Original Article

Expression of p53 as Potential Biomarker in Oral Submucous Fibrosis: An Immunohistochemical Study

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Abstract

Background: Oral submucous fibrosis (OSMF) is potentially malignant disorder known to transform into oral cancer. The malignant transformation is often associated with changes at the genetic level that in turn is reflected by the altered expression of proteins related to cell cycle, proliferation, and apoptosis. Expression of p53 tumor suppressor gene is one of the common findings in human cancers including the oral cancer. Therefore, the early detection of potentially malignant OSMF has been crucial in the inhibition of oral cancer. **Aim and Objectives:** To determine the main pathological logical factors and expression of aberrant p53 in OSMF, oral squamous cell carcinomas (OSCC) and in normal patients, to study correlation between p53 expression with clinical staging and histological grading of OSMF. **Materials and Methods:** An immunohistochemical (IHC) study was performed for p53 expression on 35 cases of OSMF, 10 cases of OSCC with history of habits and 10 normal patients without any habits. **Results:** The expression of p53 showed a significant difference between normal oral mucosa, OSMF and OSCC samples. **Conclusion:** The study demonstrated a high incidence of p53 over expression in OSMF and OSCC. The results indicate that p53 over expression may play a role in pathogenesis of OSMF and in the development of Oral squamous cell carcinoma. With early detection of the high-risk patients with OSMF, we can expect to develop more intensive treatment modalities, leading to the reduction in cancer transformation rate from OSMF.

Keywords: Immunohistochemistry, malignant transformation, oral cancer, oral submucous fibrosis, p53 biomarkers

Introduction

In Southeast Asia where oral cancer is a major public health problem, over 90% of oral malignancies are known to arise from preexisting potentially malignant lesions and conditions.[1] Oral submucous fibrosis (OSMF) is a potentially malignant disorder (PMD) which has risen rapidly in India reaching the count more than 2 millions in the last decade. The reported rate of malignant transformation (MT) in OSMF ranges from 3% to 19%.[2,3] There are approximately 600 million people worldwide amounting to 10%–20% of the world's population who chew raw areca nut alone and/or in a processed form. Areca nut is the fourth most commonly abused substance in the world (following nicotine, ethanol, and caffeine). OSMF is strongly associated with the chewing of areca nut (also referred to as betel nut) and Capsaicin and spicy food have also been suggested as etiological factors for OSMF.[4,5] The gene most frequently mutated in human cancer is p53. Evidence say that up to 80% of human Oral squamous cell carcinomas (OSCC) harbor mutated p53 tumor suppressor genes. Such a high frequency of p53 expression suggests an important role of this gene in carcinogenesis. Several studies have reported a high incidence of p53 protein expression in betel quid-associated PMD and oral cancers from India, Taiwan, Sri Lanka, Thailand, and China.[6,7] Thus, the disease is now a public health issue in many parts of the world.[4]

Although the significant roles played by oncogenes and tumor suppressor genes in the development of OSCC have been explored, there are few data on the pathogenesis of OSMF as

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well as of its MT at the molecular level.[8,9] Therefore, the early detection of potentially malignant OSMF has been crucial in the inhibition of oral cancer. Thus the present study aims to evaluate the demographic (age, education, and socioeconomic status) and pathological factors (habits with duration and frequency) for OSMF and degree and pattern of expression of p53 in OSMF, OSCC, and normal patients and to study the correlation between p53 expression with clinical staging and histological grading of OSMF.

Materials and Methods

Study design, and participants

This prospective cross-sectional observational study was conducted in Department of Oral Medicine and radiology, School of Dental Sciences KIMSDU, Karad, Satara, Maharashtra from Jan 2019 to April 2020. The patients were drawn from outpatient department of Oral Medicine and radiology using purposive/subjective sampling technique. Ethical Clearance from Institutional Ethical Committee Krishna Institute of Medical Sciences Deemed to be University (Ref No. KIMSDU/IHC/01/2018; Date: 01/2/ 2018) and the written informed consent (in local language Marathi) from all patients were obtained before the start of the study.

Ethical approval

Prior to the study, ethical approval to carry out this study was granted from the institutional ethical committee of Krishna Institute of Medical Sciences Deemed to be University (Ref. No.KIMSDU/IEC/04/2016; Dated. 29/12/2016) in accordance with the declaration of Helsinki. And written informed consent was obtained (in local language Marathi) from all patients before the start of the study.

Inclusion and exclusion criteria

The inclusion criteria of the study were individuals with clinically diagnosed as OSMF and OSCC with habits. The exclusion criteria were medically compromised patients and the patients who did not gave the written informed consent.

Samples size

Thirty-five cases of OSMF who were having habit of chewing arecanut in processed forms and others, and 10 cases of OSCC patients with habits of tobacco and other products, and 10 cases of normal oral mucosa (NOM) without any habit were considered for the study. Normal tissues were obtained from the buccal mucosa of 10 patients during the surgical removal of the third molar which served as negative controls and OSCC patients as positive controls.

Demographic details were recorded for all the patients. Diagnosis of OSMF and OSCC was made on the basis of characteristic clinical features.[10,11] All the patients were subjected to incisional biopsy and clinical diagnosis was confirmed by histopathologically. The clinical staging and histological grading of OSMF was done according to Utsunomiya H *et al*.[12] There were no bias in the selection samples observed and no drop of the study was noted.

All the biopsied tissues specimens were fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin wax using standard procedures. The biopsied tissues were cut into 3 μm thickness of tissue sections and hematoxylin and eosin (Loba Chemie Pvt. Ltd., Mumbai, India) staining was done for histopathological diagnosis for all cases. Additional sequential sections were prepared for immunohistochemical (IHC) study.

Immunohistochemistry protocol

All the biopsied tissues specimens were fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin wax using standard procedures. The biopsied tissues were cut into 3 μm thickness of tissue sections and hematoxylin and eosin staining was done for histopathological diagnosis for all cases. Additional sequential sections were prepared for (IHC) study.

All 35 cases of OSMF were available for high-quality IHC staining except 2 tissue sample in which epithelial tissue was lost for p53 staining. Formalin fixed paraffin embedded tissues were sectioned at 3 μm thick and mounted on frosted slides. The sections on frosted slides were deparafinized in xylene for 3 times 5 min each thereafter rehydrated in different concentrations of ethanol.

The antigen retrieval was carried out in Envision FLEX target retrieval solution, high pH (Dako; K8004) containing Tris-EDTA buffer pH 9 for 30 min in autoclave. After washing with distilled water at 25°C, the slides were incubated with Envision FLEX wash buffer containing Tris buffered saline solution with Tween 20 pH: 7.6 (Dako: K8007) for 20 min followed by blocking with Envision FLEX peroxidase blocking reagent containing phosphate buffer with 15 mmol/L hydrogen peroxide, sodium azide, and detergent (Dako: SM801). After 20 min H_2O_2 blocking, the sections were incubated directly with primary antibodies, Anti human p53 protein (clone DO-7) ready to use (DakoAutostrainer/ Autostrainerplus, Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup, Denmark) for 1 hour at room temperature in humidity chamber.

Thereafter, the sections are washed with wash buffer for 5 min followed by treatment with Envision FLEX/HRP goat secondary antibody against rabbit and mouse immunoglobulins coupled with peroxidase molecules (Dako: SM802). After completion of 1 h incubation with secondary antibody, sections were washed with distilled water for 5 min. The sections were stained with Envision FLEX DAB + Chromogen (3, 3' diaminobenzidine tetra hydrochloride) (Dako: DM827) in Envision FLEX substrate buffer containing hydrogen peroxide and preservative (Dako: SM803) till the brownish red color development. The sections were counter stained with hematoxylin for 2–3 min after washing with distilled water. After drying the slides, slides were dipped into 100% ethanol and thereafter xylene for clearing the sections. The slides were mounted in DPX mountant and observed under Primovert Phase contrast microscope (Carl Zeiss).

Scoring system

All images were captured under ×10 and ×20 magnification. Immunostained sections for p53 was independently examined in detail by pathologists, and the expression of p53 protein was carried out based on intensity of staining. For antibodies staining the nuclei the labeling index (LI) was calculated, for this purpose, 100 cells were counted and LI were calculated as follows:

$$LI = \frac{\text{Number of positive cells} \times 100}{\text{Number of cells}}$$

The protein expression was scored as follows:[13]

1. (−Very low) when < 5% of cells staining positive
2. (+Low) when 5%–25% of cells staining positive
3. (++Intermediate) when 25%–50% of cells staining positive
4. (+++High) when >50% of cells staining positive were considered.

Statistical analysis

The data were entered and analyzed using Statistical Package for the Social Sciences, version 21 (SPSS 21, IBM Corporation, United States). The Chi-squared test was used to analyze the differences between the intensity levels, in NOM, OSMF, and OSCC. Differences with a $P < 0.05$ were considered statistically significant.

Results

A total of 35 cases of OSMF and 10 cases of each OSCC and NOM were studied for the expression of p53 oncoprotein. Table 1 gives descriptive analysis of age, education, and socioeconomic status of OSMF, OSCC and normal patients. Most of OSMF patients were in the age range of 21–30 years and in OSCC are elderly patients. All patients attained low educational status and belongs to low to middle class socioeconomic status.

Table 2 illustrate distribution of type of habits with duration and frequency in OSMF and OSCC patients. In OSMF most of them chewed arecanut in processed form with duration of 5–10 years and frequency of 6–10 packs/day. In OSCC, tobacco chewing with other ingredients was most prevalent habit, 6–10 times a day with duration of more than 10 years.

Table 3 illustrates p53expression in OSMF, OSCC and in NOM patients. Out of 35 cases of OSMF, P53 expression was positive in 33 (94%) patients and negative in 2 (5.7%) patients as there was tissue loss. 2 (5.7%) cases showed low + (5%–25% positive stained cells) expression, 12 cases (34.2%) showed intermediate ++ (25%–50% positive stained cells) expression and 19 cases (54.2%) showed high +++ (>50% positive stained cells) expression of p53. Out of 10 OSCC Cases, 1 (10%) case showed low + expression, 2 (20%) cases showed intermediate ++ expression and other 7 (70%) showed high +++ expression of p53. Out of 10 control (NOM) Cases, 3 (30%) cases showed very low + expression and rest 7 (70%) cases showed negative expression of p53.

Table 4 illustrates correlation of p53expression with clinical and histological staging of OSMF.

However, the present study did not show the correlation between p53 expression with clinical and histological staging of the disease. The result indicates that there was no significant difference in the p53 expression with clinical and histological staging ($P > 0.05$).

Discussion

Recent epidemiological data and intervention studies suggest that OSMF is frequently noted in South-East Asian countries where areca nut chewing is popular, suggesting that this habit is the most important etiological factor in the pathogenesis of OSMF.[14]

The prevalence of OSMF has increased since 2000 (2.42 in 2000–6.42/1000/year in 2004).[15] One study recognized that OSCC originating from OSMF is clinically more invasive and also exhibits a higher metastasis and recurrence rate than OSCC not originating from OSMF.[16] Therefore, there has been much focus on investigating biomarkers for the prevention

Table 1: Descriptive analysis of age, educational and socioeconomic status, in normal oral mucosa, oral submucous fibrosis and oral squamous cell carcinomas patients

Variable	OSMF (*n*=35), *n* (%)	OSCC (*n*=10), *n* (%)	Normal (*n*=10), *n* (%)	Chi-square test	*P*
Age group					
11-20	3 (8.6)	0	0	29.91	0.0002*
21-30	22 (62.9)	0	5 (50)		
31-40	6 (17.1)	2 (20)	4 (40)		
41-50	3 (8.6)	3 (30)	1 (10)		
51 and above	1 (2.9)	5 (50)	0		
Educational status					
Graduate	12 (34.3)	2 (20)	6 (60)	5.68	0.46
Nongraduate	19 (54.3)	8 (80)	4 (40)		
Illiterate	2 (5.7)	0	0		
Not mentioned	2 (5.7)	1 (10)	0		
Socioeconomic status					
Low	14 (40)	8 (80)	3 (30)	15.91	0.0142*
Middle	18 (51.4)	2 (20)	3 (30)		
Higher	2 (5.7)	0	4 (40)		
Not mentioned	1 (2.9)	0	0		

*Significant when $P<0.05$. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinomas

Table 2: Chewing habits, duration and frequency of the habits in oral submucous fibrosis and oral squamous cell carcinomas patients

Variables	OSMF (*n*=35), *n* (%)	OSCC (*n*=10), *n* (%)	Chi-square test	*P*
Types of chewing habits in OSMF and OSCC patients				
Areca nut in processed forms (gutkha, star, mawa, khara, manikchand, vimal)	21 (60)	1	13.14	0.0014*
Arecanut+panmasala+tobacco	9 (25.7)	2 (20)		
Arecanut+pan masala+tobacco+alcohol	5 (14.3)	7 (70)		
Duration of the habit (years)				
1-5	14 (40)	2 (20)	7.26	0.27
6-10	17 (48.6)	3 (30)		
>10	4 (11.4)	5 (50)		
Frequency of the habit (packets per day)				
1-5	12 (34.2)	3 (30)	0.072	0.96
6-10	20 (57.1)	6 (60)		
10-15	3 (8.6)	1 (10)		
>15	0	0		
Total	35	10		

*Statistically significant. Chi-square test. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinomas

Table 3: Expression of p53 in normal oral mucosa oral submucous fibrosis and oral squamous cell carcinomas patients

Expression of P53	OSMF (*n*=35), *n* (%)	OSCC (*n*=10), *n* (%)	Normal (*n*=10), *n* (%)	Chi-square test	*P*
Negative	2 (5.7)	0	7 (70)	40.91	<0.0001*
Low (+)	2 (5.7)	1 (10)	3 (30)		
Intermediate (++)	12 (34.2)	2 (20)	0		
High (+++)	19 (54.2)	7 (70)	0		
Total	35 (100)	10 (100)	10 (100)		

*Highly statistically significant. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinomas

Table 4: Correlation of p53 expression with clinical and histological grading in oral submucous fibrosis patients

Parameters	OSMF patients	Expression of P3	Chi-square test	*P*
Clinical staging				
Early cases	3 (8.57)	+ Low	6.48	0.17
	1 (2.85)	++ Intermediate		
	0	+++ High		
Moderately advanced cases	2 (5.71)	+ Low		
	3 (8.57)	++ Intermediate		
	6 (17.14)	+++ High		
Advanced cases	4 (11.42)	+ Low		
	5 (14.28)	++ Intermediate		
	11 (31.42)	+++ High		
Histological grading				
Early cases	2 (5.71)	+ Low	0.95	0.62
	1 (2.85)	++ Intermediate		
	3 (8.57)	+++ High		
Moderately advanced cases	2 (5.71)	+ Low		
	4 (11.42)	++ Intermediate		
	7 (20)	+++ High		
Advanced cases	2 (5.71)	+ Low		
	4 (11.42)	++ Intermediate		
	10 (28.57)	+++ High		

*Significant when $P \leq 0.05$. OSMF: Oral submucous fibrosis

and early detection of its MT. Most reports have shown that there is an increase in the proportion of cases that show p53 abnormalities as detected by IHC from hyperplasia to dysplasia to neoplasia.[4]

In the present study, majority of them belongs to 21–30 years of age group and all OSMF patients were male. The male predominance may be because of present scenario of the younger age group possibly getting increased social exposure, economic independence and excessive freedom at an early age, which will lead to get addicted to various harmful chewing habits. Whereas females being more conscious and uncomfortable to ask the vendors in getting the gutkha products. The prevalent of OSMF was more in patients belonged to lower educational and low socioeconomic status. The result of the present were similar to the studies conducted by Yang *et al*. and Singla and Khanna.[17,18]

In a study conducted by Kamala *et al*., reported that parental education also has an affect over the development of noxious oral habits. It has been seen that higher parental education prevents the development of noxious habits in their offspring. Illiteracy or lower education status encourages the development of noxious habits.[19] In the OSCC most of patients were in above 40 years, with low education and socioeconomic status. Most of patients used (70%) used tobacco with pan masala and arecanut with duration of more than 10 years.

In the present study, the use of arecanut in processed form (60%) was more prevalent with increased duration (48.6%) and frequency (57%). There is a sudden upsurge in the use of gutkha (processed form of areca nut) recently, due to easy availability, attractive colorful packs, longer shelf life and low cost.[20] Many reports suggest that chewing areca nut starts at a young age, and it is being consumed freely by children as it helps in digestion and has got mild euphoric effects.[17,21]

Oral carcinogenesis is a multistage process arising from the accumulation of genetic events that disturb cell cycle control, proliferation, motility, survival and tumor-related angiogenesis.[21] The gene most frequently mutated in human cancer is p53, in normal cells, wild-type p53 protein has a very short half-life (6–20 min) and is present in small quantities, however mutations in the p53 gene often result in a more stable gene product and prolong the half-life of the p53 protein, causing it to accumulate within cell nuclei that it can be easily detected by means of IHC.[1,4]

To date, data on the involvement of the p53 in the pathogenesis of OSMF is lacking. The present study was thus conducted to determine the part played by p53 aberrations in the pathogenesis of OSMF and oral cancer.

In the present study 33 (94%) of OSMF cases showed positive staining with p53 expression which is very high compared to other recent studies. Manjunath *et al*., studied 30 OSMF patients irrespective of gender using indirect super sensitive polymer-HRP IHC detection system to detect p53 expression and its correlation with degree of dysplasia. Out of 30 cases 27 (90%) showed positive expression for p53, which were similar to present study.[4]

Sultana *et al*., studied 30 cases of each OSCC and OSMF to detect expression of p53 oncoprotein using DO-7 by LSAB visualization system kit. Out of 30 cases of OSMF 15 (50%) showed positive expression of for p53, and all 15 cases showed low + p53 expression. Out of 30 OSCC cases 24 (80%) showed p53 expression with 3 cases moderately ++ expression suggesting that, the p53 expression in this study were lower than the present study.[1]

Reddy *et al*., studies 10 cases of each NOM, OSCC and OSMF by IHC to detect expression of both p53and Ki67. In NOM 2 (20%) cases showed mild positivity to p53, in OSMF 6 (60%) cases showed positive staining in that 5 cases were mild (+) and 1 case was moderate (++). In OSCC all 10 (100%) cases showed positive, with 2 cases stained moderately and other 8 cases stained intense (+++) staining for p53.[22]

Out of 33 OSMF cases. 2 (5.7%) cases showed low + (5%–25% positive stained cells) expression, 12 cases (34.2%) showed intermediate ++ (25%–50% positive stained cells) expression and 19 cases (54.2%) showed high +++ (>50% positive stained cells) expression of p53. The intensity of expression of p53 was higher in our study compared to above-mentioned studies. Kerdpon *et al*., and Allison and Best, reported 94% and 100% expression of p53 respectively in their studies, by using microwave antigen retrieval technique which were similar to present study.[23,24]

In the present study all 10 (100%) OSCC cases showed expression of p53, in that 1 (10%) case showed low (+), 2 (20%) cases showed intermediate and rest 7 (70%) cases showed high p53 expression which are in accordance with Ranganathan and Kavitha (100%),[3] Humayun and Prasad (100%)[7] and Reddy *et al*., (100%).[22] However little lower percentage 93.33% were observed by Patel *et al*.[25] and Dragomir *et al*.[26] The reason for this wide range in expressibility of p53 protein may be due to the (1) Variation in the etiological factors and ethnic background of the patients. (2) Variation in the IHC technique and delay in the placement of excised tissue into fixative may reduce antigen expression. (3). Tumors may have lost both the alleles of the p53 gene and the level of p53 protein that cannot be detected by IHC.[27]

Our study did not show correlation between p53 expression with clinical staging and histological grading of OSMF. Expression of p53appears to be an early event in the development of p53positive OSCC, and once p53 expression occurs in dysplasia or carcinoma *in situ*, it appears to be persistent, with transition to invasive carcinoma appearing unpredictably months to years later.[4]

Very few authors have also shown the correlation between p53 expressions with clinical and histological grading of OSMF. Sultana *et al*.,[1] showed a statistically significant ($P < 0.05$) relationship was between p53 expressions and chewing habit in OSMF.

Baveja and Baveja,[28] in their study observed that the percentage positivity of proliferating cell nuclear antigen and p53 increased with the advancement of stage of disease. It was noted that there was an increase in the number of cases

showing IHC positivity with the clinical progression of OSMF from Stage-I to Stage-IV.

Conclusion

Expression of p53 protein may help in determining the prognosis and plan treatment modalities.

In the present study, the intensity of p53 expression is high both in OSMF and OSCC suggesting that and increased expression and pattern of intensity of p53 antigens may be useful indicators of MT. However, further studies, with more study samples and long-term follow-ups is needed.

Limitation of the study

The limitation of the present study was less sample size and lack of 1:1 ratio because only 10 cases of each OSCC and normal patients were considered for the study.

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Conflicts of interest

There are no conflicts of interest.

References

1. Sultana N, Pallagatti S, Mohamed AI. P53 expressions in oral submucous fibrosis and oral squamous cell carcinoma. Int J Oral Maxillofac Pathol 2011;2:9-14.
2. Iqbal A, Tamgadge S, Tamgadge A, Pereira T, Kumar S, Acharya S, *et al.* Evaluation of Ki-67 expression in oral submucous fibrosis and its correlation with clinical and histopathological features. J Microsc Ultrastruct 2020;8:20-4.
3. Ranganathan K, Kavitha R. Proliferation and apoptosis markers in oral submucous fibrosis. J Oral Maxillofac Pathol 2011;15:148-53.
4. Manjunath S, Himadal CG, Divakar DD, Haleem S, Mohammad Faqeeh HA, M Alshadidi MY. An immunohistochemical study of p53 expressions in oral submucous fibrosis. J Oral Maxillofac Pathol 2019;23:308.
5. Reddy V, Wanjari PV, Banda NR, Reddy P. Oral submucous fibrosis: Correlation of clinical grading to various habit factors. Int J Dent Clin 2011;3:21-ed
6. Anil S, Gopikrishnan PB, Basheer AB, Vidyullatha BG, Alogaibi YA, Chalisserry EP, *et al.* Association of poly (ADP-Ribose) polymerase 1 variants with oral squamous cell carcinoma susceptibility in a South Indian population. Asian Pac J Cancer Prev 2016;17:4107-11.
7. Humayun S, Prasad VR. Expression of p53 protein and ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas: An immunohistochemical study. Natl J Maxillofac Surg 2011;2:38-46.
8. Mondal K, Mandal R, Sarkar BC. A study of Ki-67 expression and its clinicopathological determinants in nondysplastic oral leukoplakia. Contemp Clin Dent 2016;7:493-9.
9. Rai NP, Divakar DD, Al Kheraif AA, Ramakrishnaiah R, Mustafa SM, Durgesh BH, *et al.* Outcome of palliative and radical radiotherapy in patients with oral squamous cell carcinoma – A retrospective study. Asian Pac J Cancer Prev 2015;16:6919-22.
10. Shih YH, Wang TH, Shieh TM, Tseng YH. Oral submucous fibrosis: A review on etiopathogenesis, diagnosis, and therapy. Int J Mol Sci 2019;20:2940.
11. Carreras-Torras C, Gay-Escoda C. Techniques for early diagnosis of oral squamous cell carcinoma: Systematic review. Med Oral Patol Oral Cir Bucal 2015;20:e305-15.
12. Utsunomiya H, Tilakaratne WM, Oshiro K, Maruyama S, Suzuki M, Ida-Yonemochi H, *et al.* Extracellular matrix remodeling in oral submucous fibrosis: Its stage-specific modes revealed by immunohistochemistry and *in situ* hybridization. J Oral Pathol Med 2005;34:498-507.
13. Bathi RJ, Prabhat. p53 aberrations in oral sub mucous fibrosis and oral cancer detected by immunohistochemistry. Indian J Dent Res 2003;14:214-9.
14. Bazarsad S, Zhang X, Kim KY, Illeperuma R, Jayasinghe RD, Tilakaratne WM, *et al.* Identification of a combined biomarker for malignant transformation in oral submucous fibrosis. J Oral Pathol Med 2017;46:431-8.
15. Hazarey VK, Erlewad DM, Mundhe KA, Ughade SN. Oral submucous fibrosis: Study of 1000 cases from central India. J Oral Pathol Med 2007;36:12-7.
16. Nair U, Bartsch H, Nair J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: A review of agents and causative mechanisms. Mutagenesis 2004;19:251-62.
17. Yang SF, Wang YH, Su NY, Yu HC, Wei CY, Yu CH, *et al.* Changes in prevalence of precancerous oral submucous fibrosis from 1996 to 2013 in Taiwan: A nationwide population-based retrospective study. J Formos Med Assoc 2018;117:147-52.
18. Singla C, Khanna R. OSMF and its prevalence amongst rural shop owners in Panchkula District, Haryana, India – A discriptive study. Int Healthc Res J 2017;1:23-7.
19. Kamala KA, Sankethguddad S. Influence of sociodemographic and types of chewing habits on prevalence of oral submucous fibrosis among rural population in Karad Taluka. J Crit Rev 2020;7:7079-86.
20. Nigam NK, Aravinda K, Dhillon M, Gupta S, Reddy S, Srinivas Raju M. Prevalence of oral submucous fibrosis among habitual gutkha and areca nut chewers in Moradabad district. J Oral Biol Craniofac Res 2014;4:8-13.
21. Shrikrishna BH, Jyothi AC. Prevalence of areca nut eating habits and its association with oral submucous fibrosis in preuniversity college going adolescents of Raichur in Karnataka, India: A prospective cross-sectional survey. Int J Head Neck Surg 2016;7:197-203.
22. Reddy MG, Gupta A, Kheur S, Madalli R, Dony E. Expression of p53 and ki-67 in oral squamous cell carcinoma with the background of oral submucous fibrosis. Int J Curr Res 2016;8:34094-8.
23. Kerdpon D, Rich AM, Reade PC. Expression of p53 in oral mucosal hyperplasia, dysplasia and squamous cell carcinoma. Oral Dis 1997;3:86-92.
24. Allison RT, Best T. p53, PCNA and Ki-67 expression in oral squamous cell carcinomas: The vagaries of fixation and microwave enhancement of immunocytochemistry. J Oral Pathol Med 1998;27:434-40.
25. Patel SM, Patel KA, Patel PR, Gamit B, Hathila RN, Gupta S. Expression of p53 and Ki-67 in oral dysplasia and squamous cell carcinoma: An immunohistochemical study. Int J Med Sci Public Health 2014;3:1201-4.
26. Dragomir LP, Simionescu C, Mărgăritescu C, Stepan A, Dragomir IM, Popescu MR. P53, p16 and Ki67 immunoexpression in oral squamous carcinomas. Rom J Morphol Embryol 2012;53:89-93.
27. WHO bulletin. Head and Neck Cancer. 2007:146-50.
28. Baveja S, Baveja P. Study of correlation between clinical staging and predictive molecular markers in oral sub mucous fibrosis. IOSR J Dent Med Sci (IOSR-JDMS) 2017;16:95-9.