Original Article

Immunohistochemical Expression of P16 and β-catenin in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma with or without Coexistence of Oral Submucous Fibrosis

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Abstract

Background: Oral submucous fibrosis (OSF) is a potentially malignant disorder with 0.4%–10% incidence in India and malignant transformation rate of 3%–19%. Oral squamous cell carcinoma (OSCC) coexistent with OSF exhibits distinct clinicopathological features. Hence, knowledge of the possible mechanism responsible for the epithelial–mesenchymal transition (EMT) of OSF gains importance. The study aims to assess the pattern of p16 and β-catenin expression in normal mucosa (NM), OSF, and OSCC with and without OSF, to correlate with clinicopathological parameters, and to establish association between p16 and β-catenin as markers of EMT. **Materials and Methods:** Seventy cases, 10 NM, 30 OSF, and 30 OSCC with and without OSF, were subjected to immunohistochemical staining with p16 and β-catenin. Parameters such as percentage positivity and pattern of expression were tabulated and statistically compared using Chi-square test. The combined predictive value of the biomarkers was gauged using discriminant functional analysis. **Results:** A significant increase in p16% positivity and altered pattern of p16 expression from nuclear to cytoplasmic among the groups ($P < 0.001$) and a reduced % positivity of β-catenin from NM to OSF and OSCC with and without OSF ($P < 0.001$). Localization of β-catenin expression shifted from membrane to cytoplasm among groups, which was significantly different in OSCC with and without OSF. The predictive significance of β-catenin and p16 for OSCC with and without OSF was 76.7%. **Conclusion:** The overexpression of inactivated p16 and synchronous loss of β-catenin expression can be used as an indicator of the early changes during EMT in OSF.

Keywords: *Epithelial–mesenchymal transition, oral squamous cell carcinoma, oral submucous fibrosis, p16, β-catenin*

Introduction

The prevalence of oral submucous fibrosis (OSF) in India ranges from 0.2% to 1.2% with an incidence of 0.4%–10%, the main etiology being chewing arecanut with tobacco. OSF has high rate of malignant transformation, ranging from 3% to 19%. Southeast Asia reports one-third of global cases and one-half of oral cancer-related deaths. Ranking among the three most common cancers, it is a major problem in India. Around 1% of the population have oral premalignant lesions, and the increasing incidence in younger individuals poses it imperative to identify the predictors for malignant transformation.[1-9]

The role of epithelial–mesenchymal transition (EMT) in OSF has not been clearly understood. Moreover, inadequate data exist in the literature about prognostic behavior of oral squamous cell carcinoma (OSCC) originating from OSF.[4,10-13]

Altered immunohistochemical (IHC) expressions of p16 and β-catenin are early events in the course of oral cancer. Moreover, limited studies have shown their combined expression in the prediction of malignant progression of OSF and its prognosis. Hence, we aimed to unveil the changes during malignant transformation and detect the genes or proteins altered during tumorogenesis in OSF and OSCC with and without coexistent OSF. We also aimed to study the correlation between p16 and β-catenin and compare the expression with clinicopathological parameters.

Materials and Methods

1. Tissue sample: Formalin-fixed, paraffin-embedded tissue blocks

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of 70 cases including normal mucosa (NM) as control (ten), clinically and histologically proven cases of OSF (30) as Group 1, and OSCC with coexistent OSF as Group 2A and without coexistent OSF (30) as Group 2B were retrieved from the archives of the department. For NM, patients with no relevant habit history, with a healthy mucosa and without any systemic diseases or malignancies, were included. Patients who had reported with recurrence to our institution and those under any treatment for oral carcinoma were excluded from the study. Only cases with full clinical details available in the department records were included and the clinicopathological data of all cases were recorded and tabulated.

Ethical clearance was obtained from the institutional review board before the study.

2. IHC staining and analysis: Tissue sections of 3 μm were obtained on silane-coated slides and subjected to IHC staining with $p16^{Ink4a}$ monoclonal antibody and β-catenin monoclonal antibody (Biogenex, San Roman). The procedure provided by the manufacturer was followed for staining. Brown precipitate in the nucleus/cytoplasm was taken for positive p16 expression while that in membrane/cytoplasm was considered for β-catenin. Parameters assessed were percentage positivity (PP) and pattern of expression. The number of positive cells (x) in an evenly stained area under × 40 magnification was counted in each slide out of a total of 500 cells. PP was calculated by the formula x/500 X 100. Pattern of p16 expression was noted as nuclear, cytoplasmic, or both, while β-catenin expression was taken as membranous, cytoplasmic, or both.
3. Statistical Analysis: The data were tabulated and analyzed using Chi-square test. Discriminant functional analysis was used to evaluate the combined predictive value of the biomarkers.

Results

The clinicopathological parameters assessed revealed that patients in Group 1 and Group 2A were below 40 years of age; in contrast, Group 2B were above 40 years. In all groups male predominance was noted and chewing form habit was prevalent. Predominant site of involvement for Group 2 was buccal mucosa. Clinical staging revealed most of the cases in Stage III followed by Stage II. Histopathological grading of Group 1 showed the greatest number of cases in Stage II followed by Stage III. However, in Group 2, well-differentiated SCC outnumbered moderately differentiated SCC and depth of invasion (DOI) of the tumor was largely between 5 and 10 mm.

Comparison of IHC parameters of p16 among groups

The percentage of positive cells in all control cases (100%) was <30%. In Group 1, 83.3% of cases showed 0%–30% positivity, while 16.7% showed 61%–90% positivity. An increase in PP was seen in Group 2A and B, showing 31%–60% positivity in 60.0% and 53.3% of cases respectively, and 61%–90% positivity in 40.0% and 46.7%, respectively. Highly significant difference was noted between the groups ($P \leq 0.001$). A significant difference in p16 pattern was established among the groups ($P \leq 0.001$) [Table 1].

Nuclear expression was predominant in control (80.0%) and only 20.0% of cases showed cytoplasmic expression [Figure 1]. Group 1 showed greater cytoplasmic expression (53.3%), while nuclear expression was noted in 46.7% of cases [Figure 2]. All cases in Group 2 showed cytoplasmic expression [Figure 3]. A significant difference in p16 pattern was established among the groups ($P \leq 0.001$).

Comparison of IHC parameters of β-catenin among groups

In the control group, PP was >90% in 70.0% of cases, while 30.0% had 61%–90% positivity. PP was reduced in Group 1 where 53.3% of cases showed 61%–90% of positive cells and 36.7% were >90%. Further reduction was seen in Group 2A wherein 66.7% of cases showed

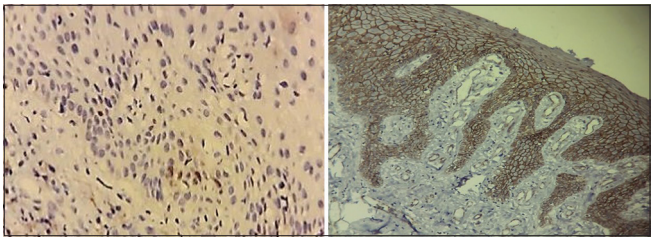

Figure 1: (Left) Nuclear p16 staining in basal layer of normal mucosa; (right) membranous β-catenin expression in all the layers of normal mucosa (×20)

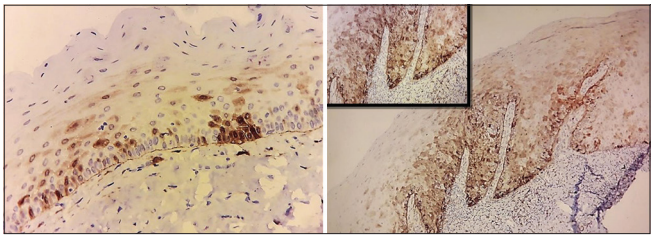

Figure 2: (Left) Nuclear and cytoplasmic p16 staining in basal/parabasal layers of oral submucous fibrosis (×40); (right) intense nuclear and cytoplasmic p16 expression from basal to spinous layer in oral submucous fibrosis with dysplasia (×10; inset × 20)

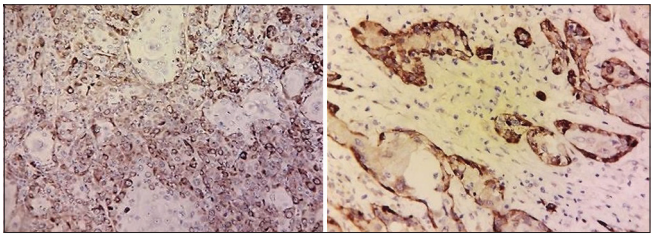

Figure 3: (Left) Intense cytoplasmic p16 staining in tumour islands of oral squamous cell carcinoma with coexistent oral submucous fibrosis; (right) intense cytoplasmic p16 expression in oral squamous cell carcinoma without coexistent oral submucous fibrosis (×40)

31%–60% positivity, while 20% and 13.3% of cases showed 61%–90% and 0%–30% positivity, respectively. Group 2B, however, revealed 66.7% of cases with 31%–60% positivity and 33.3% of cases with 61%–90% of positive cells. The variance in PP among the groups showed high statistical significance ($P \leq 0.001$). A significant difference in the pattern of expression was established among the groups ($P \leq 0.001$) [Table 2].

All control tissues showed membranous expression [Figure 1]. Group 1 also showed greater membranous expression (53.3%); however, combined membranous and cytoplasmic expression was also noted (46.7%) [Figure 4]. Group 2A revealed membranous and cytoplasmic expression in 80.0% of cases [Figure 5], while only membranous expression was reduced (20.0%). In contrast, Group 2B showed membranous expression in 73.3% of cases [Figure 5], followed by 26.7% of cases with membranous and cytoplasmic expression. A significant difference in the pattern of expression was established among the groups ($P \leq 0.001$).

Comparison of p16 and β-catenin expression with clinicopathological parameters

p16 PP showed significant association with age and habits ($P = 0.012$ and $P = 0.023$, respectively), while all other parameters were nonsignificant. In consort, the pattern of p16 expression had a significant correlation with duration of habit ($P = 0.014$) even though other parameters were nonsignificant.

PP of β-catenin showed no significant correlation with the clinicopathological parameters, namely age, sex, habits, duration, staging, site, grading, and DOI. However, pattern of expression showed association with habit ($P = 0.038$).

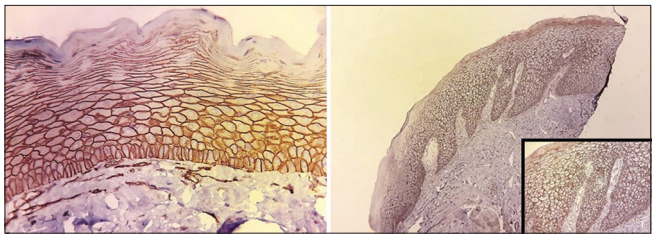

Figure 4: (Left) Intense membranous and cytoplasmic β-catenin expression in Oral submucous fibrosis (×40); (Right) Intense membrane and cytoplasmic β-catenin expression upto superficial layers in Oral submucous fibrosis with dysplasia (×10; inset × 40)

Comparison of p16 and β-catenin expression among groups

When comparing p16 expression amid Group 1 and 2A, a significant difference in PP and pattern was noted ($P < 0.001$ and $P < 0.001$, respectively). Furthermore, a significant difference between Group 1 and 2B was seen ($P < 0.001$ and $P = 0.01$, respectively). However, only PP showed a significant difference among Group 2A and B ($P = 1 < 0.001$).

β-catenin expression yielded significant difference in PP and pattern between Group 1 and 2A ($P < 0.001$ and $P = 0.033$, respectively). Only PP showed a significant difference between Group 1 and 2B ($P < 0.001$), while pattern of β-catenin expression showed a statistically significant difference among Group 2A and B [Table 3].

In spite of the simultaneous overexpression of p16 and loss of β-catenin expression among the groups, a significant association was not found between the PP or pattern of expression of these markers. A discriminant function analysis of PP and pattern of p16 and β-catenin expressions revealed a predictive significance of 76.7% for Group 2A and B [Table 4].

Discussion

OSCC and its associated potentially malignant disorders (PMD) exhibit multiple genetic and epigenetic changes in a multistep process resulting in variable expression. EMT is one such multifaceted molecular process, which is indispensable during morphogenesis as well as tumorogenesis.[13] Furthermore, the role of EMT in fibrosis has been studied in organ fibrosis and in OSF.[14,15] EMT

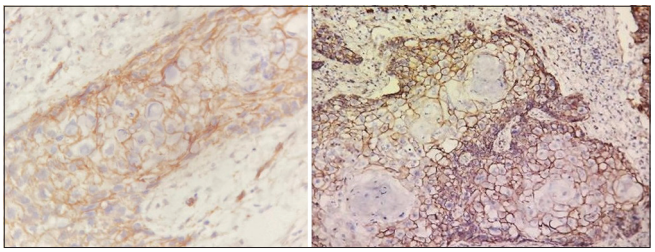

Figure 5: (Left) Moderate membranous and cytoplasmic β-catenin expression in oral squamous cell carcinoma with coexistent oral submucous fibrosis; (right) intense membranous β-catenin expression in oral squamous cell carcinoma without coexistent oral submucous fibrosis (×40)

Table 1: Comparison of immunohistochemical expression of p16 among groups

Parameters	Category	NM, *n* (%)	OSF, *n* (%)	OSF + OSCC, *n* (%)	OSCC, *n* (%)	*P*
Percentage positivity	0-30	10 (100.0)	25 (83.3)	0	0	<0.001**
	31-60	0	5 (16.7)	9 (60.0)	8 (53.3)	
	61-90	0	0	6 (40.0)	7 (46.7)	
	>90	0	0	0	0	
Pattern	Cytoplasm	2 (20.0)	16 (53.3)	15 (100.0)	15 (100.0)	<0.001**
	Nuclear	8 (80.0)	14 (46.7)	0	0	

**Highly significant. NM: Normal mucosa, OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Table 2: Comparison of immunohistochemical expression of β-catenin among groups

Parameters	Category	NM, *n* (%)	OSF, *n* (%)	OSF + OSCC, *n* (%)	OSCC, *n* (%)	*P*
Percentage positivity	0-30	0	3 (10.0)	2 (13.3)	0	<0.001**
	31-60	0	0	10 (66.7)	10 (66.7)	
	61-90	3 (30.0)	16 (53.3)	3 (20.0)	5 (33.3)	
	>90	7 (70.0)	11 (36.7)	0	0	
Pattern	Membranous	10 (100.0)	16 (53.3)	3 (20.0)	11 (73.3)	<0.001**
	Membrane and cytoplasmic	0	14 (46.7)	12 (80.0)	4 (26.7)	

**Highly significant. NM: Normal mucosa, OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Table 3: Statistical analysis of immunohistochemical parameters of β- catenin and p16 between two groups

Parameters	OSF and OSF + OSCC		OSF and OSCC		OSF + OSCC and OSCC	
	β- catenin	p16	β- catenin	p16	β- catenin	p16
Percentage positivity	<0.001**	0.001*	<0.001**	<0.001**	0.287	1<0.001**
Pattern	0.033*	<0.001**	0.190	0.001*	0.003*	0.067

*Significant, **Highly significant. OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Table 4: Discriminant functional analysis of p16 and β -catenin for oral squamous cell carcinoma with and without oral submucous fibrosis

Cases	Groups	Classification		
		Predicted group membership		Total
		OSF + OSCC	OSCC	
n	OSF + OSCC	12	3	15
	OSCC	4	11	15
	Ungrouped	3	37	40
Percentage	OSF + OSCC	80.0	20.0	100.0
	OSCC	26.7	73.3	100.0
	Ungrouped	7.5	92.5	100.0
	Result	76.7% of original grouped cases correctly classified		

OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

serves as a source of myofibroblasts and promotes paracrine signaling between epithelial cells and stromal cells.[16] The integrin linked activation of TGF-β enhances β-catenin/lymphoid enhancer factor (LEF) expression suppressing E-cadherin, thus stabilizing nuclear import of β-catenin. The nuclear import of LEF with β-catenin is considered one of the molecular steps in EMT. Effects of cell cycle regulation in OSF show that the reactive oxygen species generated by arecoline causes cell cycle arrest at G1/S phase in keratinocytes. All these events point toward the involvement of EMT in fibrosis occurring in OSF.[16,17] EMT program is a critical mechanism for the acquisition of malignant phenotypes by epithelial cells.[18] Various biomarkers have been studied to gain an insight of this mechanism including epithelial cell adhesion markers to mesenchymal markers. To pave the way for early detection of OSF transforming to OSCC, the present study was designed to evaluate the expression of β-catenin and p16 in NM, OSF with and without dysplasia, and OSCC with and without coexisting OSF. This study was an attempt to understand the role of β-catenin and p16 as diagnostic markers in these lesions.

Altered p16 expression is considered an early event in carcinogenesis. Although $p16^{Ink4a}$ is a tumor suppressor, its aberrant elevation is observed in a number of cancers.[19] Analysis of p16 expression in premalignant lesions such as leukoplakia with and without dysplasia and lichen planus has been done previously and has rendered variable results. Both reduced expression[20,21] and overexpression of p16[22,23] have been reported. The inactivation of p16 gene can be due to deletion, point mutation, and silencing by means of methylation or promoter hypermethylation.[24]

In our study, nuclear staining predominated in control and PP was <30% overall. Nuclear localization of p16 is compatible with its role as a direct inhibitor of the cyclin-dependent kinase complex. Nevertheless, mild cytoplasmic staining was noted in 2 cases. It has been reported that cytoplasmic to nuclear shuttling of the nuclear factor in normal circumstances may give rise to weak cytoplasmic staining.[25] Our findings were distinct to that of Buajeeb *et al*., who found no expression of p16 in normal tissues.[26] However, p16 positivity confined to the basal and suprabasal layer of the NM has been reported.[20] p16 expression in NM served as a control for comparison with the other groups and as a limit to assess reduced or overexpression of p16.

As per our results, Group 1 revealed p16 PP <30% with 53.3% of cases showing nuclear and cytoplasmic expression. Dysplasia is a predictor of malignant transformation; we selected OSF cases with and without dysplasia in our study. However, no noteworthy difference in p16 expression was noted among dysplastic and nondysplastic cases in Group 1, owing to lower grade of dysplasia. Similar results were found by Gologan *et al.,* where dysplastic epithelium showed no alteration in p16 expression as compared to normal.[22] Bradley *et al*. also suggested that p16 is not a reliable marker for distinguishing dysplastic lesions from normal.[20] However, in our study, 16.7% of cases in Group 1 showed

PP >30%. This overexpression of p16 could be possibly due to its inactivation. Bazarsad *et al*. proposed that p16 expression >5% could be a promising predictor of high-risk OSF cases owing to the difference in expression observed in OSF with transformation.[4] In context to our findings, we propose that the change in pattern of p16 expression to nuclear and cytoplasmic in contrast to nuclear expression in control may be indicative of a transformation process. We also propose that the cell cycle alterations in malignant transformation of OSF could be predicted with change in p16 expression and that hypoxia might be a possible reason for EMT in OSF.

OSCC arising in the background of OSF has been reported to be a clinicopathologically distinct entity with varying features.[5] Thus, p16 expression was assessed in Group 2A and B as an attempt to understand the variation in their mechanism of carcinogenesis, if any. In our study, an increase in the PP was noted in both Group 2A and B than Group 1. A significant alteration in p16 expression pattern and PP was found between Group 1 and Group 2A and B. Group 2A and B showed >30% positive cells yielding a significant difference among these groups and presented with cytoplasmic staining. A shift in the localization of p16 could be attributed to the mutation of the gene, which fails to translocate the protein to the nucleus. Nilsson *et al*. noted that invasive SCC showed nuclear and cytoplasmic p16 expression pattern, whereas some tumors had a strong cytoplasmic p16 expression.[27]

Many studies have reported loss of p16 function in OSCC accredited to the alterations in the gene either by deletion or hypermethylation.[28,29] Buajeeb *et al*. noted p16 expression in areas of microinvasion and margins of OSCC.[25] An absence or rare expression of p16 in OSCC has been proposed by Natarajan *et al*., who also postulated that p16 may be responsible for the initial invasion of tumor cells into the underlying connective tissue.[30] However, we propose that the change in the pattern of p16 expression from nuclear to cytoplasmic in group 2A may be indicative of a transformation process.

A significant correlation was noted in p16 PP with age and habits, while the pattern of p16 expression showed significant correlation with the duration of the habit. Ralli *et at.* reported that p16 expression showed a correlation with chewing habit.[31] In contrast, Smith *et al*. found an association of p16 expression with alcohol consumption and tobacco use.[32] However, Muirhead *et al*. derived a significant association of p16 with grades of tumor.[33]

β-catenin usually shows a membranous expression; however, during EMT, increased cytoplasmic levels of β-catenin are reported that can then translocate to nucleus and affect the transcription of genes associated with EMT. The effect of β-catenin could also be due to loss of cell-to-cell adhesion due to detachment of E-cadherin.[34,35]

In the present study, a progressive reduction in the expression and altered localization (membrane and cytoplasmic) was observed from Group 1 to Group 2A and B. All the control tissues showed a membranous expression largely with >90% positive cells. Chaw *et al.* reported a moderate membrane staining in NM,[10] and Ishida *et al.* noted the expression of β-catenin on the cell membrane.[35]

In our study, slight reduction in PP was noted in Group 1 and predominantly membranous staining was seen. A change in localization of β-catenin to membrane and cytoplasm was noted in 46% of cases. However, OSF with and without dysplasia showed similar staining. Reduction in β-catenin expression in OSF was observed by Bazarasad *et al.,* in their attempt to develop a combined biomarker model for malignant transformation of OSF.[4] Although the authors have found an altered β-catenin expression associated with epithelial dysplasia, in our study, no such significant difference was noted. Chaw *et al.* and Lo Muzio *et al.* have noted a cytoplasmic localization and reduction of β-catenin expression with increasing grades of dysplasia.[10,36] The disruption of cadherin–catenin complex leading to cytoplasmic accumulation of β-catenin could be responsible for the change in localization.[37] Furthermore, a mutation in Wnt signaling pathway may lead to raised β-catenin levels in cells.[38] We propose that the response of epithelial cells to EMT inducing signals could probably promote disruption of intercellular adhesion complex, which may be identified by the change in β-catenin localization and hence be used as an indicator of EMT in OSF.

In our study, a reduction in the PP was noted in Group 2A. In addition, a shift in localization was noted in 80% of cases showing cytoplasmic expression. The increased cytoplasmic localization may be due to degradation of membranous β-catenin, leading to its cytoplasmic accumulation. A significant difference was seen in PP and pattern of expression of β-catenin among the groups. Altered β-catenin levels are a frequent finding in head and neck SCC.[4,10,37] However, comparison of the increased cytoplasmic localization among OSCC arising in the background of OSF and *de novo* has not come to light. Thus, in this study, we attempted to unveil the change in the expression of β-catenin in OSCC with and without OSF. However, no significant difference among the two groups was noted. Yet, it was noteworthy that a significant increase in membranous and cytoplasmic staining was seen in Group 2B as compared to the cytoplasmic staining in Group 2A. This may indicate an altered mechanism of carcinogenesis of OSCC arising in the background of OSF and *de novo*. Furthermore, there was a significant difference in PP between Group 1 and Group 2B. A progressive increase in cytoplasmic localization of β-catenin with histologic grade of OSCC has been previously reported by Laxmidevi *et al.* and Gao *et al*.[39,40] The increased cytoplasmic localization may be due to degradation of membranous β-catenin that confers

an invasive or metastatic phenotype. Chaw *et al*. have reported a significant correlation between loss/cytoplasmic accumulation of β-catenin with Adenomatosis Polyposis Coli and vimentin expression suggesting induction of Wnt pathway in OSCC. The transcriptional activation of target genes such as vimentin leads to β-catenin accumulation in the cytoplasm, which may predispose to increased aggressiveness.[10]

An overall reduction in PP from NM to OSF to OSCC with and without OSF was derived from our study. A corresponding altered pattern of expression from membranous in NM to membranous and cytoplasmic in OSF and cytoplasmic in OSCC with OSF was statistically significant ($P \leq 0.001$).

Discriminant analysis of p16 and β-catenin for groups 2A and B revealed the combined predictive significance of these markers as 76.7%.

In our study, an increased cytoplasmic expression of p16 progressing from PMD to OSCC accompanied by a loss of membranous β-catenin expression suggested that the mutational inactivation of p16 can lead to downregulation of β-catenin. This was further confirmed by the change in cellular localization of both p16 and β-catenin from normal to OSF to OSCC. However, there was no significant association between PP or patterns of β-catenin and p16 expressions. It has been previously established that β-catenin may upregulate $p16^{Ink4a}$ in endometrial carcinoma cells in an indirect manner[41] and activated β-catenin may directly repress the expression of $p16^{Ink4a}$ by binding to its promoter and thereby contribute to the immortalization of melanocytes in melanomas.[42] Lapak and Burd found that p16 promoters have potential binding sites for β-catenin and its transcriptional factors, through which β-catenin can directly bind and activate the p16 promoter. This may result in the direct repression of p16 by β-catenin.[43] This inverse relation of p16 and β-catenin could be a part of EMT wherein inactivation of p16 and loss of β-catenin is a frequent initial finding. Although these markers have been used independently in OSF, dysplasia, leukoplakia, and OSCC, their correlation has not been documented. Thus, in-depth studies to completely understand the combined effect of β-catenin and p16 in PMDs and OSCC are necessary to mark initiation of events in EMT.

Conclusion

Loss of function/inactivation of p16 and the altered localization of β-catenin can be regarded as a possible indicator in malignant transformation of OSF to OSCC. The distinct pattern of β-catenin expression amidst OSCC with and without concomitant OSF may substantiate the disparity in the carcinogenesis. Moreover, the overexpression of inactivated p16 synchronous with the loss of β-catenin expression suggests a possible correlation among the two markers. Thus, the changes during EMT in OSF such as alterations in cell cycle and loss of cell–cell adhesion that occur as early events in malignant transformation can be gauged by means of p16 and β-catenin respectively.

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Conflicts of interest

There are no conflicts of interest.

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