Indian J Med Res 153, April 2021, pp 484-491
DOI: 10.4103/ijmr.IJMR_1409_18

Quick Response Code:

Association of E-cadherin & vimentin expression with clinicopathological parameters in lingual squamous cell carcinomas & their role in incomplete epithelial mesenchymal transition

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***Background & objectives*: Lingual squamous cell carcinomas (SCC) pose a major public health burden in India. Epithelial-mesenchymal transition (EMT) is the conversion of an epithelial cell to a mesenchymal phenotype at the invasive front (IF) enhancing invasiveness of these cells which may be studied using immunohistochemistry. The objective of this study was to assess the expression of E-cadherin and vimentin at the IF, and their correlation with the histological risk assessment score, clinicopathological parameters and lymph node metastasis.**

***Methods*: Thirty consecutive untreated patients diagnosed as lingual SCC who underwent hemiglossectomy over one year formed the study group. The immunohistochemical expression of E-cadherin and vimentin in the periphery as well as the centre of tumour islands was correlated with clinicopathological parameters, Brandwein-Gensler risk assessment score and lymph node metastasis, along with a correlation between the coexpression of two markers at the IF.**

***Results*: Loss of E-cadherin expression was seen at IF in 83.3 per cent (25/30) cases. Out of these, 20 per cent (5/25) showed a corresponding gain in vimentin expression (complete epithelial-mesenchymal transition) and 80 per cent (20/25) did not. Overall, 16.6 per cent (5/30) cases showed complete EMT. However, no correlation between E- cadherin and vimentin expression at the IF was found. No statistical significance was found between E-cadherin loss and vimentin gain at the IF, with the various parameters or the risk score.**

***Interpretation & conclusions*: The present study suggests that the cells at IF may metastasize even without a gain in vimentin expression (without classical EMT), as cohesive clusters showing incomplete EMT (E-cadh-/Vim-).**

Key words E-cadherin - epithelial to mesenchymal transition - lingual squamous cell carcinomas - risk score - vimentin

Oral cancer is the sixth-most common cancer in the world[1]. India has age-standardized incidence rates of 12.9 per 100,000 for men[2]. Tongue is the most common site in the western world with increasing incidence in India and squamous cell carcinoma (SCC) as the most common histological type[3]. Tumour cells progress from the pre-invasive stage and infiltrate the surrounding tissues and muscles before metastasizing to cervical lymph nodes, by loss of intercellular adhesion molecules and transition into mesenchymal phenotype[4]. Cells at the leading edge of a tumour have the maximum potential to infiltrate, hence the invasive

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front (IF) is the most important area to be assessed for epithelial to mesenchymal transition (EMT)[4].

E-cadherin mediates intercellular adhesion and is expressed normally in the stratum spinosum of the oral mucosa[5]. As it maintains the cohesiveness of the cells, its loss or downregulation is associated with the process of EMT[6].

Vimentin is a mesenchymal marker and a type III intermediate filament protein[7]. Vimentin is expressed on the endothelial cells, lymphocytes and fibroblasts but not by normal epithelial cells[8]. It has been associated with invasiveness and an increased metastatic potential of tumour cells[9]. It is known to be a marker of EMT[4].

Besides the histological grade, depth of invasion (DOI) and the lymph node status, a histopathological risk assessment score may depict the prognosis of these tumours. Brandwein-Gensler *et al*[10] gave a score based on the cellular pattern of invasion (POI) as proposed by Bryne *et al*[11], lymphocytic infiltrate (LI) at the tumour-host interface and perineural invasion (PNI) by malignant cells. Based on these criteria, they stratified the cases into low-risk (score 0), intermediate risk (score 1/2) and a high-risk group (Score 3-9)[10].

Although a few studies have correlated the occurrence of EMT with histomorphological parameters and lymph node metastases using immunoexpression of E-cadherin and vimentin, its correlation with such a composite risk score model is lacking[12-14].

The expression of E-cadherin and vimentin in lingual SCC at the central superficial area (CSA) and IF of the tumour islands, and its correlation with the clinicopathological parameters, the Brandwein-Gensler risk assessment score and lymph node metastasis was studied. A correlation between the co-expression of the two markers at the IF was also studied.

Material & Methods

The study was conducted in the departments of Pathology and Otorhinolaryngology, Maulana Azad Medical College and Lok Nayak Hospital, New Delhi over one year (April 2017-2018). Institutional Ethical clearance was sought before carrying out the study.

Inclusion criteria: Thirty hemiglossectomy specimens, from consenting patients above the age of 18 yr, diagnosed with lingual SCC on histopathology were included in the study. These patients were consecutively recruited.

Exclusion criteria: Specimens from patients with a history of preoperative radiotherapy or chemotherapy were excluded.

Sample size: The sample size was calculated using the formula $N= 4pq/d^2$

[where, 'p'= prevalence, 'q'= 100 − p, 'd' = precision of the estimate (10% or 20% of p)]

For E-cadherin,

$4pq/d^2 = 4 \times 75 \times 25/(10/100 \times 75)^2 = 133$ (10% precision)

For Vimentin,

$4pq/d^2 = 4 \times 30 \times 70/(10/100 \times 30)^2 = 933$ (10% precision)

Parameters assessed: Clinicopathological parameters such as age, gender, duration of symptomatology (<7 or ≥7 months)[15], history of smoking and alcohol intake, presence of a growth or ulcer and tumour size (in greatest dimension on gross examination) were assessed. Routinely processed, formalin-fixed paraffin-embedded, hematoxylin and eosin-stained sections were evaluated microscopically.

The DOI was measured from the basement membrane, of the adjacent normal mucosa and then by drawing a hypothetical 'plumb line' from this plane to the deepest point of tumour invasion[16]. Histological typing and grading were done as per the WHO classification, into well, moderate and poorly differentiated SCC[1]. Pathological staging was done as per tumour, nodes, metastases (TNM) classification[17].

POI was characterized as per Bryne *et al*[11]; (*i*) type 1-tumour invasion in a broad pushing pattern; (*ii*) type 2-broad pushing tumour islands with the stellate pattern; (*iii*) type 3-islands of >15 tumour cells; (*iv*) type 4-islands of <15 cells or cord-like or single-cell invasions (Fig. 1A-C); (*v*) type 5-tumour satellites of any size with 1 mm of intervening normal tissue (fibrosis not to be taken) at the tumour/host interface. POI type 1, 2 and 3 was assigned point 0, type 4 was given 1 point and type 5 was given 2 points[10].

If the tumour displayed more than one POI, the worst POI was considered.

LI was graded as; (*i*) type 1-continuous and dense rim of lymphocytes at the interface (point 0, Fig. 1C); (*ii*) type 2-discontinuous patches of dense lymphoid tissue (point 1) and (*iii*) type 3- limited response or no lymphoid response (point 3)[10]. PNI was assessed as absent (point 0), in smaller nerves with diameter <l mm (point 1) and in larger nerves with diameter >l mm (point 3)[10] (Fig. 1D).

An overall risk score was given and classified as score 0, score 1 or 2 and score 3-9[10].

Immunohistochemistry: Immunohistochemistry was done on sections from the tumour by the biotin-avidin technique using primary antibodies for E-cadherin (mouse monoclonal antibody, Biogenex, RTU, Fremont, CA, USA) and vimentin (mouse monoclonal antibody, Biogenex, RTU, Fremont, CA, USA). The sections were deparaffinized and blocked for endogenous peroxidase in 3 per cent H_2O_2 in methanol. Overnight incubation with primary antibody overnight (at 4° C) and, secondary and tertiary antibodies (peroxidase labelled streptavidin-peroxidase complex) for 30 minutes each was done with three washes in PBS buffer after each incubation. DAB (ES005, Temecula, California) was applied after the last wash and the reaction monitored under microscope. Counterstain with haematoxylin was done after crisp brown cytoplasm and membrane staining was seen. The adjacent mucosal epithelium was taken as a positive control for E-cadherin (Fig. 2A). Stromal cells, muscle bundles and nerve fibres was taken as positive control for vimentin (Figs 2B and D). Negative controls were obtained by omitting the primary antibodies.

Immunoexpression was evaluated at the CSA and IF of the tumour. Expression of both E-cadherin as well as vimentin was classified as described previously[4].

(*i*) <u>E-cadherin expression</u>:

(*a*) Preserved: >50 per cent epithelial tumour cells showing membranous immunostaining (brown granular membranous reaction in the tumour cells) Fig. 2C or

(*b*) Reduced: 50 per cent or less epithelial tumour cells showing membranous immunostaining. Non-specific staining of acantholytic tumour cells and keratin material was ignored.

(*ii*) <u>Vimentin expression</u>:

(*a*) Negative: <10 per cent epithelial tumour cells showing cytoplasmic immunostaining or

(*b*) Positive: 10 per cent or more epithelial tumour cells showing cytoplasmic immunostaining (membranous or cytoplasmic brown granular reaction in tumour cells away from the tumour/

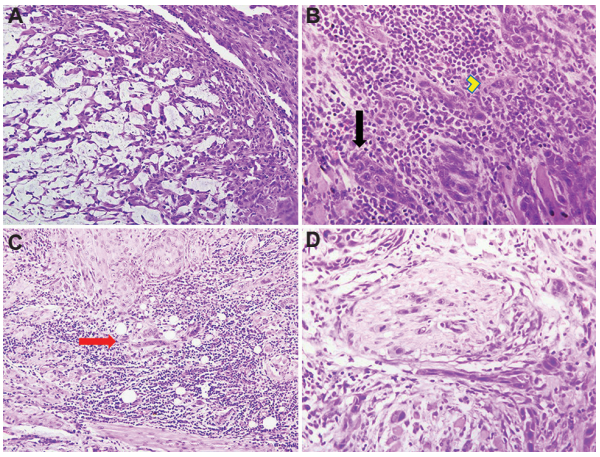

Fig. 1. (**A**) Invasive tumour front with discohesive and spindled out tumour cells, indicating EMT (H and E, ×100). (**B**) Invasive front showing tumour islands of <15 cells (arrow) and tumour buds (arrow head) with dense inflammation (upper left) composed predominantly of lymphocytes (H and E, ×100). (**C**) Invasive tumour front with tumour buds formed of 4-8 cell groups (arrow) separated by dense lymphocytic infiltrate type -1. Splayed muscle bundles seen at the bottom of picture (H and E, ×100). (**D**) Nerve bundle infiltrated and surrounded by tumour cells (H and E, ×200).

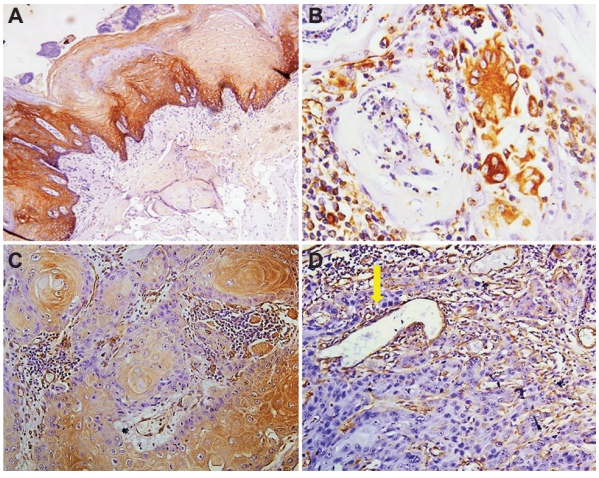

Fig. 2. (**A**) Positive control for E-cadherin: expression in the adjacent normal mucosal epithelium (×100). (**B**) Vimentin positivity in the stromal cells, few lymphocytes and histiocytic collections acting as an internal control (×200). (**C**) E-cadherin expression seen at the centre of tumour islands in the most differentiated cells with loss in the peripheral cells and tumour buds (×100). (**D**) Vimentin positivity seen only in the stromal cells and vessels (arrow) and not in the tumour cells. This positivity acted as an internal control (×100).

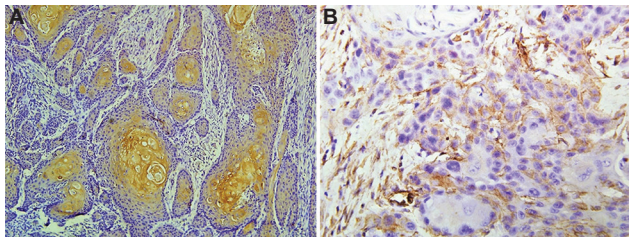

Fig. 3. (**A**) Brown granular membranous E-cadherin positivity in centre of tumour islands (×200). (**B**) Vimentin positivity in cytoplasm of tumour cells at the infiltrating edge of the tumour (×200).

stromal interface). Non-specific staining of cells abutting the stroma was ignored.

Statistical analysis: Association between E-cadherin and vimentin expression, the clinicopathological parameters, the risk assessment score and the lymph node status was expressed in simple percentages. McNemar's test was applied to assess the difference in expression of both E-cadherin and vimentin, at the IF and the central area of the tumour islands, separately. While the Spearman's co-efficient correlation was used to assess the correlation at the centre of the tumour. All data were analyzed using Statistical Package for the Social Science Software Version 16 (IBM, New Delhi).

Results & Discussion

The patients were age from 26 to 71 yr with mean of 45.73 yr. Out of 30 cases, females were 33.3 per cent (10/30) and males 66.7 per cent (20/30). Majority of the patients, 76.67 per cent (23/30) had clinical symptoms within ≤7 months. History of chewing/smoking tobacco, 93.33 per cent (28/30), 53.3 per cent (16/30) alcohol consumption, and both alcohol and tobacco with 43.3 per cent (13/30).

Presentation with an ulceroproliferative growth (56.66%, 17/30) and non-healing ulcer (43.33%, 13/30) was documented. The tumour size ranged from 0.8 to 5.2 cm with a mean of 2.33 cm and standard deviation (SD) of 1.18.

Mean DOI was found to be 6.83 mm ± 5.16. Tumour differentiation was categorically, well (33.3%, 10/30), moderate (63.3%, 19/30) and poor (3.3%, 1/30) respectively. Lymph node involvement was 36.7 per cent (11/30). TNM staging showed stage I-13 tumours, stage II-5 tumours, stage III-6 tumours and stage IV - 6 tumours. Patients presenting earlier (duration <7 months) had stage I/II disease (a lower stage) (13/18, 72.2%), whereas patients presenting later (≥ 7 months) had stage III/IV disease (7/12, 58.3%) (a higher stage), however the *P* value for this finding was insignificant (*P*=0.136).

Risk assessment score: The cases were assigned points for PNI (point 0 - 14/30, 1- 8/30, 3- 8/30), LI (point 0 - 17/30, 1-11/30, 3 - 2/30) and for POI (point 0 - 16/30, 1-14/30, 2- 0) and scored according to their risk assessment. Overall, 50 per cent cases (15/30) were intermediate score (point 0), 33.3 per cent were high risk (points 1-2) and 16.7 per cent low risk (Points 3-9). All five cases with a low-risk score showed a type 1, 2 or 3 POI and absence of PNI. No correlation was found between the risk groups and tumour size, DOI, PNI or the lymph node metastases. On grouping intermediate and high-risk categories together, 56 per cent (14 out of 25) of those with an intermediate/high score showed a type 4 POI (*P*=0.045) and 64 per cent (16 out of 25) showed the presence of PNI (*P*=0.014) (Table I).

Evaluation of immunohistochemistry (IHC) at central superficial area (CSA) and invasive front (IF): Loss of E-cadherin expression at both IF and CSA was observed in 3 tumours (10%), preserved expression at CSA and IF was seen in five tumours (16.67%) and a majority, 22 tumours (73.33%) only showed a loss at IF (Fig. 3A). None showed a loss of E-cadherin expression at CSA with preserved expression at IF. Expression of E-cadherin showed a significant decrease at the interface when compared to the expression at CSA (*P*<0.05).

Lack of vimentin expression at both IF and CSA was observed in 17 tumours (56.67%), gain in expression at IF only was seen in eight tumour (26.67%) (Fig. 3B) and positive expression at CSA was only seen in five tumour (16.66%). No significant result was found in the expression of vimentin at IF when compared with the CSA of tumour island (*P*>0.05). Also, no correlation of E-cadherin and vimentin (Table II) with clinic pathological parameters was found.

Correlation of immunohistochemistry (IHC) at the invasive front (IF): Loss of E- cadherin at IF of the tumour islands was seen in 25 cases whereas its positivity was maintained at CSA of the tumour islands in 27 cases. In three cases, loss of E-cadherin was seen at both the CSA and IF. However, a corresponding gain in vimentin expression was seen only in 8 cases.

Table I. Correlation of risk score (low vs. intermediate or high) with various clinical and pathological parameters

Risk score	Low (n=5)	Intermediate/ high (n=25)	*P* (fisher's exact)
Age (yr)			
<50	4	15	0.626
≥50	1	10	
Exophytic growth			
Present	2	15	0.628
Absent	3	10	
Tumour size (cm)			
≤4	4	24	0.310
>4	1	1	
DOI (mm)			
<4	3	7	0.300
≥4	2	18	
Differentiation of tumour			
WDSCC	0	10	0.278
MDSCC	5	14	
PDSCC	0	1	
Margin type			
Infiltrating	4	23	0.433
Pushing	1	2	
Lymph node status			
Positive	0	11	0.129
Negative	5	14	
POI			
POI (1/2/3)	5	11	0.045
POI 4	0	14	
Perineural invasion			
Present	0	16	0.014
Absent	5	9	
LI			
LI-1	5	12	0.186
LI-2	0	11	
LI-3	0	2	
TNM stage			
I/II	4	14	0.622
III/IV	1	11	

DOI, depth of invasion; POI, pattern of invasion; LI, lymphocytic infiltrate; TNM, tumour, nodes, metastases; PDSCC, poorly differentiated squamous-cell carcinoma; WDSCC, well-differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma

Of these, only five showed a corresponding loss of E-cadherin (P>0.05). A correlation between the loss of E- cadherin at IF with a corresponding gain in vimentin was not found.

Table II. Correlation of vimentin at the invasive front with various clinical and histopathological parameters and the risk score

Vimentin (IF)	Positive (n=8), n (%)	Negative (n=22), n (%)
Gender		
Male	6 (30)	14 (70)
Female	2 (20)	8 (80)
Differentiation of tumour		
WDSCC	2 (20)	8 (80)
MDSCC	5 (26.3)	14 (73.7)
PDSCC	1 (100)	0
TNM stage		
I/II	5 (27.7)	13 (72.3)
III/IV	3 (25)	9 (75)
Tumour size (cm)		
≤4	8 (28.5)	20 (71.5)
>4	0	2 (100)
Depth of invasion (mm)		
<4	2 (20)	8 (80)
≥4	6 (30)	14 (70)
Lymph node status		
Positive	3 (27.2)	8 (72.8)
Negative	5 (26.3)	14 (73.7)
POI		
POI (1/2/3)	4 (25)	12 (75)
POI 4	4 (28.5)	10 (71.5)
PNI		
Present	3 (18.7)	13 (81.3)
Absent	5 (35.7)	9 (64.3)
LI		
LI-1	5 (29.4)	12 (70.6)
LI-2	3 (27.2)	8 (72.8)
LI-3	0	2 (100)
Risk score		
Low	2 (40)	3 (60)
Intermediate	3 (20)	12 (80)
High	3 (30)	7 (70)

POI, pattern of invasion; PNI, perineural invasion; LI, lymphocytic infiltrate

Grouping intermediate and high-risk groups together, a correlation was found with the presence of

PNI and a worse POI (pattern 4). No correlation was found with lymph node metastases or stage of tumour.

Due to permanent differentiation, cells in the body perform specific functions, but activation of EMT can modify cell phenotype by transdifferentiation and convert to mesenchymal cells. Loss of normal interaction of the epithelial cell with the basement membrane enhances migratory capacity, invasiveness and resistance to apoptosis[18]. This leads to an increased chance of metastasis and secondary tumours.

E-cadherin and vimentin can demonstrate EMT at IF, as by loss of E-cadherin, the cells lose epithelial phenotype, show increased invasiveness and a tendency to metastasize by acquiring fibroblastic morphology[19]. Vimentin is increased in poorly differentiated variants of SCC and sarcomatoid differentiation[6].

A significant decrease in E-cadherin at IF compared to CSA has been reported earlier[4,8,19] with a significant gain in vimentin at IF[4], as in the present study, however, there are contrary reports[5,8].

The correlation of E-cadherin expression at the IF with clinicopathological parameters, risk score and lymph node metastasis showed no statistical significance similar to other studies[4,5,12,20], including parameters such as age, gender, size or stage of the tumour[4,5,12], worsening pattern of tumour differentiation[3,12]. Based on similar results, it shows that expression of immunomarkers such as E-cadherin may not have an impact on the biological behaviour of these tumours[20].

The expression of vimentin at IF showed no correlation in the present study, as was seen in a few other studies that showed similar lack of significant correlation with lymph node metastases[5,12], age, gender, histological differentiation, POI, size and stage of tumour[5,8]. Contrasting with a positive correlation of Vimentin positivity at IF with lymph node metastasis[8,21] degree of differentiation, clinical stage and POI[19].

At the IF, 80 per cent cases (20/25) which showed a loss of E-cadherin did not have a corresponding gain in vimentin. Only a few cases (5 out of 30) showed an actual EMT phenotype (E-cadh-/Vim+). Another 6.67 per cent cases (2/30) showed preserved E-cadherin expression with no gain in vimentin positivity (E-cadh+/Vim-) and 20 out of the 30 cases were negative for both. (E-cadh-/Vim-). This suggests that mechanisms other than EMT may be involved in the invasiveness of the tumours and loss of an epithelial phenotype may not always be associated with a gain of mesenchymal phenotype, and the two events may occur independent of each other. Tumour cells are in a particular phase of their life cycle at IF, and the phenotypes encountered could be the most common pattern during that transit, hence EMT may not be the only mode of invasion, rather an entire range of intermediate invasion phenotypes showing loss or gain of proteins may occur[22]. Complete EMT and collective cell invasion (CCI) form the ends of this spectrum[22].

Thirteen tumours showed vimentin positivity in the present irrespective of location (whether at IF or CSA), suggesting perhaps EMT is not exclusively a phenomenon at IF.

CCI is another mode of invasion where cells migrate as collective groups that detach from the primary tumour, still adherent to each other and metastasize[23]. These invade the adjacent matrix and tissues as aggregates, clusters, retaining their intercellular adhesion molecules without expressing mesenchymal protein (6.67%, 2/30 cases, E-cadh+/Vim-)[24]. Structure and arrangement of stromal elements of the parent tissue affect this invasion[22]. Organized stroma *i.e.*, muscle tissue and thick collagen as in tongue tissue, promotes CCI while short collagen fibers and fatty tissue favour single-cell invasion[22]. Hence, cells may be undergoing dedifferentiation, instead of transdifferentiation into a mesenchymal phenotype, with the existence of partial or incomplete EMT phenotypes[25]. These phenotypes lose their cell adhesion molecules but do not gain mesenchymal markers, which explains the variable expression of these proteins at IF in the present study (20 cases, E-cadh-/Vim-).

Similarly, only small number of cases in few studies showed complete EMT, giving credence to CCI as a mode of invasion and that gain in vimentin is not necessary for tumour cells to metastasize[24].

Overall in the present study, a correlation between worse POI and the presence of PNI was found in the intermediate/high-risk category group vs the low-risk group. Also, a significant loss of expression of E-cadherin at IF with only a few cases demonstrating a

corresponding gain in vimentin, was seen, suggesting that incomplete EMT/CCI may operate at IF in lingual SCC, affected by the type of stroma of parent tissue[22]. So, the sample size in the present study was small with a limited immunohistochemistry panel. Also the immunoexpression was not confirmed using PCR due to resource limitation. Despite these short comings, the present study suggests that immunoexpression at IF may not necessarily correlate with the varying degrees of tumour aggressiveness.

***Financial support & sponsorship*:** None.

***Conflicts of Interest*:** None.

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