RESEARCH ARTICLE

Reduced Expression of Limd1 in Ulcerative Oral Epithelium Associated with Tobacco and Areca Nut

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Abstract

Purpose: The aim of this study was to cast light on initiating molecular events associated with the development of premalignant oral lesions induced by tobacco and/or areca nut. Method: Immunohistochemical analyses of cell cycle regulatory proteins (LIMD1, RBSP3, p16, RB, phosphorylated RB, p53), EGFR and SH3GL2 (EGFR associated protein) were performed with inflammatory/ ulcerative epithelium and adjacent hyperplastic/mild dysplastic lesions. Results: No change in expression of the proteins was seen in inflammatory epithelium. Reduced nuclear expression of LIMD1 was evident in ulcerative epithelium. In hyperplastic lesions, reduced expression of RBSP3, p16, SH3GL2 and overexpression of p-RB and EGFR were apparent. Reduced nuclear expression of p53 was observed in mild dysplastic lesions. Conclusion: Our data suggest that inactivation of LIMD1 in ulcerative epithelium might predispose the tissues to alterations of other cell cycle regulatory and EGFR signaling proteins needed for the development of premalignant oral lesions.

Keywords: EGFR - LIMD1 - premalignant oral lesions - RB - RBSP3 - SH3GL2

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Introduction

Oral cancer is the sixth most common cancer worldwide and it accounts for 30-40% of all cancer types in Indian subcontinent (Ghosh et al., 2009). Epidemiological studies have linked tobacco habit and areca-nut (betel-nut) quid as main etiological factors for this tumor development, though the role of these factors either alone or in combination in development of the disease is not clearly established. It is evident that chronic exposure of these factors could induce multiple clinical manifestations like inflammation, ulceration, Oral submucous fibrosis (OSF), Leukoplakia, Lichen Planus, Haemangioma etc. (Pandya et al., 2009). Inflammation and ulceration are often seen in OSF, Leukoplakia, Lichen Planus, and Haemangioma along with premalignant lesions like hyperplasia and/or dysplasia (Muñoz-Corcuera et al., 2009). It seems that chronic inflammation and/or ulceration might predispose the tissues to premalignant changes followed by malignant transformation (Zhang et al., 1997). As a result, during clonal evolution of oral carcinoma, less advanced stages (inflammation/ ulceration/ hyperplasia/ dysplasia) are evident around the invasive carcinoma (Califano et al., 1996; Zhang et al., 1997). The molecular changes, if any, associated with the development of inflammation and ulceration of oral epithelium is not clearly known. Zhang et al. (1997) reported chromosomal deletions in hyperplastic lichen planus but not in inflammatory lichen planus. In the analysis of premalignant oral tumors, it was evident that over expression of EGFR and deletion of chromosomal (Chr.) 9p21 region were associated with development of hyperplastic lesions while, deletion of chr. 3p region and inactivation of p16 and p53 were associated with that of dysplastic lesions (Perez-Ordonez, 2006). In subsequent analysis of the candidate genes located in the chr.3p21-22 and chr. 9p21-22 regions, inactivation of LIMD1 (3p21.31), RBSP3 (3p22.3), P16 (9p21.3) and SH3GL2 (9p22.2) were seen to be associated with mild dysplastic oral lesions (Ghosh A et al, 2009; Ghosh S et al, 2010; Ghosh et al., 2010). LIMD1 stabilizes RB-E2F interaction, where as RBSP3 dephosphorylates RB at 807/811 residues and p16 inhibit RB phosphorylation. Thus, alterations of these genes could destabilize the RB-E2F interaction. On the other hand, SH3GL2 regulates EGFR homeostasis through membrane encapsulation followed by proteosomal degradation (Dikic I, 2003). The alterations of these genes have not been evaluated in inflammatory/ulcerative epithelium and adjacent premalignant oral lesions to find out if these genes have any role in initiation of oral carcinogenesis.

Thus, to understand the initiating events associated with oral carcinogenesis attempts have been made in this

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study to analyze expression of LIMD1, RBSP3, p16, RB, P-RB (phosphorylated RB at 807/811), p53, SH3GL2 and EGFR in inflammatory/ulcerative oral epithelium and adjacent premalignant oral lesions in same set of samples. The Immunohistochemical (IHC) analysis of these was made in the oral epithelium of inflammatory and/or ulcerative regions as well as the adjacent hyperplastic/ dysplastic regions collected from 12 individuals having tobacco and/or areca nut habits. All total 25 different microscopic fields of oral lesions were analyzed. Our data showed reduced expression of LIMD1 in chronic ulcerative lesions followed by reduced expression of p16, RBSP3 and SH3GL2 in hyperplastic lesions resulting over expression of phosphorylated RB and EGFR respectively. Nuclear expression of p53 was reduced in mild dysplastic lesions.

Materials and Methods

Patient samples:

Inflammatory/ulcerative epithelium and adjacent hyperplastic/dysplastic lesions of oral cavity were collected from 12 individual participants (Table 1) in a community based study in the rural district of East Midnapore, West Bengal, India, to evaluate prevalence of premalignant oral lesions among habitual chewers of tobacco and/or areca nut (betel-nut). Informed consent was obtained from each of the individuals for conducting biopsy. The project has been approved by institutional ethical committee. Freshly collected punch biopsy tissues were fixed in formalin and embedded in paraffin for histopathological and immunohistochemical analysis. Histopathological evaluations of the tissues were done independently by two senior pathologists. In short, 6 inflammatory, 5 ulcerative, 10 hyperplastic and 4 mild dysplastic lesions were identified from the 12-biopsy tissues. Some of the biopsy tissues showed multiple histopathological phenotypes i.e. of three sample having ulceration, one with adjacent inflammatory change though retaining histopathologically normal epithelium and rest two with hyperplastic change in the epithelium; of four hyperplastic samples, two with adjacent ulcerative and inflammatory change and rest two with inflammatory change of the epithelium only; two mild dysplastic samples with hyperplastic change (Figure 1) of the adjacent epithelium etc. (Table 1). As control specimen, buccal mucosas were collected from 4 normal individuals with similar habit who underwent surgery in the oral cavity for some other reasons and processed as above.

Anti RBSP3 polyclonal antibody production and purification:

Anti RBSP3 antibody is not commercially available. The custom made polyclonal anti- RBSP3 antibody was raised by M/s IMGENEX Biotech, Bhubaneswar, India, in rabbit against the synthetic peptide (NGGLQKGDQRQVIPIPS) which have 100% homology with human and 94% with mouse. Briefly, 220 μg of the Keyhole Limpet Hemocyanin (KLH) conjugated peptide were injected in rabbits to immunize. After that, the rabbits were given booster doses with 110 μg of the same with 7 days interval. After 6th immunization, 1st test bleeds were taken and analyzed by enzyme linked immunosorbent assay (ELISA). Similarly, after 8th and 10th immunization, 2nd and 3rd bleeds were collected and analyzed as above. The antibody was purified by affinity purification column from the serum of 3rd bleed and stored at -20^{0}C.

Immunohistochemistry:

The expression of LIMD1, RBSP3, p16, p-RB (phosphorylated RB at 807/811 position), RB, p53, SH3GL2 and EGFR was analyzed by immunohistochemistry. About 4-5µm thick sections were dewaxed, rehydrated, incubated in 10 mM citrate buffer (pH-6.0) at 90^{0}c for 30 min to retrieve the antigens and reacted overnight with primary antibodies at a dilution of 1:100 at 4°C. Mouse polyclonal IgG for p16 (sc-1661) and p53 (sc-126), rabbit polyclonal IgG for RB (sc-7905), p-RB ser-807/811 (sc-16670) and EGFR (sc-03) and goat polyclonal IgG for SH3GL2 (sc-10874) were purchased from Santa Cruz, CA, USA. The mouse monoclonal anti-LIMD1 was received from Dr. Tyson V. Sharp, University of Nottingham Medical School, UK. HRP conjugated rabbit anti-goat IgG (sc-2768), HRP conjugated goat anti-mouse IgG (sc-2005) for p16 and p53, goat anti-rabbit IgG (sc-2004) for RB, pRB and EGFR and rabbit anti-goat IgG (sc-2768) for SH3GL2 (Santa Cruz Biotechnology, CA, USA) was added at 1:500 dilutions.

The slides were developed using 3-3' diaminobenzidine as the chromogen and counterstained with hematoxylin. The staining intensity (1=weak, 2=moderate, 3=strong) and the percentage of positive cells (<1=0, 1-20 =1, 20-50=2, 50-80=3 and >80=4) were detected by two observers independently and by combining the two scores, final evaluation of expression was done (0-2=low, 3-5=intermediate, 6-7=high) (Perrone et al., 2006).

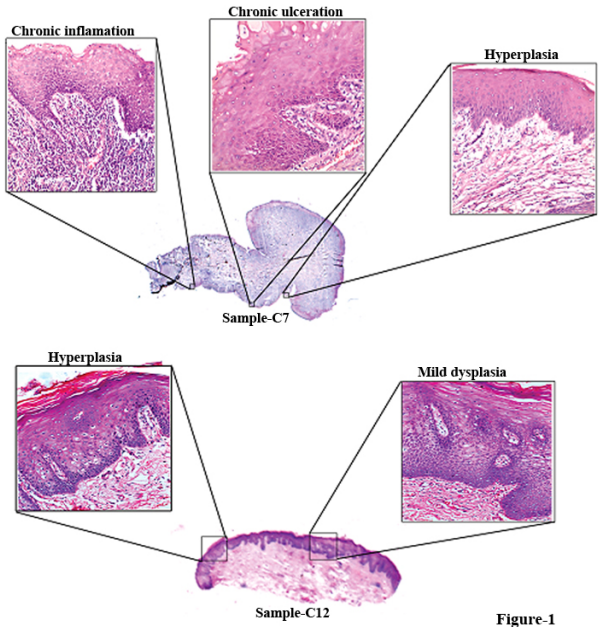

Figure 1. Multiple histopathological phenotypes of the biopsy's tissues. HE staining of multiple fields of histological phenotypes the tissue section of C-7 and C-12. Chronic inflammation, chronic ulceration and hyperplastic lesion are seen in different part of C-7 tissue section, where as C-12 shows hyperplasia and mild dysplasia.

Results

Expression analysis of RB and it's associated genes

To understand the initiating events associated with the development of premalignant oral lesions, expression of RB/ p-RB and some RB associated proteins like LIMD1, RBSP3 and p16 were analyzed at first by IHC.

In normal oral epithelium high level of nuclear expression of RB and p-RB were seen in basal and Para-basal layers followed by intermediate level of expression in the spinus layer, indicating differential expression pattern of RB/p-RB during cellular proliferation and differentiation (Figure 2). Similar pattern of their (RB/p-RB) expression was seen in both inflammatory and ulcerative epitheliums. On the other hand in hyperplastic and dysplastic lesions, reduced expression of RB was detected although intense nuclear staining of p-RB was seen in basal/parabasal layers and intermediate level in spinus layer (in 3/10 and 6/10 respectively). This suggested the importance of increased p-RB expression in the development of premalignant oral lesions.

In normal oral epithelium, intense nuclear and low cytoplasmic expression of LIMD1 was observed in basal and parabasal layers (Figure 3a). Similar trend was seen in spinus layer along with low membrane expression. In the chronic inflammatory epithelium, there was slight decrease in nuclear expression of LIMD1 in basal layer of some samples. However, majority of the chronic ulcerative epithelium (4/5) showed low nuclear and cytoplasmic expression of LIMD1 in basal and parabasal layers, whereas intermediate level of its nuclear expression was seen in the spinus layer along with distinct membrane localization (Table 2). Similarly, in hyperplastic (6/10) and mild dysplastic lesions (3/4) either low or absence of nuclear and cytoplasmic expression of LIMD1 was seen in basal and parabasal layers. Similar trend was also seen in spinus layer along with low membrane expression. This suggests that loss of expression of LIMD1 is associated with chronic ulceration of oral epithelium and subsequent oral lesions.

In case of RBSP3, low nuclear/cytoplasmic expression was observed in basal layer of normal oral epithelium followed by gradual increase in cytoplsamic expression from parabasal to spinus layers, indicating its role in cellular growth and differentiation (Figure 3b). Similar trend has also been seen in chronic inflammatory and ulcerative epitheliums along with infrequent nuclear expression in the spinus layer. However, majority of the samples (6/10) with hyperplastic epithelium showed low or absence of RBSP3 expression in basal and parabasal layers and intermediate level of cytoplasmic expression in spinus layer (Table 2). Similar phenomenon has also been seen in mild dysplastic lesions (2/4). Thus, our data suggests that loss of expression of RBSP3 is needed for the development of hyperplastic oral lesions.

High nuclear expression of p16 was detected in basal and parabasal layers, and intermediate level of expression

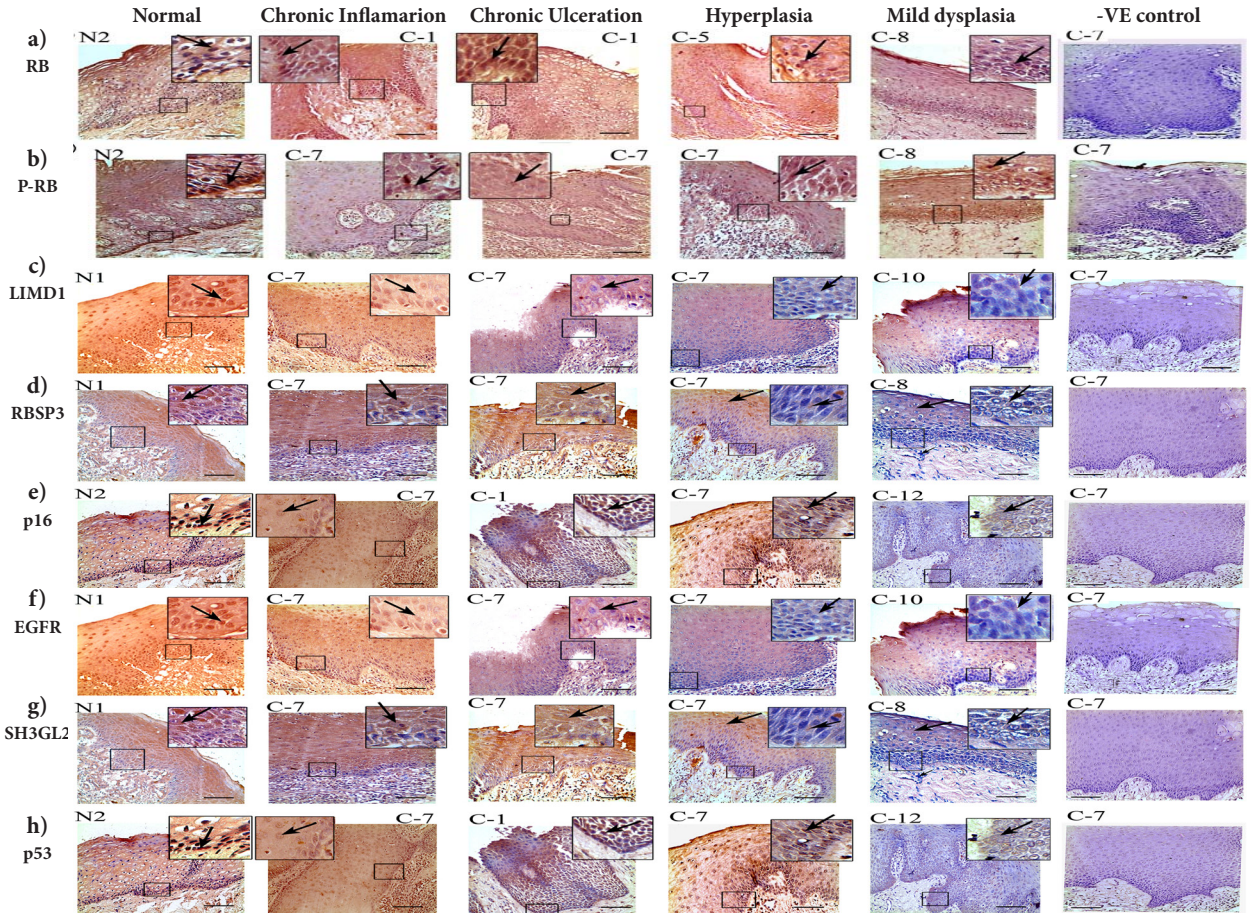

Figure 2. Immunohistochemical Analysis of a) RB, b) p-RB (phosphorylated RB), c) LIMD1, d) RBSP3, e) p16 Proteins, f) EGFR, g) SH3GL2 and h) p53 Proteins in Normal Buccal Mucosa (N) and Different Oral Lesions (C). Black arrows indicate the expression and location of the proteins Magnification (X20); Bar=100µm. Inset magnification (X40).

Table1. Clinico-Pathological Feature of the Samples

Sample	Age/Sex/Site	Clinical observation	Habit	Histopathologycal phenotypes in different fields			
				Chronic inflammation	Chronic ulceration	Hyperplasia	Mild dysplasia
N1	52/m		T+B	-	-	-	-
N2	39/m		T+B	-	-	-	-
N3	45/m		T+B	-	-	-	-
N4	60/m		T+B	-	-	-	-
C1	23/m	Fibro epithelial polyp	B	+	+	-	-
C2	46/m	Chronic ulcer	T+B	+	+	+	-
C3	59/f	Chronic ulcer	B	-	+	+	-
C4	53/m	Leukoplakia	T+B	+	-	+	-
C5	48/m	Leukoplakia	B	-	-	+	-
C6	38/m	Psudoepitheliomatous hyperplasia	B	-	+	+	-
C7	50/m	Hemangioma	B	+	+	+	-
C8	49/f	Hyperkaratosis	T+B	-	-	+	+
C9	34/m	OSF	T+B	+	-	+	+
C10	33/m	OSF	T+B	+	-	-	+
C11	61/m	OSF	B	-	-	+	-
C12	50/m	Leukoplakia	B	-		+	+

T: tobacco; B: betel nut; N: normal; C: case; M: male; F: female; BM: buccal mucosa; '-': absent; '+': present,OSF:Oralsubmucous fibrosis

Table-2: Frequency of the samples with altered expression of the proteins

Histology (No. of field)	Normal (4)			Chronic Inflamation (6)			Chronic ulceration (5)			Hyperplasia (10)			Mild dysplasia (4)		
Gene	B	PB	S	B	PB	S	B	PB	S	B	PB	S	B	PB	S
RB*	0	0	0	0	0	0	0	0	0	3(10)	3(10)	0	3(4)	3(4)	0
p-RB*	0	0	0	0	0	0	0	0	0	6(10)	6(10)	0	3(4)	3(4)	0
LIMD1*	0	0	0	0	0	0	4(5)	4(5)	0	6(10)	6(10)	1(10)	3(4)	3(4)	0
RBSP3*	0	0	0	0	0	0	0	0	0	6(10)	6(10)	0	2(4)	3(4)	0
p16*	0	0	0	0	0	0	0	0	0	3(10)	3(10)	0	4(4)	3(4)	0
EGFR*	0	0	0	0	0	0	0	0	0	5(10)	3(10)	0	3(4)	3(4)	0
SH3GL2*	0	0	0	0	0	0	0	0	0	4(10)	4(10)	0	4(4)	3(4)	0
P53*	0	0	0	0	0	0	0	0	0	0	0	0	2(4)	3(4)	0

*Frequency of alt.

in spinus layer of normal oral epithelium (Figure 3c). Similar trend was also seen in inflammatory and ulcerative epithelium. However, p16 expression was greatly reduced (low/intermediate) in hyperplastic (3/10) and mild dysplastic lesions (4/4) indicating its importance in the development of premalignant oral lesions (Table 2).

Expression analysis of EGFR and SH3GL2:

High cytoplasmic expression of EGFR was seen in the basal and parabasal layers of the normal oral epithelium followed by intermediate level of expression in spinus layer (Figure 4a). Similar trend was seen in the inflammatory epithelium and subsequent oral lesions. Moreover, in the hyperplastic (5/10) and mild dysplastic lesions (3/4) intense membrane staining of EGFR was evident along with cytoplasmic staining indicating necessity of its over-expression in these stages (Table 2).

In case of SH3GL2, intermediate level of expression was seen in cytoplasm of basal and parabasal layers followed by increase of expression in spinus layer (Figure 4b). Similar pattern was also seen in the inflammatory and ulcerative epithelium of most of the samples. However, in the hyperplastic (4/10) and mild dysplastic lesions (4/4), expression of SH3GL2 was seen to be either low or intermediate particularly in the basal and parabasal layers. This suggests that reduced expression of SH3GL2 is needed for the development of hyperplastic and subsequent oral premalignant lesions (Table 2).

Analysis of p53 expression

In normal oral epithelium, high nuclear expression of p53 was detected in basal and parabasal layers, while intermediate level of expression in spinus layer (Figure 4c). In the inflammatory and ulcerative epithelium, high/intermediate level of nuclear p53 expression was found in basal and parabasal layers followed by decrease of its expression in spinus layer, whereas in the hyperplastic lesions its expression was at the intermediate level. However, in mild dysplastic lesions nuclear expression of p53 was low in basal and parabasal layers of majority of the samples (2/4) (Table 2).

Discussion

In this study, to understand the molecular mechanism associated with the development of premalignant oral lesions, expression of some RB associated genes, p53, SH3GL2 and EGFR were analyzed in inflammatory/ulcerative epithelium and adjacent hyperplastic/dysplastic oral lesions that developed in tobacco/areca-nut habituated individuals. In inflammatory oral epithelium no change in expression pattern of these genes were seen, instead reduced expression of LIMD1 was evident in the ulcerative oral epithelium and in subsequent oral lesions suggesting its importance in initiation of oral carcinogenesis. Reduced expression of LIMD1 in ulcerative epithelium might be due to its promoter methylation, deletion, or mutation as seen in oral dysplastic and carcinoma samples (Ghosh et al., 2010). Inactivation of LIMD1 has also been reported in carcinomas of breast and lung (Sharp et al., 2008; Spendlove et al., 2008). In addition to stabilization of RB-E2F interaction, LIMD1 regulates cell-cell interaction, miRNA mediated gene silencing, cellular

differentiation etc (Foxler et al., 2011). Thus, it seems that reduced LIMD1 expression in basal layer of chronic ulcerative epithelium might modulate cellular homeostasis mechanism.

The high expression of p-RB in the hyperplastic lesions might be due to the synergistic effect of both reduced expression of RBSP3 and p16 (Figure 3b-c). Reduced expression of RBSP3 and p16 might be due to promoter methylation, deletion, and mutation as seen in the carcinomas of oral, breast etc (Sinha et al., 2008; Ghosh et al., 2009: 2010). Apart from dephosphorylation of RB, RBSP3 also inactivates RNA pol-II through dephosphorylation at C-terminal domain Serine residues (Yeo et al., 2003). It also regulates epithelial-mesenchymal transition and cell migration (Wu et al., 2009). Thus, reduced expression of LIMD1, RBSP3 and p16 might deregulate multiple cellular pathways associated with proliferation and differentiation.

The high expression of EGFR in basal and parabasal layers and intermediate level of expression in spinus layer of normal oral epithelium seemed to be in inverse association with the SH3GL2 expression (Figure 4a-b). It indicates that the feedback control of EGFR expression by SH3GL2 through endocytosis mediated degradation is needed for regulating the EGFR mediated growth signaling for cellular proliferation and differentiation (Dikic, 2003). The reduced expression of SH3GL2 in hyperplastic lesions along with high expression of EGFR indicates abrogation of its feedback control. The reduced expression of SH3GL2 might be due to its promoter methylation or deletion as seen in dysplastic and invasive oral lesions as well as in breast carcinoma (Sinha et al., 2008; Ghosh et al., 2009). It has been reported that over expression of SH3GL2 could lead to the JNK mediated death of neurons as seen in Alzheimer patients (Ren et al., 2008). Infrequent mutation of EGFR was found to be rare in HNSCC (Leemans et al., 2010), though its frequent mutation has been reported in lung cancer (Mitsudomi et al., 2007).

Low nuclear expression of p53 particularly in the basal/ parabasal layers of mild dysplastic lesions might predispose the cells in acquiring genomic instability along with deregulation of the cell cycle. The reduced expression of p53 might be due to deletion/mutation as seen in HNSCC samples (Mitra et al., 2007). Frequent genomic instability in HNSCC samples of Indian patient has also been reported (Sengupta et al., 2007). It seems that p53 inactivation in mild dysplastic lesions might provide some selective pressure for outgrowth of cells having genomic instability along with aberrations in cell cycle and other signaling pathways as mentioned above.

Thus, it was evident from our data that sequential inactivation of LIMD1 in ulcerative epithelium followed by RBSP3, P16 and SH3GL2 in hyperplastic lesions and p53 in mild dysplastic lesions might indicate the deregulation of multiple cellular pathways needed for the development of each stage of the oral premalignant lesions. However, detailed molecular analysis of the development of oral cancer is warranted to understand the molecular pathogenesis of the disease.

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Conflict of Interest statement, the authors declare that there are no conflict of interest with regard to the work presented in this article.

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