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Expression of ITG β-1, MMP9 and Vimentin in Oral Squamous Cell Carcinoma – A Real Time PCR Based Approach

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: Oral Squamous Cell Carcinoma (OSCC) is the most common malignancies which accounts for 90% of oral cancer worldwide. The 5 year survival rate of OSCC is not more than 60% due to tumor metastasis and subsequent recurrence.
Aim: To compare the gene expression of ITG β-1, MMP9 and Vimentin in OSCC tissue samples and normal tissue samples and to correlate the expression levels of these molecules with the pathological grading and survival in OSCC patients. This would facilitate the understanding of EMT in OSCC progression thereby targeting this pathway for treatment of OSCC patients in near future.
Materials and Methods: 10 OSCC samples as well as normal healthy samples were collected and RNA isolation was done using TRIR kit, and then subjected to cDNA synthesis using ITG β-1, MMP9 and Vimentin primers. Real time PCR was performed using gene specific primers at 40 cycles. The results were retrieved, tabulated and analyzed.
Results: The current research results revealed that there were up regulation of mRNA expression in ITG β-1, MMP9 and Vimentin in OSCC patients than in healthy

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individuals. On comparison, MMP9 showed highest mRNA expression levels than ITG β-1 and Vimentin

Conclusion: Over expression of ITG B-1, MMP9, Vimentin plays a crucial role in progression of oral cancer and targeting EMT molecules could be an effective targeted approach for OSCC.

Keywords: ITG β-1; MMP9; vimentin; OSCC.

1. INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is a significant reason for mortality and morbidity globally. The incidence of OSCC has greatly evolved in the last few decades with a global estimation of 377,713 new cases and 177,757 deaths in 2020 [GLOBCAN STATISTICS] [1,2]. The preponderance cause for occurrence of OSCC is the utilization of tobacco which includes both smoking & smokeless tobacco and alcohol [1]. The general public is much aware of this correlation of deleterious habits with occurrence of OSCC but still these habits are prevalent to a greater extent, especially in India. Interestingly, very person who is indulged in these deleterious habits doesn't develop oral cancer. This substantiates that alterations in genes play a vital role in the development of OSCC and this genetic alterations or mutations that supports excessive abnormal cell synthesis, proliferation, differentiation and apoptosis leading to tumorigenesis. Biological behavior of OSCC determines the prognosis through increased rate of metastasis and recurrence. The management of OSCC could be complex and controversial, with various proposed combinations and sequences of chemotherapy, radiation therapy, and/or surgery [3]. Apparently, each individual responds differently to different therapies. So, early diagnosis would facilitate early management, as later stages of malignancy would not positively respond to any type of treatment [4]. The identification at molecular level through abnormal gene expressions is the new trend in recent decade.

Integrins beta-1 (ITG β-1) is a receptor for collagen. Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells [5]. Beta-Integrins are in charge of directing Integrin dimers to the relevant sub cellular locations, which in adhesive cells are primarily focal adhesions. Mutations of ITG β-1 would have lost their ability to target focal adhesion sites [6,7].

Matrix metallopeptidase-9 (MMP-9) is a type of zinc-metalloproteinase enzyme involved in extracellular matrix degradation. MMP-9 is involved in numerous physiological processes such as embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, learning and memory, as well as in pathological processes such as arthritis, intracerebral hemorrhage and even in malignancies associated with metastasis [8,9]. Also plays a function in the tumorigenesis to cause changes during epithelial mesenchymal transition in several forms of cancers, including OSCC [10].

Vimentin is a structural protein that plays a significant role in supporting and anchoring the position of the organelles in the cytoplasm of a cell. Vimentin is one of the cytoskeleton components responsible for maintaining cell integrity such as maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeleton interactions [11].

Epithelial-Mesenchymal Transition (EMT) is a biological process involving the transition of polarized epithelial cell into mesenchymal cell phenotype. Integrin B-1 (ITG β-1) extracellular matrix receptors – Matrix Metalloprotinase 9 (MMP9) is zinc dependant endopeptidase and Vimentin is cytoskeletal protein of mesenchymal cells; all these molecules are upregulated during the process of EMT.

No other studies have evaluated the ITG β-1, MMP9 and Vimentin gene expression levels altogether in OSCC. The primary aim of this study is to compare the gene expression of ITG β-1, MMP9 and Vimentin in OSCC patients; and also to correlate the expression levels of these molecules with the pathological grading and survival in OSCC patients. This study also provides understanding of EMT in OSCC progression thereby targeting this pathway for treatment of OSCC patients in near future.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 10 samples of OSCC specimens and normal non-pathological tissues for the same patient were obtained from Saveetha Dental College & Hospitals, Saveetha University (SIMATS) in the year of 2021. All the patients had been treated surgically and were subjected for histopathological analysis and finally viable specimens suitable for this research were selected. The 10 samples selected were moderately differentiated and well differentiated squamous cell carcinoma according to the histological grading.

2.2 RNA Isolation

Total RNA was isolated from OSCC specimens using a TRIR kit – according to the manufacturer's recommendations. Optical density at 260 nm was used to determine the concentration of RNA samples. After agarose gel electrophoresis, the presence of 18S and 28S bands confirmed the quality of the RNA. The RNA samples were incubated with RNAse-free DNAse at 37 °C for 20 min to remove residual DNA contamination and then the DNAse was inactivated at 65 °C for 10 min, and RNA samples were purified using a RNA easy kit.

2.3 cDNA Synthesis

Using the Superscript II first strand cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's protocol, using oligonucleotide (dT) primers, the total RNA from each sample was used to generate cDNA. Briefly, 1 µg of DNase-treated total RNA is used as starting material, and 1 µl of oligonucleotide (dT), 1 µl of 10 mM dNTP, 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT and 1 µl amount of RNase. First mix the reactive RNA, oligonucleotides (dT) and dNTPs, then heat the contents at 65°C for 5 minutes and then chill on ice until the other ingredients are added. The samples were incubated at 42°C for 2 minutes. Next, add 1 µL of Superscript II (40 U / µL) and incubate the sample at 42°C for 50 minutes. The reaction is quenched at 70°C for 15 minutes.

2.4 Primers

The primers used are Human ITG β-1 with FW-5'-CTTCAGTTCGTGTGTGGAGACAG-3' and RW-5'-CGCCCTCCGACTGCTG-3'; Human MMP9 with FW-5'-TCGAAGGCG ACCTCAAGTG-3' and RW-5'-TTCGGTGTAGCTT TGGATCCA-3'; Human Vimentin with FW-5'-CCGGTGCAATCGTGATCTCTGGG-3' and RW-5'-ATTCAAGTCTCAGCGGGCTC-3'.The list of primers used is presented in Table 1.

2.5 PCR Procedure

Template was prepared with malignant cells of OSCC into 20-50 µl TE (10mM Tris-CI, lmM EDTA, pH8,0), 0.1% SDS (or TE, 0.1% Triton X-100), vortexed and incubated at 100°C for 5 minutes, and vortexed for few seconds. The suspension was stored at -80°C for several weeks before performing the PCR. The PCR amplification was performed using thermal Cycler. 40 cycles of denaturation at 95°C for 23 seconds, 15 seconds of annealing, and elongation for 90 seconds at 72°C with 45 seconds denaturing time in the first cycle, and 200 seconds elongation in the last cycle. A linearly decreasing annealing temperature going from 47°C in the first cycle to 40°C in cycle forty was used.

3. RESULTS

The present study revealed that there was elevated expression of these markers; ITG β-1, MMP9 and Vimentin which represents the EMT process in patients with OSCC than in healthy tissue samples which were collected from lesion free and apparently healthy adults. On comparison, the expression of MMP-9 was significantly more compared to ITG β-1 and Vimentin.

Table 1. Showing list of primers used in this research

PRIMER	SEQUENCE	
Human ITG β-1	FW-5'-CTTCAGTTCGTGTGTGGAGACAG-3'	RW-5'-CGCCCTCCGACTGCTG-3'
Human MMP9	FW-5'-TCGAAGGCG ACCTCAAGTG-3'	RW-5'-TTCGGTGTAGCTT TGGATCCA-3'
Human Vimentin	FW-5'-CCGGTGCAATCGTGATCTCTGGG-3'	RW-5'-ATTCAAGTCTCAGCGGGCTC-3'

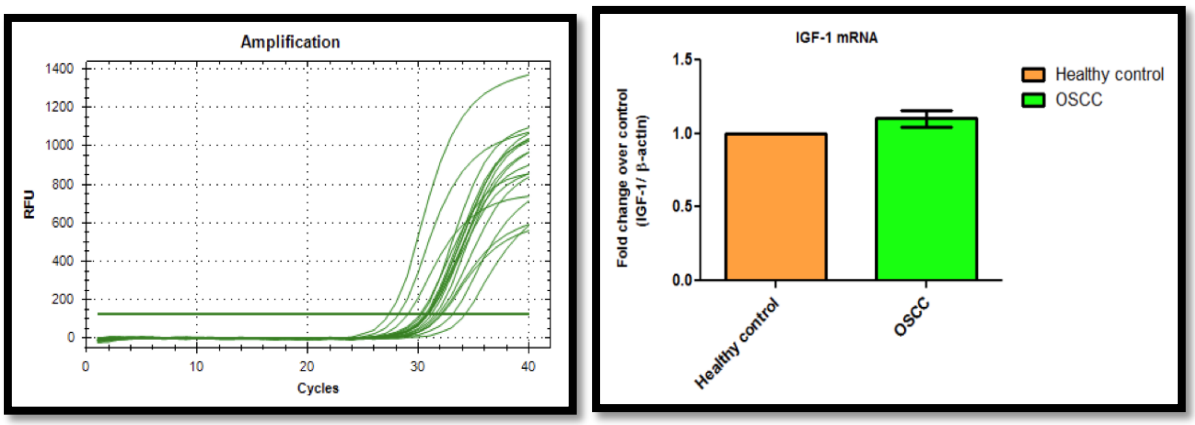
The mRNA expressions of ITG β-1 were increased in OSCC patients than in healthy individuals. The mRNA expressions of ITG β-1 are depicted in Graph 1. The fold change noticed with ITG β-1 was 1.3.

In respect to MMP-9, the mRNA expression levels of this particular gene was significantly more in OSCC patients when compared with healthy individuals. The fold change noticed was 2.6. The expression levels of MMP-9 are depicted in Graph 2.

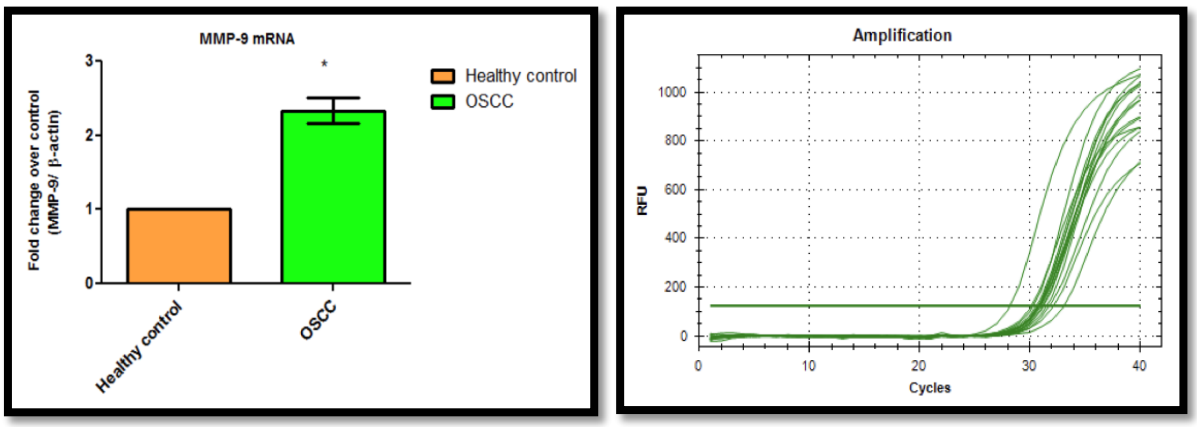
The Vimentin mRNA levels were also considerably increased in OSCC patients than in healthy control individuals. The expression levels are depicted in graph 3. The fold change was 1.6 in relation to Vimentin expression in OSCC. The expression levels of Vimentin are depicted in Graph 3.

4. DISCUSSION

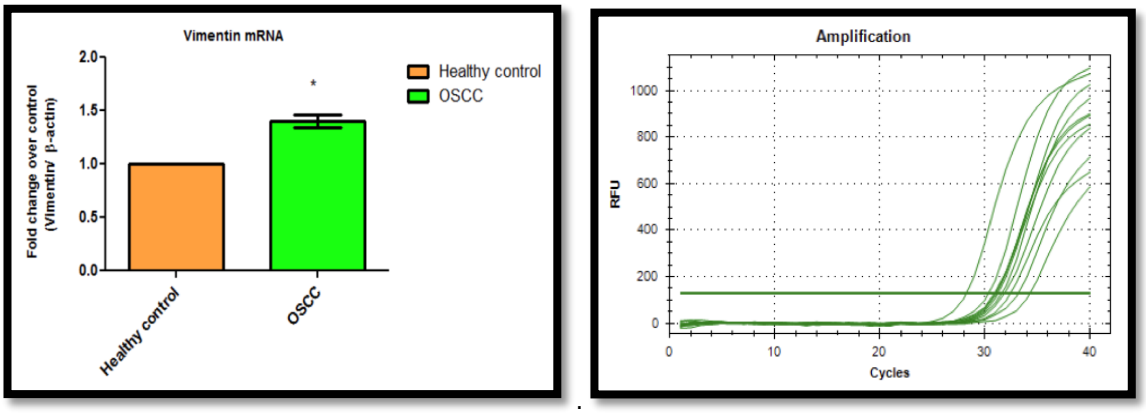
OSCC is the most common malignancies of all oral cancer worldwide. The 5 year survival rate of OSCC does not greatly support the prognosis due to tumor metastasis and subsequent recurrence [12]. During cancer invasion and metastasis, epithelial-mesenchymal transformation (EMT) is thought to play a key role. Recent advances in our understanding of EMT's role in carcinogenesis have piqued our interest, so we decided to look back at previous research on EMT in oral squamous cell carcinoma (OSCC) and also down-regulation of E-cadherin and MMP-9, epithelial phenotypes, and up-regulation of Vimentin and MMP-2, mesenchymal phenotypes. At the invasive tumor fronts, immune-localization of vimentin in the cytoplasm of OSCC cells was found [12].



Graph 1. mRNA expressions of ITG β-1 in clinically healthy and OSCC. Each bar represents Mean ± S.E.M of 3 observations. Significance at P < 0.05, ** compared with healthy control



Graph 2. mRNA expressions of MMP-9 in clinically healthy and OSCC. Each bar represents Mean ± S.E.M of 3 observations. Significance at P < 0.05, ** compared with healthy control



Graph 3. mRNA expressions of Vimentin in clinically healthy and OSCC. Each bar represents Mean ± S.E.M of 3 observations. Significance at P < 0.05, ** compared with healthy control

Our study results showed that 60% of the OSCC tissue samples with increased expression of ITG β-1, vimentin, MMP9 were diagnosed as moderately differentiated squamous cell carcinoma. Based on histological assessment were used to identify OSCC as well differentiated, moderately differentiated, or poorly differentiated. Later, this criterion was modified, and OSCC were eventually graded using multi-factorial systems that took into account tumor characteristics, the tumor-host interface, and host reactions [1]. WDSCC is potentially known to have better prognosis than moderately or poorly differentiated SCC.

We observed that in patients with upregulated ITG β-1, MMP9 and vimentin, the fold change was more in MMP9 followed by vimentin and ITG β-1 which was an average of 2.3, 1.6 and 1.3 respectively. The up regulation of MMP9 induces the up regulation of vimentin which facilitates the EMT of the oral cancer cells. The mRNA expression of MMP-9 was significantly more in our study. This is in concordance with the previous literatures. Peisker A, et al found that MMP-9 was significantly more in saliva samples of OSCC patients [13]. Shpitzer T, et al. also identified increased levels of MMP-9 in saliva. Comprehensive salivary analysis revealed an overall altered salivary composition in OSCC, indicating a compromised oral environment in these patients and suggesting salivary analysis as a new diagnostic tool for oral cancer [14,15]. MMP-9 polymorphism has a strong association with increased risk for developing oral cancer in a subset of the general population [16]. This could be because MMP-9 is essential in the early stages of tumor invasion, with its primary function being degradation and remodeling the ECM's homeostasis which in turn becomes a critical process in tumor metastasis [171]. Also decreasing the level of MMP-9 has been found to reduce the progression of tumor [17].

We also observed that in patients with recurrence and metastasis, there were 1.9 fold change expression of MMP9, 1.6 fold change expression of vimentin and 1.4 fold change expression of ITG β-1. In EMT, there is activation of matrix signaling pathway, which causes up regulation of MMP9 which facilitates the motility of the tumor cells and remodeling of the matrix (Cleaving of type IV collagen in basal lamina) thus promoting tumor invasion and phenotypic change by up regulating vimentin expression [18].

Our study results showed up regulated ITG β-1 causes the formation of ITG a3B1. This promotes tumor cell proliferation, migration, motility and disruption of basement membrane integrity since it is a receptor for extracellular matrix molecules like fibronectin, laminin and collagen. Thus, the upregulation of these molecules favors poor prognosis in these patients. There is not much of evidence to prove the increased level of ITG β-1 mRNA expression in OSCC.

Also Vimentin was found to be increased. This is also in concordance with the previous literatures. The positive expression of Vimentin was associated with tumor metastasis of oral squamous cell carcinoma, which was found by Zhou J, et al. [19]. Balasundaram P, et al. found that Vimentin immune positivity was noted with varying intensity in all cases of OSCC and concluded that the role of vimentin expression in OSCC and metastases is controversial [20]. This is because EMT is an important biological process during development and oncogenesis

and it is characterized by a reduction of epithelial polarities and production of mesenchymal phenotypes. Up regulation mesenchymal marker vimentin are hallmarks of transition [21].

5. CONCLUSION

Overexpression of ITG B-1, MMP9, vimentin plays a crucial role in progression of oral cancer and targeting EMT molecules could be an effective targeted approach for OSCC.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The specimens were collected after obtaining ethical clearance from Institutional Review Board (IRB). IRB approval number is IHEC/SDC/ OPATH-1802/21/223

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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