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E-Cadherin truncation and Sialyl Lewis-X overexpression in oral squamous cell carcinoma and oral precancerous conditions

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The present study aimed to determine significance of E-cadherin, a cell adhesion molecule, and sialyl Lewis-X (sLe^X), a cell surface antigen, in oral carcinogenesis. Expressions of E-cadherin and sLe^X were detected using western blot analysis from oral malignant (n=25), and oral precancerous tissues (OPC, n=20) and their adjacent normal tissues. An altered expression of E-cadherin (E-cad) and sLe^X was observed in malignant and precancerous tissues. E-cad western blot revealed presence of two bands, a 120 kDa (native, $E\text{-}cad^{120}$) and a 97 kDa (known as truncated $E\text{-}cad^{97}$). The accumulation of truncated $E\text{-}cad^{97}$ and sLe^X in malignant and OPC tissues compared to their adjacent normal tissues was observed. Receiver's Operating Characteristics (ROC) curve analysis showed good discriminatory efficacy of $E\text{-}cad^{97}$, $E\text{-}cad^{97:120}$ ratio and sLe^X between the malignant and adjacent. normal tissues. Further, a positive correlation of $E\text{-}cad^{97}$ and sLe^X overexpression with advanced stage of the disease and lymphnode metastasis was observed. The data suggest that E-cadherin truncation and sLe^X overexpression are early events which may facilitate the tumor cells to metastasize. Also, overexpression $E\text{-}cad^{97}$ and sLe^X in OPC tissues may be useful to predict metastatic potentials of tumors at an early stage of oral carcinogenesis.

Key words: Oral cancer, oral precancerous conditions, E-cadherin, sialyl Lewis-X, metastasis

Epithelial tumors are the predominant form of cancer metastasis and oral cancer is one of the epithelial neoplasias. A high transformation rate ranging from 6 to 36% from OPC to oral cancer have been reported [1]. Despite the number of studies aimed at the improvement of the early diagnosis and treatment of patients with oral squamous cell carcinoma (OSCC), their prognosis and survival remains poor [2]. Only about 15% of the oral cancer patients are diagnosed at an early stage. Unfavorable prognosis of OSCC is mainly due to extensive local invasion, frequent lymphnode metastasis and local recurrence of tumors. The metastatic dissemination of tumor cells is the primary cause of morbidity and mortality in cancer patients. Recent studies reported that most cancer cells in primary tumor have a 'metastatic phenotype', indicating that metastatic spread is an early event in tumorigenesis [3, 4].

Several critical steps are involved in the metastatic cascade depend on cell surface molecules such as E-cadherin and carbohydrate ligand/ receptors like sialyl Lewis-X (sLe^X). These molecules play an important role in cell-adhesion, intracellular signal transduction, cell-to-cell and cell-matrix communications, and cell migration. Cell adhesion molecules, such as E-cad, is essential for maintaining the stable structure of stratified squamous epithelium. E-cad, a 120 kDa ($E\text{-}cad^{120}$) transmembrane glycoprotein mediates homophilic adhesion in epithelial cells and is a major determinant of epithelial cell differentiation and tumor progression. It has been shown to play a role in anchorage-dependent growth and survival of SCC [5]. Suppression or post-translational changes of E-cad expression is one of the molecular events responsible for dysfunction of cell adhesion [6]. Loss of E-cad expression or functions has been demonstrated to be associated with tumor development and progression [7, 8]. Further, re-expression of native E-cad restores cell adhesion functions and reduces the invasiveness of cancer cells, which referred E-cad as the invasion suppressor gene [9]. Loss of E-cad expression is one of the characteristics for epithelial-mesenchymal transition, which increase cell motility and promote tumor invasion [10]. Thus, the control of cellular adhesion and motility is one of the crucial mechanisms during carcinogenesis as their functional loss is characterized by rapid tumor initiation and progression.

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Cell surface carbohydrate sLe^X, a tetrasaccharide (NeuAcα2 → 3Galβ1 → 4[Fucα1→3] GlcNAcβ1 → R), is characteristic of various stages of differentiation. It plays an important biological role in the recruitment and extravasation of leukocytes [11]. Several clinical studies have shown significantly increased sLe^X expression on cancer cells and correlated with disease progression [12–14]. sLe^X is frequently expressed on human cancer cells; serve as ligand for E- and P-selectin present on vascular endothelial cells [11, 15]. The degree of expression of this ligand at the cancer cells surface is thought to be correlated with the frequency of hematogenous metastasis and prognostic outcome of cancer patients [16–18]. It has been shown that expression of sLe^X in mucin-type O-glycans is highly associated with lymphatic and venous invasion [19].

Although, E-cad and sLe^X are crucial for many cellular mechanisms associated with cancer cell invasion and metastasis, there is lack of reports on their simultaneous evaluation in oral cancer and OPC. The present study evaluates E-cad truncation and sLe^X overexpression in oral cancer and the precancerous conditions as well as their correlation with various clinicopathological characteristics.

Patients and Methods

Patients. The study enrolled 25 untreated patients with OSCC and 20 patients with OPC from out patients' department of The Gujarat Cancer & Research Institute, Ahmedabad. The disease was diagnosed based on patients' clinical, radiological and histopathological examinations. The TNM staging of tumors was determined according to the guidelines from the World Health Organization (WHO) classification [20]. Distribution of oral cancer patients with regard to various clinicopathological characteristics including age, stage, tumor differentiation, nuclear grade and lymphnode metastasis is provided in table 1. All patients were diagnosed having SCC and 17/25 patients had advanced stage of the disease (Stage III+IV).

Tissue specimens. Fresh oral tumors and adjacent normal tissues (N=25) were collected immediately after surgical resection. The adjacent normal tissues were selected from the free margins at least 1-2 cm away from the tumor as defined by the pathologist. Similarly, precancerous tissues and their matched pairs (N=20) were collected at the time of biopsy. Histopathological confirmation of tumor, OPC and adjacent normal regions of each tissue were done by a pathologist. Tissues were washed with PBS to remove blood debris and immediately stored at –70°C until analyzed.

Preparation of protein lysates and western blot analysis. Tissues were washed with cold (4°C) PBS and homogenized in protein lysis buffer [50 mM Tris pH 7.5, 120 mM NaCl, 0.5% Nonidet p-40, a protease inhibitor cocktail]. This was followed by 1 hour incubation at 4°C and centrifugation. The supernatants were collected and total protein concentrations determined with Lowry method using bovine serum albumin (BSA) as a standard.

Table 1. Clinical details of the patients with oral cancer and oral precancerous conditions

Oral Cancer patients	**N = 25**
Age	
Range	19-46 years
Median	42 years
Sites	
Buccal mucosa	N = 10
Tongue	N = 7
Alveolus	N = 5
Lip	N=3
Histopathology	Squamous cell carcinoma
TNM Stage	
Stage I	N = 3
Stage II	N = 5
Early stage (I+II)	**N = 8**
Stage III	N = 5
Stage IVA	N = 10
IVB	**N = 2**
Advanced stage (III+IV)	**N = 17**
Lymphnode involvement	
No	N = 11
Yes	N = 14
Tumor differentiation	
Well	N = 10
Moderate or Poor	N = 15
Nuclear Grade	
I	
II / III	N = 11 N = 14
Patients with OPC	**N = 20**
Age	
Range	15-38 years
Median	25 years
Precancerous conditions	
Leukoplakia	N = 8
Submucous fibrosis	N = 12

OPC – oral precancerous conditions

Aliquots of whole tissue protein from each malignant, OPC and adjacent normal tissues were mixed with an appropriate volume of sample laemelli buffer (6X) containing SDS (4%), Tris (125mM) and DTT prior to being boiled at 95°C for 5 min. Equal amounts (50µg) of protein were then separated by electrophoresis using 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham). The equal protein loading and transfer onto membrane was confirmed by Ponceau S staining. After blocking with 5% non-fat milk in a buffer containing Tris-HCl (20mM, pH 7.5), NaCl (100mM) and Tween 20 (0.1%), membranes were incubated at 4°C overnight either with primary antibodies against E-cad (2µg/ml dilution) or sLe^X (1µg/ml dilution). Membranes were then washed with TBS-T for 30 min and incubated for 2 hours at room temperature with appropriate secondary antibodies conjugated to horseradish peroxidase (1:1000 dilution). Immunoreactive bands were then visualised using enhanced chemiluminescence detection system (ECL detection kit, Amersham Pharmacia, UK). The Gel documentation Bio-imaging system was used to

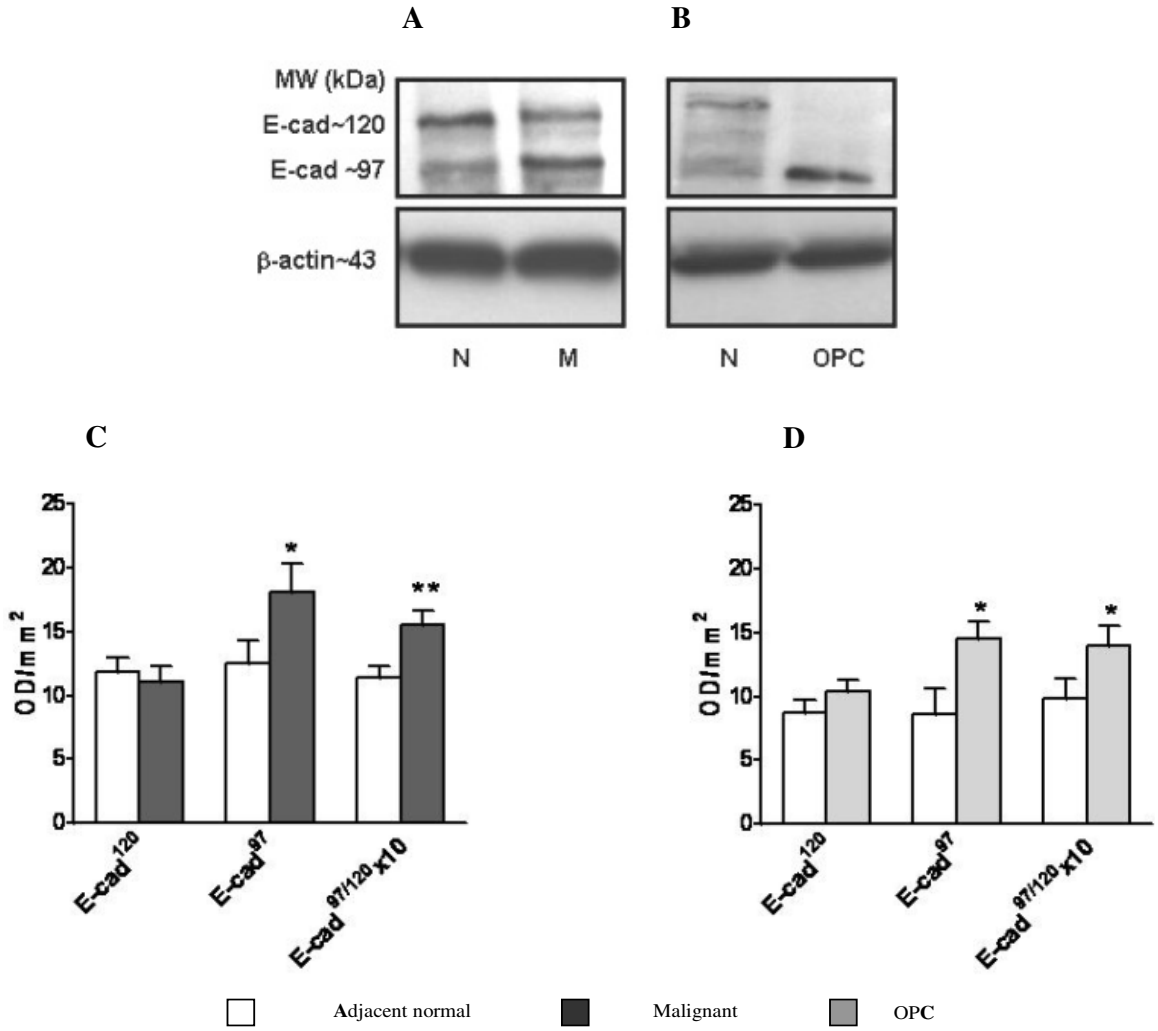

Figure 1. (A, B): Representative western blots for E-cadherin expression in patients with (A) oral cancer and (B) oral precancerous conditions N – Adjacent normal tissue, M – oral malignant tissue, OPC – oral precancerous tissue
(C, D): Comparison of full-length (E-cad^{120}), truncated (E-cad^{97}) and ratio (E-cad$^{97:120}$) between malignant (C) or OPC (D) and their adjacent normal tissues
*** < 0.05, ** < 0.01**

capture the images and densitometry analysis (Bio-Rad, USA). The band density was expressed as OD/ mm^2. The ratio for E-cad^{97}: E-cad^{120} was also calculated.

Antibodies. The HECD-1 mouse monoclonal antibody (Calbiochem, USA) was used to detect the full-length E-cad^{120} and truncated E-cad^{97}. Clone KM-93 (Chemicon, USA) mouse monoclonal antibody was used to detect the sLe^X. The anti-mouse horseradish peroxidase conjugated secondary antibody was used to detect the protein (Amersham).

Statistical analysis. Data were analyzed using the SPSS statistical software (version 10). Student's paired t-test was performed to compare E-cad^{120}, E-cad^{97}, E-cad$^{97:120}$ ratio and sLe^X in oral malignant or OPC tissues with their adjacent normal tissues. Multivariate analysis was performed to assess the correlation of E-cad^{120}, E-cad^{97} and sLe^X expression in malignant tissues with various clinicopathologic characteristics of OSCC. Receiver's operating characteristic (ROC) curves were constructed to evaluate discriminatory efficacy of E-cad^{120}, E-cad^{97}, E-cad$^{97:120}$ and sLe^X between oral malignant and adjacent normal tissues. The values were expressed as mean ± standard error of mean (S.E.M.). p values equal to or less than 0.05 were considered statistically significant.

Results

E-cadherin truncation in oral cancer and OPC tissues. The Fig. 1 is the representative western blot for E-cad in oral tumors (1A) and OPC tissues (1B) with their matched

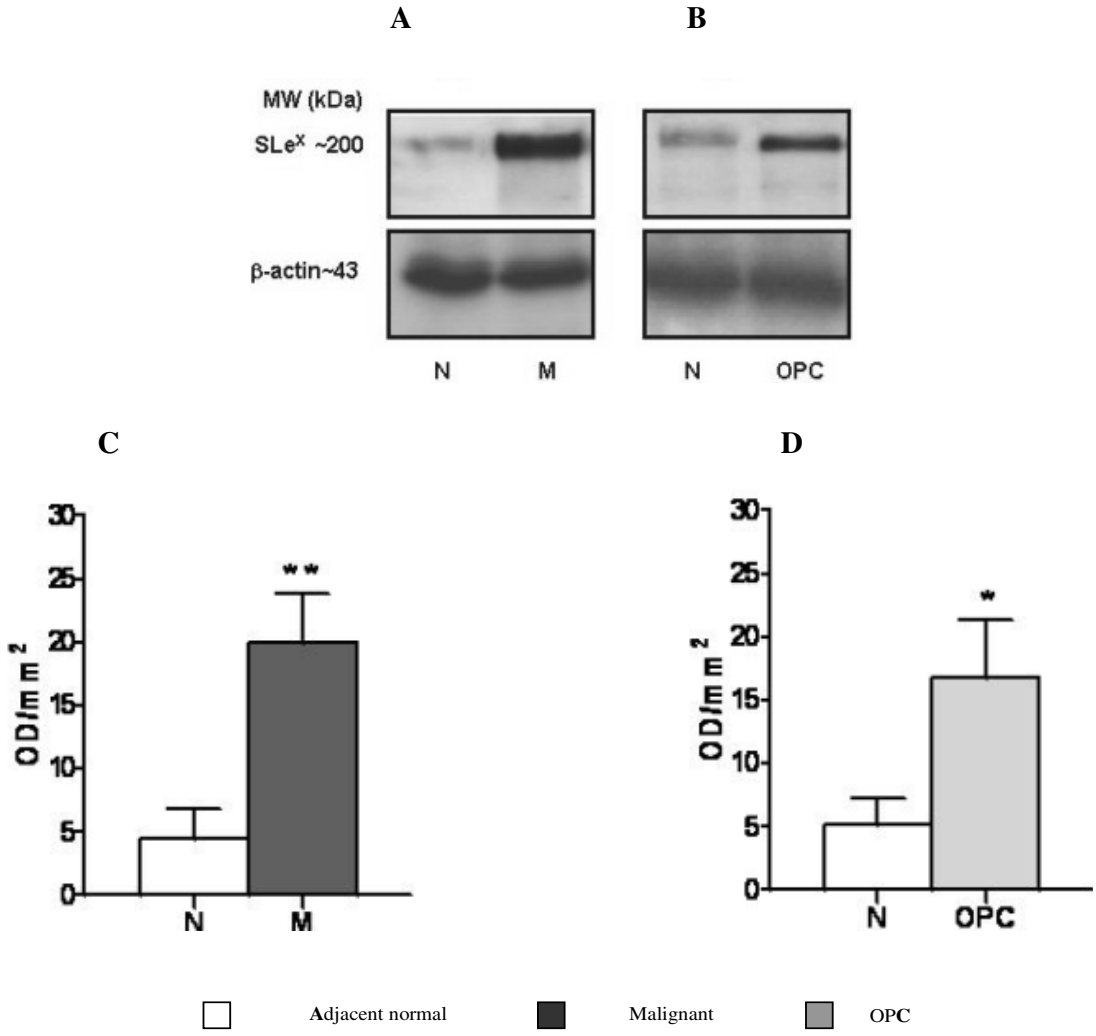

Figure 2. (A, B): Representative western blots for sialyl Lewis-X expression in patients with (A) oral cancer and (B) oral precancerous conditions
N – adjacent normal tissue, M – oral malignant tissue, OPC- precancerous tissue
Std. MW – standard molecular weight markers
(C, D): Comparison of SLe^X band intensities between malignant (C) or OPC (D) and their adjacent normal tissues
*** < 0.05, ** < 0.01**

adjacent normal pairs. The oral malignant and precancerous tissues showed higher expression of ~97 kDa protein band (also known as post-tranalationally truncated E-cad[97]) reactive with HECD-1 antibody along with native 120 kDa E-cad (E-cad[120]). The E-cad[97] band was also seen in a few adjacent. normal tissues of oral cancer and OPC. We found that the adjacent. normal tissues showing presence of truncated band had advanced stage of oral cancer. Densitometric analysis was performed and the band intensities were quantitated followed by paired-t test analysis to compare intensities between the groups. Also, apparent increase in truncated E-cad is shown by calculating the ratio of E-cad[97] to E-cad[120]. The results of densitometric analysis are documented in figures 1C and 1D. As shown in the graphs, E-cad[97] accumulation were significantly increased in malignant (1C) and OPC (1D) tissues as compared to their adjacent. normal tissues (p=0.01, p=0.033, respectively). The ratio of E-cad[97:120] were also significantly higher in malignant tissues compared to their adjacent. normal tissues (p=0.001). Similarly, E-cad[97] expression was also significantly higher in OPC tissues, whereas, native E-cad[120] did not show noteworthy difference between malignant/OPC tissues and their matched normal pairs.

Sialyl Lewis-X overexpression in oral cancer and OPC tissues. The representative western blots for sLe^X in oral malignant (Fig.2A) and OPC (Fig. 2B) tissues with their respective adjacent. normal tissues are shown in figure-2. Reactivity of anti- sLe^X antibody revealed a band of ~200

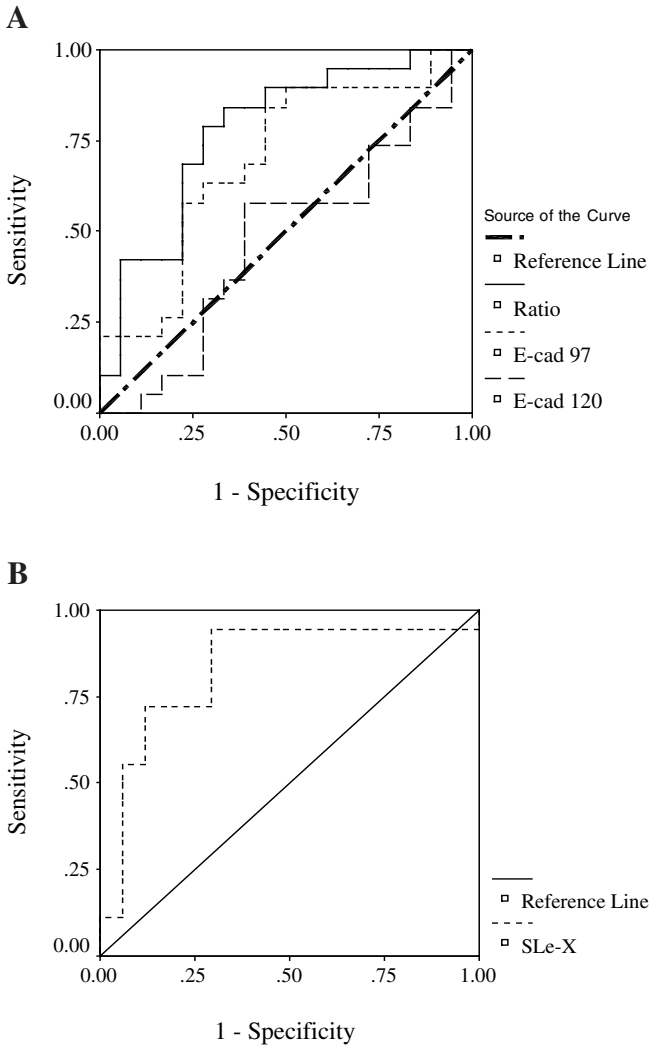

Figure 3. ROC curves for (A) E-Cad^{120}, E-Cad^{97} and E-Cad$^{97:120}$ ratio, (B) SLe^X between oral malignant and adjacent normal tissues

kDa molecular weight. sLe^X exhibit higher band intensity in tumor tissues than adjacent. normal and OPC tissues. The patterns clearly indicate that SLe^X expression was relatively low in OPC tissues as compared to oral malignant tissues and higher than their matched normal tissues. A comparison of mean OD/mm^2 obtained from densitometric analysis of tumor/OPC tissues with their respective adjacent normal tissues is shown in Figs. 2C and 2D. Accordingly, sLe^X expression was significantly higher in malignant tissues as compared to adjacent. normal tissues (p=0.001). Moreover, OPC tissues also showed markedly increased expression than adjacent. normal tissues (p=0.03).

ROC curve for E-cadherin and SLe^X. ROC curve is a statistical analysis to examine the discriminatory efficacy of the parameters between two groups under the study which simultaneously considers sensitivity and specificity of the

Table 2. Statistical data of ROC curve analysis

Variables (cut-off in OD/mm^2)	AUC	Significance	Sensitivity	Specificity
E-cad^{120}	0.477	0.808	NS	
E-cad^{97} (>11.03)	0.696	**0.007**	54%	85%
E-cad^{97}: E-cad^{120} (>1.02)	0.778	**0.0007**	61%	85%
SLe^X (>4.5)	0.83	**< 0.0001**	76%	76%

AUC – area under the curve

Table 3. Multivariate analysis for correlation of E-cadherin and SLe^X with clinicopathological parameters in oral tumors

Markers	Nuclear grade	Tumor differentiation	Stage	Lymphnode metastasis
E-cad^{120}	NS	NS	NS	NS
E-cad^{97}	0.05	0.059	0.05	0.02
E-cad$^{97:120}$	0.086	0.1	0.05	0.079
SLe^X	0.04	0.088	0.015	0.042

NS – non-significant

parameters. The ROC curves for E-cad^{120}, E-cad^{97}, ratio of E-cad$^{97:120}$ and sLe^X in adjacent. normal vs. oral malignant tissues are shown in Fig.3 (A, B), and their corresponding sensitivity, specificity and area under the curves (AUC) are presented in Tab.2. The accumulations of truncated E-cad^{97}, high E-cad$^{97:120}$ ratio and sLe^X overexpressions were statistically significant in malignant tissues than their adjacent. normal mucosa (p=0.007, p<0.0007 and p<0.0001, respectively). The cut-off values for tumor E-cad^{97} (>11.03 OD/mm^2), E-cad$^{97:120}$ ratio (>1.02 OD/mm^2) and sLe^X (>4.5 OD/mm^2) were determined from multiple points on the ROC curve which was close to the mean value in adjacent normal tissues. At the chosen cut-off, E-cad^{97} and E-cad$^{97:120}$ showed 54% and 60% sensitivity respectively with a specificity of 85% in tumor tissues. The sLe^X expression in malignant tissues also showed good discriminatory efficacy with high sensitivity (76%) and specificity (76%).

Association of E-cadherin and sLe^X expressions with clinicopathological characteristics in oral cancer patients. Multivariate analysis was performed to determine the correlation of E-cad and sLe^X in oral malignant tissues with clinicopathological parameters. As shown in table 3, poorly differentiated grade III oral tumors showed high expression of E-cad^{97} and sLe^X. Further, E-cad^{97} and sLe^X were significantly higher in tumors of patients with lymphnode metastasis and advanced disease as compared to the patients without lymphnode metastasis and early disease stage, respectively. Also, positive correlation was seen between E-cad$^{97:120}$ in tumors and various pathological parameters. However, E-cad^{120} expressions did not change when compared among the histological parameters. Thus, the data suggest a clear association of loss or truncation of E-cad and overexpression of sLe^X with disease progression and poor prognosis of the patients.

Discussion

Loss of cellular adhesion, detachment of the tumor cells from the primary sites and migration to distant organs are important steps in the metastases [21]. Earlier reports have shown the association of impaired expression of E-cad and sLe^X with various stages of cancer progression [8, 22–25]. In the present study, we demonstrate overexpression of truncated E-cad^{97} and sLe^X is an early event during oral carcinogenesis. Further, ROC curve analysis revealed good sensitivity and specificity for E-cad^{97}, E-cad$^{97:120}$ and sLe^X suggesting an early event. Also, loss of E-cad and overexpression of sLe^X showed positive correlations with oral cancer progression.

E-cad expression has been reported in various human cancers [7, 22, 26]. The mechanisms of functional loss of E-cad expressions is still inadequately understood, whether it occur at genetic levels or the abnormal synthesis or the posttranslational modification of the protein. However, much larger fraction of cancer showed reduced or absent E-cad mRNA or protein expression, yet no mutations in E-cad gene were detected [7]. In the present study, E-cad expression in oral cancer and OPC tissues was detected by western blot analysis using HECD-1 antibody. The antibody reacts with both ~120 kDa E-cad under normal conditions, and ~97 kDa truncated E-cad. Densitometry and statistical analysis revealed significant increase in E-cad truncation in tumors and OPC tissues as compared to their matched pairs, whereas, E-cad^{120} did not alter much in those tissues. This indicates that either newly synthesized E-cad by tumor cells may be truncated or the truncation of the native E-cad increases during disease progression. The present findings of E-cad truncation in oral cancer is supported by previous reports in prostate cancer suggesting a link between E-cadherin truncation and tumourigenesis [26, 27]. The cytoplasmic domains of E-cad interact strongly with a group of intracellular proteins catenins; which are essential for cellular adhesion and signaling. The truncation of the E-cad cytoplasmic domain deletes catenin binding sites leading to a loss of cadherin-mediated adhesion [28]. The loss of E-cad on cell surface linked to modulated intercellular adhesion which may facilitate detachment of tumour cells from primary tumor.

In this study, E-cad truncation accompanied overexpression of sLe^X in oral tumors and OPC tissues. The presence of sLe^X on cancer cells serves an efficient ligand for endothelial cells. sLe^X overexpression may induce vascular E-selectin expression during hematogenous metastasis which facilitates cancer cells to travel to the distant organs via blood stream. Interaction of cancer cells with endothelial cells via complex formation of sLe^X-E-selectin may have physiological relevance in hematogenous metastasis of cancer cells [29]. We observed increased expression of sLe^X in oral cancerous and OPC tissues. The approximate band size of carrier protein expressing sLe^X epitope in oral tissues was 200 kDa and this carrier protein could be mucin. According to Hanski et al. mucins MUC1 and MUC2 serve as carriers of sLe^X in colon carcinomas [30]. In support to this, Mann and colleagues suggested several unidentified mucins which may carry significant amount of this carbohydrate epitope [29]. However, other comparative study indicated MUC1 and sLe^X as independent prognosticators for colon cancer [31]. sLe^X expressions have been reported as predictors of invasiveness and metastatic potentials in cancer patients [14, 18, 19]. Farmer et al. have shown that sLe^X has important role in metastasis of head and neck carcinoma [32]. A study on B16-F1 melanoma cells showed that fucosyltransferase III is responsible for sLe^X expression and high metastatic potentials [24]. Furthermore, tumor formation could be inhibited by pre-incubation of those cells with anti-sLe^X antibody. The increased accessibility of sLe^X epitopes in carcinoma has been reported to be associated with altered sialylation and/or fucosylation pattern of cell surface molecules [11]. The data from our laboratory revealed overall increased sialylation [33] and fucosylation of proteins in oral cancerous and precancerous tissues and serum. In addition, we found increased activity of total fucosyltransferase in oral tumors (unpublished data).

E-cad truncation and sLe^X overexpression in OPC tissues demonstrate an early event during oral cancer progression. Also, their expression was seen in few adjacent. normal tissues which may be suggestive of the process of field cancerization. The apparently adjacent normal tissues might have undergone certain genetic and biochemical changes even though these cells may yet be histopathologically normal. This suggests an early event or the presence of occult malignant disease even after surgical resection of the tumor. However, local recurrence may occur even with histopathologically negative surgical margins. Hence, comparison of malignant tissues with their corresponding adjacent normal tissues would help understanding activation of malignant phenotypes.

The present study demonstrates a good discriminatory efficacy of E-cad and sLe^X using semi-quantitative western blot. The efficiency may be improved by ELISA-based quantitative analysis; however, this method is unable to detect truncated form of E-cad. Our data suggest E-cad^{97}, E-cad$^{97:120}$ and sLe^X may be useful to detect early changes during oral carcinogenesis. The E-cad^{97}and sLe^X may be neo-expressed by cancer cells and not present in normal tissues. E-cad and sLe^x expression in the present study was detected from whole protein lysates of tumor tissue. Lysate is a mixture of heterogenous cell populations comprises clusters of premalignant or malignant cells, inflammatory infiltrates and adjacent stromal cells especially fibroblasts and endothelial cells. The use of miscrodessection method helps overcome the drawbacks of tissue complexity by isolation of different cell types including tumor and surrounding stromal cells. The expression of truncated E-cad^{97} and sLe^X in particular cell types could be determined using microdissection in conjunction with molecular techniques. There are different types of microdissection techniques available that has advanced the ability to acquire pure cells population or single cell for molecular analyses and, genome and proteome expression profiling [34–36] It also

helps analyzing early time points during tumorigenic process [37]. However, we could not use mechanical or laser miscrodissection in the present study due to availability of a very small size of tumors.

Multivariate analysis showed the correlation of loss or truncation of E-cad and sLe^X overexpression with oral cancer progression and prognostication. An increased expression of $E\text{-}cad^{97}$, $E\text{-}cad^{97:120}$ and sLe^X was seen in tumor tissues with lymphnode metastasis and advanced disease (stage III+IV) compared to the patients without lymphnode metastasis and early disease (stage I+II), respectively, whereas $E\text{-}cad^{120}$ remained unaltered. Studies have shown E-cad as a prognosticator in various cancers [23, 38–40]. Elzagheid et al. have reported that the survival of lymphnode positive breast cancer patients can be predicted by E-cad index [39]. High levels of soluble E-cad are reported to be a potentially valuable pre-therapeutic prognosticator and correlated with inoperability/ palliative treatment and lymphnode metastasis in gastric cancer [40].

Recently, an inverse correlation between sLex and E-Cadherin has been reported in canine mammary carcinomas [18]. The presence of carbohydrate receptor sLe^X in tumors or serum has been associated well with poor prognosis of various cancers [12, 14, 16, 17] and suggested no other carbohydrate epitope examined to date has equal prognostic value. sLe^X expressions in colorectal and non-small cell lung cancer has been correlated with advanced disease, increased distant metastasis and decreased survival [41]. An *in vitro* study identified molecules capable of interfering metastatic process by inhibiting sLe^X-mediated adhesion between tumor cells and activated endothelial cells [42]. Apart from its use as tumor marker, antibodies raised towards sLe^X may be used as a passive tumor vaccine. Studies have reported the importance of sLe^X and its analogs in cancer treatment [24, 43].

In conclusion, the present study showed association of $E\text{-}cad^{97}$ truncation and sLe^X overexpression with invasive and metastatic potentials of oral cancer and an inverse correlation among them. Increased E-cad truncation and sLe^X expression represents functional loss of cell-adhesion properties, which suggest that these molecules may facilitate tumor cells to escape and metastasize. Further, these alterations in OPC could be an early event during malignant transformation. Overall, these findings propose $E\text{-}cad^{97}$ and sLe^X may be valuable for early detection and better prognostication of oral cancer as well as in understanding the biology of progression of oral cancer metastasis.

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