Journal of Pharmaceutical Research International

33(44A): 182-188, 2021; Article no.JPRI.73993
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Comparative Evaluation on the Expression of Pi3 Kinase - AKT- mTOR Signalling Molecules in Oral Squamous Cell Carcinoma - A Real Time PCR Based Approach

Krishnapriya Umashankar[1*], J. Selvaraj[2] and Pratibha Ramani[3]

[1]Saveetha Dental College and Hospitals, Velapanchavadi, India.
[2]Department of Biochemistry, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences, Velappanchavadi, Chennai-600 077, India.
[3]Department of Oral Pathology, Saveetha Dental College and Hospitals, Velapanchavadi, Chennai – 600077, India.

Authors' contributions

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

Article Information

DOI: 10.9734/JPRI/2021/v33i44A32605
Editor(s):
(1) Dr. Giuseppe Murdaca, University of Genoa, Italy.
Reviewers:
(1) Harpreet Singh, BJS Research Institute, India.
(2) Dunia Wadeea Sabea, Iraqia University, Iraq.
(3) Razzagh Mahmoudi, Qazvin University of Medical Sciences, Iran.
Complete Peer review History: https://www.sdiarticle4.com/review-history/73993

Original Research Article

Received 03 July 2021
Accepted 13 September 2021
Published 17 September 2021

ABSTRACT

Background: Oral squamous cell cancer (OSCC) develops as a result of the accumulation of many genetic mutations that are influenced by genetic predisposition. Upon acquisition of genetic predisposition, the precancerous cells will transform into malignant cells culminating into carcinomas. Advances in genetic research over the past few decades have rendered early detection possible.
Aim: To compare the gene expression of Pi3 Kinase, AKT and mTOR in OSCC and to correlate the expression levels of these molecules with the survival in OSCC patients. Also to understand the role of Pi3 Kinase pathway in OSCC progression thereby attempting targeted therapy in OSCC patients.

**Corresponding author: E-mail: krishnapriya91@gmail.com;*

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 then subjected to cDNA synthesis using Human TGF-β1, Human GSK-3β 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 The PI3K/AKT/mTOR in OSCC patients than in healthy individuals. On comparison, mTOR showed highest mRNA expression levels than AKT and PI3K.
Conclusion: Overexpression of Pi3 kinase, AKT, mTOR plays a crucial role in progression of oral cancer and targeting Pi3 kinase/mTOR pathways could be a novel and targeted approach for OSCC.

Keywords: PI3K; AKT; mTOR; OSCC.

1. INTRODUCTION

Oral squamous cell cancer (OSCC) is a multi-factorial malignancy that involves accumulations of several genetic alterations that are modulated by genetic predisposition. Oncogenesis and tumour suppressor genes are the major category of genes involved in tumorigenesis [1]. OSCC is caused by the malignant transformation of a single precursor cell, which leads to the development of a monoclonal cancer cell population through proliferation. The precursor cancer cells tend to have increased proliferation and decreased rate of apoptosis, resulting in excessive growth of malignant tissue [2,3]. Upon acquisition of genetic predisposition, the precancerous cells will transform into malignant cells culminating into carcinomas [3]. These alterations are assessed through researches at molecular levels, which provide information about the particular cancer and correlating its genetic susceptibility with patients, thus predicting the overall prognosis and survival of the patients. OSCC is one of the intense and lethal diseases of carcinomas which do not show much signs and symptoms at an early stage. The stage of malignancy of must be considered, as later stages would almost certainly have a poor prognosis and survival. Advances in genetic research over the past few decades have rendered early detection possible.

Phosphoinositide 3-kinases (PI3Ks), also known as phosphatidylinositol 3-kinases, are a family of enzymes that play a role in cellular functions such as cellular progression, survival, angiogenesis, and intracellular trafficking, all of which play a role in tumorigenesis [4]. Many tumors lack Phosphatidylinositol [3,4,5] P3 trisphosphate [3,4,5] P3 phosphatase antagonists of PI3K signalling, resulting in hyperactivation of phosphoinositide 3-kinase (PI3K) signalling cascades that causes excessive proliferation of malignant cells. There is evidence that the Pi3K pathway is activated in about 30%–50% of carcinomas, according to multiple sources [5].

Activated Pi3 kinase signaling pathway promotes activation of AKTby phosphorylation. Overexpression of p-AKThas been shown to be associated with shorter disease-free survival independently of classification and nodal status [6,7]. Upon activation of AKT, it can produce variety of downstream effects; one such is the activation of mTOR [8].

mTOR is a member of the PI3K related kinase family of protein kinases [9]. Over activation of mTOR signalling is linked to tumour initiation and progression, and mTOR activity has been shown to be disrupted in a variety of cancers, including breast, prostate, lung, melanoma, bladder, brain, and renal carcinomas. Additionally, mTOR activity is deregulated in many cancers as a result of increased activity of PI3K or AKT [10].

Biologically based therapies that directly target the pathways responsible for malignant transformation and progression are desperately required [11]. The PI3K/AKT/mTOR signalling pathway is an intracellular signalling pathway that plays a role in cell cycle regulation. As a result, it's linked to cellular quiescence, proliferation, cancer, and resilience. AKTis phosphorylated and activated by PI3K activation, which causes it to be localised in the plasma membrane [11].

Immunohistochemical expression of Pi3 kinase, AKT, mTOR are done in previous literature. No other researches have been performed to assess the activation of PI3K/AKT/mTOR signalling pathway in OSCC. This study is first of its kind.

The aim of the present research is to compare the gene expression of Pi3 Kinase, AKT and mTOR in OSCC and to correlate the expression levels of these molecules with the survival in OSCC patients. Also to understand the role of Pi3 Kinase pathway in OSCC progression thereby attempting targeted therapy in OSCC patients.

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 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°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 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-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 fourty was used.

2.6 Statistical Analysis

Chi-square analysis was done to compare the gene expression among case and control groups using SPSS 20 software.

Table 1. Showing list of primers used in this research

PRIMER	SEQUENCE	
Human AKT	FW-5'-TTGTCATGGAGTACGCCAACG-3'	RW-5'-ACAGCCCGAAGTCTGTGATCTT-3'
Human mTOR	FW-5'-CCAATCATTCGCATTCAGTCC-3'	RW-5'-AACAAACTCATGTCCGTTGCTG-3'
Human PI3K	FW-5'-ATGCCTGCTCTGTAGTGGTGG-3'	RW-5'-CATTGAGGGAGTCGTTGTGC-3'

3. RESULTS

The present study results showed that the mRNA expression levels of Pi3 kinase, AKT, mTOR are significantly increased in OSCC patients than in healthy individuals. On comparison, mTOR showed highest mRNA expression levels than Pi3 kinase and AKT. There was also no significant difference in the statistical mean values.

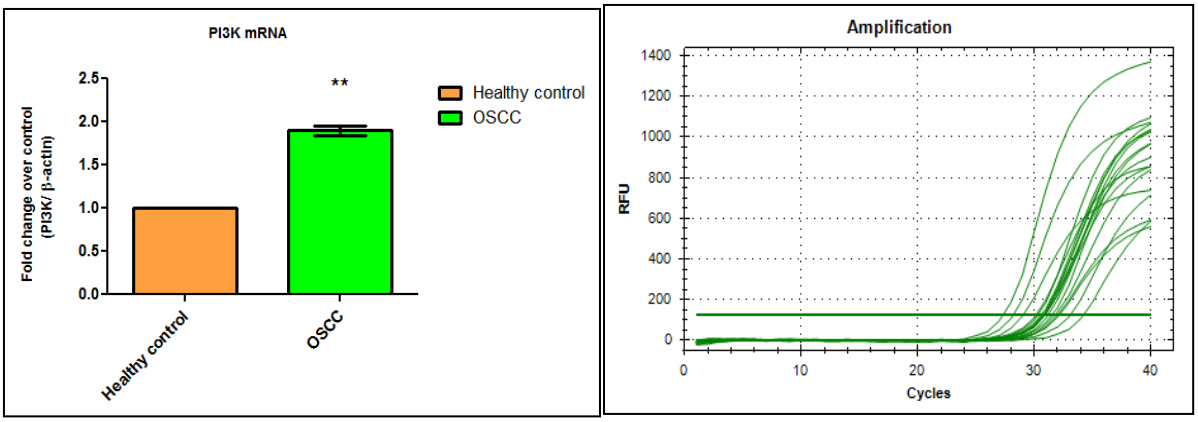
The mRNA expressions of PI3K were increased in OSCC patients than in healthy individuals. We also observed that in OSCC patients', 1.5 fold times expression of Pi3 kinase. The mRNA expression levels are depicted in Graph 1.

In respect to AKT, the mRNA expression of AKT was noticeably increased in OSCC patients when compared to healthy individuals. The expression levels are depicted in Graph 2. 1.8 fold times expression of AKT was noticed.

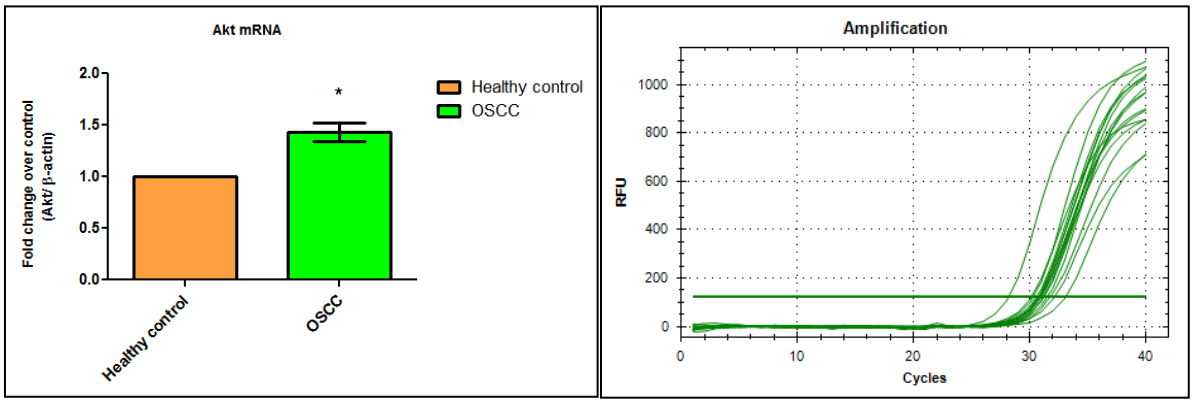
The mTOR mRNA levels were also considerably increased in OSCC patients than in healthy control individuals. The expression levels are depicted in Graph 3. 2.1 fold times expression of mTOR was noticed.

4. DISCUSSION

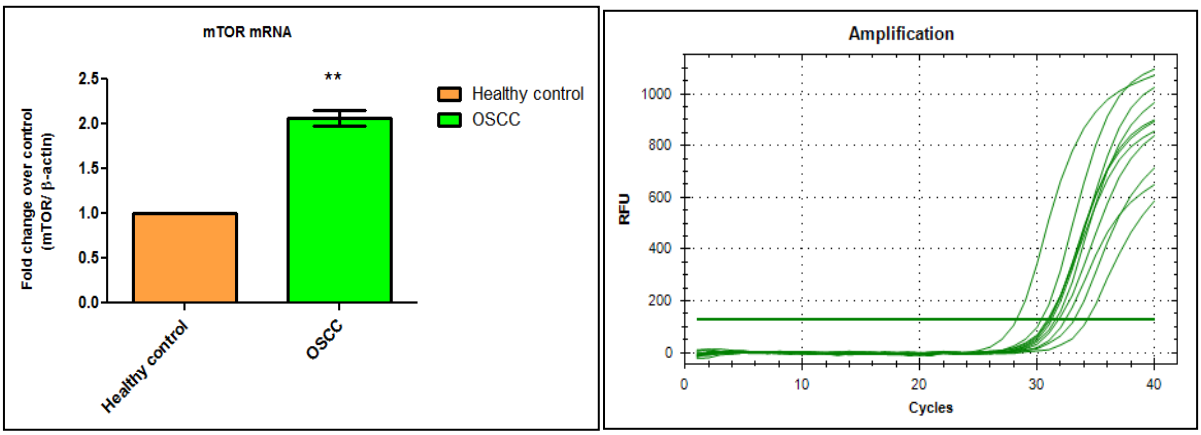
OSCC is the most common malignancies in head and neck region which accounts for 40%. Since there is increased tobacco use in developing countries like India, there is increased incidence of OSCC in the last decade. The 5 year survival rate of OSCC ranged between 35% to 44% in patients with recurrence. There is a need to assess the biological behaviour of OSCC in order to predict the prognosis of OSCC patients.



Graph 1. mRNA expressions of Pi3 kinase mRNA expression 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 AKT mRNA expression 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 mTOR mRNA expression 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 demonstrated elevated expression of Pi3 kinase, AKT and mTOR in OSCC patients than in healthy individuals. 50% of the OSCC tissue samples with increased expression of Pi3 kinase, AKT, mTOR were diagnosed as moderately differentiated squamous cell carcinoma. 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 [12].

Activated Pi3 kinase signaling pathway promotes activation of AKT by phosphorylation. Overexpression of p-AKT has been shown to be associated with shorter disease-free survival independently of classification and nodal status (6, 7). Increase in PI3K increases AKT, thereby suggesting a positive correlation between PI3K and ATK. 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 [13]. Multiple types of tumor malignancies have been identified to activate the PI3K/AKT pathway [14]. Qin L et al, the PI3K/AKT pathway was associated with migration and invasion of gastric cancer [15]. Baek S, et al found that the inhibition of PI3K/AKT/mTOR signaling pathway suppresses the progression of lung cancer and that provides a source of potential therapeutic compounds to control the metastatic dissemination of tumor cells [16]. PI3K/AKT signalling pathway is a multistep process which involves numerous up regulation/ down regulation and activation/ inactivation of multiple genes, proteins and enzymes; all these contributes to the tumorigenesis in OSCC.

PI3K/AKT/mTOR is one of the most frequently deregulated signalling pathways in cancer [17]. AKT, a serine/threonine kinase, is a key mediator of the PI3K pathway, and its activation phosphorylates a number of proteins that control cellular responses such as apoptosis, metabolism, cell proliferation, and cell development [4]. This previous existing theory of PI3K/AKT/mTOR pathway is concordant with our present study. Our study results showed predominant elevation of mTOR than the PI3K/AKT in OSCC patients than in healthy individuals. Also we observed that in patients with recurrence and metastasis, there was 2.1 fold times expression of mTOR, 1.8 fold times expression of AKT and 1.5 fold times expression of Pi3 kinase. In oral carcinogenesis, activation of Pi3 kinase due to cytokines and interferons causes upregulation of AKT and mTOR which promotes the survival and metastasis of cancer cells thereby affecting the prognosis.

5. CONCLUSION

Overexpression of Pi3 kinase, AKT, mTOR plays a crucial role in progression of oral cancer and targeting Pi3 kinase/mTOR pathways could be a novel and targeted approach for OSCC.

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 number “IHEC/SDC/OPATH-1802/21/221”)

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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Pérez-Sayáns, Pérez-Sayáns. Genetic and molecular alterations associated with oral squamous cell cancer (Review). Oncology Reports. 2009;22.
   Available:http://dx.doi.org/10.3892/or_00000565
2. Sayed SI, Dwivedi RC, Katna R, Garg A, Pathak KA, Nutting CM, et al. Implications of understanding cancer stem cell (CSC) biology in head and Neck Squamous Cell Cancer . Oral Oncology. 2011;47:237–43.
   Available:http://dx.doi.org/10.1016/j.oraloncology.2011.02.009
3. Feller LL, Khammissa RR, Kramer BB, Lemmer JJ. Oral squamous cell carcinoma in relation to field precancerisation: Pathobiology. Cancer Cell International. 2013;13:31.
   Available:http://dx.doi.org/10.1186/1475-2867-13-31
4. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nature Reviews Drug Discovery. 2009;8:627–44.
   Available:http://dx.doi.org/10.1038/nrd2926
5. Tanaka T, Ishigamori R. Understanding Carcinogenesis for Fighting Oral Cancer. Journal of Oncology. 2011;2011:1–10.
   Available:http://dx.doi.org/10.1155/2011/603740
6. Massarelli E, Liu DD, Jack Lee J, El-Naggar AK, Lo Muzio L, Staibano S, et al. AKT activation correlates with adverse outcome in tongue cancer. Cancer. 2005; 104:2430–6.
   Available:http://dx.doi.org/10.1002/cncr.21476
7. Lee C-H, Hung P-F, Liu K-J, Chung H-L, Yang W-C, Hsu K-C, et al. LDOC1 Suppresses Microbe-Induced Production of IL-1β in Human Normal and Cancerous Oral Cells through the PI3K/AKT/GSK-3β Axis. Cancers. 2020;12: 3148.
   Available:http://dx.doi.org/10.3390/cancers12113148
8. Rafalski VA, Brunet A. Energy metabolism in adult neural stem cell fate. Progress in Neurobiology. 2011;93:182–203.
   Available:http://dx.doi.org/10.1016/j.pneurobio.2010.10.007
9. Mitra A, Luna JI, Marusina AI, Merleev A, Kundu-Raychaudhuri S, Fiorentino D, et al. Dual mTOR Inhibition Is Required to Prevent TGF-β-Mediated Fibrosis: Implications for Scleroderma. Journal of Investigative Dermatology. 2015;135: 2873–6.
   Available:http://dx.doi.org/10.1038/jid.2015.252
10. Xu K, Liu P, Wei W. mTOR signaling in tumorigenesis. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2014;1846:638–54.
    Available:http://dx.doi.org/10.1016/j.bbcan.2014.10.007
11. King D, Yeomanson D, Bryant HE. PI3King the Lock. Journal of Pediatric Hematology/Oncology. 2015;37:245–51.
    Available:http://dx.doi.org/10.1097/mph.0000000000000329
12. Almangush A, Mäkitie AA, Triantafyllou A, de Bree R, Strojan P, Rinaldo A, et al. Staging and grading of oral squamous cell carcinoma: An update. Oral Oncology. 2020;107:104799.
    Available:http://dx.doi.org/10.1016/j.oraloncology.2020.104799
13. Ibrahim MY, Nunez MI, Harun N, Jack Lee J, El-Naggar AK, Ferrarotto R, et al. PI3-kinase pathway biomarkers in oral cancer and tumor immune cells. Head & Neck; 2018.
    Available:http://dx.doi.org/10.1002/hed.25350
14. Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: Divergent roles of isoforms, modes

of activation and therapeutic targeting. Nature Reviews Cancer. 2015;15:7–24. Available:http://dx.doi.org/10.1038/nrc3860

15. Qin L, Jia Z, Xie D, Liu Z. Knockdown of long noncoding RNA urothelial carcinoma–associated 1 inhibits cell viability, migration, and invasion by regulating microRNA-182 in gastric carcinoma. Journal of Cellular Biochemistry. 2018; 119:10075–86. Available:http://dx.doi.org/10.1002/jcb.27344
16. Baek SH, Ko J-H, Lee JH, Kim C, Lee H, Nam D, et al. Ginkgolic Acid Inhibits Invasion and Migration and TGF-β-Induced EMT of Lung Cancer Cells Through PI3K/AKT/mTOR Inactivation. Journal of Cellular Physiology. 2017; 232:346–54. Available:http://dx.doi.org/10.1002/jcp.25426
17. Simpson DR, Mell LK, Cohen EEW. Targeting the PI3K/AKT/mTOR pathway in squamous cell carcinoma of the head and neck. Oral Oncology. 2015;51: 291–8. Available:http://dx.doi.org/10.1016/j.oraloncology.2014.11.012

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/73993