## Original Research Article

# Does epithelial mesenchymal transition markers snail and slug correlate with clinical stage of oral cancer: a clinico-pathological study of 258 patients

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### ABSTRACT

**Background:** Head and neck cancers are the 6th most common cancers worldwide. In contrast, according to population-based cancer registries these constitute the commonest cancer in Indian men and third most common cancer in women. Oral cancer is the commonest head neck cancer, attributed to rampant smoking and chewing of tobacco and areca nut in India. Epithelial mesenchymal transition (EMT) is the process in which committed epithelial cells undergo transformation into mesenchymal phenotype, that has invasive properties and thus contribute to metastatic potential of cancers.
**Methods:** The current study was undertaken to correlate the expression of EMT markers snail and slug with tumor stage at the time of presentation.
**Results:** In present study, snail positivity was 60% in patients presenting at stage 1, 73% in stage 2, 85% in stage 3 and 90% in stage 4 patients. There was no significant association between clinical staging and snail positivity (p=0.549). Slug positivity was 40% in patients presenting at stage 1, 26.7% in stage 2, 59% in stage 3 and 77% in stage 4 patients. There was significant association between clinical staging and Slug positivity (p=0.002). Either snail or slug expression was found in 89.53% patients.
**Conclusions:** We found that EMT marker slug expression was significantly associated with advanced clinical stage of oral cancer at the time of presentation.

**Keywords:** Epithelial mesenchymal transition, Oral cancer, Oral squamous cell carcinoma, Neck metastasis

## INTRODUCTION

Cancers are a major global public health problem. It is predominantly disease of developed world with an incidence of 350 to 500 per 100,000.[1] Head and neck cancers are the 6th most common cancers worldwide.[2] In contrast, according to population based cancer registries these constitute the commonest cancer in Indian men and third most common cancer in women.[3] The high incidence of head and neck cancer in India is a consequence of widespread use of tobacco in multiple forms-smoked, chewed, applied topically on the gums, and inhaled. Amongst head and neck cancers, oral cancers are the commonest and constitute 40% to 70%.[2]

Chewing tobacco accounts for 95% of oral cancers in women and two-thirds in men. While tongue is the predominant sub site involved by cancer in the West,

mouth cancers (alveolus, buccal mucosa, and retro molar trigone) occur predominantly in India (age adjusted ratio (AAR) of 1.68 to 17.13 and 1.79 to 12.15 for mouth and tongue cancers, respectively).[3]

Of oral cancers, there is a predilection for the gingivobuccal sulcus subsite, which is attributed to the habit amongst Indians of keeping the betel quid or tobacco mixture between the cheek and the lower alveolus. However, there has been a rise in the incidence of tongue cancer in the recent past as reported from some series.[4] The traditional classification of tumors has been anatomical; tumors are categorized on the basis of tumor , nodes and metastases (TNM) classification (currently 8th edition).[5] The purpose is to stratify tumors based on the prognosis and requirement of adjuvant therapy. The major shortcoming of this classification is it does not take into consideration the individual molecular characteristics of tumors but rely only on the anatomical extent of the tumors.

With the advent of molecular markers of cancers and immune based therapy, molecular markers have been added to help care providers in better understanding the tumor biology. Specific molecular markers can identify more aggressive tumors, which require more intense therapy. This molecular profiling can spare adverse effects of therapy by choosing less intense therapy in favorable tumors with less chances of recurrence.

Epithelial mesenchymal transition (EMT) is the process in which committed epithelial cells undergo transformation into mesenchymal phenotype, that has invasive properties and thus contribute to metastatic potential of cancers. The reverse process of mesenchymal to epithelial transition takes place at the distal site, where tumor colonizes and produce nodal and distal metastasis (Figure 1).
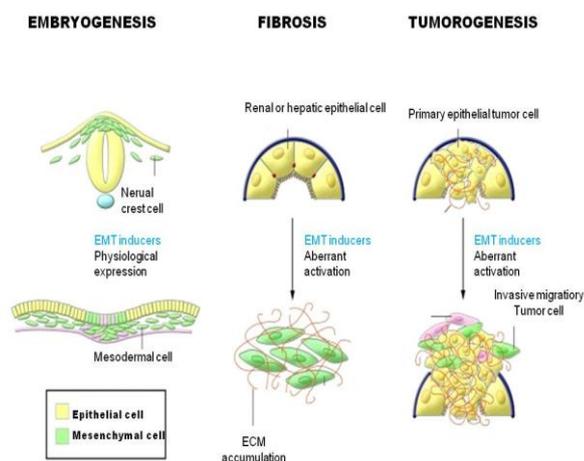


**Figure. 1: EMT during embryogenesis, fibrosis and tumour progression.**

The physiological process of EMT is important for embryogenesis and healing process.

Snail and slug proteins are markers of EMT and can be detected by immune histochemistry. The current study was undertaken to correlate the expression of EMT markers snail and slug with tumor stage at the time of presentation. This can potentially open up the thought process of adding molecular markers to traditional TNM classification and evaluate its utility in predicting tumor progression.

## METHODS

### Setting

An observational, cross sectional study was conducted in the Department of Otorhinolaryngology and Department of Pathology at University College of Medical Sciences & GTB Hospital, Delhi, a tertiary care university hospital from January 2017 to January 2019. Institutional ethical committee approved of this research work.

### Sample size

Consecutive 258 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, poor general condition and unfit for surgery, refused consent were excluded from the study.

Detailed clinical history was taken and head & neck and systemic examination were 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 American Joint Committee on Cancer and patients were divided into two groups based on the clinical stage as in group 1, patients with early stage oral cancer (stage I and 2), in group 2, patients with advanced stage oral cancer (stage 3 and 4).

All patients underwent biopsy from oral primary lesion that included at least 1mm of the apparently normal tissue. Formalin fixed, paraffin embedded archival tissue blocks of biopsy specimen or surgically removed oral squamous cell carcinoma specimen were included in study. The H & E stained slides were reviewed and the report noted. Immunohistochemistry was done for EMT associated proteins Snail and Slug by the following technique.

Four-micron thick sections were cut. Section was de-waxed in xylene and rehydrated through graded concentration of alcohol. Antigen retrieval was done by heating in 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 humid chamber at room temperature for 60 min. and section was washed with buffer. Secondary and tertiary antibody was applied successively for 30 minutes each and washed with buffer in between. Diaminobenzidine tetrahydrochloride (DAB) was used as chromogen. All sections were counterstained with haematoxylin.

All immunohistochemistry (IHC) slides showing snail or slug positivity (Figure 2 and 3). Were interpreted on basis of location of stain (nuclear or cytoplasmic) and percentage of positive cells. Grading was done on basis of percentage of positive cells for snail or slug. Grade 1 was assigned, when less than 10% cells are positive, grade 2, when 10 to 50% cells are positive and grade 3, when more than 50% cells are positive.
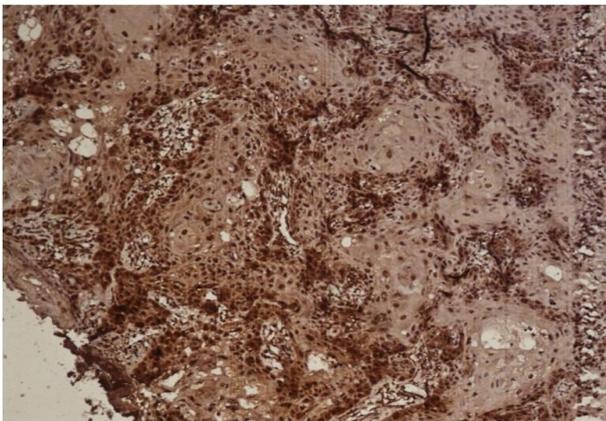


**Figure 2: Slug IHC (100X) showing cytoplasmic positivity grade 3 in SCC buccal mucosa.**
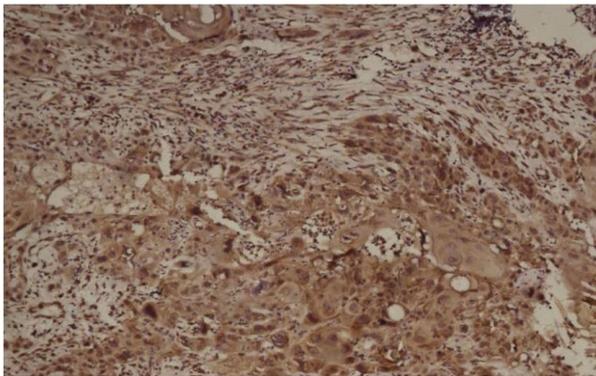


**Figure 3: Snail IHC (100X) showing cytoplasmic positivity grade 3 in SCC buccal mucosa**

Statistical methods were employed for determining the differential expression of EMT associated proteins Snail or slug and its correlation with clinical stage and its statistical significance.

***Statistical analysis***

Data was entered in MS excel and analyzed 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.

## RESULTS

Buccal mucosa has been the most common site of oral cancer in our series, followed by tongue. Mandibular alveolus, retro molar trigone and floor of mouth were relatively fewer common sites. Demographic characteristics was given in Table 1.

**Table 1: Patient characteristics (n=258).**

| Age in years | Mean=41.3 (range 21-78) | |
|---|---|---|
| **Male:female ratio** | 4.5:1 | |
| **Site of oral cancer** | **Number of patients** | **Percentage (%)** |
| Buccal mucosa | 117 | 45.3 |
| Tongue | 90 | 34.8 |
| Lip | 18 | 6.9 |
| Maxillary alveolus and hard palate | 18 | 6.9 |
| Mandibular alveolus | 3 | 1.1 |
| Floor of mouth | 6 | 2.32 |
| Retromolar trigone | 6 | 2.32 |
| **TNM stage (AJCC 8th edition)** | | |
| Stage 1 and 2 (early disease) | 33 | 33.3 |
| Stage 3 and 4 (advanced disease) | 53 | 61.6 |

**Table 2: Distribution of clinical stage (n=258).**

| Stage | No. of patients |
|---|---|
| 1 | 15 |
| 2 | 45 |
| 3 | 81 |
| 4 | 117 |
| **Total** | 258 |

In our study, majority of patients (45%) presented at stage 4. In present study, snail positivity was 60% in patients presenting at stage 1, 73% in stage 2, 85% in stage 3 and 90% in stage 4 patients.

We did not find significant association between clinical staging and snail positivity (p value=0.549).

In present study, slug positivity was 40% in patients presenting at stage 1, 26.7% in stage 2, 59% in stage 3 and 77% in stage 4 patients.

**Table 3: Relationship between clinical stage and EMT associated protein snail.**

| Stage | Grade of snail positivity | | | | Total number of patients with snail positivity | P value |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | | |
| 1 | 6 | 0 | 0 | 9 | 9 | |
| 2 | 12 | 0 | 9 | 24 | 33 | 0.549 |
| 3 | 12 | 3 | 18 | 48 | 69 | |
| 4 | 12 | 0 | 30 | 75 | 105 | |

**Table 4: Relationship between clinical stage and EMT associated protein slug.**

| Stage | Grade of slug positivity | | | | Total number of patients with slug positivity | P value |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | | |
| 1 | 9 | 0 | 3 | 3 | 6 | |
| 2 | 33 | 6 | 3 | 3 | 12 | 0.002 |
| 3 | 33 | 6 | 12 | 30 | 48 | |
| 4 | 27 | 12 | 3 | 75 | 90 | |

We found significant association between advanced clinical stage clinical staging and slug positivity (p value=0.002).

## DISCUSSION

There have been significant advances in management of head and neck cancers over the past decades, but with little effect on survival outcomes. Oral cancer is characterized by poor prognosis and survival rates of 90-45.5%, based on clinical stage (TNM) at the time of presentation.[6,7]

In patients presenting at advanced stage, there is high incidence of invasion to surrounding tissues, lymph node and distant metastasis.[6] Lymph node metastasis is one of most important prognostic factors for head and neck squamous cell cancer. Survival rates fall by 50% once lymphatic metastasis occurs irrespective of treatment modality.[8,9] The factors that contribute to metastasis in oral cancer are still unclear.

EMT is a process by which epithelial cells convert to mesenchymal cells having invasive properties, leading to invasion of tumour cells to underlying tissues, regional and distal metastasis.[10] The present study was undertaken to evaluate the expression of EMT associated markers snail and slug in oral cancer patients with and without neck lymph nodes metastasis.

The mean age of patients at presentation was 41.3 years (range 21-78), In several studies the commonest age group affected was fifth to 7th decade.[11-13] our patients presented at a younger age because of rampant exposure to oral tobacco early in life in India.

Male to female ratio was 6:1 in present study. These findings are consistent with other studies. This is attributed to habit of tobacco and alcohol consumption in males in our country. The commonest cancer in our series has been buccal cancer. This is in contrary to the west western literature where tongue is the commonest sub site. Tobacco consumptions by smoking is common in west, and hence tongue is directly exposed. Tobacco abuse by placing a mixture of tobacco and slaked lime in lower gingivo buccal sulcus is rampant in Indian subcontinent, where cancer typically develops.

Majority of our patients (76.74%) presented at stage III and IV in present study. This finding was consistent with other studies. Oral cancer presents at an advanced stage. Brandizzi et al in his study found 65% patients of oral cancer presented at advanced stages (III and IV). The main reason of delayed presentation in our patients is owed level of awareness and lack of access to healthcare facility.[13]

In our study, snail positivity was observed in 216 (83.72%), slug positivity was observed in 156 cases (60.46%) and either of two protein expressions was observed in 231 cases (89.53%).

We found that slug expression was significantly associated with clinical stage of oral cancer at the time of presentation.

Zheng et al studied snail and slug expression in human oral tongue squamous cell carcinoma cell lines by IHC. They concluded snail or slug or two protein co-expressions was significantly associated with T stage and lymph node metastasis.[14] In our study, we also found snail expression was significantly associated with clinical T stage.

Kuroika et al concluded that Slug expression correlates with local invasiveness of ameloblastoma.[15] In our study on oral cancer patients with and without neck metastasis, we found that slug positivity correlates with T staging, N staging and clinical stage. Though histology of tumour is different in both the studies but EMT may represent a common step in tumorigenesis, depicting the invasive properties and aggressiveness of tumour.

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.[16] We found similar results that slug positivity correlates with T, N stage and clinical stage.

Verrucous carcinoma is a well differentiated variant of squamous cell carcinoma. Regional and distant metastasis is rare in this cancer. Most common site of oral verrucous carcinoma is buccal mucosa.[17] Verrucous carcinoma did not show positivity for either snail or slug on IHC.

Negative reaction of verrucous carcinoma on IHC can be interpreted that snail and slug positivity on correlate with aggressive and metastatic behaviour of cancer. 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.

Slug expression correlates with invasiveness and metastatic potential of oral cancer and hence higher stage at the time of presentation. Molecular markers should be added to the TNM classification of cancer to better prognosticate and predict the tumour behaviour. This will stratify tumours besides their anatomic extent into a biological classification that can be a better predictive tool for patient outcome than TNM alone. This has the potential of changing the treatment trajectory, where less aggressive tumours can be treated with a de-escalated treatment protocol and limiting adverse effects. On the contrary more aggressive tumours as predicted by molecular markers can be treated and followed up with standard protocol. EMT markers snail and slug have the potential to be included in such a composite prognostic classification. The other potential application is in follow up of patients after treatment, where detection of these markers in body fluids (serum and saliva) can help, predict recurrence before there is a clinical recurrence. These early recurrences can be better managed by interventions when tumour load is less and can lead to a better overall survival.

## CONCLUSION

This study evaluated the epithelial mesenchymal transition (EMT) marker snail and slug in oral cancer. There was a positive correlation between advanced stage at the time of presentation and expression EMT marker slug. Further validation studies are required correlating EMT markers with patient outcomes and overall survival of patients. These outcome and survival studies will pave a way for inclusion of EMT markers, snail and slug in routine pre-treatment workup of patients with oral cancer along with anatomical prognostic staging TNM.

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