Original Research Article

DOI: https://dx.doi.org/10.18203/issn.2454-5929.ijohns20204953

Evaluation of epithelial mesenchymal transition markers snail and slug as predictor of nodal metastasis in oral squamous cell carcinoma

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Received: 20 October 2020 Revised: 27 October 2020 Accepted: 28 October 2020

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ABSTRACT

Background: Presence of regional neck node metastasis in head and neck cancer is a major determinant of overall survival. In patients presenting with neck node metastasis, there is a fifty percent decrease in overall survival, irrespective of the treatment modality. Tumor, nodes, metastases staging system, based on the anatomical extent of disease is used to predict patient prognosis and need for adjuvant treatment. Advent of immune based therapy has led to development of new molecular markers which can predict the disease aggressiveness by predicting lymph node and distal metastasis. Epithelial mesenchymal transition (EMT) in cancer is thought to convert the stable epithelial cells to mesenchymal cells that acquire properties of invasion with regional and distal metastasis.

Methods: In the current study we evaluated the expression of EMT markers snail and slug in oral squamous cell carcinoma with and without neck node metastasis in 86 patients.

Results: In this study, snail positivity was observed in 72 cases (83.72%), slug positivity was observed in 52 cases (60.46%) and either of the two expressions was observed in 77 cases (89.53%). Found that snail was significantly associated with clinical nodal status (p=0.037) and post-op histopathological nodal status (p=0.003). Also found that slug was significantly associated with clinical nodal status (p<0.001), post-op histopathological nodal status (p=0.001) and perineural invasion (p=0.003).

Conclusions: Snail and slug positivity correlates with clinical and post-op histopathological nodal status and thus can be used as a predictor of nodal metastasis in oral squamous cell carcinoma.

Keywords: Head neck cancer, Oral cancer, EMT, Snail and slug

INTRODUCTION

Cancer and other non-communicable diseases (NCDs) are now widely recognized as a threat to global development. In 2017, there were 24.5 million incident cancer cases worldwide and 9.6 million cancer deaths. Head and neck cancers are a major public health problem in India, mainly due to widespread use of tobacco. In contrast to the West, where it is the 6th most common cancer, head and neck squamous cell carcinoma in India is the

commonest cancer in males.² Out of all head and neck cancers, oral cancers are the commonest and constitute 40-70%. Age standardized rate (ASR) of 7.2.³ Smokeless form of tobacco consumption is the predominant cause of these cancers. Chewing tobacco accounts for 95% of oral cancers in women and two-thirds in men.⁴ Widespread use of tobacco and lime mixture in India is the cause of very high incidence of lower gingivobuccal cancer, where the tobacco mixture is typically placed. Other risk factors for oral cancer are alcohol consumption and poor orodental hygiene.

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At present, oral cancer is risk stratified on the basis of the tumor, nodes, metastases (TNM) system of classification proposed by AJCC (8th edition).⁵ The system is based on tumor extent, regional lymph node involvement and distal metastasis. This system has been used to predict prognosis and need for adjuvant treatment. However local and regional recurrences are common after treatment and predominant cause of death in oral cancer. Currently there is a need for molecular markers that can predict the nodal and distal spread of disease, possibly changing the trajectory of cancer treatment.

A major disadvantage of the TNM classification is that it does not incorporate biomarkers. Addition of validated tumor markers is a novel and attractive concept that has the potential to increase the prognostic and predictive value of TNM. Refinement in TNM based on biological markers of tumor micro-metastatic spread can better define tumor behavior and prevent under and over treatment of these patients.

EMT is the capacity of cells to switch between epithelial and mesenchymal cellular states and has been fundamental in the generation of complex body patterns and embryogenesis. EMT is a conserved epigenetic program that generates motile invasive mesenchymal cells from epithelial sheets under physiologic conditions.⁶ EMT is activated by signaling pathways that up-regulate expression of EMT inducing transcription factors.⁷ In cancer, increased EMT activity has proven to be associated with increased metastatic potential and apoptosis and resistance to chemotherapeutic agents in preclinical studies. Though EMT has been consistently associated with poor outcomes, mesenchymal signature is not considered in deciding adjuvant chemotherapy. The current study was aimed at evaluating the expression of EMT associated proteins as a predictor of nodal metastasis and hence tumor aggressiveness of oral cancer.

METHODS

Settings

An observational, cross sectional study was conducted in the department of otorhinolaryngology and department of pathology at university college of medical sciences an GTB hospital, New Delhi, a tertiary care university hospital, from November 2017 to April 2019. Approval was obtained from institutional ethical committee for research on human subjects.

Sample size

Eighty-six consecutive patients of oral squamous cell cancer irrespective of neck nodal metastasis were included in the study.

Exclusion criteria

Patients who have received any form of previous treatment in the form of surgery, radiation and

chemotherapy, patients in poor general condition and unfit for surgery or those who refused consent were excluded from the study.

Detailed clinical history was taken and head and neck and systemic examination was done. Contrast enhanced CT scan of the primary and neck and chest for evaluation of metastatic lymph nodes and staging was done. TNM staging was done as per 8th edition of AJCC and patients were divided into two groups based on the clinical /radiological evidence of neck node metastasis. Group 1: oral cancer without neck nodes and group 2: oral cancer with neck nodes.

All patients underwent biopsy from oral primary lesion that included at least 1 mm of the apparently normal tissue all around. Formalin fixed; paraffin embedded archival tissue blocks of surgically removed oral squamous cell carcinoma specimens were included in study. The H and E stained slides were reviewed and the report noted. Immunohistochemistry was done for EMT associated proteins snail and slug on biopsies by the following technique using polyclonal antibodies against snail and slug manufactured by G-biosciences, St Louis, USA

Four-micron thick sections were cut. Section was dewaxed in xylene and rehydrated through graded concentration of alcohol. Antigen retrieval was done by heating in a microwave oven for 30 minutes in citrate buffer. Endogenous peroxidase activity was blocked with hydrogen peroxide solution in methanol for 30 minutes after washing with buffer solution for 5 minutes, primary antibody was applied in a humid chamber at room temperature for 60 min. and sections were washed with buffer. Secondary and tertiary antibody was applied successively for 30 minutes each and washed with a buffer in between. DAB (diaminobenzidine tetrahydrochloride) was used as a chromogen. All sections were counterstained with haematoxylin.

All IHC slides showing snail or slug positivity (Figure 1-3) were interpreted on the basis of location of stain (nuclear or cytoplasmic) and percentage of positive cells. Grading was done on the basis of percentage of cells positive for snail or slug. No detectable staining was given grade 0, <10% positivity was given grade 1, 10 to <50% as grade 2 and >50% as grade 3. EMT associated proteins snail and slug demonstrates cytoplasmic staining on immunohistochemistry.

Statistical methods were employed for determining the differential expression of EMT associated proteins snail or slug and its correlation with cT stage, cN stage, clinical stage, post-op histopathological nodal status, perineural invasion and its statistical significance.

Statistical analysis

Data was entered in MS excel and analysed using SPSS 20.0 software. The expression of EMT associated

proteins snail and slug was presented as proportions. Comparison of expression of EMT associated proteins snail and slug between oral cancer patients with and without neck metastasis was done using chi-square test. P value of less than 0.05 was considered statistically significant.

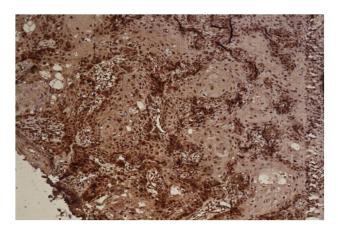


Figure 1: Slug IHC (100X) of cytoplasmic positivity grade 3 in SCC buccal mucosa.

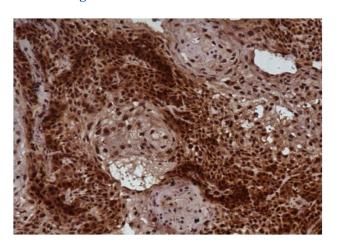


Figure 2: Slug IHC (200X) of cytoplasmic positivity grade 3 in SCC buccal mucosa.

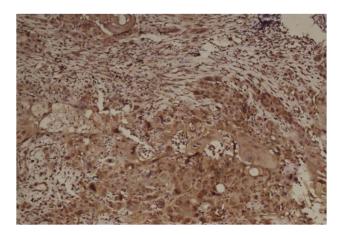


Figure 3: Snail IHC (100X) of cytoplasmic positivity grade 3 in SCC buccal mucosa.

RESULTS

This observational, cross sectional study was conducted on 86 patients of proven oral cavity cancer.

Patient characteristics

Patient characteristics are summarized in (Table 2). In this study population, age of patients ranged between 22-75 years. Mean age of patients was 46.2 years. Most of the patients were in the 31-40 years of age group.

Found tongue as most common site of oral cancer followed by buccal mucosa. Mandibular alveolus was least commonly involved site (Table 1). Not found any significant association between primary cancer site and snail positivity (p=0.702) or slug positivity (p=0.29).

Squamous cell carcinoma was predominant histopathology of study. Amongst squamous cell carcinoma, majority (56.4%) were moderately differentiated.

Table 1: Patient characteristics (n=86).

Variables	Number of patients (%)		
Age (year)	Mean=46.2 (range 22-75) M:F ratio 6:1		
Site of oral cancer			
Tongue	39 (45.35)		
Buccal mucosa	30 (34.88)		
Lip	6 (6.98)		
Maxillary alveolus and hard palate	6 (6.98)		
Mandibular alveolus	1 (1.16)		
Floor of mouth	2 (2.32)		
Retromolar trigone	2 (2.32)		
T stage			
T1 and T2 (Early	33 (38.3)		
disease)	33 (30.3)		
T3 and T4 (Locally advanced disease)	53 (61.6)		

In present study, snail positivity in early (T1 and T2) and advanced disease (T3 and T4) was 82 and 85% respectively. We didn't find any association between T stage and Snail positivity (p value=0.970).

We found slug positivity is significantly associated with T stage (p value=0.029) (Table 2).

In our study, 50% patients had palpable neck nodes at the time of presentation. Patients were assigned N staging as per AJCC 8th edition. There was significant association between N staging and snail positivity (p value=0.037).

Patients were divided into two groups on the basis of presence or absence of neck nodes. Group I characterized by absence of neck nodes and group II with presence of neck nodes.

Table 2: Association between T stage and EMT associated protein slug.

T stage	Grad	de of s	lug pos	itivity	Total number of patients with slug positivity (%)	P value
	0	1	2	3		
T1 and T2 (Early disease)	18	3	4	8	15 (45)	
T3 and T4 (Locally advanced disease)	16	5	3	29	37 (70)	0.029

Table 3: Snail expression between group I (N_0) and group II (N_+) .

Grade of snail positivity						Davolaro
Nodal status	odal status 0	1	2	3	Total number of patients with snail positivity (%)	P value
No (Group I)	12	1	11	19	31 (72)	0.002
N ₊ (Group II)	2	0	8	33	41 (95)	0.003

Table 4: Association between pN stage (post-op pathological nodal status) and EMT associated proteins snail.

Post-op nodal	Grade	e of sna	il positi	vity	Total number of patients with Snail positivity (%)	Dyalya
status	0	1	2	3	Total number of patients with Shan positivity (78)	r value
pN_0	11	1	8	14	23 (67)	0.002
pN_{+}	3	0	11	38	49 (94)	0.003

Table 5: Association between pN stage (post-op nodal status) and EMT protein slug.

Post-op nodal	Grad	e of slug	g positi	vity	Total number of nationts with Slug positivity (0/)	Dyrolys
status	0	1	2	3	Total number of patients with Slug positivity (%)	r value
pN_0	19	4	5	6	15 (44)	0.001
pN ₊	15	4	2	31	37 (71)	0.001

Table 6: Association between perineural invasion and EMT associated protein slug.

PNI	Grade	of slu	g positi	vity	Total number of patients with slug positivity (%)	P value
0	0	1	2	3		
Present	5	0	0	16	16 (76)	0.002
Absent	29	8	7	21	36 (55)	0.003

There was a significant difference of snail expression between group I (N_0) and group II (N_+) $(p \ value=0.003)$ (Table 3).

In present study, we found significant association between N staging and slug positivity (p value<0.001). There was a significant difference of slug expression between group I and group II (p value<0.001). N staging was significantly associated with nail/slug positivity.

There was a significant difference of snail/slug expression between group I and group II (p value=0.002).

There was significant association between post-op nodal status and snail positivity (p value=0.003).

There was significant association between post-op nodal status and slug positivity (p value=0.001). There was significant association between post-op nodal status and

snail/slug positivity (p value=0.001). We did not find any association between PNI and snail positivity (p=0.725).

There was significant association between PNI and slug positivity (p value=0.003).

Snail positivity acts as an independent predictor for postop nodal status after adjusting age, sex and slug positivity.

DISCUSSION

Oral cancer is characterized by poor prognosis and survival rates of 90-45.5%, based on clinical stage (TNM) at the time of presentation.

In patients presenting at an advanced stage, there is a high incidence of invasion to surrounding tissues, lymph nodes and distant metastasis.^{8,9} Lymph node metastasis is

one of the most important prognostic factors for head and neck squamous cell cancer. Survival rates fall by 50% once lymphatic metastasis occurs irrespective of treatment modality. ^{10,11} The factors that contribute to metastasis in oral cancer are still unclear.

EMT is a process by which epithelial cells convert to mesenchymal cells, leading to invasion of tumor cells to underlying tissues with regional and distal metastasis.⁶

During EMT, epithelial cells lose adherent junction, desmosomes and tight junctions that connect them laterally with neighboring cells and with underlying basement membrane which maintain their apical-basal property. As a result, cells lose epithelial phenotype and gain mesenchymal phenotype; acquiring migratory abilities. These tumor cells invade basement membrane, enter lymphatic vessels and become circulatory tumor cells. These cells proliferate to establish nodal metastasis.

The present study was undertaken to evaluate the expression of EMT associated markers snail and slug in oral cancer patients with and without neck metastasis.

The mean age at presentation was 46.2 years; in several studies the commonest age group affected was fifth to 7th decade. 12–14 Our patients presented at a younger age because of rampant exposure to oral tobacco early in life.

Male to female ratio was 6:1 in present study. These findings are consistent with other studies. This is attributed to the habit of tobacco and alcohol consumption in males in our country.

Squamous cell carcinoma predominant was histopathology of our study. Amongst squamous cell carcinoma, majority (56.4%)were moderately differentiated. Bhattacharjee et al in his study found that squamous cell cancer was commonest histological type in head and neck cancer. Bhat et al found that squamous cell carcinoma as the most common histology of oral and oropharyngeal cancer. Majority were moderately differentiated. Brandizzi et al also found squamous cell cancer is the most common histological type of oral cancer.

Majority of our patients (76.74%) in present study presented in stage III and IV. This finding was consistent with other studies. Oral cancer presents at an advanced stage. Brandizzi et al in his study found 65% of patients with oral cancer presented at advanced stages (III and IV). In our study, snail positivity was observed in 72 cases (83.72%), slug positivity was observed in 52 cases (60.46%) and either of two protein expressions was observed in 77 cases (89.53%).

We found that snail or slug or snail/slug expression was significantly associated with clinical nodal status and post-op histopathological nodal status. We also found that slug positivity was associated with clinical stage and T

stage. In our study, there was no association of snail positivity with T stage and clinical stage. We also found that slug positivity was associated with perineural invasion.

Zheng et al studied snail and slug expression in human oral tongue squamous cell carcinoma (OTSCC) cell lines by immunohistochemistry. They concluded snail or slug or two protein co-expressions were significantly associated with T stage and lymph node metastasis. In our study, we also found snail or slug or snail/slug expression was significantly associated with lymph node metastasis and Slug positivity associated with T stage and clinical stage.

Kuroika et al in 2016 concluded that slug expression correlates with local invasiveness of ameloblastoma. ¹⁶ In current study on oral cancer patients with and without neck metastasis, we found that slug positivity correlates with T staging, clinical and post-op histopathological N staging and clinical stage. Though histology might be different in this comparison, EMT may represent a common step depicting the invasive properties and aggressiveness of tumors.

Tang et al found that slug expression was significantly associated with TNM stage, perineural invasion, local regional recurrence and distant metastasis of patients with adenoid cystic carcinoma of salivary gland. ¹⁷ We found similar results that slug positivity correlates with T, clinical and post-op histopathological N stage and clinical stage and perineural invasion.

Verrucous carcinoma is a well differentiated variant of squamous cell carcinoma. Regional and distant metastasis is rare in verrucous carcinoma. Most common site of oral verrucous carcinoma is buccal mucosa. Verrucous carcinoma did not show positivity for either snail or slug on IHC. Negative reaction of verrucous carcinoma on IHC may be interpreted that snail and slug positivity on IHC may correlate with aggressive metastatic behavior. However, there were only two cases in our study and a further study with a larger sample size is needed to validate this finding. We could not find any such study in literature comparing the verrucous histology and correlation with EMT markers snail and slug.

CONCLUSION

Lymph node metastasis is the most important factor that determines the outcome and survival rates fall by 50% once metastasis occurs, irrespective of treatment modality. Therefore, the present study was done to evaluate the expression of epithelial mesenchymal transition associated proteins snail and slug in oral cancer and its correlation to cervical lymph node metastasis. Slug positivity was significantly associated with clinical N staging (p<0.001). We also found slug positivity was significantly associated with post-op pathological nodal status (p=0.001) and perineural invasion (p=0.003). We

concluded that snail, slug or snail/slug expression correlates with invasiveness and metastatic potential of oral cancer. Molecular markers should be added to the TNM classification of cancer to better prognosticate and predict the tumor behavior. This will stratify tumors besides their anatomic extent into a biological classification that can be a better predictive tool for patient outcome than TNM alone.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: The study was approved by the

Institutional Ethics Committee

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Cite this article as: Arya S, Arora V, Taneja HC, Gogoi P. Evaluation of epithelial mesenchymal transition markers snail and slug as predictor of nodal metastasis in oral squamous cell carcinoma. Int J Otorhinolaryngol Head Neck Surg 2020;6:2183-8.