EXPRESSION OF EPITHELIAL MESENCHYMAL TRANSITION MARKERS AT INVASIVE TUMOR FRONT IN PRIMARY HEAD AND NECK SQUAMOUS CELL CARCINOMA

Pathology

Dr. Shankar M*	Post Graduate student Department of Pathology, Department of E.N.T. *Corresponding Author
Dr. Agarwal K	Director Professor Department of Pathology, Department of E.N.T.
Dr. Kumar. S	Director Professor Department of Pathology, Department of E.N.T.
Dr. Singh S	Associate Professor Department of Pathology, Department of E.N.T.

ABSTRACT

BACKGROUND: The head and neck cancers is the sixth cancer by incidence worldwide.[1] Few studies showed that reduced E-cadherin expression and positive vimentin expression is associated with decrease in differentiation and stage disease and hence poor prognosis of the patient. [2,3]

METHODS: Sixty newly diagnosed HNSCC cases were included. H&E staining and immunohistochemistry for E-cadherin and Vimentin was performed.

RESULTS: Most common site was oral cavity 71.6%. Out of 60 cases 67% cases showed reduced E-cadherin expression (p=0.013), 58% cases showed positive vimentin expression at ITF (p=0.245). Reduced E-cadherin and positive vimentin expression at ITF seen more in TNM stage I and II and also seen more in well differentiated tumor (p<0.001).

CONCLUSION: Most common site involved was oral cavity and significant correlation was found between reduced E-cadherin expression, but no significant correlation was found with positive vimentin expression at ITF.

KEYWORDS

E-cadherin, Epithelial to mesenchymal transition (EMT), Invasive tumor front (ITF) and Vimentin.

INTRODUCTION

The annual incidence of head and neck cancers worldwide is around 6,86,000 cases with around 3,76,000 deaths each year.[1] It is the sixth cancer by incidence worldwide.[2] About 90% of all head and neck cancers are squamous cell carcinomas (HNSCC). [3]

Its prognosis depends upon various factors like size and thickness of tumor, degree of differentiation, deepest area of tumor invasion or Invasive tumor front (ITF) and metastasis into regional lymph node etc.[4,5,6] Invasive tumor front is defined as the deepest three to six cell layers or detached tumor cell groups at the advancing edge of the tumor. Cancer cells located at the ITF have been suggested to be more aggressive in terms of metastatic potential.[6]

Few studies in head and neck squamous cell carcinoma showed that reduced E-cadherin expression and increased vimentin expression, Epithelial to mesenchymal transition markers(EMT) in oral squamous cell carcinoma had been found to be associated with decrease in differentiation, deeper invasion, lymph node metastasis and recurrence of the disease and hence poor prognosis of the patient. [7,8,9,10]

The two hallmark EMT proteins, E-cadherin and vimentin are tightly controlled during EMT through multiple signal transduction pathways.[11] The transmembrane glycoprotein E-cadherin is the major molecule responsible for cell-cell adhesion in epithelial tissues and plays an essential role in the establishment and maintenance of its polarity and structural integrity.[12,13,14] A loss of E-cadherin expression increases the mobility of epithelial cells and its ability for local invasion. Vimentin is an intermediate filament expressed at sites of cellular elongation and found to be associated with a migratory phenotype.[15]

Thus expression of these markers at the ITF can be used to predict lymph node involvement, tumor differentiation, invasion and metastasis leading to tailored management.

AIM: To study the immunohistochemical expression of E-cadherin and vimentin at the invasive tumor front in patients with primary HNSCC.

MATERIALS AND METHODS

Sixty newly diagnosed histopathologically proven primary head and neck squamous cell carcinoma were included in the study after taking their informed written consent. This study was approved by institutional ethics committee, New Delhi. Patients already on therapy for head and neck squamous cell carcinoma and malignancies other than squamous cell carcinoma were excluded from the study. This study was conducted in the Department of Pathology in collaboration with Department of Otorhinolaryngology, Lady Hardinge Medical College and Associated hospitals, New Delhi during November 2015 to March 2017.

Biopsy tissue were taken and paraffin sections (4 μm) were prepared and stained with H&E staining. The immunohistochemistry were performed **E-Cadherin**: Monoclonal Mouse anti human E cadherin clone NCH-38(Anti –E cadherin ,NCH-38) English Code M 3612 (DAKO recommended dilution 1:50). Anit body diluent used (code S0809). **Vimentin** Monoclonal Mouse anti Vimentin, clone V9, (Code No./Code/Code-Nr.M0725)Edition/Ausgabe 18.12.02. (DAKO recommended dilution1:100). DAKO REAL ᵀᴹ Envision ᵀᴹ Detection System, Peroxidase/DAB⁺, Rabbit/Mouse.Code K5007, LOT-20024970. HRP Rabbit/Mouse (ENV). Carcinoma breast and Leiomyoma of uterus was taken as positive control for E cadhrin and Vimentin respectively. All the section were assessed by two independended pathologists who were not aware of the clinical data. IHC Scoring for of E –cadherin (membranous stain) more then 90 % of tumor cells was taken as preserved expression and scoring for Vimentin (cytoplasmic stain) more 10% staining of tumor cells was taken as positive expression.

STATISTICAL ANALYSIS

Data was transformed /coded and entered in SPSS version 20.0. Pearson chi square was used for categorical varibles and pearson correlation was used to see the relationship between measurement scale variable, p value of< 0.05 was considered as significant at 95% confidence level.

RESULTS

we analysed 60 patients with age ranged from 15 to 75 years. Tumor were located in the oral cavity 43 cases, larynx 15 and external auditory canal 2 cases. Forty cases showed significantly reduced E- cadherin expression and 20 cases showed preserved expression at ITF **(p=0.013).** More no of cases (35/60) showed positive vimentin expression however it was not statistically significant (p=0.205).

Table-1 shows clinicopathological parameter of patients. Maximum no of cases 58.33% (35/60) showed reduced E-cadherin and positive Vimentin expression while 33.3% (20/60) showed preserved E-cadherin and negative vimentin expression and 8.33% (5/60) cases showed reduced E-cadherin and negative vimentin expression(both negative) at ITF. However, reduced E-cadherin and positive Vimentin expression at the ITF was not statistically significant (p=0.839). Table

3 and 4 shows correlation of Ecadherin and vimentin expression with prognostic factors. Stage I & II and stage III &IV were combined for statistical calculation. Stage I & II and stage III were less in stage III. There was only one case of poorly differentiated carcinoma hence it was combined with moderately differentiated carcinoma for statistical calculation.

Table 1: Clinicopathological parameter

		(N=60)	PERCENTAGE %
1	AGE [YEARS]	< 20	(1) 1.60%
		21-40	(21) 35.00%
		41-60	(22) 36.66%
		>60	(16) 26.66%
2	Sex	Male	(52) 86.66%
		Female	(8) 13.33%
3	Addiction	Present	(28) 46.66%
		Absent	(32) 53.33%
4	Site	Tongue	(16) 26.6%
		Buccal	(14) 23.3%
		Larynx	(7) 11.6%
		Others	(23) 38.5
5	Tumor size	T 1	(43/60)71.6%
		T 2	(15/60)25.0%
		T 3	(2/60)3.3%
		T 4	0
6	Lymph node	Cervical	(6/8) 75%
		cervical & submandibular	(2/8)25%
7	TNM Stage	Stage I	(39) 65%
		Stage II	(11)18.3%
		Stage III	(2)3.3%
		STAGE IVa	(8)13.3%
8	Grade	WDSCC	(40) 66.66%
		MDSCC	(19)31.7%
		PDSCC	(1)1.66%

Table 2 – Correlation of E cadherin and Vimentin expression at ITF with prognostic factors

	Reduced E-cadherin & positive Vimentin n=35	Preserved E-cadherin & negtive vimentin n=20	Both negative n=5	p-value
Stage				
Stage I and II	28	17	5	0.517
Stage III and IV	7	3	0	
Lymph node involvement				
Yes	5	3	0	0.655
No	30	17	5	
Histological grade				
Well	23	13	4	0.803
Moderately/poorly	12	7	1	

Table 3: Reduced E-cadherin & positive Vimentin expression with TNM stage, size, lymph node and histological grade.

		Reduced E-cadherin & positive Vimentin (N=35/60)	Pearson chi square value	p-value
Stage 'T'	T1	24/35 (68.57%)	0.257	0.063
	T2 and T3	11/35 (31.42%)		
Stage 'N'	Involved N2 Stage	5 /35(14.28%)	17.857	<0.001
	Uninvolved N0 Stage	30/35 (85.71%)		
TNM Stage	Stage I & II	28/35 (80%)	12.600	<0.001
	Stage III & IV	7/35 (20%)		
Grade	WDSCC	23/35(65.71%)	20.800	<0.001
	MDSCC& PDSCC	12/35 (34.28%)		

DISCUSSION

The incidence of head and neck cancers in India is higher as compared to developed countries. [1] Overall incidence and mortality attributed to HNSCC is increasing and it is one of the most difficult malignancies to control because of its high propensity for local invasion and lymph node dissemination. The prognosis of patients suffering from HNSCC has been poor despite much scientific effort to improve the therapy.

In the epithelial mesenchymal transition process, epithelial cells switch to mesenchymal like cell exhibiting high mobility. This acquired migratory phenotype is important during invasion and metastasis and thus overall survival of patients.

Invasive tumor front has been identified as the region chiefly responsible for the clinical behaviour of tumor. So evaluation of these EMT marker at invasive tumor front is important to predict invasion, tumor differentiation, lymph node involvement and metastasis which may leads to better and timely management of patients.

The cases included in our study ranged in age from 15 to 75 years. Maximum number of cases were in the range of 41 to 60 years (36.66%). In studies by Kyu Ho Kim et al [10] and Zhou J et al [16] age of patients ranged from 27 to 94 years and 34 to 86 years respectively. Risk of HNSCC increases with increasing age.

Most of cases in our study were male (86.66%). Numerous studies[10,17]also found most of HNSCC cases predominantly in males (85.6%, 90%) respectively. This difference may be attributed to predominance of risk factors in males as compared to females. In this study addiction in the form of tobacco/smoking /alcohol was present in 46.66% cases. Kyu Ho Kim et al [10] found 66.10% were addicted to smoking. Other studies [18,19,20,21] also showed association of the cancer with tobacco, smoking and alcohol.

In this study most common tumor site was oral cavity(71.6%) followed by larynx. Other studies like Lai-Kui Liu et al [9], Costa et al [17] and Zhou J et al [16]Kyu Ho Kim et al [10] also found maximum cases involving the oral cavity followed by larynx. In our study 71.6% cases were in T1 stage, 25% cases were in T2 stage, 3.3% cases in T3 stage and no cases belonged to T4 stage. However, Lai-Kui Liu et al [9] found maximum cases belonging to T2 (38.55%) followed by T4, T1 and T3 (26.50%, 24.09% , 10.84%) respectively. Kyu Ho Kim et al [10] found maximum cases belonged to T2 followed by T1, T4 and T3 (43.55%, 24.09%, 16.10%, 15.25%) respectively.

In the present study only 8 patients showed lymph node involvement (13.33%) and belonged to N2 stage. Lai-Kui Liu et al [9], Kyu Ho Kim et al [10]and Costa et al [17] found more lymph node metastasis than the present study (38.55%, 48.30% and 35%) respectively. Maximum number of patients (65%) belonged to stage I while 18.3% and 13.3% belonged to stage II and IV respectively. Only 3.3% cases belonged to stage III. Our results are comparable with other study done by Afrem M C et al [7] who observed most of the cases belonged to 46.66% stage I, followed by stage III, equally in stage II, IV (33.33% , 20%, 20%) respectively. However, Lai-Kui Liu et al [9] found maximum cases of stage II (36.14%) then stage IV, I and III (26.50%, 22.89%, 14.45%) respectively. Contrary to our study Costa et al [71]observed maximum cases of stage IV 30% followed by equally in stage I and II ,than III (25%, 25%, 20%) respectively.

In our study, majority of cases (66.66%) were well differentiated squamous cell carcinoma (WDSCC) while 31.70% were moderately differentiated (MDSCC) and 1.66% were poorly differentiated squamous cell carcinoma (PDSCC). Our results are comparable to the study done by Lai-Kui Liu et al [9] who also found most of the cases belonged to well differentiated squamous cell carcinoma (59.03%) than moderately and poorly differentiated carcinoma (36.14%, 4.81%) respectively. Whereas Kyu Ho Kim et al [10] and showed most of the cases in moderately and poorly differentiated (69.49%) followed by well differentiated tumor (30.50%) . Afrem M C et al [7] andZhou J et al [16] observed most of the cases in moderately (53.33%, 50%) followed by well /poorly differentiated tumor (26.66%, 20% and 28.57%, 21.42%) respectively .

E-cadherin and vimentin expression at invasive tumor front

Out of 60 Head and Neck squamous cell carcinoma (HNSCC) cases 67% cases showed reduced E-cadherin expression and 33% showed preserved E-cadherin expression at invasive tumor front which was statistically significant **(p=0.013).** Our results are supported by study of Cheng Wang et al [22] Costa et al [17] and Zhou J et al [16] who also showed significant correlation of E-cadherin expression at ITF (p<

0.05). Lai-Kui Liu et al [9] found that in cancerous tissue from oral squamous cell carcinoma patients, 48 (58%) of 83 tumors showed membranous or cytoplasmic E-cadherin expression [low expression, 17 (35.4%) cases; high expression, 31 (64.5%)cases]. The remaining 35 (42%) were negative. At the invasive front of the tumor, the E-cadherin expression was frequently lost. Forty five (45) cases (54%) showed zero expression, 16 cases (19.2%) and 22 cases (26.5%) showed low and high expression respectively. Kyu Ho Kim et al [10] found that, eighty-six cases (72.9%) showed strong membranous E-cadherin expression. The loss of E-cadherin was found in 32 cases (27.1%). Afrem M C et al [7] observed E-cadherin reactivity was more in the center of tumor and in the superficial part of tumors as compared to advancing edge.

In this study out of 58% cases showed positive vimentin expression and 42% showed negative expression at invasive tumor front which was not statistically significant (p=0.245). Our findings are supported by study by Costa et al [17] who also observed, no statistically significant difference for vimentin expression when the ITF region was compared to the central/superficial area region (p>0.05). Zhou J et al [16] also observed vimentin positive expression rate to be lower in center area as compared to infiltrative margin but p value was significant(p < 0.05).

Similar to the present study Lai-Kui Liu et al [9] observed 57(69%)weak or strong cytoplasmic expression for vimentin [low expression, 29 cases (34.9%) high expression, 28 cases (33.7%)] and negative expression in 26 (31%) at the invasive front however, they did not calculate the p value. Afrem M C et al [7] observed that vimentin reactivity was more at the periphery of tumor and at the advancing edge but they did not mention the values. On the contrary Kyu Ho Kim et al [10] found that only twenty-nine cases (24.6%) to be focal or diffusely positive for vimentin at the invasive tumor front.

Association of stage, lymph node and tumor grade with E-cadherin & Vimentin expression at ITF : In this study, lymph node involvement was seen in 8/60 cases. No significant correlation was found between E-cadherin and vimentin expression with Lymph node, N stage of tumor (p=0.655). Out of 35 cases with reduced E-cadherin expression and positive vimentin expression, 14.28% cases showed lymph node involvement and 85.71% did not show lymph node involvement. Reduced E-cadherin and positive vimentin expression was seen more in lymph node, (N0) stage (p<0.001). Hence, reduced E-cadherin and positive vimentin expression did not correlate with lymph node metastasis. Our results are consistent with the study of Lai-Kui Liu et al [9], Kyu Ho Kim et al [10] and Costa et al [17] who showed no correlation between E-cadherin and vimentin expression with Lymph node involvement (p=0.349, p=0.092;p=0.136, p=0.709;<0.05) respectively. On the other side, Zhou J et al [16] found significant correlation between E-cadherin and vimentin expression with Lymph node (p<0.05).

28/50 cases belonging to stage I, II showed reduced E-cadherin expression and positive vimentin expression, 17/50 cases of stage I, II showed preserved E-cadherin and negative vimentin expression whereas 5/50 cases belonging to stage I, II showed reduced E-cadherin and negative vimentin expression at ITF. 7/10 cases belonging to stage III and IV showed reduced E-cadherin expression and positive vimentin expression, 3/10 cases of stage III and IV showed preserved E-cadherin and negative vimentin expression at ITF No case belonged to stage III and IV showed reduced E-cadherin and negative vimentin expression. No significant correlation was found between E-cadherin and vimentin expression with TNM Staging (p=0. 517).

Out of 35 cases with reduced E-cadherin expression and positive vimentin expression, 80% cases belonged to stage I and II whereas 20% cases belonged to stage III and IV. Reduced E-cadherin and positive vimentin expression at ITF seen more in TNM stage I and II (p<0.001). Hence no correlation was observed with higher TNM stage. Our results are supported by the other studies (Lai-Kui Liu et al[9] and Costa et al [17]) who showed no correlation between E-cadherin and vimentin expression with TNM stage of tumor (p=0.371, p=0.481, and p>0.05) respectively. Afrem M C et al [7] found E-cadherin expression also decreased with increase in the TNM stage of the tumor and cases which showed negative expression of vimentin, the tumors were in stage I. Vimentin reactivity was seen higher in more advanced stages (III–IV).

23/40 patients of well differentiated carcinoma showed reduced E-cadherin expression and positive vimentin expression, 13/40 patients of well differentiated carcinoma showed preserved E-cadherin expression and negative vimentin expression. 4/40 patients of well differentiated carcinoma showed reduced E-cadherin expression and negative vimentin expression. No significant correlation was found between E-cadherin and vimentin expression with grade of tumor (p= 0.803). Out of 35 cases with reduced E-cadherin expression and positive vimentin expression, 65.71% patients belonged to well differentiated carcinoma and 34.28% belonged to moderate, poorly differentiated carcinoma. Reduced E-cadherin and positive vimentin expression at ITF seen more in well differentiated carcinoma (p<0.001) hence it did not correlate with tumor differentiation. Our results are consistent with other study by Lai-Kui Liu et al [9] who showed no correlation between E-cadherin and vimentin expression with tumor grade (p=0.83, p=0.90).

On the contrary, Kyu Ho Kim et al [10] and Zhou J et al [16] observed significant correlation of E-cadherin expression with tumor differentiation (p=0.016 and p=0.022) respectively. Whereas both found no correlation of vimentin expression with tumor differentiation (p=0.944, p=0.064) respectively. Afrem M C et al [7] found that membranous E-cadherin reactivity decreased and vimentin reactivity increased with decrease in tumor differentiation.

CONCLUSION
Most common site involved was oral cavity and significant correlation was found between reduced E-cadherin expression at invasive tumor front. But no significant significant correlation was found with positive vimentin expression. Reduced E-cadherin and positive vimentin expression at ITF did not correlate with prognostic factors like tumor size, lymph node involvement, higher TNM stage and higher grade of tumor.

LIMITATION OF THE STUDY: In our study sample size was small, so study with large sample size should be done to see the significance of these markers at invasive tumor front in head and neck squamous cell carcinoma.
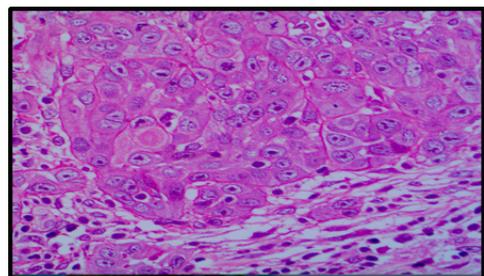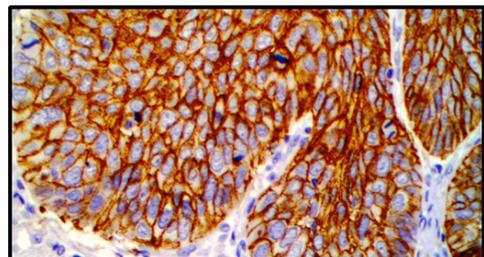
Fig1 : Invasive tumor front

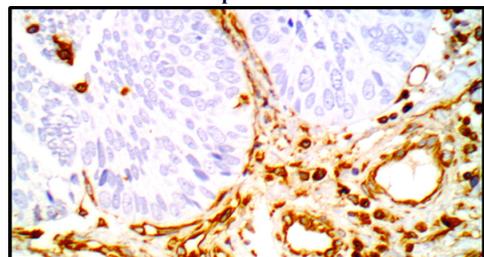**Fig 2: Preserved E cadherin expression at ITF**

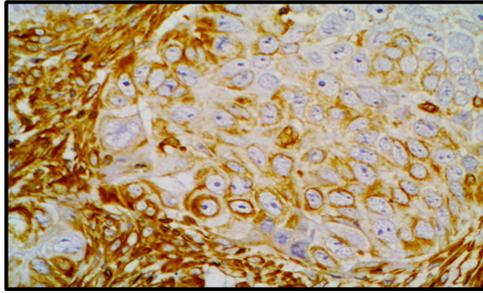**Fig 3 Reduced Ecadherin expression at ITF**

Fig 4: Positive vimentin expression at ITF

REFERENCE

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M   et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer 2015; 136:E359–E386.
2. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular   biology of head and neck cancer. Nat Rev Cancer 2011;11(1):9-22.
3. Johnson NW, Warnakulasuriya S, Gupta PC, Dimba E, Chindia M, Otoh EC et al. Global Oral Health Inequalities in Incidence and Outcomes for Oral Cancer: Causes and Solutions. Adv Dent Res 2011:23(2):237-246.
4. Dragomir LP, Simionescu C, Margaritescu CL, Stepan A, Dragomir IM, Popescu MR. p53, p16 and Ki-67 Immunoexpression in oral squamous carcinomas. Rom J Morphol Embryol 2012;53(1):89–93.
5. Singh H, Verma RP. Expression of p53 protein and Ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas: An immunohistochemical study. Natl J Maxillofac Surg 2011;2(1):38–46.
6. Manjula BV, Augustine S, Selvam S, MathanMohan A. :Prognostic and Predictive Factors in Gingivo Buccal Complex Squamous Cell Carcinoma: Role of Tumor Budding and Pattern of Invasion. Indian J Otolaryngol Head Neck Surg 2015 Mar;67(Suppl 1):98-104.
7. Afrem MC, Mărgăritescu C, Crăițoiu MM, Ciucă M, Sarlă CG, Cotoi OS. The immunohistochemical investigations of cadherin "switch" during epithelial-mesenchymal transition of tongue squamous cell carcinoma. Rom J Morphol Embryol 2014; 55(3 Suppl):1049–1056.
8. Nijkamp MM, Span PN, Hoogsteen IJ, van der Kogel AJ, Kaanders JHAM, Bussink   J. Expression of E-cadherin and vimentin correlates with metastasis formation in head and neck squamous cell carcinoma patients. Radiotherapy   and Oncology 2011;vol99(3):344–348.
9. Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL and Jiang HB. Upregulation of vimentin and aberrant expression of E-cadherin/β-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. Modern Pathology 2010;23:213–224.
10. Kim KH, Choi LKSJ, Han JY, Kim JYHJM,   Kim YCCYM, Lim ISPJH. The Clinicopathological Significance   of Epithelial Mesenchymal Transition Associated Protein Expression in Head and Neck Squamous Cell Carcinoma. Korean J Pathol 2014;48:263-269.
11. Smith A, Teknos TN, Pan Q. Epithelial to Mesenchymal Transition in Head and Neck Squamous Cell Carcinoma. Oral Oncol 2013April;49(4):287–292.
12. Berx G, Van Roy F. Involvement of members of the cadherin superfamily in cancer. Cold Spring Harb Perspect Biol 2009;1:a003129.
13. Stemmler MP. Cadherins in development and cancer. Mol Biosyst 2008;4:835-50.
14. Scanlon CS, Van Tubergen EA, Inglehart RC, D'Silva NJ. Biomarkers of epithelial-mesenchymal transition in squamous cell carcinoma. J Dent Res 2013,92:114-21.
15. Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci 2011;68:3033-46.
16. Zhou J, Tao D, Xu Q, Gao Z, Tang D . Expression of E-cadherin and vimentin in oral squamous cell carcinoma. Int J Clin Exp Pathol 2015;8(3):3150-3154.
17. Costa LCMC, Leite CF, Cardoso SV, Loyola AM, Faria PR, Souza PEA, Horta MCR. Expression of epithelial-mesenchymal transition markers at the invasive front of oral squamous cell carcinoma. J Appl Oral Sci. 2015;23(2):169-78.
18. Mehrotra R, Yadav S. Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations. Indian J Cancer. 2006;43(2):60-66.
19. Merchant A, Husain SS, Hosain M, Fikree FF, Pitiphat W, Siddiqui AR, et al. Paan without tobacco: an independent risk factor for oral cancer. Int J Cancer. 2000 Apr 1;86(1):128-131.
20. Blot WJ. Alcohol and cancer. Cancer Res. 1992 Apr 1;52(7 Suppl):2119s-2123s.
21. Harty LC, Caporaso NE, Hayes RB, Winn DM, Bravo-Otero E, Blot WJ, et al. Alcohol dehydrogenase 3 genotype and risk of oral cavity and pharyngeal cancers. J Natl Cancer Inst. 1997 Nov 19;89(22):1698-1705.
22. Wang C, Huang H, Huang Z, Wang A, Chen X, Huang L et al. Tumor budding correlates with poor prognosis and epithelial-mesenchymal transition in tongue squamous cell carcinoma. J Oral Pathol Med 2011;40(7):545–551.