ORIGINAL RESEARCH

Gene Expression of GSK3, TGF β-1 and PI3 Kinase in Oral Squamous Cell Carcinoma – A Real Time PCR Based Approach

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most predominant type of oral cancer which has a poor prognosis, with 5-year survival rates less than 50%. Clinical characteristics such as tumor position, TNM classification and method of treatment, as well as histological grades have all been studied as OSCC prognostic factors but evaluating the genetic expression is the evolving trend in early diagnosis.

Aim: To compare the gene expression of TGF-β-1, GSK3, Pi3 kinase in OSCC and normal tissue samples and to correlate the expression levels of these molecules with the pathological grading and survival in OSCC patients. Also to understand the role of GSK3 in Pi3 kinase pathway and TGF-β signaling pathway in OSCC progression thereby attempting targeted therapy in OSCC patients.

Materials and Methods: 10 OSCC samples as well as normal healthy samples were collected and RNA isolation was done using RNA easy kit from Qiagen (Valencia, CA), and thensubjected to cDNA synthesis using Human TGF-β1, Human GSK3β and Human Pi3 kinase 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 GSK3, TGF β-1 and Pi3 kinase in OSCC patients than in healthy individuals. On comparison, Pi3 kinase showed highest mRNA expression levels than GSK3 and TGF β-1.

Conclusion: The expression of GSK3 and its role in activation of Pi3 kinase pathway plays a crucial role in progression of oral cancer and targeting GSK3βcould be a novel and targeted approach for treating OSCC.

Key words: GSK3, TGF β-1, Pi3 Kinase, OSCC

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Introduction

Oral cancer is the 6th most common cancer with prevalence of 16.1% noticed in men and 10.4% in women [Globocan cancer statistics, 2018].[1] OSCC is the most predominant type of oral cancer which is often associated with increased local recurrence and poor survival.[2] OSCC is a cause for high rates of morbidity and mortality. Development of OSCC is a multistep carcinogenesis mechanism that includes the evolution of malignant changes over time as a result of the accumulation of multiple genetic mutations within cells. These genetic mutations cause hyper plastic conditions, dysplastic cellular appearance, unorganized deregulated cell formation, and ultimately carcinoma. Damage to the certain genetic material causes epithelial carcinogenesis by causing changes in particular genes.[3] In this current situation of present-day medication and novel treatments, malignancies actually stay to be one of the reasons for the demise of individuals globally. Majority of these treatments are expensive and are related with unlikely results.[4] The treatment of any carcinoma at later stages becomes highly questionable, so an early diagnosis might facilitate early management that in turn increases the survival rate. But early diagnosis of OSCC through conventional biopsies is not highly supportive. The molecular level investigations such as identification of abnormal gene expressions and alterations would facilitate such early diagnosis of OSCC.

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The CMGC family of kinases corresponds to the cyclin-dependent kinases (CDK), mitogen-activated protein kinases (MAPK), glycogen synthase kinase (GSK3) and CDC-like kinase (CLK) group of protein kinases.[5] GSK3 is largely a cytosolic proteinand widely expressed member of the CMGC family of protein kinases and well known for its multiple physiological processes. Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase that is a key signaling molecule that induces neurodegeneration and deficits in memory formation. [6,7] GSK3 is a constitutively dynamic chemical in normal cells and has the ability for rapid inhibition upon stimuli. Its perplexing function as a tumor suppressor is currently being investigated in many neoplastic diseases[6,8].

TGF β-1 is transforming growth factor beta 1, a cytokine that belongs to the transforming growth factor beta superfamily which regulates a variety of cellular functions like synthesis, proliferation, differentiation, and apoptosis[9]. TGF β-1 is elevated in many head and neck carcinomas especially in OSCC and induces tumorigenesis which primarily begins as severe oral epithelial dysplasia. Also, metastatic carcinomas show higher levels of TGF β-1 in comparison with primary or recurrent localized carcinoma[10,11].

Phosphoinositide 3-kinases (PI3Ks), also called phosphatidylinositol 3-kinases, are a family of signaling enzymes which is similar to TGF β-1 involved in a variety of cellular functions such as cellular progression, survival, angiogenesis and intracellular trafficking, which in turn are involved in tumorigenesis[12]. The Phosphatidylinositol[3,4,5] P3 trisphosphate P3 phosphatase antagonists of PI3K signaling are apparently absent in many tumors which causes hyperactivation of phosphoinositide 3-kinase (PI3K) signaling cascades. There is multiple evidence to substantiate that the Pi3K pathway is activated in about 30%–50% of carcinomas[4].

Previous literature evidences to substantiate the gene expression of GSK3, TGF β-1 and PI3Kin variety of tumors have been performed earlier but no research has been performed to evaluate the hyperactivity of GSK3, TGF β-1 andPI3K, altogether in OSCC. This research is first of its kind.

The aim of this current research is to compare the gene expression of TGF B-1, GSK3, and Pi3 kinase inOSCCand normal tissue samples and to correlate the expression levels of these molecules with the pathological grading and survival in OSCC patients. This research helps to understand the role of GSK3 in PI3 kinase pathway and TGF-β signaling pathway in OSCC progression thereby attempting targeted therapy in OSCC patients.

Table 1: List of primers used in this research

PRIMER	SEQUENCE	
Human TGF-β1	FW-5′-TCGCCAGAGTG-GTTATCTT-3′	RW-5′-TAGTGAACCC-GTTGATGTCC-3′
Human GSK3β	FW-5′- GACTA-AGGTCTTCCGACCCC-3′	RW-5′-AAGAGTGCAG-GTGTGTCTCG-3′
Human Pi3 kinase	FW-5′- ATGCCTGCTCT-GTAGTGGTGG-3′	RW-5′-CATT-GAGGGAGTCGTTGT-GC-3′

Materials and Methods:

Sample collection:

A total of 10 samples of OSCC specimens and normal non-pathological tissues for the same patient were obtained in the year of 2021. Biopsies were subjected for histopathological analysis and finally viable specimens suitable for this research were selected. The 10 samples selected were either moderately differentiated and well differentiated squamous cell carcinoma according to the histological grading. The specimens were collected after obtaining ethical clearance from Institutional Review Board (IRB).

RNA isolation:

Total RNA was isolated from OSCC specimens using a RNA easy kit from Qiagen (Valencia, CA), 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^{0}C for 20 min to remove residual DNA contamination and then the DNAse was inactivated at 65^{0}C for 10 min, and RNA samples were purified using a RNA easy kit.

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.

Primers:

The primers used in the current study are asenlisted in Table 1:

PCR procedure:

Template was prepared with malignant cells of OSCC into 20-50 µl TE (10mM Tris-Cl, 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.

Results

The current research results revealed that there was up regulation of mRNA expression in GSK3, TGF β-1 and Pi3 kinase in OSCC patients than in healthy individuals. On comparison, Pi3 kinase showed highest mRNA expression levels than GSK3 and TGF β-1. There was also no significant difference in the statistical mean values.

The mRNA expressions of GSK3were increased in OSCC patients than in healthy individuals. The patient characteristics of the samples included in this research along with its fold change of GSK3 are presented in table 2 and mRNA expression of GSK3 are depicted in graph 1. The GSK3 fold changes were slightly altered with respect to each patient and found that GSK3 fold change ranged between 1.0-1.7.

In respect to TGF β-1, the mRNA expression of TGF β-1 was noticeably increased in OSCC patients when compared to healthy individuals. The expression levels are depicted in graph 2.

The Pi3 kinase mRNA levels were also considerably increased in OSCC patients than in healthy control individuals. The expression levels are depicted in graph 3.

Discussion

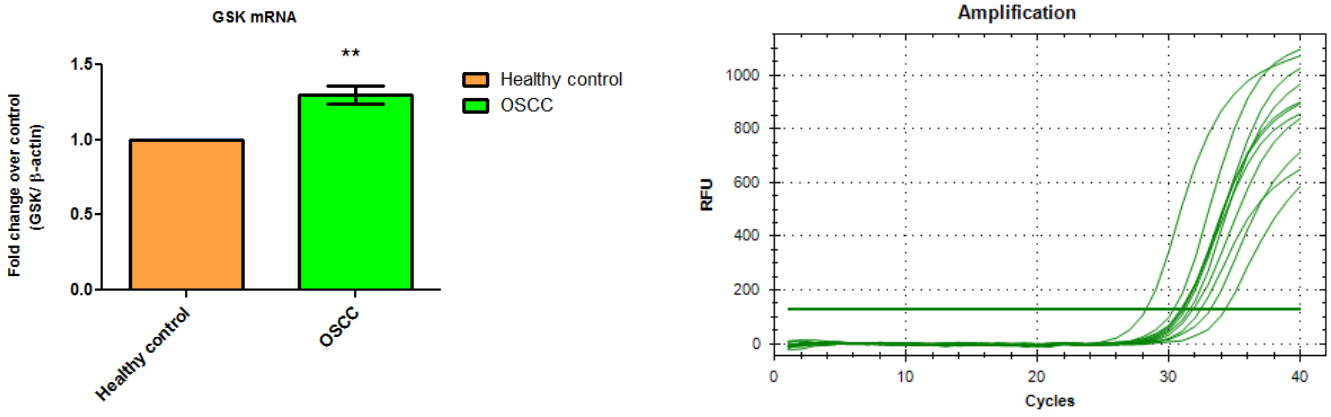
OSCC constitutes 16% to 40% of all malignancyin the head and neck region witha 5 year survival rate of OSCC ranging from 35% to 44% in patients with recurrence (13). There is a need to assess the biological behavior of OSCC in order to predict the prognosis of OSCC patients. This research was performed to assess the expression of GSK3, TGF β-1 and PI3K using RT-PCR and to identify the role of GSK3 β in the PI3 kinase signaling pathway and TGF beta signaling pathway. GSK3β is found to demonstrate either a proor ananti-tumor role in head and neck cancer. However its role in the progression of OSCC is still unclear. Our study demonstrated elevated expression of GSK3 β, TGF β 1 and PI3 kinase in OSCC patients than in healthy control individuals.

Our study results revealed 40% of the OSCC tissue samples with increased expression of GSK3 were diagnosed as WDSCC and 60% were diagnosed as MDSCC. However, the fold change in WDSCC was found to be increased (1.5) than MDSCC (1.2).

Broder's criteria were used to classify OSCC as well differentiated, moderately differentiated and poorly differentiated based on histological evaluation. Later this criterion was modified and finally OSCC were graded based on the multi-factorial systems considered features of the tumor, the tumor-host interface and host reactions[14]. WDSCC is potentially known to have better prognosis than moderately or poorly differentiated OSCC. Bilim V, et al. found that inhibiting GSK3, decreases the expression of NF-B target genes Bcl-2 and XIAP, as well as an increase in apoptosis in renal cancer cells which indicates GSK3 is a positive regulator of renal cancer cell proliferation and survival[15]. This is consistent with our research, but

Table 2: OSCC patient details with fold change of GSK3

S.NO	AGE	GENDER	SITE	HISTOPATH DIAGNOSIS	SURVIVAL	FOLD CHANGE
1	58	Male	Buccal mucosa	MDSCC	Death	1.0
2	46	Male	RMT	MDSCC	Recurrence	1.2
3	52	Male	RMT	WDSCC	Metastasis	1.2
4	44	Male	Tongue	WDSCC	Disease free	1.4
5	52	Male	Buccal mucosa	MDSCC	Recurrence	1.3
6	56	Male	RMT	WDSCC	No follow-up	1.6
7	46	Male	Tongue	WDSCC	Death	1.7
8	51	Male	Buccal mucosa	MDSCC	Recurrence	1.0
9	50	Male	Tongue	MDSCC	Death	1.3
10	54	Male	Buccal mucosa	MDSCC	Recurrence	1.3

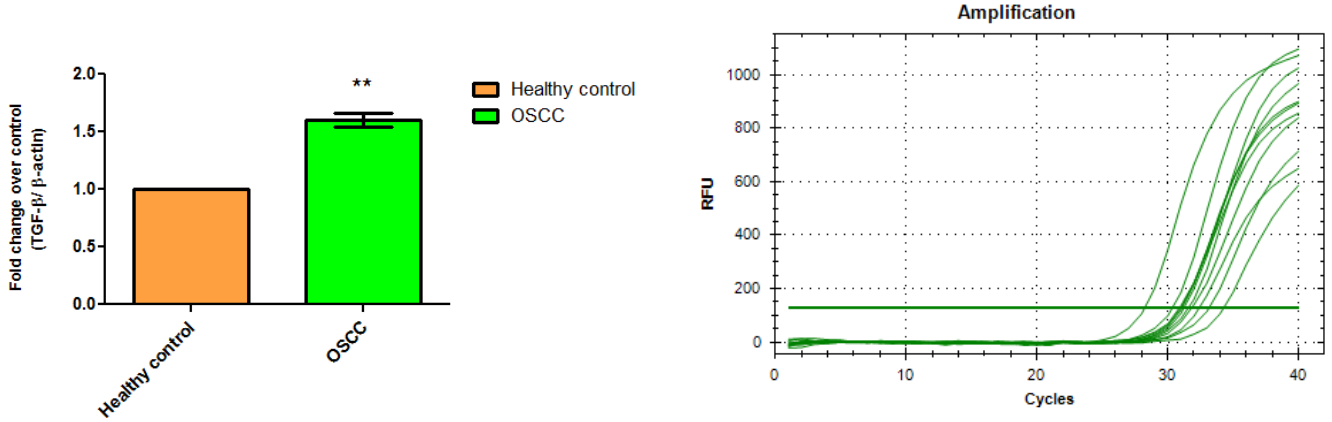


Graph 1: mRNA expressions of GSK3in clinically health and OSCC. mRNA expressions of GSK3were assessed by Real Time-PCR. Each bar represents Mean ± S.E.M of 3 observations. Significance at $P < 0.05$, **- compared with healthy control.

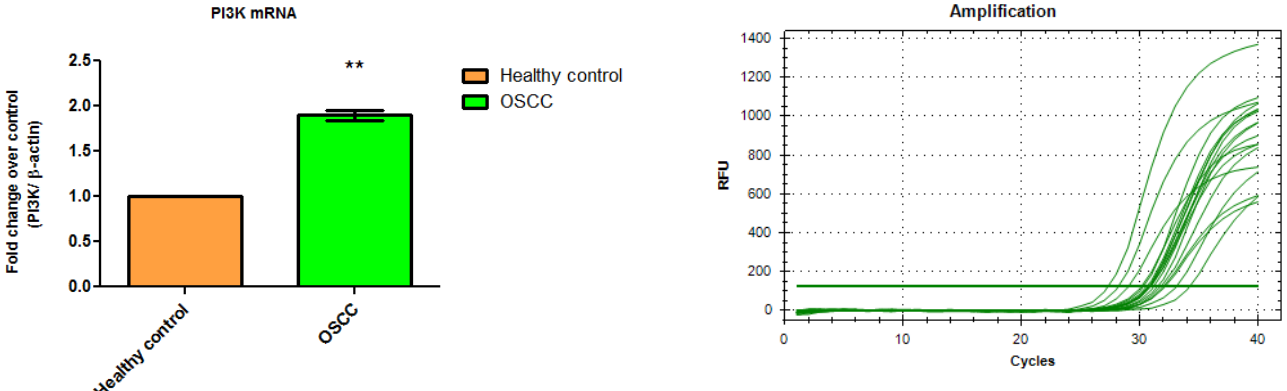
with respect to OSCC, there is definitely an increase in the mRNA expression of GSK3.

Activated Pi3 kinase signaling pathway promotes activation of Akt by phosphorylation. This activated Akt causes inhibition of GSK3 which maintains cell cycle regulators such as cyclin D1 and c-Myc. Overexpression of p-Akt has been shown to be associated with shorter disease-free survival independently of classification and nodal status[16,17]. Ibrahim M, et al found that activation of the PI3-kinase pathway in cancer cells was associated with higher risk of recurrence and/or death through activation of p-Akt pathway[18]. Mishra, et al identified increased expression of GSK3 correlated positively with cyclin D1 inprogression of carcinoma of the tongue and demonstrated that the inactivation of GSK3 is an important event in OSCC[6]. These previous results are concordant with our findings, thus proving that Pi3 kinase promotes Akt-phosphorylation thereby inhibiting GSK3. We also observed that in patients with recurrence and metastasis, there was 1.2 times expression of GSK3 and 1.6 fold change of Pi3 kinase. In oral carcinogenesis, activation of Pi3 kinase due to cytokines and interferons causes upregulation of Akt and downstream regulation of GSK3 which promotes the survival and metastasis of cancer cells thereby affecting the prognosis. In this study GSK3 gene was upregulated in OSCC particularly in cases of moderately differentiated squamous cell carcinoma. It was also noted that the GSK 3 upregulation was associated with upregulation of TGFβ and PI3 kinase.

We observed that patients showed elevated expression of TGF β-1 (1.6) in patients with recurrence and metastasis, as well asin cases of MDSCC. There was inverse correlation of GSK3 and TGF β-1 in these patients. Less expression value of GSK3 (1.2) was seen associated with higher value TGF β-1 (1.6). Not much of an increase was noted in GSK3 (1.2) and the controls (1.0). Alterations in the TGF-β1 signaling pathway can contribute to the development of many cancers and appear to play a role during oral carcinogenesis[19]. Weissheimer C, et al found that TGF-β1 was significantly increased in OSCC compared to normal oral mucosa but apparently TGF-β1 had no prognostic value and appears to maintain its suppressive role concerning cell proliferation[20]. Taghavi N, et al from Iran demonstrated no association of TGF-β1 with OSCC patients' survival[21] but a study performed by Chen MF, et al found that TGF-β1 is capable to predict a shorter survival time of OSCC patients as the disease advances[22]. This inconsistency in the results could be due to the inconsistency in the proliferative behavior of OSCC and also molecular level changes depending on each patients' biological behavior. TGF-β 1 can preserve its anti-proliferative activity while also acting as a pro-tumorigenic factor by facilitating the epithelial-mesenchymal transition (EMT)[20].



Graph 2: mRNA expressions of TGF-β in clinically health and OSCC. mRNA expressions of TGF-β were assessed by Real Time-PCR. Each bar represents Mean ± S.E.M of 3 observations. Significance at P < 0.05, **- compared with healthy control.



Graph 3: mRNA expressions of Pi3 kinase in clinically healthy and OSCC. mRNA expressions of Pi3 kinasewere assessed by Real Time-PCR. Each bar represents Mean ± S.E.M of 3 observations. Significance at P < 0.05, ** compared with healthy control.

Conclusion

Expression GSK3βand its role in activation of Pi3 kinase pathway plays a crucial role in progression of oral cancer and targeting GSK3β could be a novel and targeted approach in treating OSCC.

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