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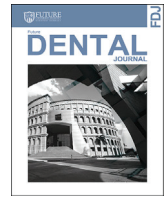
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# Expression of Vimentin, $\alpha$ -SMA and TGF- $\beta$ in Different Grades of Oral Squamous Cell Carcinoma

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## ABSTRACT

**Background:** Oral Squamous cell carcinoma (OSCC) is typically discovered at an early stage; nevertheless, regional lymph node metastases are prevalent; therefore, surgical excision of the primary tumor is commonly coupled with neck dissection and radiotherapy. Cancer cells become invasive by acquiring a mesenchymal phenotype, a process known as the epithelial-to-mesenchymal transition. Vimentin,  $\alpha$ -SMA and TGF- $\beta$  expressions are believed to take part in EMT and tumor progression. Aim: this work aimed to evaluate the expression of Vimentin,  $\alpha$ -SMA and TGF- $\beta$  on OSCC progression and hence propose potential role as potential prognostic markers or therapeutic targets. Materials and methods: twenty-four specimens of different OSCC grades were divided according to Broader's classification into three groups I, II and III. Mean area fraction of immunopositivity for Vimentin,  $\alpha$ -SMA and TGF- $\beta$  expressions as well as clinical lymph-node involvement was evaluated for each group. Results:  $\alpha$ -SMA and TGF- $\beta$  were significantly upregulated in poorly differentiated OSCC and  $\alpha$ -SMA expression correlated with lymph node involvement. Vimentin expression was highest in moderately differentiated OSCC and didn't correlate with lymph-node involvement. Conclusion:  $\alpha$ -SMA is suggested to be a better prognostic factor than vimentin for OSCC progression.

## 1. INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents a substantial threat to public health, accounting for around two thousand deaths worldwide each year. The treatment of OSCC is determined by the disease stage, possible complications associated with each therapeutic approach, and the patient's quality of life. Surgery and radiotherapy are mainstay treatment modalities for patients suffering from early-stage OSCC. Since advanced oral carcinomas have a high risk of lymph node (LN) metastasis, neck dissection is an important part of tumor eradication. The size, number, and location of the LN involved influence the extent of neck dissection<sup>(1, 2)</sup>.

Due to the patient's consciousness of the presence of a mass as well as symptoms, OSCCs are typically discovered at an early stage; nevertheless, regional LN metastases are prevalent; therefore, surgical excision of the primary tumor is commonly coupled with neck dissection and radiotherapy. Owing to the morbidity associated with this line of treatment, there is a need to find molecular biomarkers to foresee LN metastases<sup>(3)</sup>.

Cancer cells become invasive by acquiring a mesenchymal phenotype, a process known as the epithelial-to-mesenchymal transition (EMT)<sup>(4)</sup>. This process occurs when the apical-basal polarity and cell-cell adhesions of the epithelial cells are lost. This leads to enhanced cell migration<sup>(5)</sup>.

EMT is intricately regulated via signaling between tumor cells and their neighboring extracellular-matrix (ECM) and the tumor-microenvironment (TME)<sup>(6)</sup>. A chronic inflammatory, pro-angiogenic, and immunosuppressive environment is driven within the TME. Over the past ten years, the TME has gained recognition as an environment with a wealth of targets for the development of novel anticancer medicines<sup>(7)</sup>. Cancer-associated fibroblasts (CAFs), that are elongated cells that remodel and build up the structure of the ECM, are one of the most dominant components in the TME<sup>(8)</sup>.

The most frequent origin of CAFs, which are frequently discovered in the TME, is assumed to be the trans-differentiation of local fibroblasts. TGF-upregulation and the generation of a markedly contractile phenotype (similar to myofibroblasts) concurrent with the production of alpha-smooth muscle actin ( $\alpha$ -SMA) are assumed to be the catalysts for this trans-differentiation. It has been demonstrated that CAFs frequently alter genetically and react to external chemicals like cytokines and growth hormones<sup>(9)</sup>.

The most commonly identified CAFs marker is  $\alpha$ -SMA. It has been shown that  $\alpha$ -SMA expression in CAFs is associated with a greater number of lymph node metastases and a poor clinical outcome in a variety of cancer patients.  $\alpha$ -SMA positive CAFs also influenced tumor growth in vivo and were linked to an increased incidence of cancer stem cells<sup>(10)</sup>. In addition to being considered a sign of EMT, vimentin is the main protein component of intermediate filaments in both healthy and malignant mesenchymal cells<sup>(11, 12)</sup>.

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It is generally acknowledged that the TGF- $\beta$  mediated signaling pathway controls the EMT process. TGF- $\beta$  stimulates intracellular signaling molecules like SMAD-2 and SMAD-4 upon binding to the potential receptors, which in turn activates the transcriptional repressors of the E-cadherin gene <sup>(13)</sup>.

TGF- $\beta$  has different effects depending on the cell type and environment. There is mounting evidence that TGF- $\beta$  signaling plays a dual role in the development of tumors. TGF- signaling pathway activation promotes cell-cycle arrest and death in healthy cells and early-stage malignant cells, whereas in late-stage malignancies, TGF- $\beta$  signaling works as an oncogene to increase metastasis and drug resistance. <sup>(14)</sup>

The current work was performed to evaluate the expression of  $\alpha$ -SMA, vimentin and TGF- $\beta$  in different histopathological grades of OSCC and association of their expressions with lymph node involvement.

**2. MATERIAL AND METHODS**

**Clinical Lymph node involvement and immunohistochemical Vimentin,  $\alpha$ -SMA and TGF- $\beta$ , gene expression in different OSCC grades:**

Twenty-four formalin-fixed paraffin-embedded tissue samples were acquired retrospectively, from different OSCC grades, after receiving patients' informed consent. The blocks were obtained from the archives of the Oral Pathology Department, Faculty of Oral and Dental Medicine, Future University in Egypt and the Faculty of Dentistry, Ain-Shams University, (from 2011–2022). The study protocol was approved by our Institutional ethics committee.

49 years was the mean age of patients included in this work, of which nine (37.5 %) were female and 15 (62.5%) were male. Cases were evaluated histopathologically using H&E staining by two independent oral pathologists as per Broader's classification. Eight (33.3 %) of the cases were found to be well differentiated (WD-OSCC), nine (37.5%) moderately differentiated (MD-OSCC), and seven (29.2%), poorly differentiated (PD-OSCC). Immunohistochemical expression of the chosen markers was assessed and associated with clinical lymph node involvement.

Clinically the mean tumor size was 21 mm x 15 mm in greatest dimension, 5 of the included cases exhibited lymph node involvement while none of the included cases exhibited distant metastases, as reported by the referring surgeons.

Immunohistochemical staining procedure following the peroxidase-anti-peroxidase method and the biotin-streptavidin system, was performed at order, the Oral Biology Department Lab at the Faculty of Dentistry at Ain Shams University used monoclonal antibodies against vimentin,  $\alpha$ -SMA and TGF- $\beta$ . Four micrometer-thick sections of the blocks were cut, mounted, and set on glass slides that were positively charged. Following xylene, sections were deparaffinized, rehydrated, microwaved in citrate buffer then stained. A generic kit (Lab Vision) was utilized for immunostaining. In order to decrease endogenous peroxidase activity, hydrogen peroxide 3% was applied to sections and then they were treated with the immunolabeled primary antibody (Lab Vision) overnight. Sections were cleaned with Phosphate buffer saline (PBS) and then dyed.

Quantifying the area fraction of immunohistochemical expression of vimentin,  $\alpha$ -SMA and TGF- $\beta$  was performed using photomicrographs from digital camera (C5060, Olympus) attached with a C-mount to a light microscope (BX60, Olympus) (Image J, 1.41a, NIH).

**3. RESULTS**

**Clinical Lymph node involvement and immunohistochemical Vimentin,  $\alpha$ -SMA, and TGF gene expression in different OSCC grades:**

**A. Assessment of the gene expressions**

Using One Way Analysis of Variance (ANOVA) and Chi-square test, it was found that there was a statistically significant differences in mean area fraction of Vimentin,  $\alpha$ -SMA, TGF- $\beta$ , expressions and Lymph node involvement among the studied groups. (Table 1 and fig. 1)

Independent t-test revealed that there was a statistically significant difference between the mean area fraction of expression of Vimentin,  $\alpha$ -SMA and TGF- $\beta$  and Lymph node involvement in the studied groups. (Table 2 fig. 2)

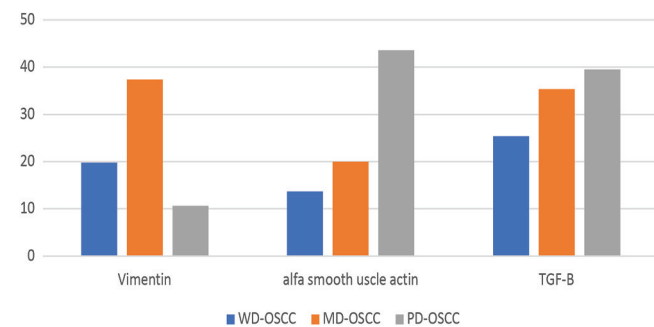
**Table (1)**

Descriptive analysis and ANOVA regarding Vimentin,  $\alpha$ -SMA, TGF- $\beta$ , expressions and Lymph node involvement among the studied groups

Variables	Group (I) / WD-OSCC (n=8)	Group (II) /MD-OSCC (n=9)	Group (III) / PD-OSCC (n=7)	P value
Vimentin	19.8 ± 3.3	37.3 ± 4.9 <sup>a</sup>	10.6 ± 1.4 <sup>ab</sup>	<0.001**
$\alpha$ -SMA	13.6 ± 2.1	20.0 ± 4.3	43.5 ± 11.4 <sup>ab</sup>	<0.001**
TGF- $\beta$	25.4 ± 2.4	35.3 ± 1.7 <sup>a</sup>	39.4 ± 3.4 <sup>ab</sup>	<0.001**
No lymph node involvement	8={100 %}	8={88.9%}	3={42.9%}	0.016*
Lymph node involvement	0={0%}	1={11.1%}	4={57.1%}	

Post-HOC: (a) significant difference with group (I) , (b) significant difference with group (II)

\*\*p-value <0.001 HS \*p-value <0.05 S; p-value>0.05 NS;



**Figure (1) — Column chart showing the expression of Vimentin,  $\alpha$ -SMA, TGF- $\beta$ , expressions in the different grades of OSCC included in the study.**

**Table (2)**

Independent t-test comparing the expression of Vimentin,  $\alpha$ -SMA and TGF- $\beta$  and Lymph node involvement in the studied groups.

Markers	Lymph node involvement		p-value
	involved (n=5)	Not involved (n=5)	
Vimentin	14.8±9.8	26.0±11.5	0.026*
$\alpha$ -SMA	36.8±11.2	21.5±13.3	0.019*
TGF- $\beta$	37.7±2.7	32.0±6.5	0.029*

Independent Sample t-test; significance at \*p-value <0.05

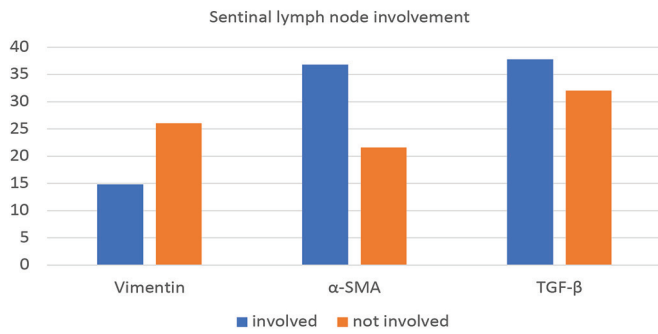


Figure (2) — Column chart showing Vimentin,  $\alpha$ -SMA, TGF- $\beta$ , expressions and Lymph node involvement in the studied groups

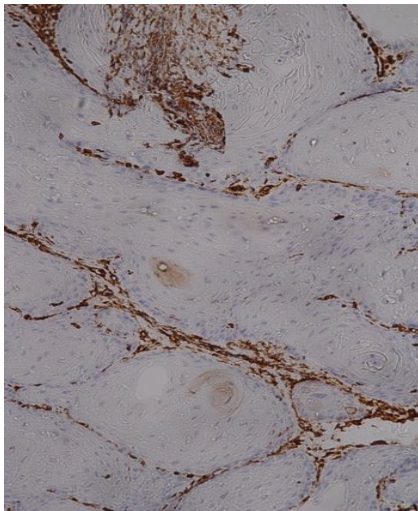


Figure (3) — Photomicrograph of WD-OSCC specimen exhibiting immunopositive stromal cells (green arrow) and immunonegative neoplastic epithelial cell nests (yellow arrow) (Vimentin, original magnification x20).

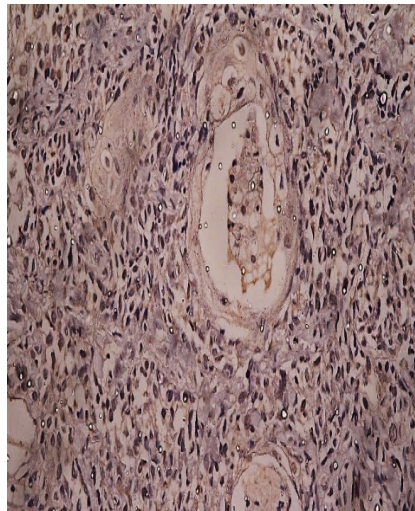


Figure (4) — Photomicrograph of WD-OSCC specimen exhibiting few immunopositive stromal cells (green arrow) (TGF- $\beta$ , original magnification x40).

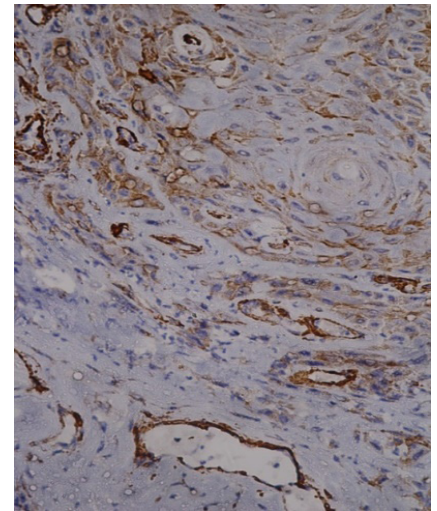


Figure (5) — Photomicrograph of a WD-OSCC specimen showing immunopositive stromal cells (red arrow) and immunonegative neoplastic epithelial cell nests (yellow arrow) ( $\alpha$ -SMA, original magnification x40).

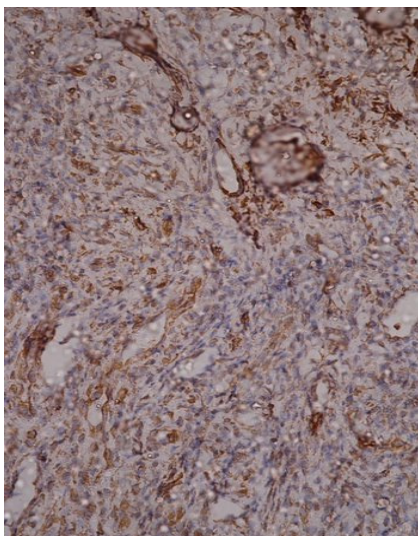


Figure (6) — Photomicrograph of PD-OSCC specimen note immunonegative neoplastic epithelial cells (green arrow) with few immunopositive cells (yellow arrow) Few spindle stromal cells showing immunopositivity (red arrow) were also noted (vimentin original magnification x20).

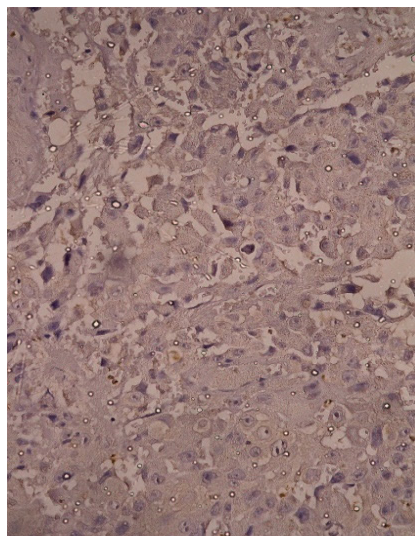


Figure (7) — Photomicrograph of PD-OSCC specimen showing cytoplasmic and membranous immunopositive reaction in the majority of the neoplastic epithelial cells (yellow arrow). The nuclei of the neoplastic epithelial cells were mostly immunonegative (red arrow) (TGF- $\beta$ , original magnification x40).

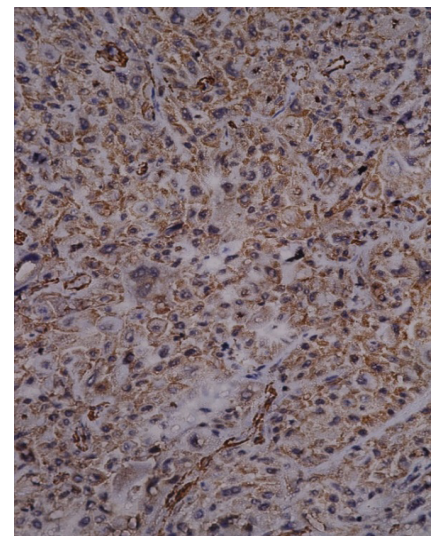


Figure (8) — Photomicrograph of a PD-OSCC specimen where majority of neoplastic epithelial cells exhibited membranous immunopositivity and few exhibited some cytoplasmic reaction (red arrow). Few stromal spindle cells exhibited immunopositivity (yellow arrow) ( $\alpha$ -SMA, original magnification x40).

#### 4. DISCUSSION

OSCC has a high propensity to metastasize, and LN metastasis is among the most significant prognostic factors: One positive LN can result in a 50% reduction in the 5-year disease-free survival (15). The continuous cross-talk between the cancer cells and TME is thought to take part in enabling cancer cells to invade, migrate and eventually metastasize. However, the molecular mechanism of this interaction between TME and OSCC remains obscure (2).

In our study, immunohistochemical evaluation of WD-OSCC specimens showed that the TME, rather than the tumor cells, expressed  $\alpha$ -SMA and TGF- $\beta$  and that these two proteins were expressed in the stroma right next to the invading epithelial cells. In contrast, the transcription of those same markers was raised in the tumor cells in PD-TSCC, and there were also more occurrences of LN involvement. This could be explained by the idea that  $\alpha$ -SMA overexpression is required for EMT in SCC (1; 9; 10). TGF- $\beta$  expression was raised in MD-OSCC compared to both the WD-OSCC and PD-OSCC. It may be hypothesized that this is due to the dual function of TGF- $\beta$  as a promoter and a repressor of carcinogenesis depending on the stage of carcinogenesis or that it takes part initially in upregulation of  $\alpha$ -SMA and CAFs trans-differentiation and EMT then may be the process is self-driving. Similar hypotheses were introduced by Yoshida K, et al, 2013 (16; 17). However, the exact explanation of the reason will require further investigation.

Vimentin however, exhibited a different pattern of expression according to the results of our study. It was found that it was expressed was higher in the connective tissue both in the WD-OSCC and the PD-OSCC. The results also show that vimentin upregulation did not correlate with increased lymph node involvement and that its expression was significantly lowered in the PD-OSCC specimens.

Even though Vimentin and  $\alpha$ -SMA expressions in malignant epithelial cells are both associated with EMT according to previous research (12; 17), our results demonstrated that  $\alpha$ -SMA – and not vimentin- correlated significantly with LN involvement and tumor grade. Microscopically the majority of the invading malignant epithelial cells in the PD-OSCC expressed  $\alpha$ -SMA, which was not seen when specimens were stained for vimentin.

It should also be noted that the expression of both these proteins was seen in the stromal cells however,  $\alpha$ -SMA is far more specific to CAFs and CAFs are believed to be a strong driving factor in Cancer progression. Statistical assessment in our study showed that the expression of  $\alpha$ -SMA was higher than vimentin in PD-OSCC however, it should be emphasized that the expression was predominantly seen in the epithelial cells for the  $\alpha$ -SMA and predominantly in stromal cells for vimentin. Similar findings were reported by Ding L, et al, 2014, who concluded that  $\alpha$ -SMA may be associated with enhanced EMT of tumor cells and lymphogenesis (11).

#### 5. CONCLUSION

$\alpha$ -SMA is suggested to be a better potential prognostic marker/ therapeutic target than vimentin for OSCC progression.  $\alpha$ -SMA is significantly increased in higher-grade OSCC and its expression is associated with increased lymph node involvement. Further investigations are required to understand the exact mechanism behind  $\alpha$ -SMA role in OSCC.

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