Original Article

Assessment of epithelial–mesenchymal transition signatures in oral submucous fibrosis

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Abstract

Background: Recently, the concept of field cancerization has questioned the accuracy of biopsy site selection clinically. Oral submucous fibrosis (OSMF) has a global malignant transformation rate of 7.6% despite having less dysplastic changes clinically or histologically. Hence, this study was undertaken to evaluate the expression of vimentin, epithelial-cadherin (E-Cad) and collagen IV in OSMF, using immunohistochemistry and polymerase chain reaction (PCR).

Materials and Methods: One hundred and eighty- five patients with OSMF (61), with habits and no OSMF (61) and patients without habit and OSMF (63) were subjected to biopsy for sample collection. The samples were analyzed immunohistochemically for vimentin, E-Cad and collagen IV. The PCR values for vimentin and E-Cad were also done.

Results: Vimentin expression was increased in OSMF patients, whereas E-Cad expression was decreased in OSMF patients.

Conclusion: Epithelial–mesenchymal transition signatures are definitely positive in OSMF cases.

Keywords: Collagen-IV, epithelial-cadherin, epithelial–mesenchymal transition markers, immunohistochemistry, oral submucous fibrosis, vimentin

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Received: 24.07.2018, **Accepted:** 09.03.2019

INTRODUCTION

The concept of most invasive oral cancers arising from potentially malignant lesions such as leukoplakia, erythroplakia and oral submucous fibrosis (OSMF) is well accepted. Studies have found that over 5 million people are affected by oral cancers in India alone (0.5% of the Indian population).[1] A plethora of studies have been taken up for the early detection of oral cancer due to the limited ability of the current clinical/histological methods to predict high-risk precursor lesions for the conversion of malignant transformation and lack of adequate early predictive markers. With the recent advances in technology, focus is shifted from clinical to biological behavior and underlined molecular events to get better outcomes in detecting, treating and preventing the disease.[2] The concept of field cancerization further questions the validity of biopsy reports which is always debatable, as a seemingly normal tissue can have genetic changes which can eventually turnout to be malignant particular part

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/jomfp.JOMFP_177_18

How to cite this article: Prasad RS, Pai A, Shyamala K, Bhadranna A, Shenoy S, Yaji A. Assessment of epithelial–mesenchymal transition signatures in oral submucous fibrosis. J Oral Maxillofac Pathol 2019;23:308.

of the tissue because a seemingly normal tissue can also have genetic changes which can eventually turn out to be malignant in future.[3]

The gold standard for the diagnosis of lesions ranging from PMDs to full-blown cancers is biopsy. The prognosis and treatment are based on the degrees of dysplasia and other histopathological changes seen in biopsy samples. Numerous studies emphasize on early genetic changes that eventually transform into malignancy without any clue, which is too early to be detected histopathologically.

Considering this, epithelial–mesenchymal transition (EMT) markers such as vimentin, E-Cad and collagen IV can be diagnostic in the early changes which cannot be detected from the biopsy. It is well known that frank dysplastic changes are seldom seen in OSMF, but yet considered to have a malignant potential of 7.6% globally.[4]

Epithelial-cadherin (E-Cad) expression is an important parameter in assessing the morphology and homeostasis of epithelial tissues. Loss of epithelial integrity is related to the lower expression of E-Cad which is an EMT marker.[5] Apart from E-Cad, connective tissue EMT markers such as vimentin are also involved in the morphology and regulation of cell behavior.[6] Recently, the hallmark EMT signatures with low E-Cad and high vimentin have shown greater metastasis in primary head-and-neck squamous cell carcinomas. Basement membrane (BM) integrity is often questioned in the process of carcinogenesis. Several degrees of discontinuity are noted in invasive carcinomas. Immunostaining for BM proteins such as collagen IV can reveal BM discontinuity.[7]

Considering the above facts, this study was undertaken to evaluate the expression of vimentin, E-Cad and collagen IV using immunohistochemistry (IHC) and polymerase chain reaction (PCR) in OSMF.

MATERIALS AND METHODS

The study was conducted in the outpatient department of Oxford Dental College with institutional ethical clearance. One hundred and eighty-five sample size were selected, wherein 63 were normal healthy patients without any habits, 61 patients were considered with a habit history (chewing gutkha and other forms of areca nut) without OSMF and the other 61 patients had a habit history with OSMF. Patients clinically diagnosed as OSMF and healthy controls with or without habits that consented to participate in the study were included in the study. Patients with systemic disorders which will contraindicate biopsy and extraction were excluded from the study.

After initial screening, patients' tissue samples were collected and stored in formalin for transportation. Tissues were dehydrated with a graded concentration of isopropyl alcohol and xylene. They were embedded in wax blocks and cut into 4-μ thin sections, transferred onto the slide and stained with hematoxylin and eosin stain. To identify epithelium and connective tissue on the slide and the tissue block, initially, a slide was observed under a compound microscope followed by naked eye. Later, the slides were overlapped on the tissue block to identify the epithelium and the connective tissue part. The previous microscopic image of epithelium and connective tissue was kept as reference and markings were done. After making the notches on the block, the block was mounted onto the soft-tissue microtome, and the ribbon tissues produced were picked up and run over a water bath.

On the water bath, using a forceps, the tissue was separated at the "line of separation" created by the notches made on the tissue block. Finally, the tissue was divided into epithelial part and connective tissue part, and the separated tissue bits were transferred into labeled bullets for molecular analysis.

- Preparation of Aminopropyl triethoxysilane-coated slides – Before coating the glass slides with silane, they were subjected to pretreatment to enhance adherence to the tissue. Glass slides were washed well and rinsed for 2 h in Laxbro-soap solution, scrubbed well with a brush to remove the grease from the slides and then washed well again. The slides were then soaked in 1/10 N hydrochloric acid overnight. The slides were subjected to a thorough wash in autoclaved distilled water the next day and incubated at 56°C–60°C in an incubator overnight
- The slides so pretreated were then coated with silane for adhesion of the tissues as follows: Glass slides were first dipped in acetone for 3 min and then in 2% silane, i.e., 2 ml of 3 aminopropyl triethoxysilane solution in 98 ml of acetone for 5 min and then the slides were dipped in distilled water and dried using hot air oven at 55°C–60°C for 48 h. Then, the slides were stored in refrigerator till use.

Preparation of wash buffer

Tris-buffer-silane (TBS) used in this study was prepared by adding 6.6 g of Tris buffer to 1 L of distilled water and then the pH was adjusted to 7.4–7.6 by adding hydrochloric acid or sodium hydroxide. The pH range was checked using a digital pH meter.

Preparation of citrate buffer for antigen retrieval

2.94 g of sodium citrate was added to 1 L of distilled water and the pH was adjusted to 6.0–6.4 by adding hydrochloric acid or sodium hydroxide solution. The pH range was checked using a digital pH meter. Formalin-fixed, paraffin-embedded sections of OSMF were stained by hematoxylin and eosin, and the serial sections of the same were stained by immunohistochemical reagents. All the antibodies in the procedure were incubated at room temperature, in a moist chamber.

Immunohistochemistry procedure for vimentin, E-Cad and collagen IV

The sections of 4 μm thickness were cut and mounted on the 3-aminopropyltriethoxysilane-coated glass slides. The slides were incubated at 55°C–60°C overnight before the day of staining. The sections were deparaffinized in two changes of xylene for 5 min each, and then the slides were hydrated through different grades of iso-propyl alcohol (100%, 50% for 3 min each) and brought to distilled water.

The tissues were then incubated with 3% hydrogen peroxide block for 5–10 min in room temperature to block the endogenous peroxidase activity. The tissues were then washed in TBS for 5 min and subjected to antigen retrieval. The slides were then antigen retrieved using a solution of sodium citrate buffer in a microwave oven for three cycles of 800 W for 6 min, 800 W for 4 min and 200 W for 14 min and then allowed to cool back to room temperature; the sections were subjected to two washes of TBS.

The sections were then incubated with a protein block to eliminate background staining. The sections so treated were then incubated with primary antibody for 60 min. The slides were then washed with TBS twice for 5 min each. Subsequently, they were incubated with BIOGENEX SS Polymer for 30 min. The slides were then washed as before in TBS and incubated with fresh DAB chromogen for 4–5 min. DAB chromogen was prepared by adding DAB to the buffer at the ratio of 1:2. The slides were then washed in water to stop the chromogen reaction and remove the excess DAB and counterstained with hematoxylin provided in the kit for 1 min. The slides were then dehydrated through iso-propyl alcohol, cleared using xylene and mounted with DPX. Similarly, E-Cad and collagen IV were assessed using appropriate primary and secondary antibody kits. Dako IHC kit for vimentin and E-Cad and Biogenx kit for collagen IV were used.

Evaluation of staining for e-cadherin, vimentin and collagen IV markers

The criteria used to define E-Cad-positive cells were brown staining in cell membrane of epithelial cells; for vimentin, brown staining in the cytoplasm of epithelial cells and for collagen IV, brown staining along the BM [Figures 1-3, respectively].

The percentage of positively stained cells was scored as well as staining intensity was assessed in comparison with the positive control tissue and scored accordingly [Table 1].

Qualitative analysis is made by intensity of staining and quantitative analysis is made by percentage of cells stained; hence, both are considered.

Polymerase chain reaction for epithelial tissue-specific expression of E-cadherin and vimentin

Samples of 40 μm-sized shavings of paraffin-embedded epithelial tissue were provided after separating the connective tissue and epithelial tissue, for tissue-specific E-Cad and vimentin expression by real-time PCR. Tissue sections were deparaffinized with 500 μl 100% xylene under a fume hood. Then, contents were vortexed for 1 min and left in a 65°C water bath for 15 min. The xylene solvent was decanted immediately, and the procedure was repeated two more times to remove the wax content completely. After this to remove the residual xylene, the samples were washed five times with ethanol first by adding 1 ml of absolute ethanol and mixed by vortex for 10 s and removed after 10 min. Then, 1 ml of absolute ethanol was added

Table 1: Scoring for percentage of positive cells and intensity of cell staining

Percentage of positively stained cells	Score	Intensity of cell staining	Score
No cells	0	Negative	0
≤10%	1	Weak	1
11%-50%	2	Moderate	2
51%-80%	3	Strong	3
≥81%	4		

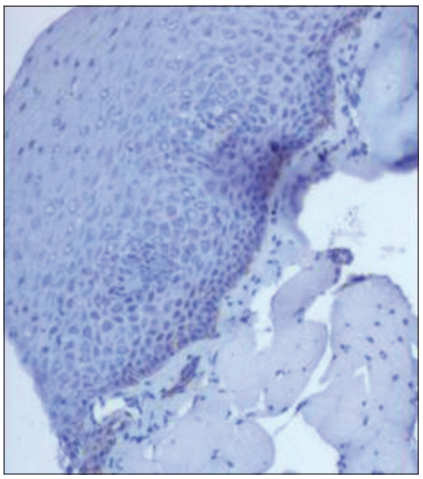

Figure 1: Epithelial cadherin-negative expression in oral submucous fibrosis cases

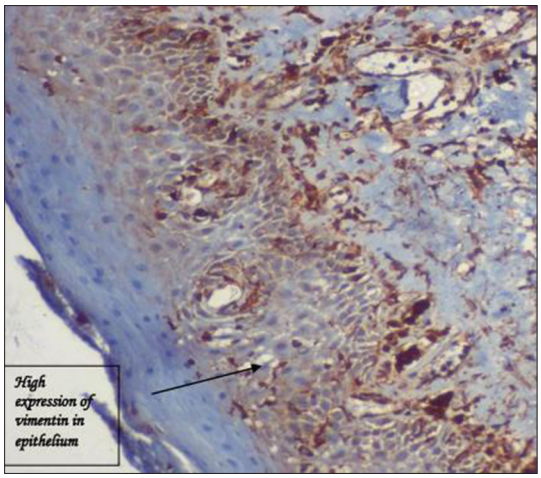

Figure 2: High expression of vimentin in epithelium

and mixed by vortex for 10 s and removed after 30 min. Later, the ethanol concentration was gradually decreased by adding 1 ml of 90% ethanol and mixed by vortex for 10 s and after 20 min, the tube was centrifuged at 8000 rpm and then ethanol was removed out. This was followed by adding 1 ml of 70% ethanol, mixed by vortex for 10 s and after 20 min, the tube was centrifuged at 8000 rpm and then ethanol was removed out. Finally, 1 ml of 50% ethanol was added and mixed by vortex for 10 s and after 20 min, the tube was centrifuged at 8000 rpm and then ethanol was removed out.

The microtubes were then left in a 40°C oven to dry the tissues and to evaporate the residual ethanol. After the tissues were dried, 500 µl lysis solution was added to each microtube (40 mM Tris, 1 mM ethylenediaminetetraacetic acid, 0.5% Tween 20, 0.5 µg/µl proteinase k, pH, 8); the proteinase k was added after pH adjustment and the microtubes were left in a 60°C water bath and were inverted every 30 min until the tissue was completely lysed (temperatures over 60°C will inactivate the enzyme rapidly). Then, the resulting cell lysate was heated at 95°C for 8 min to inactivate the proteinase k.

For the DNA extraction from cell lysate, phenolchloroform was used as follows: An equal volume of trissaturated phenol (pH, 8) was added and the tubes were left on a rotating wheel for 10 min and then centrifuged at 12,000 for 2 min. Then, 300 µl of the upper phase was transferred to a new microtube and an equal volume of phenol: chloroform mixture (1:1) the tube contents were mixed on the rotating wheel for 10 min and were centrifuged at 12,000 ×*g* for 2 min. The upper phase was transferred to a new microtube and 2.5 µl of cold absolute ethanol was added. For DNA precipitation, the microtube was left overnight at 20°C. Then, tubes were centrifuged for 30 min at 4 and ethanol

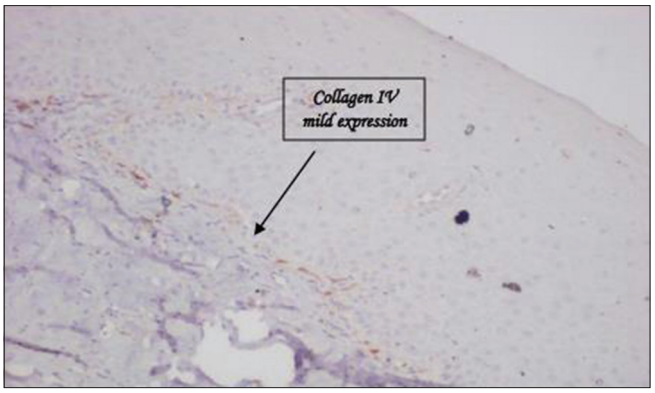

Figure 3: Mild expression of collagen IV in oral submucous fibrosis

was decanted. The DNA pellet was gently washed two times with cold 70% ethanol and then the pellet was dried completely at room temperature. The pellet was dissolved in 30–70 µl of sterile distilled water, depending on the size. To complete the solubilization, the microtubes were put in 40°C water bath for 1 h. To check the extracted DNA, 5 µl of the solution was run on a 1% agarose gel.

Agarose gel electrophoresis

0.8 g of agarose powder was measured and added to a 250-ml conical flask. To the flask, 100 ml TAE (1x) buffer was added and mixed properly. The agarose was melted in a microwave oven or hot water bath until the solution became clear. The solution was allowed to cool to about 50°C–55°C with swirling of the flask occasionally to cool evenly. 1–2 µl of ethidium bromide solution was added to the melted agarose gel and mixed well. Ends of the casting tray were sealed and combs were placed. Molten agarose solution was poured into the casting tray without forming air bubbles and let to cool until it attained solid state. The combs were carefully pulled out, the tape was removed and the gel was placed in the electrophoresis chamber. Sufficient amount of 1x TAE buffer was added so that there is about 2–3 mm of buffer over the gel. 6 µl of 6x sample loading buffer was added to each 25 µl of the DNA sample. Each sample/DNA ladder was carefully pipetted into separate wells in the gel. Electrode wires were connected to the power supply, making sure that the positive (red) and negative (black) are connected correctly. Power supply was turned on to about 100 V (should not exceed 5 V/cm between electrodes). After sufficient run, the power pack was turned off, and the gel was removed by using gloves. The DNA in the gel was visualized with ultraviolet (UV) light, photographed and documented using UV trans-illuminator.

No band of DNA was present in the samples isolated. As there was no band detected in the gel, the DNA was quantified using spectrophotometer, and all the samples

had no nucleic acid detected. This could be because of the smaller size of the samples. As there are wax removing steps and further purification steps, the concentration of DNA would have reduced to detect in the PCR. Further, PCR done for Ecad and vimentin along with human actin PCR did not yield an expected amplification. This confirmed the lack of required concentration of DNA for the primers to probe the complementary region in the DNA.

Later, 50-μm tissue shavings from the paraffin blocks were used for DNA isolation as per the above mentioned method. The isolated DNA was checked on the agarose gel, and the presence of DNA was detected for the samples [Figure 4].

Primer designing

The primers for E-Cad and vimentin were designed using Primer Express software, synthesized and purified by high-performance liquid chromatography for real-time PCR purpose.

- E-Cad forward: 5'-TGC CCA GAA AAT GAA AAA GG-3'
- Reverse: 5'-GTG TAT GTG GCA ATG CGT TC-3'
- Vimentin forward: 5'-AAA GCG TGG CTG CCA AGA AC-3'
- Reverse: 5'-GTG ACT GCA CCT GTC TCC GGT A-3'.

The primers were validated for the samples, and it was observed that, when PCR was performed with two randomly picked samples, both the primers yielded the expected band, i.e., 240 bp as shown in Figure 4.

Real-time PCR (RT-PCR quantification) was performed in Applied Biosystems StepOne Real Time PCR (Foster City, CA, USA). All reaction components were procured from Life Technologies, USA. Standard reaction volume 10 μl contains 1x SYBR Green master mix, 50 ng DNA template and 100 nM of oligonucleotide primer. The initial step of RT-PCR was 2 min hold at 95°C. Cycles (n = 40) consisted of a 10 s melt at 95°C, followed by a 30 s annealing and extension at 60°C. All reactions were performed in duplicates against a serially diluted standard. PCR amplicons of E-Cad and vimentin were cloned into the plasmid and used as a standard for the quantification of the sample. Threshold cycle (Ct) analysis of all samples was either set at 0.5 relative fluorescence units or left to automatic detection by the system.

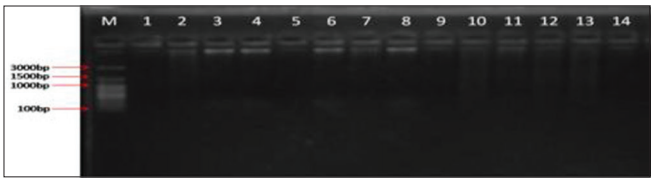

Figure 4: Gel image showing the DNA bands from the tissue shavings

Absolute quantification analysis

A standard curve with the highest R2 value was constructed based on the values generated by the quantitative PCR, and the quantity of E-Cad and Vimentin in each sample was calculated against the standard values.

Using the above criteria, intensity of staining and percentage of cells stained were calculated by a single observer.

RESULTS

Males dominated the three study groups compared to females. The mean age of the study population in each group was comparable and was in the third decade [Table 2].

The percentage of positive vimentin cells between 11% and 50% was maximum in OSMF group (72.1%) as compared to other groups. OSMF group was the only group to have 1.6% of cells above 50%. When compared with other two groups, these were statistically significant. OSMF cases had maximum intensity of 39.3% in moderate group, followed by 16.4% in strong group and 1.6% in very strong group which was higher intensity compared to other groups. Maximum number of negative staining was seen in normal cases. Weak staining was maximum in cases with a habit of chewing gutkha and other forms of areca nut. Intergroup comparison showed statistical significance [Table 3].

OSMF group showed marked reduction in E-Cad levels with 68.9% below or equal to 10% of positive cells as compared to other groups. Normal cases (healthy) showed 61.9% in 51%–81% category. Group with a habit history also showed marked reduction in E-Cad levels, with 72.1% at below or equal to 10% of positive cell category [Table 3].

Collagen IV analysis showed OSMF group had maximum cases in negative (19.7%) and ≤10% (68.9%) category followed by the habit cases (39.3% in ≤10% category).

Table 2: Age and Gender comparison between different groups

Variables	Categories	OSMF		Habits		Normal		*P*
		n	%	*n*	%	*n*	%	
Gender	Males	47	77.0	46	75.4	47	74.6	0.95[a]
	Females	14	23.0	15	24.6	16	25.4	
		Mean	SD	Mean	SD	Mean	SD	
Age	Mean & SD	34.1	13.4	37.3	17.1	32.8	11.0	0.95[b]
	Median	30.0		28.0		30.0		
	Range	20-80		20-85		20-79		

Table 3: Comparison of percentage of positive cells and level of intensity for vimentin, E cadherin and collagen IV between the three groups using Chi-square test

Variable	Categories	OSMF, *n* (%)	Habits, *n* (%)	Normal, *n* (%)	c^2	*P*
Comparison of percentage of positive cells for vimentin and its level of intensity between the three groups using Chi-square test						
Percentage of positive cells	Negative	1 (1.6)	14 (23.0)	57 (90.5)	128.379	<0.001*
	≤10%	15 (24.6)	26 (42.6)	4 (6.3)		
	11%-50%	44 (72.1)	21 (34.4)	2 (3.2)		
	51%-80%	1 (1.6)	0 (0.0)	0 (0.0)		
	≥81%	0 (0.0)	0 (0.0)	0 (0.0)		
Intensity	Negative	2 (3.3)	14 (23.0)	57 (90.5)	126.870	<0.001*
	Weak	24 (39.3)	31 (50.8)	5 (7.9)		
	Moderate	24 (39.3)	16 (26.2)	0 (0.0)		
	Strong	10 (16.4)	0 (0.0)	1 (1.6)		
	Very strong	1 (1.6)	0 (0.0)	0 (0.0)		
Comparison of percentage of positive cells for E-cadherin and its level of intensity between the three groups using Chi-square test						
Percentage of positive cells	Negative	4 (6.6)	2 (3.3)	1 (1.6)	102.871	<0.001*
	≤10%	42 (68.9)	44 (72.1)	0 (0.0)		
	11%-50%	10 (16.4)	11 (18.0)	21 (33.3)		
	51%-80%	5 (8.2)	4 (6.6)	39 (61.9)		
	≥81%	0 (0.0)	0 (0.0)	2 (3.2)		
Intensity	Negative	3 (4.9)	2 (3.3)	1 (1.6)	73.090	<0.001*
	Weak	24 (39.3)	17 (27.9)	0 (0.0)		
	Moderate	1 (1.6)	13 (21.3)	15 (23.8)		
	Strong	28 (45.9)	25 (41.0)	16 (25.4)		
	Very strong	5 (8.2)	4 (6.6)	31 (49.2)		
Comparison of percentage of positive cells for collagen-4 and its level of intensity between the three groups using Chi-square test						
Percentage of positive cells	Negative	12 (19.7)	0 (0.0)	0 (0.0)	115.979	<0.001*
	≤10%	42 (68.9)	24 (39.3)	0 (0.0)		
	11%-50%	6 (9.8)	32 (52.5)	43 (68.3)		
	51%-80%	1 (1.6)	5 (8.2)	14 (22.2)		
	≥81%	0 (0.0)	0 (0.0)	6 (9.5)		
Intensity	Negative	12 (19.7)	0 (0.0)	0 (0.0)		
	Weak	15 (24.6)	16 (26.2)	0 (0.0)	101.511	<0.001*
	Moderate	34 (55.7)	23 (37.7)	13 (20.6)		
	Strong	0 (0.0)	18 (29.5)	44 (69.8)		
	Very strong	0 (0.0)	4 (6.6)	6 (9.5)		

*Statistically significant. OSMF: Oral submucous fibrosis

Normal cases showed maximum of 68.3% positive cells in 51%–80% category. Intergroup results were statistically significant. The above results indicate a very early event in breach of the BM depicted by collagen IV in OSMF group followed by habit group [Table 3].

PCR values for percentage of positive cells stained for vimentin were statistically significant in OSMF group. This implied that PCR values corresponded to the aberrant vimentin expression in OSMF [Table 4]. However, the intensity of staining and PCR values were not statistically significant in all the three groups. No statistically significant results were seen in comparison of PCR values and intensity and the number of positive cells stained for E-Cad in all the three groups.

DISCUSSION

Being a member of the classical cadherins, E-Cad is a large single-pass transmembrane glycoprotein (120 kDa) involved in calcium-dependent cell–cell adhesion.[8,9] CDH1 which is located at chromosome 16q22.1 encodes E-cad whose tail end is associated with many different cytoplasmic proteins. These proteins are mainly composed of the a-, b- and p120-catenins.[9]

Different E-Cad-binding partners mediate and regulate the activity of E-Cad, particularly its association with the actin–myosin cytoskeleton, its transport and recycling and interactions with the epithelial cells. The cadherin switch plays an important role in cancer propagation.[9]

In OSMF, changes in oral epithelium and BM thickness with numerous alterations in molecular level are noted. E-Cad is considered an indicator of EMT induction in OSMF. Increased expressions of p-63 gene and its oncogenic isoform 1 with reduction in E-Cad levels and beta catenin levels are seen in OSMF.[10]

Vimentin, a type III intermediate filament protein, is another EMT marker involved in a number of diseases and conditions, including cancer, inflammation and congenital cataracts.[11] It is predominantly expressed in mesenchymal,

Table 4: Comparison of mean polymerase chain reaction values between percentage of positively stained cells and for vimentin and E-cadherin using Kruskal-Wallis test

Comparison of mean PCR values between percentage of positively stained cells for vimentin using Kruskal-Wallis test							
Variable	**Group**	**Percentage of cells positive**	***n***	**Mean**	**SD**	***H***	***P***
Percentage of positive cells	OSMF	≤10%	15	52,593,033.1	19,342,266.9	8.689	0.01*
		11%-50%	44	66,853,597.2	19,316,288.6		
		51%-80%	1	95,945,634.0			
	Habits	≤10%	26	33,925,948.0	21,876,678.3	1.080	0.30
		11%-50%	21	29,068,668.5	24,424,343.7		
	Normal	≤10%	4	2,994,180.5	2,324,071.1	1.929	0.17
		11%-50%	2	445,137.5	342,405.9		
Comparison of mean PCR values between percentage of positively stained cells for E-cadherin using Kruskal-Wallis test							
Variable	**Group**	**Percentage of cells positive**	***n***	**Mean**	**SD**	***H***	***P***
Percentage of positive cells	OSMF	≤10%	42	48,141,755.4	67,417,718.2	0.729	0.70
		11%-50%	10	41,099,803.1	24,010,725.0		
		51%-80%	5	44,763,071.6	11,010,989.7		
	Habits	≤10%	44	82,538,624.1	144,392,713.4	0.795	0.67
		11%-50%	11	66,197,742.1	69,696,270.7		
		51%-80%	4	57,318,521.0	16,767,064.7		
	Normal	11%-50%	21	126,090,938.1	172,175,545.4	3.976	0.14
		51%-80%	39	212,256,268.2	250,829,433.9		
		≥81%	2	32,153,145.0	13163.5		

*Statistically significant. PCR: Polymerase chain reaction, SD: Standard deviation, OSMF: Oral submucous fibrosis

many undifferentiated and cultured cells and also associated with the metastasis of cancer.[12-14]

The role of vimentin is increasingly recognized in carcinogenesis and is seen to be upregulated in many invasive cancers. Vimentin might play a key role in EMT associated with stress or pathological situations.[12]

Aberrant vimentin expression of oral dysplastic and fibrotic tissues such as OSMF has been reported in literature. In addition, vimentin has shown a significant correlation with aggressive phenotype and survival of the OSCC patients.[12]

Loss of E-Cad and gain of vimentin could be used as important EMT markers in the progression of carcinoma *in situ* to microinvasive or invasive squamous cell carcinoma.[13] Hence, the expression of E-Cad and vimentin using PCR in OSMF cases was considered in our current research.

Sawant *et al.* studied 71 samples of OSMF using IHC and reported aberrant vimentin expression in the epithelial cells to be suggestive of EMT. They also observed 227 cancer cases which showed positive EMT.[15] An IHC study conducted on 100 biopsy samples of normal, dysplastic and squamous cell carcinoma cases by Chaw *et al.* revealed decreased E-Cad expression but increased vimentin expression that correlated with increased disease severity.[16]

Fernández *et al.* reported downregulation of E-Cad in dysplastic cells by evaluating the expression of E-Cad, vimentin, CD31 and CD117 in 16 oral epithelial dysplasia, 19 oral squamous cell carcinoma and 16 normal mucosa samples. In addition, epithelial cells expressed vimentin which is a mesenchymal marker.[17] Our IHC observations showed similar vimentin and E-Cad expressions.

Our study results showed that percentage of cells stained for vimentin was maximum in OSMF group followed by habit group. PCR values for vimentin were highest in OSMF group. The expression of E-Cad was less in OSMF group when compared to habit group.

Tosios *et al.* conducted an IHC study using mouse monoclonal antibodies for analyzing laminin and collagen IV in 30 dysplastic tissue (leukoplakia) and 50 invasive squamous cell carcinoma tissue specimens. The study revealed alterations in the distribution of laminin and type IV collagen indicating the loss of continuity of the subepithelial BM which in turn implies the neoplastic transformation process in oral epithelium.[18] Agarwal *et al.* evaluated the expression of type IV collagen in three histological grades of oral squamous cell carcinoma in comparison with that in normal oral mucosa.[19] IHC using monoclonal mouse antibodies noted discontinuity of BM in squamous cell carcinoma. Normal mucosa showed no breach in the BM. OSMF and habit groups in the present study showed discontinuity of BM.

CONCLUSION

This observational study suggests that EMT signatures are definitely positive in OSMF cases. Surprisingly, habit cases also showed positive EMT signatures even before the manifestation of any disease. These signatures occur even before dysplastic changes are evident histopathologically.

Exposure to the habit of areca nut chewing may initiate EMT signatures long before clinical manifestations irrespective of the duration or frequency of the habit. Hence, this study gives valuable inputs for initial changes instigating disease process before classic clinical or histopathologic changes.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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