Original paper

Altered immunoexpression of SOX2, OCT4 and Nanog in the normal-appearing oral mucosa of tobacco users

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Abstract

Background. Tobacco use is causatively associated with various human cancers, including oral carcinoma. A number of pathways have been delineated to describe its etiopathological link with oral carcinogenesis, including alterations in the expression of stem cell markers. Embryonic stem cell markers, such as sex-determining region Y-box 2 (SOX2), octamer-binding protein 4 (OCT4) and homeobox protein Nanog, which are mainly involved in the maintenance of stemness and pluripotency, have been positively associated with the pathogenesis of oral potentially malignant disorders and oral cancers. In this context, we attempted to explore the subcellular impact of tobacco through examining the expression of these stem cell markers in normal and normal-appearing oral mucosa in non-tobacco users and tobacco users.

Objectives. The aim of the study was to analyze the immunoexpression of SOX2, OCT4 and Nanog in the normal-appearing oral mucosa (NAOM) of tobacco users as compared to the normal oral mucosa (NOM) of non-tobacco users.

Material and methods. The tissue samples of tobacco users and non-tobacco users ($n = 50$ per group) were immunohistochemically stained to assess the expression of SOX2, OCT4 and Nanog.

Results. In the oral mucosa of non-tobacco users, a peculiar parabasal expression pattern of SOX2 and OCT4 was observed, whereas Nanog was non-reactive. The grade of inflammation was found to be a predictive variable influencing the expression of the 2 markers. In tobacco users, variables such as male gender, mixed habit and basilar hyperplasia significantly controlled the basilar and suprabasilar expression of SOX2, OCT4 and Nanog. The expression of SOX2 and OCT4 was higher in tobacco users; in particular, OCT4 positivity was significantly increased ($p < 0.001$) in comparison with non-tobacco users.

Conclusions. The altered expression of the examined stem cell markers could be an indication of early molecular changes in NAOM under the influence of tobacco.

Keywords: tobacco, stem cells, oral mucosa

Cite as
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Introduction

Tobacco use is one of the leading contributors to untimely deaths and associated economic damage globally, as it has been consistently linked with the etiopathogenesis of several human cancers, including oral cancer. Various forms of tobacco, including smoking and smokeless, or some combination of both, are thought to be involved in the multistep process of oral carcinogenesis.[1] Around 60 chemicals, including pro-carcinogens that are present in tobacco products, can cause genetic and epigenetic aberrations in oral mucosal cells through DNA damage and chromosomal instability, and thus contribute to field cancerization. These subcellular changes appear to be early events in multistep progression from clinically normal and histopathologically non-dysplastic mucosa (normal oral mucosa (NOM)), through oral potentially malignant disorders, to oral cancer.[2] The deleterious effects of tobacco at the cellular level need to be detected early through biomarkers so that individuals at risk of developing clinical lesions can be identified and managed accordingly.

A well-characterized transcription factor called sex-determining region Y-box 2 (SOX2) is known to play a crucial role in the maintenance of pluripotency and the self-renewal capability of human embryonic stem (ES) cells.[3] Another transcription factor, octamer-binding protein 4 (OCT4) – a member of the POU-family of DNA-binding proteins, also contributes to the regulation of pluripotency of ES cells through cooperative interactions with SOX2.[4] Homeobox protein Nanog is another member of core pluripotency regulators that acts on the downstream targets of OCT4, and hence plays a key role in cell fate determination, mainly in the differentiation of ES cells.[5] Several clinical studies have reported on the association of these master regulators of ES cell pluripotency at various stages of carcinogenesis and the overall prognosis of various human malignancies.[6–8] The expression of SOX2, OCT4 and Nanog have been studied in combination with or without other stem cell markers as predictors of the risk of malignant transformation in oral potentially malignant disorders and with regard to the clinical outcomes of oral cancer. Despite the conflicting results, the overexpression of these transcription factors has been observed to play a vital role in oral carcinogenesis.[9–11] Furthermore, a number of studies have also noticed an increased expression of these markers in normal-appearing oral mucosa (NAOM) adjacent to the lesional carcinoma tissue, supporting the association of SOX2, OCT4 and Nanog in the field cancerization of oral mucosa under the influence of tobacco.[9,12] Based on this evidence, we evaluated the expression of SOX2, OCT4 and Nanog in the NOM of non-tobacco users in comparison with that of tobacco users by means of immunohistochemistry to assess the influence of tobacco on oral mucosa prior to any evidence of clinically established lesions.

Methodology

Patients and tissue sample collection

The research protocol was reviewed and ethically approved by the institutional Ethical Review Board at the MGM Institute of Health Sciences, Navi Mumbai, India (No. of approval: MGMIHS/RES./02/2018-19/63). Healthy patients, with or without tobacco habits, were selected from among the individuals who visited the dental hospital for periodontal, orthodontic or surgical treatment, such as crown lengthening, ridge augmentation, pericoronitis, or periodontal flap surgery. After obtaining informed consent from the participants, the discarded NAOM samples taken from the abovementioned surgical procedures were collected. A total of 100 tissue samples were obtained from non-tobacco users (n = 50) and tobacco users (n = 50) for the present study.

Histopathological assessment

All tissue samples were fixed in 10% neutral buffered formalin, followed by tissue processing and staining with the use of hematoxylin and eosin (H&E). The histopathological assessment of the samples was carried out by 2 independent oral pathologists for the presence of microscopic changes, such as basilar hyperplasia and inflammation. Inflammation in the samples was scored as follows: 0 – no inflammation; 1 – mild (less than 25 inflammatory cells); 2 – moderate (more than 25 and less than 125 inflammatory cells); and 3 – severe (more than 125 inflammatory cells).[13]

Immunohistochemical staining

From each paraffin-embedded block, tissue sections were cut to a thickness of 3 µm with a rotary microtome (Leica RM 2245; Leica Camera, Wetzlar, Germany). The sections were dewaxed in xylene, and then rehydrated in absolute alcohol, 95% alcohol and 85% alcohol. Antigen retrieval was performed by immersion in Tris-EDTA (0.1 M, pH 9) in a decloaking chamber at 125°C for 10 min. Endogenous peroxidase activity was blocked by using 3% hydrogen peroxide in methanol for 30 min. The slides were then incubated with an anti-SOX2 rabbit monoclonal antibody (prediluted, Clone EP103, Cat. No. PR071; PathnSitu Biotechnologies, Livermore, USA), an anti-OCT4 rabbit monoclonal antibody (prediluted, Clone EP143, Cat. No. BSB2029; Bio SB, Santa Barbara, USA) and an anti-Nanog mouse monoclonal antibody (prediluted, Clone 5A10; Novus Biologicals, Centennial, USA) diluted in Tris-buffered saline (TBS) and 5% bovine serum albumin (BSA) at 4°C overnight in a wet chamber. For the substrate chromogenic reaction, the sections were immersed in a freshly prepared solution of 0.03% diaminobenzidine for 2 min at room temperature, followed by

counterstaining with Mayer's hematoxylin. The samples of colon carcinoma, esophageal carcinoma and seminoma were used as positive controls, whereas the substitution of the primary antibody with TBS served as an internal negative control for each batch of staining.

Immunohistochemical analysis and scoring

Two pathologists independently reviewed the stained slides without any access to clinical or demographic data of the patients. The nuclear expression of SOX2, OCT4 and Nanog was observed in the epithelial cells of both tobacco users and non-tobacco users. A total of 1,000 cells were counted in a ×400 magnification field per specimen, using the ImageJ software (National Institutes of Health (NIH), Bethesda, USA; https://imagej.nih.gov/ij/). The percentage positivity of all markers was calculated by dividing the total number of positively stained cells by the total number of cells in the section (minimum 1,000 cells) in high-power fields ×100%. Staining intensity was observed and graded as weak, moderate or strong. The scoring of percentage positivity (<5% of cells – 0; 5–24% – 1; 25–49% – 2; 50–74% – 3; and >75% of cells – 4) and intensity (no cells – 0; weak – 1; moderate – 2; and strong – 3) was done according to the methodology proposed by Vijayakumar et al.[14] The final scoring of each section was calculated by adding the scores of percentage positivity and intensity (0–3 – low expression; and 4–7 – high expression). If any disagreement occurred (intensity score discrepancy >1), the slides were re-evaluated by a third independent pathologist along with previous observers to obtain a consensus diagnosis.[9]

Statistical analysis

The observations were noted and recorded in a Microsoft® Excel sheet (Microsoft Corporation, Redmond, USA). The results were compared for statistical significance using IBM SPSS Statistics for Windows, v. 24.0 (IBM Corp., Armonk, USA). Student's independent *t* tests were used to analyze the intra- and intergroup variations in SOX2, OCT4 and Nanog expression. Linear regression analysis was applied to explore independent predictor variables influencing the expression of stem cell markers.

Results

The study participants constituted 2 groups: group I comprised healthy controls with NOM, without any tobacco habit (n = 50); and group II consisted of individuals with NAOM with a tobacco habit (n = 50). The association of the expression patterns of the stem cell markers with the demographic data of both groups are depicted in Tables 1–4.

Expression patterns of SOX2, OCT4 and Nanog, and their correlation with clinicopathological factors in non-tobacco users (group I)

The nuclear expression of SOX2 and OCT4 was observed only in the parabasal layer of NOM, with mild to moderate intensity of immunoreactivity observed for both markers. Nanog was found to be negative for all NOM samples (Fig. 1). The association between the percentage expression of SOX2 and OCT4 and the clinicopathological features of this group, including age, gender and the grade of inflammation, was examined with the independent *t* test. The percentage expression of SOX2 and OCT4 was statistically significantly different among the grades of inflammation ($p < 0.001$), whereas age and gender did not seem to influence their expression (Table 1). Linear regression analysis showed the grade of inflammation to be a significant and independent predictor of expression of these 2 stem cell markers (Table 2).

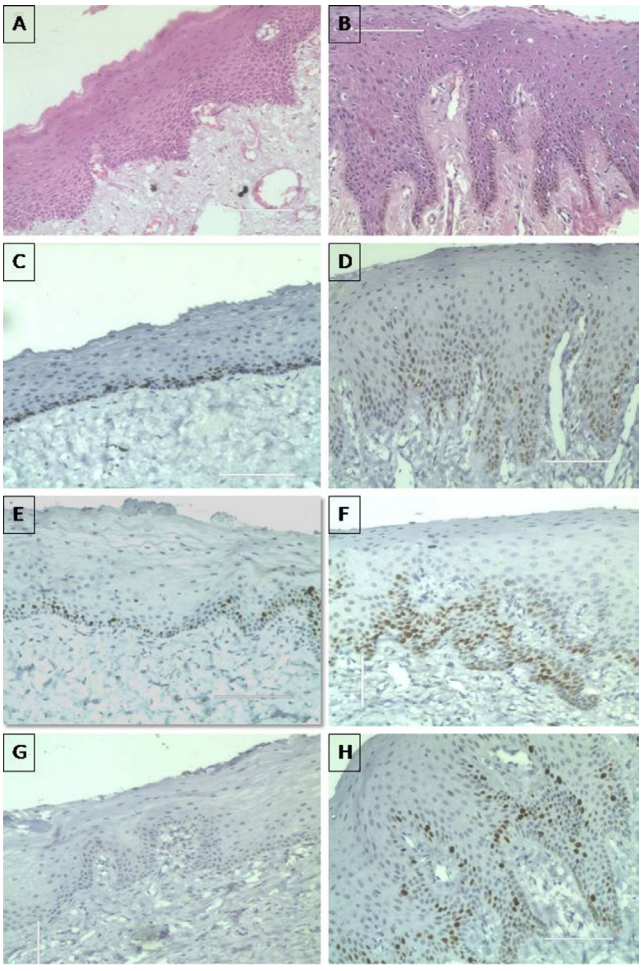

Fig. 1. Histopathological staining of oral mucosa (hematoxylin and eosin (H&E); ×400 magnification) in non-tobacco users (A) and tobacco users (B). In non-tobacco users, the immunohistochemical expression of SOX2 (C), OCT4 (E) and Nanong (G) were found to be limited to the parabasal layer (if positive for staining), whereas the immunoreactivity of all these markers increased up to the basal and suprabasal layers and above in tobacco users (D, F and H for SOX2, OCT4 and Nanong, respectively)

Table 1. Correlation between demographic features and the percentage expression of SOX2 and OCT4 in normal oral mucosa (NOM) in non-tobacco users

Variables		Sample size (n = 50)	Percentage expression of SOX2 [%]	p-value	Percentage expression of OCT4 [%]	p-value
Age [years]	≤40	28	3.12 ±1.59	0.842	0.93 ±0.93	0.853
	>40	22	3.03 ±1.72		0.98 ±1.07	
Gender	male	38	2.83 ±1.64	0.053	0.92 ±0.98	0.723
	female	12	3.88 ±1.40		1.04 ±1.07	
Grade of inflammation	no	34	2.58 ±1.43	<0.001*	0.65 ±0.83	<0.001*
	mild	8	3.21 ±1.56		0.93 ±1.03	
	moderate	8	5.06 ±0.93		2.62 ±0.41	

Data presented as mean ± standard deviation ($M \pm SD$). * statistically significant (unpaired two-sample t test).

Table 2. Multiple regression analysis showing the association of predictive variables with the percentage expression of SOX2 and OCT4 in the normal oral mucosa (NOM) of non-tobacco users

Model		Unstandardized coefficients		Standardized coefficient β	t	p-value	95% CI for β	
		β	SE				lower bound	upper bound
SOX2 expression	(constant)	2.002	0.843	–	2.374	0.022	0.303	3.700
	age	–0.248	0.401	–0.076	–0.618	0.539	–1.055	0.559
	gender	0.622	0.485	0.164	1.284	0.206	–0.354	1.599
	grade of inflammation	0.890	0.331	0.415	2.692	0.010*	0.224	1.556
OCT4 expression	(constant)	0.710	0.527	–	1.347	0.185	–0.352	1.772
	age	0.000	0.242	0.000	0.001	0.999	–0.487	0.488
	gender	–0.286	0.294	–0.125	–0.975	0.335	–0.878	0.305
	grade of inflammation	0.668	0.190	0.513	3.522	0.001*	0.286	1.050

SE – standard error; CI – confidence interval; * statistically significant.

Expression patterns of SOX2, OCT4 and Nanog, and their correlation with clinicopathological factors in tobacco users (group II)

In NAOM, the nuclear expression of all 3 markers was noticed in both basal and suprabasal layers of oral mucosa. The immunoexpression of these markers was analyzed along with the clinicopathological parameters of tobacco users, including age, gender, type of habit (smoking, smokeless and mixed, i.e., with alcohol), duration of habit, and tobacco contact time, and histopathological features, such as basilar hyperplasia and the grade of inflammation. Male gender, mixed habit and the presence of basilar hyperplasia significantly contributed to the alteration of the percentage expression of SOX2, OCT4 and Nanog (Table 3). For SOX2 expression, the type of tobacco habit was found to be a significant predictive variable, whereas age, duration of habit, basilar hyperplasia, and the grade of inflammation emerged as independent predictive variables for OCT4 expression in linear regression analysis. Gender, tobacco contact time and the grade of inflammation were observed to have significantly influenced Nanog immunoexpression (Table 4). The intensity of immunoreactivity of all markers was noticed to increase along with an increase in the grade of inflammation.

Intergroup comparison of SOX2, OCT4 and Nanog expression

The mean value of percentage expression of OCT4 (9.74 ±4.79%) was found to be statistically higher in tobacco users when compared to non-tobacco users (0.95 ±0.99) ($p < 0.001$). A similar increasing trend was observed in SOX2 expression under the influence of tobacco, though the result was not statistically significant. In the case of Nanog, the 2 groups were not compared, as this marker was expressed only in tobacco users and was absent in the NOM of non-tobacco users.

Co-expression of SOX2, OCT4 and Nanog

The co-expression of SOX2, OCT4 and Nanog was assessed by evaluating the percentage expression in the same area of the tissue specimens. In tobacco users, a higher co-expression of SOX2 and OCT4 (76%) was observed in comparison with non-tobacco users (30%), though the statistical correlation was found to be non-significant (Spearman' coefficient $\rho = 0.120$; $p = 0.408$). The co-expression of all 3 markers was observed in 58% of all tobacco users. The overall intensity of immunoreactivity of all markers was observed to be higher in tobacco users in comparison with non-tobacco users.

Table 3. Correlation between demographic features and the percentage expression of SOX2, OCT4 and Nanog in normal-appearing oral mucosa (NAOM) in tobacco users

Variables		Sample size (n = 50)	Percentage expression of SOX2 [%]	p-value	Percentage expression of OCT4 [%]	p-value	Percentage expression of Nanog [%]	p-value
Age [years]	≤40	28	5.06 ±6.20	0.559	9.85 ±7.14	0.908	3.96 ±4.65	0.291
	>40	22	4.13 ±4.62		9.60 ±8.07		2.54 ±4.66	
Gender	male	41	5.38 ±5.82	<0.001*	10.71 ±7.50	0.034*	4.07 ±4.89	0.016*
	female	9	1.37 ±1.37		5.32 ±5.93		0.00 ±0.00	
Type of habit	smoking	15	3.11 ±1.56	<0.001**	12.66 ±7.11	<0.001**	3.71 ±3.58	0.003**
	smokeless	23	2.81 ±4.66		4.54 ±4.04		1.24 ±1.71	
	mixed	12	10.10 ±6.98		15.38 ±6.90		6.72 ±7.21	
Duration of habit [years]	≤5	26	5.94 ±6.20	0.196	12.09 ±7.52	0.060	4.46 ±5.39	0.136
	6–10	16	3.72 ±5.22		7.69 ±6.55		2.71 ±3.90	
	>10	8	2.32 ±1.72		6.22 ±7.35		0.90 ±1.76	
Tobacco contact time [h]	≤1	36	3.84 ±4.37	0.197	9.44 ±7.63	0.662	3.54 ±5.41	0.609
	>1	14	6.73 ±7.57		10.49 ±7.33		2.78 ±1.63	
Basilar hyperplasia	absent	20	1.98 ±1.85	0.001*	5.44 ±5.80	<0.001*	1.45 ±1.72	0.016*
	present	30	6.44 ±6.42		12.60 ±7.17		4.59 ±5.55	
Grade of inflammation	no	9	3.33 ±3.09	0.535	5.96 ±8.01	0.025*	1.91 ±1.92	0.260
	mild	15	4.73 ±6.52		6.09 ±6.11		1.98 ±1.77	
	moderate	24	4.68 ±4.96		12.37 ±7.05		4.63 ±6.27	
	severe	2	9.80 ±13.85		16.30 ±8.02		4.25 ±0.63	

Data presented as mean ± standard deviation (*M* ±*SD*). * statistically significant (unpaired two-sample *t* test); ** statistically significant (one-way ANOVA (two-tailed)).

Table 4. Multiple regression analysis showing the association of predictive variables with the percentage expression of SOX2, OCT4 and Nanog in the normal-appearing oral mucosa (NAOM) of tobacco users

Model		Unstandardized coefficients		Standardized coefficient	t	p-value	95% *CI* for β	
		β	*SE*	β			lower bound	upper bound
SOX2 expression	age	−0.636	1.886	−0.058	−0.337	0.738	−4.448	3.176
	gender	−2.913	2.903	−0.204	−1.004	0.322	−8.781	2.954
	type of habit	3.259	1.254	0.436	2.599	0.013*	0.724	5.793
	duration of habit	0.701	1.451	0.095	0.483	0.632	−2.232	3.633
	tobacco contact time	−0.972	2.078	−0.080	−0.468	0.642	−5.171	3.227
	basilar hyperplasia	2.075	1.710	0.186	1.214	0.232	−1.381	5.531
	grade of inflammation	0.343	1.090	0.052	0.315	0.754	−1.860	2.547
OCT4 expression	age	4.971	2.131	0.333	2.332	0.025*	0.663	9.278
	gender	−4.440	3.302	−0.230	−1.347	0.185	−11.120	2.225
	type of habit	0.552	1.644	0.055	0.336	0.739	−2.771	3.875
	duration of habit	−4.020	1.634	−0.403	−2.463	0.018*	−7.329	−0.722
	tobacco contact time	−5.060	2.676	−0.307	−1.892	0.066	−10.470	0.346
	basilar hyperplasia	4.460	1.937	0.295	2.304	0.027*	0.547	8.378
	grade of inflammation	4.302	1.223	0.477	3.517	0.001*	1.830	6.773
Nanog expression	age	0.694	1.464	0.075	0.474	0.638	−2.265	3.653
	gender	−4.752	2.049	−0.395	−2.320	0.026*	−8.893	−0.612
	type of habit	1.739	1.028	0.276	1.691	0.099	−0.340	3.817
	duration of habit	−0.904	1.125	−0.145	−0.804	0.426	−3.177	1.369
	tobacco contact time	−5.407	1.590	−0.526	−3.400	0.002*	−8.621	−2.193
	basilar hyperplasia	1.089	1.322	0.115	0.824	0.415	−1.582	3.760
	grade of inflammation	2.132	0.839	0.380	2.540	0.015*	0.436	3.829

* statistically significant.

Discussion

The cumulative evidence of a positive correlation between the immunoexpression of SOX2, OCT4 and Nanog and various oral potentially malignant disorders and oral carcinoma indicates the putative role of these proteins in oral carcinogenesis. Though previous research has reported on the presence or absence of these pluripotent stem cell markers in normal mucosa as compared to that of the lesional tissues, an exclusive study targeting their expression in adult stem cells in NOM has not been conducted until now. In this context, the present study observed only SOX2 and OCT4 expression in NOM, which is in agreement with the observations of Qiao et al.,[10] whereas all the samples showed negative immunoreactivity toward Nanog.[10,15] SOX2 is thought to be a guardian of the embryological development of the head and neck region through its expression in neural crest cells. Since most of the oral and orofacial structures are derived from the migrated neural crest cells, the presence of SOX2 in the NOM of non-tobacco users explains its putative role in oral mucosa differentiation through a precise cell flow.[16]

The lower mean value of OCT4 percentage expression as well as the absence of Nanog expression in NOM could be explained by the regulatory and repressing effect of SOX2 on OCT4 and Nanog during specific lineage differentiation.[11,17] We observed the parabasal layer expression of these pluripotent stem cells, in contrast to the basal layer expression reported by many authors. As NOM is thought to have its stem cell population distributed in the quiescent basal cell and active parabasal cell layers, this justifies the peculiar pattern of parabasal layer expression of SOX2 and OCT4 rather than the basal cell layer expression, as observed in previous studies.[18] The presence of inflammation not only was found to be significantly associated with an increased immunoreactivity of these markers, but also emerged as a predictive variable for the same in the NOM of non-tobacco users. Inflammation has an inducible effect on stem cell proliferation through cytokine production. In NOM, even sterile inflammation (non-microbial), which may result from chemical and physical insults, can cause the proliferation of stem cells. At the site of injury, inflammatory cells recognize danger-associated molecular patterns and secrete molecules that prime the tissue restoration via stem cell induction.[19]

In tobacco users, an increased expression of SOX2 and OCT4 and the appearance of Nanog in tobacco-affected oral mucosa were observed. Naini et al. reported similar results in the adjacent non-tumor oral tissue, which indicated the potential role of these markers in the early molecular stages of carcinogenesis.[12] Fu et al. also observed a similar higher expression of Nanog in the corresponding tumor-adjacent normal tissues as compared to their normal counterparts, which supports its underexplored behavior in carcinogenesis.[9] Significant associations between tobacco-related parameters, including the type of habit and tobacco contact time, and an increased immunoreactivity of all these markers indicate a critical role of nicotine in oral carcinogenesis through the regulation of the expression and stemness of SOX2, OCT4 and Nanog.[20] In addition, a mixed habit that includes alcohol use was shown to be associated with an increased expression of all of these ES cells markers, which is in accordance with the experimental findings showing the alcohol-activated induction of cancer stem cells.[21]

Histopathological factors, including basilar hyperplasia, were correlated with an elevated expression of stem cell markers in the present study. Under the influence of tobacco, the shift and expansion of the stem cell niche from the parabasal cell layer (active stem cell population) to the basal cell layer (quiescent stem cell population) was evident from the basal cell hyperplasia observed in histopathological examinations and the criteria depicting architectural changes in epithelial dysplasia. In addition, the grade of inflammation also correlated positively with an increased expression of stem cell markers. Both smokeless and smoke tobacco can cause genetic and proteomic alterations through the dysregulation of inflammation-associated pathways, such as MAPK/ERK as well interferon signaling in oral keratinocytes.[22]

The mean percentage expression values of all of the markers were significantly elevated in tobacco users in comparison with non-tobacco users, which may indicate the cumulative effect of long-term tobacco exposure on oral keratinocytes by inducing the expansion of the embryonic stem cell population. The co-expression of SOX2 and OCT4 was found to be increased in the oral mucosa of tobacco users, similar to the observations made by Qiao et al., who reported an elevated $SOX2^{+}OCT4^{+}$ profile in the transforming oral mucosa of the rat and in precancerous lesions of humans that displayed simple hyperplasia.[10] The co-expression of all 3 markers in over a half of tobacco users could be a result of the mechanical and chemical impact of tobacco on oral keratinocytes.

Conclusions

The use of tobacco and the tobacco habit can induce early molecular changes at the cellular level through the expansion of the stem cell niche, which could be recognized histopathologically as basilar hyperplasia. Increased expression and co-expression of pluripotent stem cell markers SOX2, OCT4 and Nanog in NAOM may indicate another molecular pathway of tobacco-induced oral carcinogenesis. The observations of the present study could be used as a baseline for further research on the impact of tobacco on oral mucosal stem cells in the development of various oral potentially malignant disorders and oral cancer.

Ethics approval and consent to participate

The research protocol was reviewed and ethically approved by the institutional Ethical Review Board at the MGM Institute of Health Sciences, Navi Mumbai, India (No. of approval: MGMIHS/RES./02/2018-19/63). Informed consent was obtained from all participants.

Data availability

All data generated and/or analyzed during this study is included in this published article.

Consent for publication

Not applicable.

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