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ROLE OF ALPHA SMOOTH MUSCLE ACTIN IN ORAL SQUAMOUS CELL CARCINOMA PROGRESSION

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ABSTRACT

Squamous cell carcinoma (SCC) is the most common type of oral cancer. Malignant epithelal cells undergo cytological changes by a process referd to as epithelial mesenchymal transition (EMT). The cancer associated fibroblasts (CAFs) in the tumor micro-environment are now the focus of intense research and are believed to correlate with poor prognosis. They are characterized by alpha smooth muscle actin (α-SMA) expression, which is a myofibroblastic marker. Physiologically myofibroblasts are not as abundant as fibroblasts in the oral cavity, they have limited locations such as blood vessels walls in the oral mucosa. **Aim:** evaluation of the potential role of α-SMA in the progression of SCC. **Materials and methods:** qRT-PCR was performed to evaluate α-SMA gene expression in tongue SCC cell line (SCC 25), normal fibroblasts cell line (Wi-38) and in a coculture of both cells. Qualitative and quantitative immunohistochemical analysis of α-SMA expression with clinico-pathological correlations was performed for 24 SCC specimens of different grades. **Results:** qRT-PCR results showed that there was α-SMA expression in the epithelial cells, and that co-culturing resulted in an overall increase in α-SMA expression. Immunohistochemical evaluation of the specimens revealed that the expression of α-SMA increased with tumor grade and correlated with lymph node involvement. **Conclusion:** α-SMA can be used as a prognostic marker and a potential target for cancer therapy.

KEY WORDS: Squamous cell carcinoma; myofibroblasts; alpha smooth muscle actin; cancer associated fibroblasts; epithelial mesenchymal transition.

INTRODUCTION

Squamous cell carcinoma (SCC) is the most prevalent cancer affecting the oral and oropharyngeal mucosa. It was considered to be a disease of the elderly. However, recently, more and more patients are being diagnosed with SCC, before the age of forty[1].

Several trails have been attempted in order to be able to accurately predict the prognosis of SCC. Broader`s classification for histological grading and

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the TNM staging system, are the most widely utilized. The most important prognostic factor is the presence or absence of metastases[1, 2]. Invasion and metastases involve epithelial to mesenchymal transition (EMT). EMT is activated during development and wound healing, however, aberrant activation is associated with cancer progression[3].

Normal epithelial cells are identified based on their histomorphological features and their immunohistochemical profile, where they express cytokeratins and absence of mesenchymal markers like α-SMA. Malignant epithelial cells establish EMT as they down regulate their junctions and adopt new signaling pathways through genetic/ epigenetic alterations. Eventually, they confer a mesenchymal phenotype that is able to invade. The "T" in EMT denotes the transitional quality of this process as the epithelial cells are able to revert back to their original form through mesenchymal epithelial transition (MET). To establish motility, epithelial cells rearrange their actin cytoskeleton and develop membrane projections "lamellopodia/ invadopodia" made of membrane bound actin. They are then able to alter the surrounding stoma and the extracellular matrix (ECM), as well[4, 5].

The normal stroma contains an array of cells, the most abundant of which are the fibroblasts. Fibroblasts main function is to provide support to the surrounding structures through the production of several ECM components. Myofibroblasts however, are less abundant and are mainly located intra-orally in the walls of the blood vessels. They recognized by α-SMA expression[6].

Stroma surrounding the tumorous mass is referred to as, the tumor micro-environment (TME). Fibroblasts in the TME have received much attention recently, as they are now recognized to transdifferentiate into α-SMA expressing cells, which are refered to as cancer associated fibroblasts (CAFs), by a process known as mesenchymal-mesenchymal transition, MMT. α-SMA is encoded by the ACTA2 gene (10q22-q24). α-SMA is used as a myofibroblastic marker and is involved in cells structural integrity, contractility and motility. Alteration in this gene expression has been associated with several diseases such as heart diseases, lung disease, multisystemic smooth muscle dysfunction syndrome, cancer progression and EMT[7,8].

In the setting of a malignant neoplasm, α-SMA may be also noted in the epithelial cells during EMT as well in the endothelial cells. It has been documented that endothelial cells may normally exhibit some α-SMA immunopositivity but in TME the α-SMA expression is upregulated due to what is known as endothelial to mesenchymal transition (End-MT)[9]. The source of the α-SMA expressing CAFs in the TME has been debated for so long and is know believed to be from one of the folowing sources; resident fibroblasts, tumor cells, endothelial cells or from the bone marrow.

It is postulated that there is cross talk between the malignant epithelial cells in the TME, which favors CAFs differentiation with consequent release of cytokines from CAFs which inturn enhance tumor progression and EMT. During cancer progression, tumor cells secrete a variety of growth factors, one of which is the TGF-β. TGF-β trans-differentiates resident fibroblasts into CAFs, some authors also suggest that the oxidative stress in the tumor environment plays a role in CAFs trans-differentiation[10]. CAFs, subsequently, release more growth factors like IL-6, FGF, HGF, CCL2, CCL5, CCL7, CXCL16 and SDF1, enhancing carcinogenesis even further. Moreover, CAFs release matrix metallo-proteinases (MMPs), SNAIl and TWIST which alter the architecture of the TME through the creation of passages and the parallel arrangement of fibers in the ECM, to aid cancer cell movement and migration (haptotaxis)[10-12].

Aim of the study:

This study was done to evaluate the expression of α-SMA by qRT-PCR in a co-culture of SCC and fibroblasts, to assess the cross talk between

these two cell lines regarding α-SMA expression. Moreover, α-SMA expression in different grades of SCC immunohistochemically was performed and the α-SMA area fraction of immunopositivity was correlated to lymph node involvement.

PATIENTS/ MATERIALS AND METHODS

Cell cultures preparation

Human male tongue squamous cell carcinoma (SCC-25) and human lung fibroblast (WI-38) cell lines were obtained from ATCC, Wesel, Germany. SSC-25 and WI-38 cell lines were cultured separately or co-cultured for 7 days in 75 ml flasks with DMEM (Invitrogen, USA) supplemented with 10% FBS (Hyclone, USA), 10 μg/ml insulin (Sigma, USA) and 1% penicillin-streptomycin. Flasks were maintained in an incubator at 37 °C in a humidified atmosphere and 5% CO_2.

qRT-PCR for α-SMA expression

Total RNA extraction was done using RNeasy micro kit Cat.no 74004, Qiagen, USA. A total of 1x106 cells from each cell line and their co-culture was disrupted in buffer RLT and homogenized. Ethanol was then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The samples were then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high quality RNA is eluted in RNase-free water. All bind, wash and elution steps were performed by centrifugation in a micro-centrifuge.

RNA reverse transcription and amplification was done using BIORAD iScriptTM One-Step RT-PCR Kit with SYBR® Green on Rotogen-S, korea.

Primers sequences used were as follows:

α SMA: F 5'- GCATCCACGAAACCACCTA-3'R 5'- CACGAGTAACAAATCAAAGC -3'

GAPDH: F 5'- GAAGGTGAAGGTCGGAGT -3' R 5'- CATGGGTGGAATCATATTGGAA -3'

The cell lines were grouped as follows:

Group I: SCC25 cell line

Group II: WI-38 cell line

Group III: co-culture of both SCC 25 and WI-38 cell lines

Tissue samples from patients

Paraffin blocks from 24 different oral SCC patients, were obtained from the archives of the Oral Pathology department (from 2011-2019), Faculty of Dentistry, Ain-Shams University and the Oral Pathology department, Faculty of Oral and Dental Medicine, Future University in Egypt. Fifteen (62.5%) of the patients were male and nine (37.5%) were females, and the mean age of the patients included in the study was (±) 49 years. Histopathologically, the cases were graded according to broader's classification using H&E stained sections and were found to be divided as follows; 8 (33.3 %) well, 9 (37.5%) moderately and 7 (29.2%) poorly differentiated SCC.

Immunohistochemistry

Immunohistochemical techniques using monoclonal antibodies against α-SMA was employed in this study. The blocks of paraffin were cut into four micrometer thick sections and then mounted on positively charged glass slides. Sections were deparaffinized with xylene and gradually rehydrated in serial alcohol concentrations. Sections were immersed in citrate buffer and treated in a microwave then stained. For immunostaining, a universal kit (Lab Vision, USA) was used. Peroxidase-antiperoxidase method of immunostaining using the biotin-streptavidin system was performed. Hydrogen peroxide (3%) was applied to the sections to block endogenous peroxidase activity. The sections were then immunolabelled using the primary antibody α-SMA (Lab Vision, USA) and incubated

overnight at room temperature. Sections were then rinsed with phosphate buffer saline (PBS) and covered by the link antibody, followed by streptavidin labeling antibody. Sections were then rinsed again with PBS and diaminobenzidine chromogen was applied followed by the counterstain. Sections were dehydrated in graded alcohol, cleared in xylene and mounted.

Photomicrographs were captured at a magnifications of x20 and x40. All steps for immunohistochemical quantitative evaluation were carried out on photomicrographs captured at a magnification of x20 using image analysis software (Image J, 1.41a, NIH, USA).

STATISTICAL ANALYSIS

The collected data from the qRT-PCR and histomorphometric analysis for α-SMA gene expression were tabulated using Microsoft Excel (Microsoft Office 2008). All cases were included in the statistical analysis. The data were tabulated using the Statistical Package for Social Science (SPSS 15.0) Software. The mean values and standard errors were calculated for each time point from the pooled normalized data. The statistical tests performed included the One Way ANOVA and Post hoc test for comparison of means. The results were considered significant when the P value was ≤ 0.05. Graphs were performed using. Microsoft word software (Microsoft Office 2008).

RESULTS

q-RT PCR results

The highest mean α-SMA gene expression was noted in group III and the least expression was in group II (fig.1). Statistically significant differences were seen using ANOVA, while the post hoc test revealed that group III had a significantly higher α-SMA gene expression than group I and II. At the same time no significant difference in expression was noted between groups I and II (table 1).

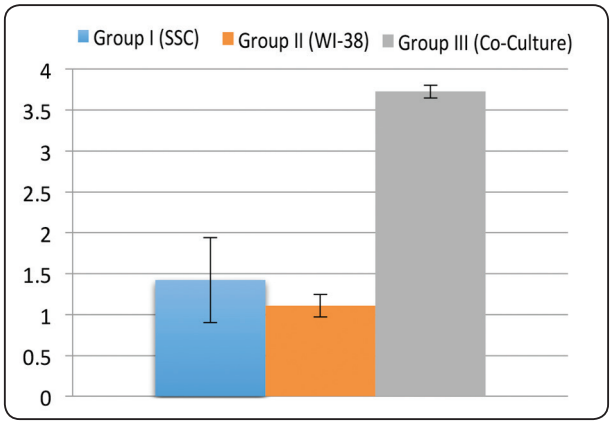

Fig. (1) Column chart of the mean values of α-SMA gene expression in groups I, II and III

TABLE (1) One Way ANOVA and Post hoc test of the mean values of α-SMA gene expression between groups I, II and III

Compared groups		Sig. (p-value)	ANOVA
Group I	Group II	.786	F= 80.019 Sig.= 0.000
Group II	Group III	.000	
Group I	Group III	.000	

The mean difference is significant at the 0.05 level

α-SMA Immunohistochemical results

Microscopic evaluation

All the studied specimens exhibited immunopostivity with anti α-SMA anti-body. The well differentiate SCC (WD-SCC) specimens exhibited focal immunopositivity in the TME and the malignant epithelial cells, as well. The majority of the neoplastic cells were immunonegative only some of the cells at the periphery of the cell nests exhibited immunopositivity. The blood vessels showed nuclear and cytoplasmic immunopositivity (fig. 2 and 3).

The moderately differentiated SCC (MD-SCC) showed immunopositivity in the tumor cells at the peripheries of the nests while the cells at the center of the nests appeared to be immunonegative. The immunopositivity was granular cytoplasmic and/or

membranous, the nuclei were immunonegative. The CAFs appeared spindle in morpholgy, exhibited both nuclear and cytplasmic immunopositivity and were located near the periphery of the nests. The blood vessels walls were also immunopositive (fig. 4-6).

Immmunopositivity in the poorly differentiated SCC (PD-SCC), appeared to be in the majority of the malignant epithelial cells. The reaction was granular, cytoplasmic and membranous, and the nuclei were all immunonegative. CAFs were scarcely noted (< 10/HPF) (fig. 7).

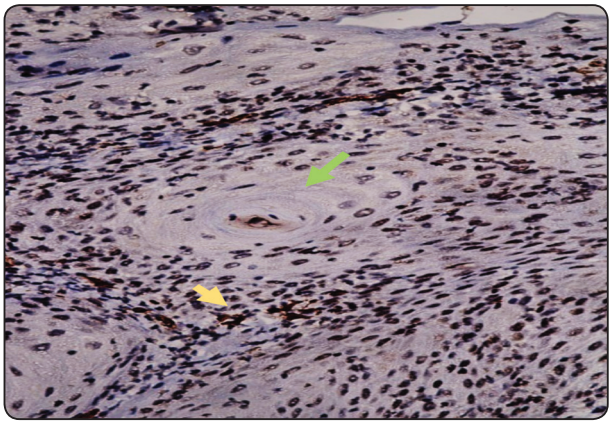

Fig. (2) Photomicrograph of WD-SCC exhibiting immunonegative epithelial cells (green arrow) and focal immunopositivity in the TME (yellow arrow). Note: the stromal cells revealed nuclear and granular cytoplasmic immunopositivity. WD-SCC: well differentiated squamous cell carcinoma (α-SMA , orig. mag.x20)

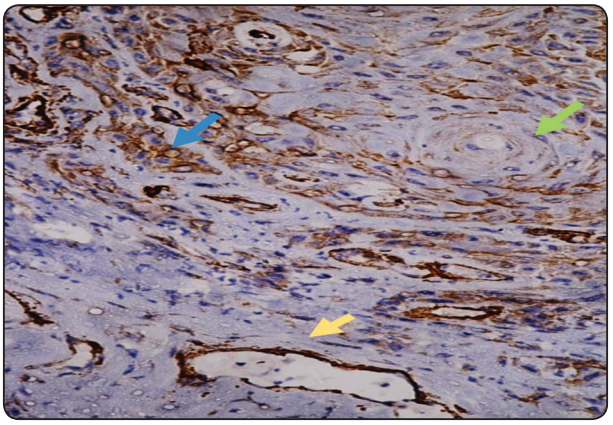

Fig. (3) Photomicrograph of WD-SCC showing some immunonegative (green arrow) and some immunopositive malignant epithelial cells (blue arrow), where the immunopositivity was membranous and cytoplasmic. The blood vessels walls exhibited nuclear and cytoplasmic immunopositivity (yellow arrow). WD-SCC: well deffirentiated squamous cell carcinoma (α-SMA , orig. mag.x20)

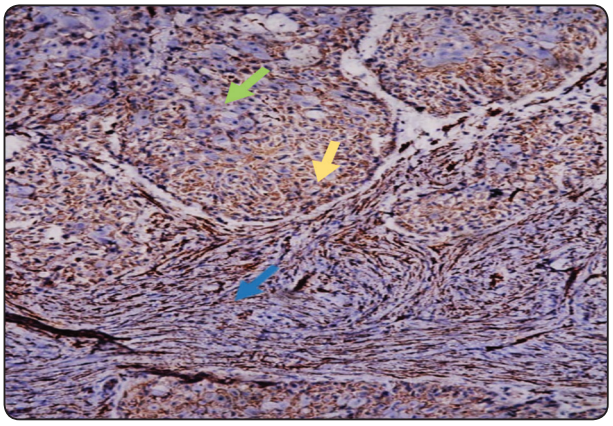

Fig. (4) Photomicrograph of MD-SCC. The cell nests demonstrated few immunonegative (green arrow) and some immunopositive malignant epithelial cells, the immunopositive cells were situated more at the periphery of the nests. (yellow arrow). Note: immunopositive CAFs in the TME. MD-SCC: moderately differentiated squamous cell carcinoma; CAFs: cancer associated fibroblasts. TME: tumor microenvironment (α-SMA, orig. mag. x20)

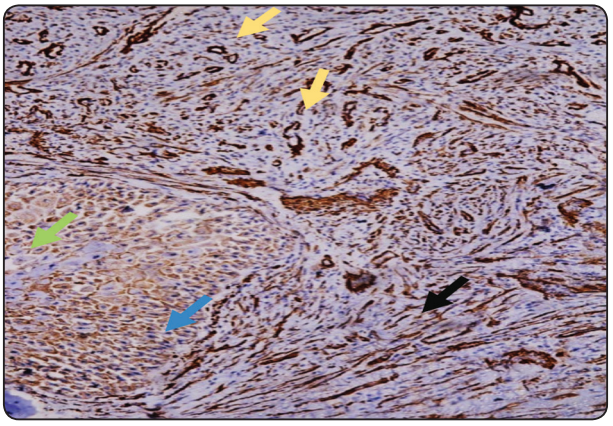

Fig. (5) Photomicrograph of MD-SCC showing, some immunonegative (green arrow) and some immunopositive malignant epithelial cells (blue arrow). The blood vessels walls (yellow arrow) appeared numerous, small and immunopositive. CAFs neighbouring the cell nests (black arrow). MD-SCC: moderately differentiated squamous cell carcinoma; TME: tumor microenvironment; CAFs: cancer associated fibroblasts. (α-SMA, orig. mag. x20)

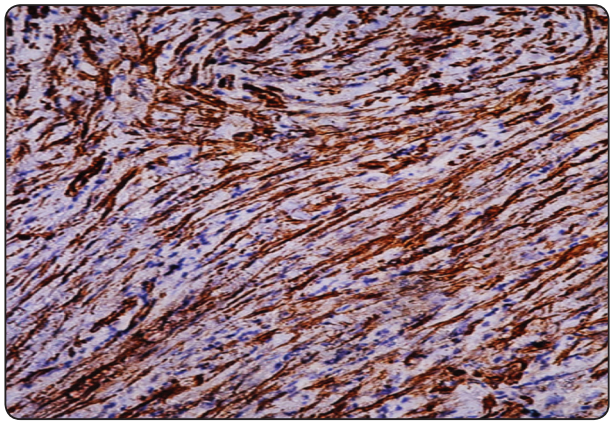

Fig. (6) Photomicrograph of immnuopositive myofibroblasts exhibiting a "network pattern" in the TME of SCC. They appeared to be spindle in morpholgy and exhibited both nuclear and cytoplasmic immunopositivity. TME: tumor microenvironment; SCC: squamous cell carcinoma (α-SMA, orig. mag. x40)

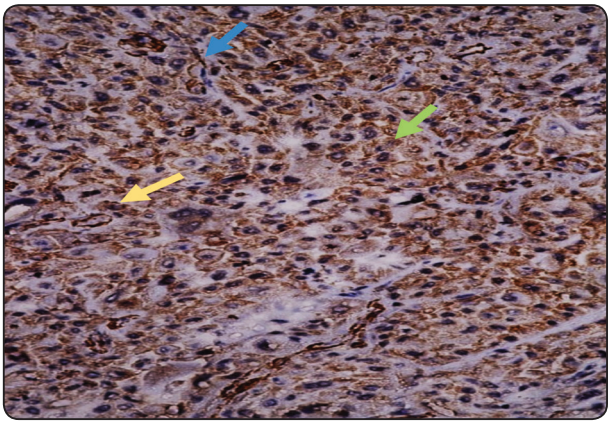

Fig. (7) Photomicrograph of a PD-SCC. The invading malignant epithelial cells demonstrated membranous and a diffuse, granular cytoplasmic immunopositivity, while the nuclei were immunonegative (green arrow). The blood vessels walls exhibited intense immunopositivity (yellow arrow). Scarce immunopostive CAFs were seen (blue arrow) TME. (TME: tumor microenvironment) (α-SMA, orig. mag. x40)

Immunohistochemical analysis and correlation between mean values of α-SMA gene expression and lymph node involvement

Table 2 and fig. 8, show the mean area fraction and standard deviation of immunopositivity of α-SMA, in the SCC specimens. The highest mean expression was in the PD-SCC followed by the MD-SCC then the WD-SCC. One way ANOVA showed that there are significant differences among the studied groups (Table 2). Using post hoc test (Table 2) it was found that there is a significant difference in area fraction of α-SMA expression between the PD-SCC and either of the WD-SCC and the MD-SCC ($p \leq 0.05$). While the difference in α-SMA area fraction was insignificant between the WD-SCC and the MD-SCC.

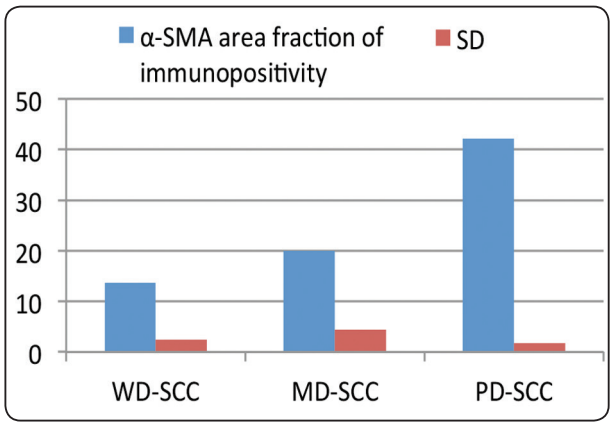

Fig. 8: Column chart showing Area fraction of immunopositivity with α-SMA antibody in different grades of SCC (WD-SCC: Well differentiated squamous cell carcinoma; MD-SCC: Moderately differentiated squamous cell carcinoma; PD-SCC; Poorly differentiated squamous cell carcinoma)

Clinically, of all the 24 cases included in the study, only six cases showed lymph node involvement. Statistically, there was a positive correlation between α-SMA area fraction and lymph node involvement (Table 3).

TABLE (2) One way ANOVA and post hoc test results between the studied groups

SCC Grades		P.Sig.	ANOVA
WD	MD	.264	F= 5.559 Sig.= 0.001
	PD	0.001	
MD	WD	.264	
	PD	0.001	
PD	WD	0.001	
	MD	0.001	

TABLE (3) Correlation between α-SMA and lymph node involvement

Nodes	N	Mean SMA	± SD
Negative	18	0.20	0.10
Positive	6	0.40	0.15

t-test = 3.537, P value = 0.002 (Significant)

DISCUSSION

SCC is the most common intra-oral cancer and its numbers have been increasing gradually over the last five decades, consequently it is the most studied oral cancer type. Yet, and in spite of the intense research and advances in diagnosis and treatment modalities, the 5-year survival rate of SCC is still about 50%, which is lower than several other cancer types which have shown improvements in survival rates and recurrences[13]. Accordingly, new therapeutic approaches are investigated which aim to control the TME along with the tumor cells. The TME and the immune system, are the new focus of cancer research[14, 15]

CAFs in the TME are believed to have a role in tumor progression. They are identified by the expression of different markers, the most notorious of which is the α-SMA. The α-SMA expressing CAFs in the TME are postulated to be derived from; resident fibroblasts (via MMT), bone marrow cells, tumor epithelial cells (via EMT) or endothelial cells (via End-MT). No matter where they came from, in context of a malignant tumor, CAFs are associated with cancer progression and poor prognosis[15-17].

With the idea that α-SMA expressing CAFs may serve as an available potential target for therapy/adjuvant therapy for cancer, this study focused on the assessment of α-SMA expression in tongue squamous cell carcinoma (TSCC), SCC25. First qRT-PCR was performed and the results showed that there was α-SMA expression in SCC25 cells. This expression may be attributed to the fact that malignant epithelial cells do express α-SMA during EMT. It has been reported that, malignant epithelial cells undergo a "cadherin switch" from E-cadherin to N-cadherin, upregulate mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, release TGF-β, develop cytoskeletal changes and express α-SMA (normally not expressed by epithelium). It should be noted as well, that in the present work, not only did the SCC25 cells express α-SMA but, the expression was even higher than that of the Wi-38. This may be explained by the fact normal fibroblasts do not always express α-SMA. These results were consistent with those of Alt. E, et al 2011, who stated that Wi 38 is positive for collagen I, HSP47 (heat shock protein 47), vimentin, FSP (fibroblast surface protein)[18] and may exhibit little α-SMA positivity[19, 20].

To determine the paracrine effects of TSCC on fibroblasts in its milieu and vice-versa, regarding α-SMA expression, the SCC25 cells were co-cultured with WI-38 (fibroblasts) for 7 days. α-SMA was there-after assessed and compared to its expression in SCC25 and WI-38, each on its own. Co-culturing SCC and normal fibroblasts for 5-7 days was documented to result in trans-differentiation of fibroblasts into α-SMA expressing CAFs[21, 22].

The expression of α-SMA after 7 days of co-culturing was significantly higher than either that of the SCC25 or the WI-38 cell lines. This upregulation is thought to be due to the trans-differentiation of fibroblast into α-SMA expressing CAFs, which is now believed to be an early event in carcinogenesis, that is mediated by the release of factors like IL-6, PDGF and TGF-B by the tumor cells, along with the oxidative stress state created by carcinogenic process. CAFs trans-differentiation is followed by changes in SCC25 cells with de-novo α-SMA expression [23, 24].

Once differentiated, CAFs aid cancer progression through several mechanisms. Cirri P. and Chiarugi P., 2011, suggested that besides the released factors discussed earlier, there is a metabolic benefit gained by the tumor cells from CAFs as the latter undergo aerobic glycolysis providing cancer cells

with substrates like pyruvate or lactate for the tricarboxylic acid cycle and hence promoting their survival, growth and eventual spread[25].

The qRT-PCR of α-SMA in this study, support the fact that, SCC25 cells and WI-38 cells exert a paracrine effect on each other, and that malignant epithelial cells do express α-SMA. Nevertheless, the exact cellular distribution of α-SMA expression, the difference in the pattern of expression between the malignant epithelial cells and fibroblasts, and clinic-pathological correlation could not be assessed through PCR. To overcome this, in the present work, immunohistochemical evaluation α-SMA in different SCC grades was done and correlated with lymph node involvement.

Immunohitochemical evaluation showed that all cases included in the study exhibited immunopositivity with α-SMA. Immunohistochemical analysis revealed that there was a significant difference in α-SMA expression between different SCC grades. The expression seemed to increase with increased cytological atypia, since with decreased differentiation, α-SMA expression by the epithelial cells was elevated. It should be noted though that the difference in expression between the WD-SCC and the MD-SCC cases was insignificant. It was also observed that the CAFs appeared to be less evident in the PD-SCC, inspite of significantly higher α-SMA expression, compared to the WD-SCC and MD-SCC.

Tumor cells support the recruitment and transdifferentiation of CAFs through paracrine effects of TGF-B, the differentiated CAFs in turn, present a helping hand to the tumor cells through the release of several factors (eg; MMP, IL-6)[10, 26] that mediate angiogenesis and matrix re-organization allowing for EMT and metastasis[27]. CAFs secret VEGF and several members of MMPs family degrading the ECM, cleaving growth factors and adhesion molecules, collectively resulting in EMT[28], angiogenesis and eventually metastases. In addition, CAFs have been documented to enhance invasiveness of the malignant epithelial cells through upregulation of SNAIL and TWIST[29], and the induction of stemness (upregulation of CD133 and CD44 gene expression)[30].

In the present work, assessment of the WD-SCC and MD-SCC showed that the immunopositive cells were located more at the invading front and the periphery of the nests rather than at the center of these nests. In addition, the CAFs were located mostly juxta-epithelial which again validates the theory that there is cross-talk going on between CAFs and the SCC cells[31].

Moreover, in this study, the nuclei of the tumor cells were always immunonegative with α-SMA, irrespective of the tumor grade. The immunopositive reaction was noted to be granular, cytoplasmic and/or membranous in the studied specimens, possibly denoting the previously described cytoskeletal and membranous changes and the development of invadopodia in preparation for EMT. Albiges-Rizo C., et al, 2009, who documented that invadopodia tend to develop as granules, outside but close to the nucleus and the Golgi apparatus, which may explain the above mentioned immunohistochemical findings[32].

On the other hand, CAFs exhibited intense nuclear and cytoplasmic immunopositivity, which to be expected due to the fact that they are of myofibroblastic lineage and α-SMA is inherent in them. The endothelial cells as well exhibited intense immunopositivity with α-SMA which is suggested to be due to upregulation of TGF-β and the activation of the Notch and Wnt-signalling pathways, which work synergistically in inducing End-MT. It should be noted however, that under normal physiological conditions the endothelial cells may exhibit some immunopositivity with α-SMA[33].

The clinical and immunohistochemical correlation showed that area fraction of α-SMA immunopositivity correlated with histopathological grade of the tumor and lymph node involvement. It was noted as well that the increased α-SMA immunopositivity especially, in the malignant epithelial cells, may be a significant prognostic factor to consider regarding EMT and lymph node involve-

ment. Histopathological evaluation of the invading malignant epithelial cells in the studied specimens were found to be predominantly immunopositive in the cases that exhibited positive lymph node involvement.

Even though α-SMA expression was highest in the PD-SCC, it should be pointed out that, this increase beyond the WD and MD- SCC cases in the present study, is thought to be due to the increased expression of α-SMA by the maliganat epithelial cells rather than by the CAFs. This concept concurs with the previously reported fact that CAFs trans-differentiation is an early event in carcinogenesis[23,24].

However, from the assessment of α-SMA expression in this study, it is thought that the CAFs differentiation might be down regulated with carcinogenesis progression and that after the initial increase once the malignant cells gain sufficient genetic mutations, they over take the TME and CAFs numbers diminish. This observation is put forward, as within the PD-SCC specimens studied here-in, the invading epithelial cells barely had any CAFs in-between.

CONCLUSION

α-SMA is a promising prognostic marker. Therapy directed against α-SMA in theory should combat both CAFs trans-differentiaon in the TME and EMT of the malignant epithelial cells. Anti-α-SMA therapy offers an inexpensive and clinically tried and approved solution, as there are already several commercially available drugs that act against α-SMA which can be repurposed for cancer treatment.

RECOMMENDATIONS

Anti- α-SMA therapy for cancer patients offers a promising treatment option, however, further preclinical and clinical research is required to reach the best mode of administeratio, dose and duration, for different cancer types.

Conflict of interest:

None to declare

Ethical statement:

All work has been approved by the ethical committees of the Faculty of Dentistry, Ain-Shams University, Faculty of Oral and Dental Medicine, Future University in Egypt, and VACSERA

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