Original Research Paper

Volume - 10 | Issue - 11 | November - 2020 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Pathology

TUMOR BUDDING AND ITS CORRELATION WITH EPITHELIAL-MESENCHYMAL TRANSITION MARKERS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Dr. Meenakshi Shankar*	Post Graduate Student, Department of Pathology, Lady Hardinge Medical College S.S.K.H, Delhi. *Corresponding Author
Dr. Kiran Agarwal	Director Professor, Department of Pathology, Lady Hardinge Medical College S.S.K.H, Delhi.
Dr. Sunil Kumar	Director Professor, Department of E.N.T., Lady Hardinge Medical College S.S.K.H, Delhi.
Dr. Smita Singh	Associate Professor, Department of Pathology, Lady Hardinge Medical College S.S.K.H, Delhi.

ABSTRACT **Background:** Recently in many cancers, tumor budding has been recognized as an adverse prognostic factor. However, the prognostic value of tumor budding in head and neck squamous cell carcinoma (HNSCC) has not been reported yet.

Aims: The purpose of this study is to assess the correlation of tumor budding with the clinicopathologic features, EMT markers like E- cadherin and Vimentin expression in primary head and neck squamous cell carcinoma.

Setting And Design: Hospital based descriptive observational cross sectional study, conducted in the Department of Pathology, Lady Hardinge Medical College, New Delhi.

Material And Methods: 60 newly diagnosed HNSCC cases between 1st November 2015 to 31stMarch 2017 were included. H&E staining and immunohistochemistry for E-cadherin and Vimentin was performed.

Results: Out of 60 cases 36 cases had tumor buds. Most common tumor site was tongue (33.3%). The patients ranged in age from19 to 69 years. Maximum number of cases were in the range of >60 years (41.7%). Among 36 cases of HNSCC cases that we examined, 20 cases (55.5%) revealed high-intensity tumor budding (≥ 5 tumor buds) ahead of the invasive front, 16 cases (45.5%) revealed low-intensity budding (<5 tumor buds). We found significant correlations among addiction, tumor size, (pT), and clinical stage and grade of tumor with tumor budding. But no correlation was found with lymph node metastasis, which may due very less no of patients had lymph node involvement. Statistical analysis revealed that the high-intensity tumor budding is associated with reduced E-cadherin expression ($P<0.05$) and enhanced Vimentin expression ($P<0.05$).

Conclusion: Hence, we concluded tumor budding, is associated with epithelial-mesenchymal transition and also significantly correlated with prognostic factors like size, clinical stage, grade of tumor in head and neck squamous cell carcinoma.

KEYWORDS : E-cadherin , Epithelial to mesenchymal transition (EMT), Head and Neck Squamous Cell Carcinomas (HNSCC), Invasive tumor front (ITF) and Vimentin.

INTRODUCTION:

The annual incidence of head and neck cancers worldwide is around 6,86,00 cases.[1] [2] It is the sixth cancer by incidence worldwide.[3-5] Majority of head and neck cancers histologically are squamous cell carcinomas.[6] (HNSCC) Main prognostic factors for survival of patients are size, thickness of tumor, degree of differentiation, invasive tumor front, (ITF) tumor budding, metastasis into regional lymph node etc.

Invasive tumor front is defined as 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.[5] Epithelial to mesenchymal transition (EMT) is a dynamic cellular process that is essential for the development of metastatic disease. During EMT, a tumor cell with epithelial characteristics transits to a tumor cell with mesenchymal characteristics through modulation of cell polarity and adhesion. There are many EMT proteins e.g E-cadherin, desmoplakin, cytokeratins, claudins, occludin, beta- catenin and overexpression of mesenchymal markers such as N-cadherin, vimentin and fibronectin. The two hallmark EMT proteins, E-cadherin and vimentin are tightly controlled during EMT through multiple signal transduction pathways.[7] Loss of E-cadherin expression increases the mobility of epithelial cells and hence leads to local invasion. Vimentin is an intermediate filament expressed at sites of cellular elongation and found to be associated with a migratory phenotype.[8]

Tumor budding is defined as the presence of small cell clusters up to four or isolated single cells scattered in the stroma ahead of the ITF.[9]The presence of tumor buds has been considered to be characteristic of aggressive cancer. Budding indicates cellular discohesion and active invasion (feature of malignancy). In cancers likes colorectal, esophageal, lung and ampullary adenocarcinoma, laryngeal cancers tumor budding has been demonstrated as a valuable prognostic marker.[10-20] However, to our knowledge, the effectiveness of this relatively straight-forward histopathological assessment and its prognostic value for HNSCC have not been investigated so far.

The aims of this study was to investigate the possible association of tumor budding and clinicopathologic features and the EMT status of the cancer cells in the tumor buds in patients with HNSCC.

MATERIALS AND METHODS

Study Design Hospital based descriptive observational cross sectional study.

Study Area This study was conducted in the Department of Pathology in collaboration with Department of Otorhinolaryngology, Lady Hardinge Medical College and Associated hospitals, New Delhi.

Sample Size 60 newly diagnosed cases of Head and neck squamous cell carcinoma.

Duration of study 1st November 2015 to 31st March 2017.

Selection of cases (inclusion criteria) Newly diagnosed histopathologically proven primary head and neck squamous cell carcinoma were included in the study after taking their informed written consent. Exclusion criteria Patients already on therapy for head and neck squamous cell carcinoma. Malignancies other than squamous cell carcinoma.

Methodology: Biopsy tissue was sent from Department of Otorhinolaryngology in formalin and received in Department of Pathology. Paraffin sections (4µm) were routinely prepared and stained with H&E. Immunohistochemistry for E-cadherin and Vimentin were performed using appropriate kit & standard technique. Antibodies used: **E-cadherin:** Monoclonal mouse clone NCH 38. (DAKO CODE X0931) **Vimentin:** Monoclonal mouse Clone v9 (DAKO LSAB TM+/HRP kit, code no.K 0679). Analysis of immunostaining: IHC Scoring for of E–cadherin (membranous stain) < 90 % of tumor cells considered as reduced expression and >90 % of tumor cells were taken as preserved expression. Vimentin (cytoplasmic stain) when negative <10 % staining of tumor cells

>10% staining of tumor cells were considered as positive. Tumor budding was defined as the presence of isolated single tumor cells or small clusters (< 5 cancer cells) ahead of the invasive front.(Figure 1&2) H&E slides were scanned at the ×4 objective lens (and ×10 ocular) to see the ITF and select the areas with the highest tumor budding density. In that selected area tumor budding was counted using the ×20 objective lens, and the highest count per slide was noted. Budding index was classified as low (< 5 buds/field) or high (≥ 5 buds/field) intensity.

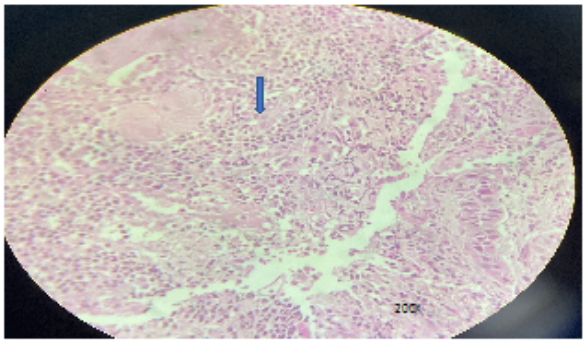

Figure1: H&E Section Showing Tumor Budding

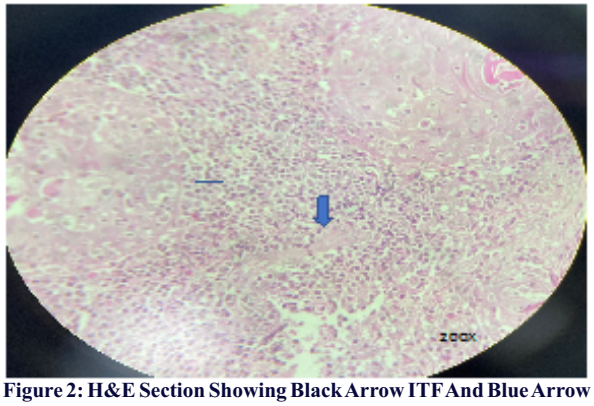

Figure 2: H&E Section Showing Black Arrow ITF And Blue Arrow Showing Tumor Budding

Statistical Analysis

Data was coded and entered in SPSS version 23.0 for windows. Pearson chi square was used for categorical variables and pearsons 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

H&E staining was performed on 60 HNSCC cases and out of 60 cases 36 cases had tumor buds. Most common tumor site was tongue (33.3%). The patients ranged in age from19 to 69 years. Maximum number of cases were in the range of >60 years (41.7%). Maximum number of cases were male (86.1%). Addiction in the form of tobacco/smoking /alcohol was present in 77.8% patients.[Table -1]

Table1: Clinicopathological Parameters

VARIANTS	N=36	Percentage
AGE(YEARS)	<20	2.5%(1/36)
	21-40	25%(9/36)
	41-60	30.5%(11/36)
	>60	41.7%(15/36)
SEX	MALE	86.1%(31/36)
	FEMALES	13.9%(5/36)
ADDICTION(Tobacco chewing /smoking/alcohol)	PRESENT	77.8%(28/36)
	ABSENT	22.2%(8/36)
SITE	TONGUE	33.3%(12/36)
	BUCCAL	22.2%(8/36)
	LARYNX	19.4%(7/36)
	OTHERS	25%%(9/36)

Maximum number of (52.7%) belonged to stage T1. Most common lymph node group involved was cervical (75%), followed by combined involvement (cervical and submandibular) 25%. Maximum number of patients (41.6%) belonged to stage I while 30.5% and 22.2% belonged to stage II and IV respectively. Only 5.5% cases belonged to stage III. Stage IV cases belonged to stage IVa. Majority of cases (52.8%) were moderately differentiated squamous cell carcinoma(MDSCC) while 44.4% were well differentiated (WDSCC) and 2.7% were poorly differentiated squamous cell carcinoma (PDSCC).[Table-2]

Table 2: Prognostic Factors

Prognostic Factors	N=36	Percentage
Tumor size	T1	52.7%(19/36)
	T2	41.6(15/36)
	T3	5.5%(2/36)
	T4	
Lymph nodes	Cervical	75%(6/8)
	Cervical and Submandibular	25%(2/8)
TNM Stage	Stage I	41.6%(15/36)
	Stage II	30.5%(11/36)
	Stage III	5.5%(2/36)
	Stage IV	22.2%(8/36)
Grade	WDSCC	44.4%(16/36)
	MDSCC	52.8%(19/36)
	PDSCC	2.7%(1/36)

Intensity Of Tumor Budding And Its Correlation With Clinicopathological Parameters

On H &E section tumor buds can be easily identified. Out of 60 cases of HNSCC 36 had tumor buds. Among 36 cases that we examined, 20 cases (55.5%) revealed high-intensity tumor budding (≥ 5 tumor buds) before the invasive front; 16 cases (45.5%) revealed low-intensity budding (<5 tumor buds). Of the 60 no tumor bud was observed in 24 cases.

High-intensity tumor budding is associated with reduced E-cadherin expression and enhanced Vimentin expression in HNSCC The expression pattern of E-cadherin within the centre/superficial tumor parts was almost like that within the adjacent non-cancerous epithelium. In particular, at the tumor budding site before ITF, a loss of E- cadherin expression was frequently observed. Cytoplasmic Vimentin expression was observed within the stromal cells of the adjacent non-cancerous tongue tissues, but not within the epithelium. No staining or weak staining (<10% of tumor cells) was found in the center/superficial tumor parts. Positive vimentin expression was seen in cells of tumor budding.

Correlations were tested among tumor budding and clinical parameters of the HNSCC cases (Table-3). We found significant correlations among addiction, tumor size, pT, and clinical stage and grade of tumor. But no correlation was found with lymph node metastasis, which may due very less no of patients had lymph node involvement.

Table 3: Correlation Of Clinicopathological Parameters With Tumor Budding

Variants	Correlation coefficient	P value
AGE	3.65	0.161
GENDER	2.83	0.774
ADDICTION	4.33	0.033
TUMOR SIZE	1.51	0.021
LYMPH NODE	2.94	0.791
TNM STAGE	5.33	0.028
GRADE	4.57	0.048

Increased expression of Vimentin was detected in tumor budding (80%, P<0.05). Statistical analysis showed that the high-intensity tumor budding is associated with reduced E-cadherin expression (P<0.05) and enhanced Vimentin expression (P <0.05) (Table 4).

Table 4: E Cadherin And Vimentin Expression At Tumor Budding

		Tumor budding intensity		No of patients n=36	P value
		LOW(<5 buds) n=16 (44.5%)	HIGH(>5 buds) n=20 (55.5%)		

E CADHERIN	Preserved	4	1	5 (13.8%)	<0.05
	Reduced	12	19	31 (86.2%)	
VIMENTIN	Negative	5	2	7 (19.4%)	<0.05
	Positive	11	18	29 (80.6%)	

DISCUSSION

The cases included in our study ranged in age from 19 to 69 years. Maximum number of cases were in the range of > 60years. Risk of HNSCC increases with increasing age. Our study reinforces the data obtained from the other studies.[21-23] Lai-Kui Liu et al found in their study of 83 cases that maximum cases (69.87%) were above 50 years of age, Afrem M C et al and Costa et al observed the mean age of 63 years and 54.2 years respectively.[21,24,25]Most of cases in our study were male (86.6%). Numerous studies also found most of HNSCC cases predominantly in males (85.6%, 90%) respectively. [23,24]

Addiction in the form of tobacco/smoking /alcohol was present in 77.8% patients. Kyu Ho Kim et al found 66.10% were addicted to smoking.[22]In this study most common tumor site was tongue (33.3%), which similar to other studies.[21,23] Variable distribution of cancer at various site suggest difference in risk factors. Carcinoma of buccal mucosa and tongue are frequently seen in betal quid chewer because the quid is compressed against the buccal mucosa. In India betal quid chewer constitute an important risk population and carcinoma of buccal mucosa and tongue are most commonly seen in Indian population. But Kyu Ho Kim et al found maximum cases involving the oral cavity (33.9%) followed by larynx (28.0%).[22]

Maximum number of patients (41.6%) belonged to stage I while 30.5%and 22.2% belonged to stage II and IV respectively. Only 5.5% cases belonged to stage III. Our results are comparable with other study done by Afrem M C et al 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.[25] However, Lai-Kui Liu et al found maximum cases of stage II (36.14%) then stage IV, I and III (26.50%, 22.89%, 14.45%) respectively.[21] Contrary to our study Costa et al observed maximum cases of stage IV 30% followed by equally in stage I and II ,than III (25%, 25%, 20%) respectively.[24]

In the present study, majority of cases (52.8%) were moderately differentiated squamous cell carcinoma (MDSCC) while 44.4% were well differentiated (WDSCC) and 2.7% were poorly differentiated squamous cell carcinoma (PDSCC). Our results are comparable to the study by Kyu Ho Kim et al and showed most of the cases in moderately and poorly differentiated followed by well differentiated tumor.[22] Zhou J et al and Afrem M C et al observed most of the cases in moderately(50%, 53.33%) followed by well /poorly differentiated tumor (28.57%, 21.42% and 26.66%, 20%) respectively.[25,23]

In many solid tumors ITF gives valuable prognostic information. Malignancy grading system of ITF was developed in 1992 and according to it grading is based on degree of keratinization, nuclear polymorphism, pattern of invasion and infiltration of lymphocytes.[25-27]

Role of ITF in prognosis of tumors was confirmed by few researchers[28-30,21] Brandwein-Gensler et al in their study combined ITF with perineural invasion and lymphocytic host response to assess the aggressiveness of head and neck cancer.[32,33]Recently in few tumors, tumor budding, has been suggested as a potential index of aggressiveness and poor prognosis.[9,10] However, little is known about the prognostic value of tumor budding in patients with HNSCC.

In this study, high-intensity tumor budding is compared to patients with low-intensity budding. This is in agreement with studies on other solid tumors (e.g.larynx and esophageal cancer, colorectal cancer) showing strong associations of tumor budding with a prognosis factors.[13,14,19,20] Advantage of tumor budding-based index as prognostic indicator is the simplicity and reproducible measurement of the budding and without the need for additional cost-demanding techniques. This feature is may have therapeutic and clinical benefits for the patients with HNSCC. According to Bryne et al the tumor budding was classified into grade 4 (the pattern of invasion is defined as wide spread and marked cellular dissociation in small groups or in single cells.[25,26]The tumor cells showed a high tendency to metastasize to regional lymph nodes compared with those that invade in pushing fronts or in bands , strands or in cords. Brandwein-Gensler's et al in two consecutive studies found these similar observation in which they found that the worst pattern of invasion 4 (tumor budding can be classified into this group) and 5 significantly associated with lymph node metastasis and overall survival. [32,33]

In our study we found no associations of tumor budding with lymph node metastasis which is not in agreement to these studies. Tumor cells at ITF and tumor buds exhibit distinct morphological features, including loss of cell-cell adhesion and dedifferentiation. This fibroblast-like morphological appearance is characteristic of cells undergoing epithelial to mesenchymal transition, characterized at the molecular level by loss of E-cadherin and the increase in expression of Vimentin(mesenchymal marker). In our study we observed no significant correlation of tumor budding with age, gender ($p<0.05$) which is in agreement with the study by Cheng Wang et al.[34]However, we found significant correlation with the addiction, size (pT), stage and grade of tumor, our results are in concordance with the study by Cheng wang et al.[34] But we found no association with the lymph node metastasis which may be due less number of patients had lymph node involvement which was not seen in the study by Cheng Wang et al. [34]

Our results showed expression of E-cadherin is significantly reduced in cells located at tumor buds (86.2%) when compared with those located in the central/superficial portions of the tumor samples. We also found there is an increase in Vimentin expression with reduction in E-cadherin in budding cells. Our results are in agreement with previous observations of reduced E-cadherin expression in ITF and tumor budding of Oral SCC. [31,34] Hence, these findings demonstrate that cancer cells located in the tumor buds underwent EMT, which is associated with enhanced metastatic potential. However, additional studies required to further investigate the molecular events associated with tumor cells that reside in the ITF/budding areas, which will lead to a better understanding of HNSCC invasion and metastasis. It will be helpful for making potential targeted therapeutic strategies and which may leads to better and timely management of patient.

CONCLUSION:

Hence, we concluded tumor budding, is associated with epithelial-mesenchymal transition and also significantly correlated with prognostic factors like size, clinical stage, grade of tumor in head and neck squamous cell carcinoma.

Limitations And Recommendations:

This is cross-sectional study, hence we cannot determine a causal link. The sample size of this study is small and more studies are recommended with greater sample size.

Financial Support And Sponsorship: Nil.

Conflicts Of Interest

There are no conflicts of interest.

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