservative (Dako; SM803) until a brownish-red color developed. The sections were counterstained with hematoxylin for 2-3 minutes after washing with distilled water. Once dried, the slides were dipped in 100% ethanol and then xylene to clear the sections. The slides were mounted in DPX mountant and observed under a Primovert Phase contrast microscope (Carl Zeiss).

Scoring system

The immunostains were evaluated by a pathologist based on the percentage of positive cells and the intensity of the staining. They were categorized as follows:

• Negative (-): Less than 5% of cells staining positive.

• Low (+): Between 5% and 50% of cells staining positive.

• Intermediate (++): Between 50% and 75% of cells staining positive.

• High (+++): More than 75% of cells staining positive.

Statistical analysis

Data were entered and analyzed using the Statistical Package for the Social Sciences, version 21 (SPSS 21, IBM Corporation, United States). The chi-squared test was employed to analyze the differences in intensity levels among NOM, OSMF, and OC. Differences with a probability value of ≤0.05 were considered statistically significant.

Results

Distribution of respondents by demographic variables

Table 1 provides a descriptive demographic analysis. The majority of OSMF patients were in the age range of 21-30 years (28 patients, 46.7%), followed by those aged 31-40 years (25 patients, 41.7%). In the case of OC, the majority were aged 41-50 years (20 patients, 33.3%), followed by those aged 51-60 years (19 patients, 31.7%). All OSMF and OC patients had attained low levels of education and belonged to the low to middle socioeconomic classes.

Expression of p53 in OSMF, OC and in Controls

Table 2 illustrates the expression of *p53* in OSMF, OC, and control groups. Approximately 30 (50.0%) OSMF cases exhibited high expression of *p53*, followed by 18 (30.0%) with intermediate expression, and 5 (8.3%) with low expression. Tissue loss was observed in 2 (3.3%) cases, and 5 (8.3%) cases showed negative expression. In OC, 49 (81.7%) cases showed high expression, 6 (10.0%) cases showed low expression, and 4 (6.7%) cases showed intermediate expression. Among the control groups, 54 (90.0%) cases displayed negative or very low expression, and 6 (10.0%) cases exhibited low expression. The expression of *p53* in OSMF, OC, and controls was found to be highly statistically significant (Figure 1, 2 and 3).

Expression of Ki67 in OSMF, OC and in Controls

Table 3 illustrates the expression of *Ki67* in OSMF, OC, and control groups. About 51 (85.0%) OSMF cases exhibited high expression of *Ki67*, while 3 (5.0%) cases each showed low and intermediate expression. Tissue loss was observed in 2 (3.3%) cases, and 1 (1.7%) case showed negative expression of *Ki67*. In OC, 57 (95.0%) cases exhibited very high expression, and only 3 (5.0%) cases showed intermediate expression. Among the control

Table 1. Distribution of Respondents by Demographic Variables

Variable	OSMF (60)	OC (60)	Controls (60)	Total (180)	Pearson Chi-Square
Gender	n (%)	n (%)	n (%)	n (%)	
Male	53 (88.3)	50 (83.3)	38 (68.3)	141 (78.3)	p=0.002
Female	7 (11.7)	10 (16.7)	22 (36.7)	39 (21.7)	
Age					
<20	3 (5.0)	0 (0.0)	0 (0.0)	3 (1.7)	p<0.001
21-30	28 (46.7)	0 (0.0)	30 (50)	58 (32.2)	
31-40	25 (41.7)	8 (13.3)	25 (41.7)	58 (32.2)	
41-50	1 (1.7)	20 (33.3)	5 (8.3)	26 (14.4)	
51-60	3 (5.0)	19 (31.7)	0 (0.0)	22 (12.2)	
>60	0 (0.0)	13 (21.7)	0 (0.0)	13 (7.2)	
Socioeconomic status					
Low	27 (45.0)	19 (31.7)	13 (21.7)	59 (32.8)	p< 0.001
Middle	29 (48.3)	38 (63.3)	29 (48.3)	96 (53.3)	
Higher	4 (6.7)	3 (5)	18 (30)	25 (13.9)	
Educational status					
Illiterate	4 (6.7)	5 (8.3)	4 (6.7)	3 (7.2)	p= 0.026
Below graduates	38 (63.3)	32 (53.3)	21 (35)	91 (50.6)	
Graduates	18 (30)	23 (38.3)	35 (58.3)	76 (42.2)	

Table 2. Expression of *p53* in OSMF, OC and in Controls

p53 expression	OSMF =60 n(%)	OSCC=60 n(%)	Control=60 n(%)	Total=180 n(%)
Negative/very low	5 (8.3)	1 (1.7)	54 (90.0)	60 (33.3)
Low	5 (8.3)	6 (10.0)	6 (10.0)	17 (9.4)
Intermediate	18 (30.0)	4 (6.7)	0 (0.0)	22 (12.2)
High	30 (50.0)	49 (81.7)	0 (0.0)	79 (43.9)
Tissue loss	2 (3.3)	0 (0.0)	0 (0.0)	2 (1.1)
Total	60 (100)	60 (100)	60 (100)	180 (100)

Pearson Chi-Square = p< 0.001

Table 3. Expression of *Ki67* in OSMF, OC and in Controls

Ki67 expression	OSMF =60 n (%)	OSCC=60 n (%)	Control=60 n (%)	Total=180 n (%)
Negative/very low	1 (1.7)	0 (0.0)	57 (95.0)	55 (30.6)
Low	3 (5.0)	0 (0.0)	3 (5.0)	9 (5.0)
Intermediate	3 (5.0)	3 (5.0)	0 (0.0)	6 (3.3)
High	51 (85.0)	57 (95.0)	0 (0.0)	108 (60.0)
Tissue loss	2 (3.3)	0 (0.0)	0 (0.0)	2 (1.1)
Total	60 (100.0)	60 (100.0)	60 (100.0)	180 (100.0)

Pearson Chi-Square = p< 0.001

Table 4. Correlation of Expression of *p53* in Relation to Habits, duration and Frequency in OSMF Patients

Chewing Habits	Very low(%)	Low (%)	Intermediate (%)	High (%)	Tissue loss (%)	Total (%)	Pearson Chi-Square
AN + pan	0 (0.0)	3 (37.5)	0 (0.0)	4 (50.0)	1 (12.5)	8 (100)	p< 0.034 (S)
AN in processed form+pan+tobacco +alcohol	1 (7.7)	0 (0.0)	6 (46.2)	6 (46.2)	0 (0.0)	13 (100)	
AN in processed form	4 (10.3)	2 (5.1)	12 (30.8)	20 (51.3)	1 (2.6)	39 (100)	
Total	5 (8.3)	5 (8.3)	18 (30.0)	30 (50.0)	2 (3.3)	60 (100)	
Duration of chewing habits							
1-4yrs	2 (9.1)	4 (18.2)	5 (22.7)	10 (45.5)	1 (4.5)	22 (100)	p< 0.338 (NS)
5-9 yrs	2 (6.9)	1 (3.4)	12 (41.4)	13 (44.8)	1 (3.4)	29 (100)	
≥ 10yrs	1 (11.1)	0 (0.0)	1 (11.1)	7 (77.8)	0 (0.0)	9 (100)	
Total	5 (8.3)	5 (8.3)	18 (30.0)	30 (50.0)	2 (3.3)	60 (100)	
Frequency of chewing habits							
1-4p/d	4 (22.2)	4 (22.2)	7 (38.9)	3 (16.3)	0 (0.0)	18 (100)	p=0.006(S)
5-9p/d	1 (3.0)	1 (3.0)	7 (21.2)	22 (66.7)	2 (6.1)	33 (100)	
≥ 10 p/d	0 (0.0)	0 (0.0)	4 (44.4)	5 (55.6)	0 (0.0)	9 (100)	
Total	5 (8.3)	5 (8.3)	18 (30.0)	30 (50.0)	2 (3.3)	60 (100)	

yrs, years; p/d, packets per day; S, significant; NS, nonsignificant

group, 57 (95.0%) cases displayed negative or very low expression, and 3 (5.0%) cases exhibited low expression. The expression of *Ki67* in OSMF, OC, and controls was found to be highly statistically significant (Figure 1, 2 and 3).

Correlation of expression of p53 in relation to habits, duration and frequency in OSMF patients

When the expression of *p53* was correlated with chewing habits, duration, and frequency, the following patterns emerged (Table 4). Among the 60 OSMF cases,

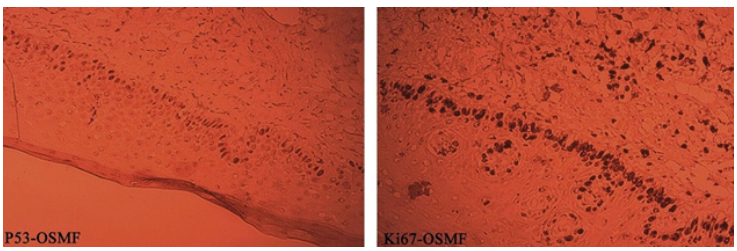

Figure 1. Expression of *p53* and *Ki67* in OSMF

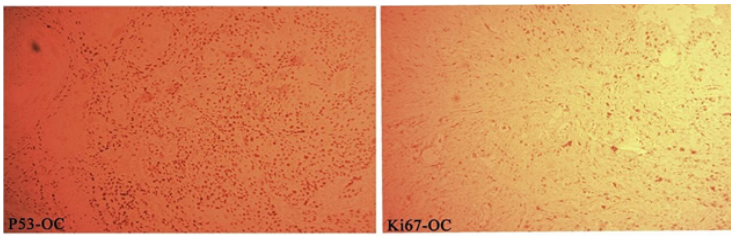

Figure 2. Expression of *p53* and *Ki67* in OC

Table 5. Correlation of Expression of *Ki67* in Relation to Habits, duration and Frequency in OSMF Patients

	Very low (%)	Low (%)	Intermediate (%)	High (%)	Tissue loss (%)	Total (%)	Chi-Square
Chewing Habits							
AN in processed forms	1 (2.56)	2 (5.12)	2 (5.12)	32 (82.05)	2 (5.12)	39 (100)	p<0.001 (S)
AN + pan	1 (12.5)	5 (56.5)	1 (12.5)	1 (12.5)	0 (0.0)	8 (100)	
AN processed form +pan +tobacco+ alcohol	0 (0.0)	1 (7.96)	10 (76.92)	2 (15.38)	0 (0.0)	13 (100)	
Total	2 (3.33)	8 (13.33)	13 (21.66)	35 (58.33)	2 (3.33)	60 (100)	
Duration of Chewing Habits							
1-4yrs	2 (9.09)	8 (36.36)	7 (31.81)	5 (22.72)	0 (0.0)	22 (100)	p=0.004 (S)
5-9 yrs	0 (0.0)	0 (0.0)	5 (17.24)	23 (79.31)	1 (3.44)	29 (100)	
≥ 10yrs	0 (0.0)	0 (0.0)	1 (11.11)	7 (77.77)	1 (11.11)	9 (100)	
Total	2 (3.33)	8 (13.33)	13 (21.66)	35 (58.33)	2 (3.33)	60 (100)	
Frequency of Chewing Habits							
1-4p/d	2 (11.11)	6 (33.33)	6 (33.33)	4 (22.22)	0 (0.0)	18 (100)	p=0.0043 (S)
5-9p/d	0 (0.0)	2 (6.06)	6 (18.18)	24 (72.72)	1 (3.03)	33 (100)	
≥ 10 p/d	0 (0.0)	0 (0.0)	1 (11.11)	7 (77.77)	1 (11.11)	9 (100)	
Total	2 (3.33)	8 (13.33)	13 (21.66)	35 (58.33)	2 (3.33)	60 (100)	

yrs, years; p/d, packets per day; S, significant

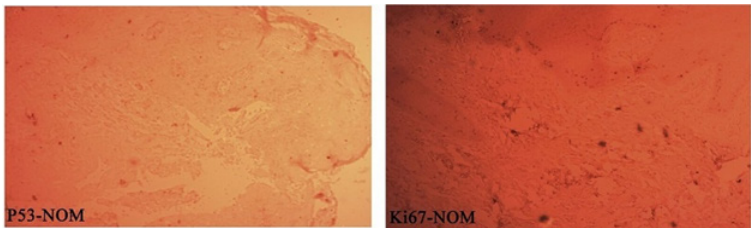

Figure 3. Expression of *p53* and *Ki67* in NOM

more than half (39 cases, 65%) chewed processed AN. Within this group, 20 cases (51.3%) showed high *p53* expression, followed by 12 cases (30.8%) with intermediate expression. Of the 29 cases (48.33%) who had been chewing for 5-9 years, 13 cases (44.8%) showed high expression and 12 cases (41.4%) showed intermediate expression. Among the 33 cases (55%) who chewed 5-9 packets per day, 22 cases (66.7%) exhibited high *p53* expression. Both habits and frequency showed a statistically significant correlation, indicating that as the habits and duration of chewing increased, the expression of *p53* also increased.

Correlation of expression of Ki67 in relation to habits, duration and frequency in OSMF patients

When the expression of *Ki67* was correlated with chewing habits, duration, and frequency (Table 5), it was observed that 32 cases (82.05%) showed high expression when using processed AN. Among the 60 OSMF cases, 23 cases (79.31%) exhibited high *Ki67* expression when they had chewed AN in processed form for 5 to 9 years, and 24 cases (72.72%) showed high expression when chewing AN 5 to 9 times daily. These correlations were statistically significant.

Discussion

Epidemiological data indicate a significant increase in the prevalence of OSMF. Approximately 80% of OCs in India are associated with the use of processed AN, betel quid, and tobacco, along with other ingredients [4]. The present study observed a male predominance in both OSMF and OC, with a male-to-female (M:F) ratio of 7.6:1 in OSMF and 5:1 in OC. Similar male predominance was reported in studies conducted by Panday et al. [16] with a ratio of 6.8:1 and by Goel et al. [17] with a ratio of 5:1.

In cases of OSMF, the majority of patients were aged 21-30 years (28 cases, 46.7%), followed by those aged 31-40 years (25 cases, 41.7%). In contrast, most OC cases occurred in the 41-50 years age group, followed by those aged 51-60 and above 60 years. The predominance of younger OSMF patients highlights a shifting trend towards involving younger age groups. This could be due to increased social interactions and economic freedom at this age in a rapidly developing nation like India. Another contributing factor might be the commercialization and easy availability of processed AN products, along with peer pressure. Similar findings were reported by Anuradha et al. [18], Sharma et al. [19], and Choudhary et al. [20]

Most of the subjects in both OSMF and OC belonged to middle and low socioeconomic statuses and had low levels of education. Various studies have indicated that the use of AN, tobacco, and their products is inversely related to education level and socioeconomic status [21]. Chatterjee et al. [22] stated in their study that low-income, less-educated population groups such as farmers, fishermen,

and truckers are at the highest risk of developing OC due to extensive exposure to various risk factors. Kamala et al. [23] reported that parental education also influences the development of harmful oral habits, with higher parental education levels helping to prevent these habits in their offspring. McGurk and Crag, [24] studied the Asian community settled in the United Kingdom and found that most of the OSMF patients were from low- or middle-income groups..

In the present study, 58 cases (96.66%) exhibited expression of both *p53* and *Ki67* in OSMF. Of these, 30 cases (50.0%) showed high or intense expression of *p53*, while 51 cases (85.0%) demonstrated high or intense expression of *Ki67*, followed by intermediate levels of expression. This pattern of expression was statistically significant, being most prominent in OC patients, followed by OSMF patients, and least in NOM patients. The results of this study are consistent with findings from other studies.

Ranganath and Kavita, [25] analyzed 50 cases of OSMF and 10 cases each of OC and NOM for the expression of *p53*, *Ki67*, *bcl2,* and bax using IHC. The results showed that the labeling indices (LI) were highest in OC, followed by OSMF, and lowest in NOM. The authors concluded that the profiles of *p53*, *Ki67*, and bax in OSMF and OC were altered compared to normal tissues, suggesting that these markers could be used as surrogate markers for MT in OSMF.

Bazarsad et al. [26] studied 36 cases of OSMF and 6 cases of NOM to analyze the expression of *Ki67*, *p53*, cyclin D1, p16, β-catenin, c-Jun, c-Met, and IMP3. The aim was to identify useful biomarkers for predicting high-risk patients with OSMF. The combined biomarkers showed significantly different expressions between patients with MT and those without MT. The authors concluded that identifying high-risk patients in OSMF could help develop more intensive treatment modalities, potentially reducing the OC transformation rate.

Humayun and Prasad, [7] conducted IHC on 4 cases each of OC, OSMF, and leukoplakia, and on 2 cases of NOM to assess the expression of *p53* and *Ki67*. The intensity of staining was moderate to intense. In most OSMF cases, staining was confined to the basal layer, whereas in OC, staining was observed in all layers. The expression of *p53* and *Ki67* increases as NOM becomes dysplastic and undergoes MT. The authors concluded that *p53* and *Ki67* biomarkers might serve as prognostic tools for the early detection of oral premalignant lesions and conditions.

Reddy et al. [8] assessed 10 cases each of OSMF, OC and OC with an OSMF background for the expression of *p53* and *Ki67* biomarkers using IHC. The results showed statistically significant differences among the three groups, with moderate to severe expression ($p<0.05$) of both markers. The authors concluded that these biomarkers could be useful in assessing the MT in oral precancerous conditions and may serve as intermediate points for cancer prevention programs.

Manjunath et al. [10] postulated that mutant *p53* overexpression is an early event in the multistep process of head and neck cancer. Verma et al. [27] concluded from their study that higher *p53* labeling values may indicate biological malignancy with increased proliferative activity. According to Dwivedi et al. [28], the expression of Ki-67 LI increased in the basal and suprabasal layers as the degree of dysplasia progressed from non-dysplastic to dysplastic. Reddy et al. [8] reported that *Ki67* expression was higher in OSMF compared to controls, but lower compared to OC. This suggests the possibility of mutations in the *Ki67* genes, leading to uncontrolled cell proliferation, further genetic abnormalities, the development and progression of OSMF, and eventually malignancy. The *Ki67* biomarker is reliable and can be widely used as a predictive marker.

The present study found a statistically significant correlation between the expression of *p53* and *Ki67* with the duration and frequency of chewing habits. As the duration (in years) and frequency (packets per day) of these habits increased, the expression of both *p53* and *Ki67* also increased. The consumption of AN begins at a young age in India, according to data from the Global Adult Tobacco Survey 2016–2017, AN usage was 18.3% among individuals aged 15–18 years and 21.5% among those aged 19–23 years [29]. Nandhini et al. [30] reported that the duration and frequency of chewing habits have a significant relationship and are directly related to the severity of the disease. Reddy et al. [8] reported an increased presence of *p53* and *Ki67* positive cells in patients who chewed processed AN and those who consumed AN with pan and tobacco. Sultana et al. [3] found a correlation between *p53* expression and chewing habits, concluding that the accumulation of *p53* protein might reflect a persistent response to DNA-damaging agents present in AN and/or tobacco. Several authors have suggested that the high expression of *p53* in OSMF may be due to the high copper content in AN, which binds to *p53*, potentially leading to a copper-mediated etiopathogenic mechanism for the genetic aberrations found in OSMF [31, 32]. Kaur et al. [33] reported that a mixture of betel leaf, AN, and tobacco induces a high rate of *p53* mutations. Nair et al. [34] suggested that nitrosonornicotine (NNN) may play a key role in initiating oral tumors in tobacco and betel-quid chewers by generating reactive oxygen species that can cause oxidative damage to the DNA of buccal mucosa cells.

In another study, the author stated that the generation of oxygen radicals is known to modify guanine residues in DNA at the C-8 position and induce 8-hydroxydeoxyguanosine in DNA in betel-quid chewers, potentially causing specific genetic changes, including mutations of the *p53* gene. This suggests that betel-quid chewing may be a critical environmental factor in the development of OC [35]. Conversely, Yan et al. [36] reported that Taiwanese patients without a betel quid chewing habit had a higher rate of *p53* overexpression than heavy chewers.

In conclusion, the expression of *p53* protein and *Ki67* antigen can help identify high-risk OSMF patients, indicating biological malignancy. These biomarkers can be used as tools in predicting MT in OSMF cases. From the study, we conclude that using these biomarkers (*p53* and *Ki67*) can enable early detection of high-risk OSMF

patients, potentially preventing further MT in these patients. However, further studies with larger sample sizes and long-term follow-up using additional biomarkers are recommended.

Author Contribution Statement

Both authors have made substantial contributions to the work participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data (when applicable), as well as the writing of the manuscript, their names are listed in the manuscript.

Acknowledgements

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If it was approved by any scientific Body/ if it is part of an approved student thesis

This work is NOT approved by scientific Body/ and it is NOT part of an approved student thesis.

Any conflict of interest

Authors declare that there are no conflicts of interest in this study.

How the ethical issue was handled (name the ethical committee that approved the research)

Ethical Clearance from institutional ethics committee Krishna Institute of Medical Sciences, Deemed to be University, Karad (Ref. No. KIMSDU/IEC/01/2018) was obtained before the start of the study. Ethical clearance certificate has been Uploaded.

Availability of data (if apply to your research)

The data (patients were) was drawn from outpatient department (OPD) of Oral Medicine and radiology (KIMSDU, Karad) using purposive/subjective sampling technique.

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