ORIGINAL ARTICLE

Clinico-pathological correlation of E-cadherin expression at the invasive tumor front of Indian oral squamous cell carcinomas: An immunohistochemical study

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ABSTRACT

Background: Recent studies have indicated that although malignant cells at the invasive tumor front, bare morphological resemblance to the cells at central portion of the tumor, their molecular character differs significantly. E-cadherin is a cell-cell adhesion molecule that connects epithelial cells. This study attempts to correlate the E-cadherin expression at the invasive tumor front with tumor differentiation along with its clinico-pathological parameters. **Materials and Methods:** Immunohistochemical staining with E-cadherin was carried out on archival cases of primary oral squamous cell carcinomas (n = 30). The E-cadherin expression at the invasive tumor front was analyzed and was linked to clinico-pathological parameters including patient prognosis. **Results:** The downregulation of E-cadherin expression at the invasive tumor edge when compared with patient's prognosis yielded a significant correlation (P = 0.041) but its correlation with the degree of differentiation determined was not significant (P = 0.27). Also, its association with tumor size and lymph node status was negative. **Conclusions:** Loss of E-cadherin expression at the invasive tumor front is an important event in the progression of oral squamous cell carcinomas. Tumors with a loss of expression of E-cadherin are those which had a poor prognosis

Key words: Differentiation, E-cadherin, invasive tumor front, oral cancer, prognosis

INTRODUCTION

Oral squamous cell carcinomas (OSCC) constitute the sixth most common cancer diagnosed worldwide.[1] In India, it is the leading cancer among men and the third most common among women. An estimate of 75,000-80,000 new cases are reported annually.[2,3] Although, TNM staging is widely used to determine treatment options, it is not sufficient for optimal prognostication.

A distinctive feature of OSCC is that amongst patients with same stage and site of the tumor, some patients do better than others.[4] This is because OSCC usually exhibit a heterogenous cell population with probable differences in the degree of differentiation; invasive and metastatic behavior.[5-7] Recent studies have demonstrated that cells present at the invasive tumor front (ITF) of carcinomas have different molecular characteristics when compared with those in the superficial areas of the tumor. It is believed that greater prognostic information can be deduced from the ITF where the deepest and presumably the most aggressive tumor cells reside and where crucial molecular interactions take place.[8-10]

Cell adhesion molecules regulate the growth and differentiation of epithelial cells and play a pivotal role in maintaining the structural integrity of the stratified epithelia. E-cadherin is a 120 kDa, calcium-dependent glycoprotein that connects epithelial cells via homotypic interactions.[11] The cytoplasmic terminus of the E-cadherin molecule has shown to be linked to the actin cytoskeleton via α-catenin and β-catenin.[12]

Extensive research has shown that reduced or loss of expression of E-cadherin/catenin complex is a frequent event in several human cancer including oral cancers. This reduced expression of E-cadherin has been associated with parameters

such as aggressiveness of the tumor, enhanced invasive and metastatic potential of the tumor.[13,14] However, the reports on clinical significance of overall E-cadherin expression in whole tumor sections of OSCC are controversial. Decreased expression of E-cadherin was found to correlate significantly with poor prognosis by some researchers, while others could not obtain such results from their work.[15-20] In addition, the prognostic significance of the altered expression of E-cadherin at the invasive tumor front (ITF) in these tumors has not been widely explored.[21]

There has been an inference that the clinico-pathological and molecular profile of Indian carcinomas shows significant differences from oral cancers in several developed countries.[22] Earlier, the expression of E-cadherin at the superficial/central areas of the tumor had been assessed in OSCC from India.[23] Thus, the objectives of this study were two-fold; (i) to examine the expression of E-cadherin at the ITF of the neoplasm, (ii) to correlate the expression of E-cadherin with tumor grade and with patient prognosis.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed and paraffin-embedded tissue blocks of 30 cases of primary OSCC, 10 each of well, moderate and poorly-differentiated OSCC that underwent surgery as the only mode of treatment and with a follow-up record of 5 years in the period of 1995-2001 were drawn from the archives of the department. The clinical data regarding age, gender, site and TNM staging were also noted from the patient's files. The 5-year follow-up data after surgery was also recorded and those cases that did not present with recurrence/death due to OSCC over this period were considered as the good prognosis group (11 cases) and those that did were categorized as poor prognosis group (19 cases). Five tissue specimens from the normal oral mucosa were included in the study to serve as positive controls.

Immunohistochemistry

Three fresh sections of 4-μm-thickness were cut from formalin-fixed and paraffin-embedded tissue blocks. The sections were taken onto 3-aminopropyltriethoxysilane (APES)-coated (slide adhesive) micro-slides. The sections were deparaffinized, washed in tris-buffer and treated with 0.3% hydrogen peroxide solution for 20 min in a humid chamber to block endogenous peroxidase. Antigen retrieval was done in tri-sodium citrate buffer (pH: 6.0-6.2) in a pressure cooker (2 whistles followed by cooling to room temperature). After blocking with protein block serum-free for 20 min, the sections were treated with pre-diluted, unlabeled primary antibody i.e. mouse-anti human E-cadherin (Clone No. 36, Biogenex Life Sciences Private Limited, CA, USA) at 37⁰C temperature, for 60 min in a humid chamber.

The sections were then incubated with secondary-linking antibody (biotinylated anti-immunoglobulins/super enhancer) at room temperature, in a humid chamber for 30 min to enhance the effect of subsequent polymer step. The sections were incubated with pre-diluted secondary antibody, i.e. conjugate (enzyme-conjugated streptavidin) at room temperature for 30 min. This was followed by incubation with diaminobenzidine tetra hydrochloride and counterstained with Mayer's hematoxylin. For negative control tissue, sections were treated with all the reagents except the primary antibody. The normal salivary glands tissue served as the internal positive control. The expression of E-cadherin protein was evaluated by two independent observers using a light microscope.

Immunohistochemical analysis

Semi-quantitative analysis was carried out by two authors under a light microscope (x400 original magnifications). The expression of E-cadherin in all the cases of primary OSCC was assessed at the ITF. Membranous expression of E-cadherin were graded into four subgroups according to Bankfalvi *et al.,*[15] and is given in Table 1.

As for statistical analysis, A, B, C and D were defined as negative, weak, moderate and strong E-cadherin expression. Frequency and percentage were used to summarize distribution of prognosis across histopathology grade, tumor differentiation and E-cadherin expression. Chi-square test was used to test whether the distribution of prognosis across histopathology grade and E- cadherin expression was statistically significant. Analysis was carried out using Statistical Package for the Social Sciences software (SPSS 15.0) A P value of less than 0.05 was considered to be statistically significant.

RESULTS AND OBSERVATIONS

The study group comprised of 24 men and 6 women with an age range of 28-85 years (mean 60.3 years). As per the TNM staging (1987 UICC/AJCC classification), the cases of early stage (T1 and T2) were 10 and the advanced stages (T3 and T4) were 20 cases. Lymph node metastasis was present in 21 cases. Distant metastasis was not evident in any of the patients at the time of diagnosis.

Among the 10 cases of early stage tumors (T1 and T2), 6/10 (60%) of cases showed a moderate expression for E-cadherin. While, among the 20 cases of advanced stage

Table 1: Grading of membranous expression of E-cadherin into four subgroups according to Bankfalvi *et al.*

	Score	E-cadherin expression
A	0	No detectable expression
B	1 (+)	Expression in ≤10% of tumor cells
C	2 (++)	Expression in 11 to 75% of tumor cells
D	3 (+++)	Expression in ≥75% of tumor cells

tumors (T3 and T4), equal number of cases 9/20 (45%) showed moderate and strong E-cadherin expression. Among the nine cases that did not present with lymph node metastasis, five (55%) cases showed a moderate expression for E-cadherin. Among the 21 cases that were positive for lymph node metastasis, 10 cases (47%) showed a moderate expression for E-cadherin. No significant data was revealed from the staging system [Table 2].

All the tissue section of the normal oral epithelium showed complete cellular membranous staining of E-cadherin in the basal, parabasal and lower parts of the spinous layers [Figure 1]. Among the well-differentiated squamous cell carcinomas, 6 cases (60%) showed a moderate and 4 cases (40%) showed strong membranous expression of E-cadherin [Figure 2]. Among the moderately differentiated squamous cell carcinomas, 1 case (10%) showed a week expression, 4 cases (40%)

moderate [Figure 3] and 5 cases (50%) showed strong membranous expression of E-cadherin. Among the poorly differentiated squamous cell carcinomas, 3 cases (30%) showed a week expression [Figure 4], 5 cases (50%) showed moderate and 2 cases (20%) showed strong membranous expression of E-cadherin. There was no significant ($P = 0.27$) loss of expression associated with the degree of differentiation [Table 3].

When the expression of E-cadherin was correlated with patient outcomes, 7/11 cases (63.6%) that had a good prognosis showed a strong positive expression of E-cadherin, while 11/19 (57.8%) cases of poor prognosis showed a moderate positive expression of E-cadherin [Table 4]. There was a significant ($P = 0.041$) reduction in the expression of E-cadherin among the cases with poor prognosis when compared with those of a good prognosis.
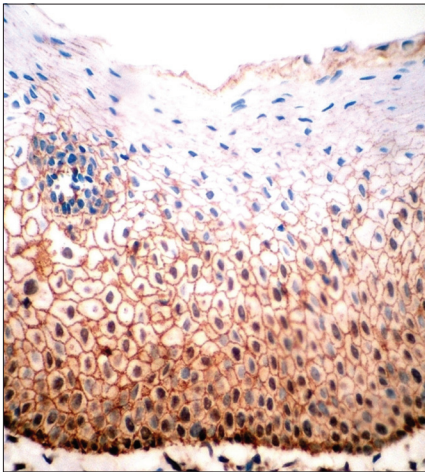

Figure 1: Photomicrograph shows complete membranous expression of E-cadherin in all the cells of the basal, parabasal and superficial spinous layers of the normal oral mucosa (IHC stain, ×400)
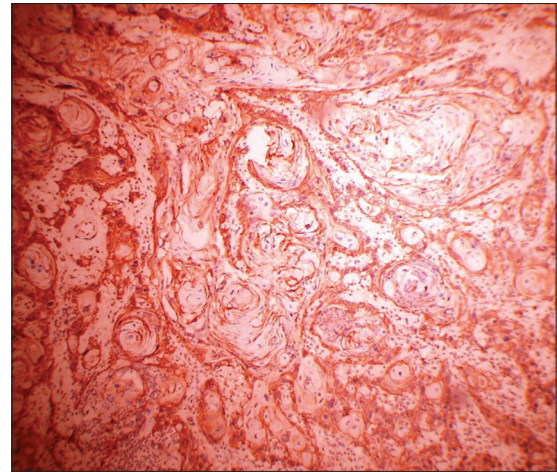

Figure 2: Photomicrograph shows all the cells of the tumor island at the invasive tumor front exhibit complete membranous expression of E-cadherin in well-differentiated OSCC (IHC stain, ×100)
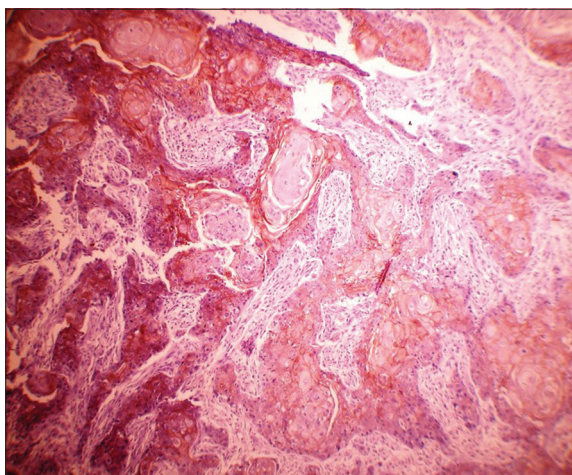

Figure 3: Photomicrograph shows most of the tumor cells at the invasive tumor front exhibit complete membranous expression while few cells exhibit reduced expression of E-cadherin in moderately-differentiated OSCC (IHC stain, ×100)
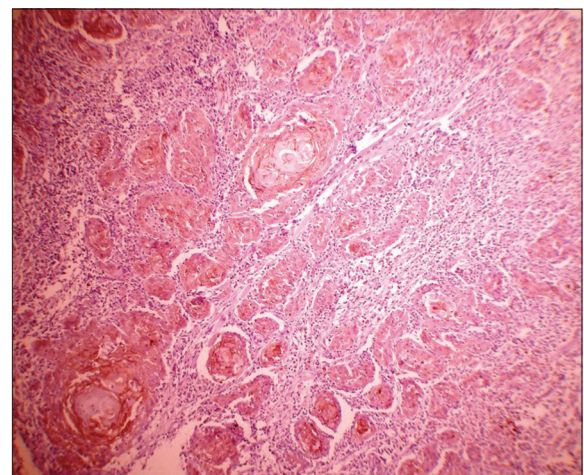

Figure 4: Photomicrograph shows few tumor cells at the invasive tumor front exhibit complete membranous expression while others are negative for the expression of E-cadherin in poorly-differentiated OSCC (IHC stain, ×100)

A summary of the E- cadherin expression between the different prognostic groups and within the different grades of the tumor is given in Table 5.

DISCUSSION

Cell-cell adhesion molecules play an important role in regulating the growth and differentiation of normal stratified squamous epithelia. E- cadherin is a primary mediator of these cell- cell adhesions between epithelial cells. It plays a crucial

Table 2: Correlation of clinical parameters with E-cadherin expression

	−/+	++	+++
Tumor size			
T1-T2 (10 cases)	2	6	2
T3-T4 (20 cases)	2	9	9
Lymph node metastasis			
N0 (9 cases)		5	4
N1-N3 (21 cases)	4	10	7

−/+: Negative or mild expression, ++: Moderate expression, +++: Strong expression

Table 3: Tumor differentiation and pattern of E-cadherin expression

Tumor differentiation (no of cases)	−/+ (%)	++ (%)	+++ (%)	Total no of cases
Well-differentiated	0 (0)	6 (60)	4 (40)	10
Moderately-differentiated	1 (10)	4 (40)	5 (50)	10
Poorly-differentiated	3 (30)	5 (50)	2 (20)	10

Chisquare=5.173, df=4, *P*=0.27. −/+: Negative or mild expression, ++: Moderate expression, +++: Strong expression

Table 4: Correlation of E-cadherin expression and patient's prognosis

Prognosis	−/+	++	+++	Total no of (cases)
Good prognosis (*n*=11)	0	4 (26.7)	7 (63.6)	11
Poor prognosis (*n*=19)	4 (100)	11 (73.3)	4 (36.4)	19
Total	4 (100)	15 (100)	11 (100)	30

Chi-square=6.407, df=2, *P*=0.041*. −/+: Negative or mild expression, ++: Moderate expression, +++: Strong expression

Table 5: Correlation of tumor grade with patient outcome and extent of E-cadherin expression

Tumor differentiation (10 cases)	Prognosis	−/+ (%)	++ no (%)	+++ no (%)	Total
Well-differentiated	Good	0	1 (25)	3 (75)	4
	Poor	0	5 (83)	1 (17)	6
Moderately-differentiated	Good	0	1 (33.3)	2 (66.6)	3
	Poor	1 (16)	3 (42)	3 (42)	7
Poorly-differentiated	Good	0	2 (50)	2 (50)	4
	Poor	3 (50)	3 (50)	0	6

−/+: Negative or mild expression, ++: Moderate expression, +++: Strong expression

role in normal tissue morphogenesis, including segregation of cell types and differentiation.[21,24]

In the normal epithelium, E-cadherin is usually present in cells that have divided and moved into the differentiated layers. We found membranous expression of E-cadherin in the basal/parabasal and lower parts of the spinous layers of the normal oral mucosal tissues that served as control tissues for this study. In the superficial layers of the epithelium the expression of E-cadherin was reduced. Although, the mechanisms of regulation of E-cadherin expression in the upper layers of the epithelium are not understood, it is thought that the loss of expression may play a role in normal desquamation of the epithelium.[15,24-28]

In the present study, we found that most of the well-differentiated cases expressed a moderate or strong membranous expression for E-cadherin. Among the poorly differentiated cases, there were few cases that showed a negative or weak expression of E-cadherin. Thus, similar to Bankfalvi *et al's*[15] study, no statistically significant result was recorded in our study. William *et al.*,[27] in their study found that 15/19 carcinoma cases showed loss of membranous staining for E-cadherin. All the four poorly differentiated cases in their study showed a loss of E-cadherin expression. This could be due to the limited number of cases that were analyzed and/or the differing profile at the ITF of Indian population.

Previous *in vivo* experiments have shown that the absence or loss of function of E- cadherin leads to the disappearance of epithelial characteristics of the cells and generates higher invasiveness for extracellular matrices and embryonal heart tissue.[28,29]

In this study, the size of tumor as per the WHO staging did not have a significant influence on the expression of E-cadherin. Wang *et al.*, found a significant difference in the expression between T1/T2 tumors and T3/T4 tumors.[21] Bagutti *et al.*, have shown that the least differentiated tumors showed a reduced expression of E-cadherin in the later stages and these tumor cells are said to acquire invasive phenotype.[19]

Detachment of tumor cells from the primary site is assumed to be the initial and most important step in the invasive and metastatic process.[30,31] E-cadherin negative tumors migrate from the primary site and colonize in the neighboring lymph nodes. Alternatively, lymphatic vessels might have better access to E-cadherin negative cells and these cells may be preferentially mobilized.[14]

Hubner *et al.*, found that downregulation of E-cadherin expression in cancer cell is associated with occult metastasis in oral cavity and oropharyngeal squamous cell carcinomas. These findings support the function of E-cadherin in tumor suppression and lymphogenous metastasis *in vivo*.[12]

Along with downregulation of E-cadherin, Bryan *et al.* has summarized a phenomenon called "cadherin-switching" namely the upregulation of other members of the cadherin family like N-cadherin or P-cadherin has been found, which is also associated with worse outcome in case of bladder carcinoma.[32]

Similarly, increased N-cadherin expression was significantly correlated with malignant behaviours and cadherin switching was well correlated with histological differentiation, pattern of invasion and lymph node metastasis in head and neck OSCC cases.[33]

In the current study, we found 5/9 (55%) cases did not present with lymph node metastasis and showed a strong E-cadherin expression at the ITF. While 10/21 (47%) cases of tumors that presented with lymph node metastasis showed only a moderate expression of E-cadherin. Schipper *et al.* and Shiozaki *et al.*, have reported that reduced staining for E-cadherin correlates with an invasive and metastatic behavior of OSCC.[14,34]

In vivo studies have shown that carcinoma cell lines with an epitheloid phenotype were non-invasive and expressed E-cadherin, while those with a fibroblastoid phenotype were invasive and had lost E-cadherin expression.[14] Thus, this significant loss of E-cadherin in metastasis reflects the "invasion suppressor function of this molecule".[34,35]

The underlying molecular mechanism for E-cadherin downregulation in OSCC is not known. The *CDH1* gene that is located on chromosome 16q22.1 encodes the epithelial specific cadherin glycoprotein. Mutations in this structural gene or due to indirect suppression of E-cadherin gene can result in the reduced expression of E-cadherin in malignancy.[36]

An intact E-cadherin/β-catenin complex is required for the maintenance of normal intercellular adhesion. Disturbances in the expression or function of these complexes result in loss of intercellular adhesion. The reduction/loss of cell-cell junctions as in E-cadherin could be either due to the mutations/abnormalities seen in the E-cadherin and/or its associated molecular complex involving catenins and actins, leading to disturbances in the normal architecture of the epithelium. Inversely, disturbed architecture, which occurs due to some other causes/mutations, can lead to disruption in the cadherin/catenin complex.[35,37]

When the prognostic significance of E-cadherin expression was examined, we found that all the cases 4/4 (100%) which showed a negative or mild E-cadherin were those that had a poor prognosis. Whereas, 7/11 (63.3%) cases that showed a strong E-cadherin expression were those that had a good prognosis and 11/15 (73.3%) cases that showed a moderate E-cadherin expression were those with a poor prognosis. Wang *et al.*,[21] also reported E-cadherin downregulation at the ITF in the poor prognosis group when compared with the good prognosis group. Sorscher *et al.*,[38] also suggested that the loss of expression was a marker for metastatic disease and was associated with a poorer prognosis of the tumor.

As the adhesive function of E-cadherin crucially depends on their association with cytoplasmic catenins, additional studies focusing on the ITF on a larger sample and along with these related cell adhesion molecules is necessary to evaluate the biological value of loss of E-cadherin in the progression of this neoplasm. These studies should focus on assessing the cadherin-catenin complex as well as cadherin switch at the ITF and attempt to understand the mechanisms by which disruption of these complexes occur.

CONCLUSION

This study shows that the loss of E-cadherin appears to be associated with poor prognosis of OSCCs. However, we could not find a correlation between clinico-pathological features and the loss of expression of E-cadherin. This could be attributed to limited sample size of the study or the different molecular profile of the tumor cells at the ITF of OSCC, in the Indian population. Studies need to be carried out on larger number of tumor specimens and the expression of E-cadherin should be assessed along with other molecules that play an important role in cell-cell contact between the epithelial cells.

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