Research Paper

Assessment of salivary IKKB and NfkB levels in patients with OSCC with different grade of cancer

Running title: Assessment of salivary IKKB and NfkB in patients with OSCC

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ABSTRACT

Background

Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. It is estimated that more than 90% of all oral neoplasms are OSCC. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that controls transcription of DNA. IKK is composed of a heterodimer of the catalytic IKKα and IKKβ subunits.

Aim

The aim of the study is to assess salivary IKKB and NfKB levels in patients with OSCC with different grade of cancer.

Method

Human Nuclear factor-kappa B (NF-kB) ELISA Kit employs a two-site sandwich ELISA to quantitate NF-kB in samples.

Results and Discussion

When comparing the levels of salivary IKKB and NfkB in patients with oral squamous cell carcinoma and healthy individuals, patients with OSCC showed higher levels of the protein when compared to normal individuals.

Conclusion

It was concluded that salivary IKKB and NfkB levels in patients with OSCC are higher when compared to normal individuals.

Keywords: salivary IKKB and NfKB, OSCC, Cancer.

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INTRODUCTION: Squamous-cell carcinomas (SCCs), also known as epidermoid carcinomas, comprise a number of different types of cancer that begin in squamous cells (1). These cells form on the surface of the skin, on the lining of hollow organs in the body, and on the lining of the respiratory and digestive tracts. About 90% of cases of head and neck cancer (cancer of the mouth, nasal cavity, nasopharynx, throat and associated structures) are due to SCC (2). Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms (3). It is estimated that more than 90% of all oral neoplasms are OSCC (4,5). One of the real dangers of this neoplasm is that in its early stages, it can go unnoticed. Common sites for OSCC to develop are on the tongue, lips and floor of the mouth (6). Some OSCCs arise in apparently normal mucosa, but others

are preceded by clinically obvious premalignant lesions, especially erythroplakia and leukoplakia. Usually, OSCC presents as an ulcer with fissuring or raised exophytic margins (7). It may also present as a lump as a red lesion, as a white or mixed white and red lesion, as a non-healing extraction socket or as a cervical lymph node enlargement, characterized by hardness or fixation. OSCC should be considered where any of these features persist for more than two weeks (8).

The transcription factor NF - κB was first discovered in 1986 by David Baltimore as a factor in the nucleus that binds the promoter of the kappa chain of immunoglobulin in B Cells (9). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that controls transcription of DNA, cytokine production and cell survival (10). Activation of the NF-κB is initiated by the signal-induced degradation of IκB proteins. This occurs primarily via activation of a kinase called the IκB kinase (IKK)(11). IKK is composed of a heterodimer of the catalytic IKKα and IKKβ subunits. There are many different types of human tumors that have been mis regulated (12). Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis (13). In cancer, proteins that control NF-κB signaling are mutated leading to defective coordination between the malignant cell and the rest of the organism. There are different activation pathways of NF- κB that cause expression of proteins which promotes apoptosis or inhibits apoptosis. This depends on the cell type or the type stimulation that determines which signaling pathways are activated (13,14).

Many chemical and physical carcinogens implicated in the promotion of human cancer activate NF – κB and Ikb levels. Nicotine and carcinogens in tobacco are associated in the pathogenesis of head and neck malignancies (15).

The main aim of the study is to assess salivary Ikb and NfkB levels in patients with oral squamous cell carcinoma (OSCC) with different grades of cancer.

MATERIALS AND METHODS:

Sample collection & storage:

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Tissue homogenates :

For general information, hemolysis blood may affect the result, so you should rinse the tissues with ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces which will be homogenized in PBS with a glass homogenizer on ice. (The volume depends on the weight of the tissue, 9mL PBS would be appropriate for 1 gram tissue pieces. Some protease inhibitor is recommended to add into the PBS.) To further break the cells, you can sonicate the suspension with an ultrasonic cell disruptor or subject it to freeze -thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to collect the supernatant.

Cell culture supernatants and other biological fluids :

Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay immediately or store samples in aliquot at -20 °C or -80 °C for later use. Avoid repeated freeze/thaw cycles.

Serum - Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4 °C before centrifugation for 20 minutes at approximately 1000×g. Assay freshly prepared serum immediately or store samples in aliquot at -20 °C or -80 °C for later use. Avoid repeated freeze/thaw cycles.

Plasma :

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8 °C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20 °C or -80 °C for later use. Avoid repeated freeze/thaw cycles.

Bring all reagents to room temperature before use. If crystals were formed in the Buffer Concentrates, warm them gently until they completely dissolved.

Wash
buffer
Dilut
e with
Distil
led or
deion
ized
water
.
Stand
ard :

Pipette 150 µL of Standard Diluent into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard. Standard Diluent serves as the zero standard.

Assay procedure

1. Prepare all reagents before starting the assay procedure. It is recommended that all Standards and Samples be added in duplicate to the microplate.
2. Add standard: Set Standard wells, testing sample wells. Add diluted standard 50 µL to

standard well.

3. Add Sample: Add sample diluent 40 µL to testing sample well. Then add sample 10 µL to the testing sample well, Blank well doesn't add anything.
4. Cover with a plate cover and incubate for 45 minutes at 37 °C.
5. Aspirate each well and wash, repeating the process four times for a total of five

washes, 1-3 minutes per time. Wash by filling each well with a Wash buffer (250 µL) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add HRP-Conjugated detection antibody 50 µL to each well, except blank well.
7. Cover with plate cover. Incubate for 30 minutes at 37 ℃.
8. Repeat the aspiration/wash process for five times as in step 5.
9. Add chromogen solution A 50 µL and chromogen solution B 50 µL to each well. Gently mix and incubate for 15 minutes at 37°C. Protect from light.
10. Add 50 µL Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader within 15 Minutes.

DATA ANALYSIS :

Typical data -

The standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

RESULT:

The salivary enzyme nfkb and ikkb was significantly upregulated in oral cancer tissues, predicting OSCC prognosis. In this study, salivary IKKB was compared with the normal group and the group having OSCC. Similarly NfKB was compared with normal groups and groups with OSCC. As a result patients with OSCC had significantly high salivary IKKB and NfKB levels when compared with the normal group. After each observation both the group showed a statistically significant value (p<0.05).

Salivaray IKKB in healthy and OSCC subjects

FIGURE 1 : Each bar represents Mean±SEM of 3 observations (n=10). Significance considered as P<0.05; * -compared to Healthy Control. Values are expressed in ng/ml.

Salivaray NFKB in healthy and OSCC subjects

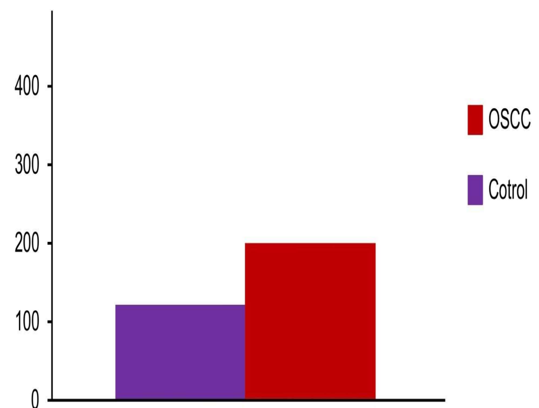

FIGURE 2 : Each bar represents Mean±SEM of 3 observations (n=10). Significance considered as P<0.05; * -compared to Healthy Control. Values are expressed in ng/L.

DISCUSSION :

Many chemical and physical carcinogens implicated in the promotion of human cancer activates NF – κB. Nicotine and carcinogens in tobacco and betel nut are associated in the pathogenesis of head and neck malignancies11. Chemotherapy and radiation induced DNA damages activates NF – κB through the signaling mechanism of IKK complex. Classical NF – κB pathway, that is the IKK – β-dependent NF – κB activation pathway, might show the molecular

link between inflammation and tumor promotion.
In a previous study the authors examined the expression levels of proteins related to NF – κB activation and IκK activity in head and neck cancer cells and also in normal oral epithelium and salivary gland cells. They had shown that enhanced NF – κB activity is caused by the phosphorylation and degradation of IκB α protein. There was no significant difference in the
expression levels of IκK β or IκK γ in cancer lines, compared to those in normal cells (16). .Our team has extensive knowledge and research experience that has translate into high quality publications (17–26)

Another study investigated the role of NF – κB in oral cancer progression . Expression of NF – κB and IκB was evaluated using IHC. The present study demonstrated that nuclear localization of NF – κB and IκB protein was increased with histological progression from normal to cancer tissue. NF – κB expression had a negative correlation in the evaluation of surgical margins, both p50 and p65 had a decreasing cytoplasmic expression from tumor to surgical margin to extra marginal tissue when evaluated by IHC(16,27).

In another study it was concluded that the result showed there is increased expression of NF – κB in OSCC when compared to normal. Although staining intensity and mean labeling index did not show any significant difference between OSCC and OSF, further studies on a larger sample will help in ascertaining the significance of NF – κB expression in OSF samples (28). In a study, they analyzed the expression of NF – κB and COX -2 in oral tissues in hyperplastic, dysplastic lesions and also in OSCC by IHC and correlated it with tobacco habits (29)

In a recent study it shows that NF – κB has a pro-apoptotic role. It plays an essential role in activation of wild type p53 to initiate pre-apoptotic signaling in response to ROS accumulation 38 ((30) . In a different study it was said that Cytokines including IL – 1β and TNF α can also activate the NF – κB pathway, which can result in amplification of inflammatory response and the persistence of chronic inflammation at local sites41. These metabolites contribute to the pathogenesis of inflammatory processes. Although NF – κB acts as an initiator of inflammation and suggests that it is also involved in the resolution of inflammation 42 (31).

When comparing the levels of salivary IKKB and NfkB in patients with oral squamous cell carcinoma and healthy individuals, patients with OSCC showed higher levels of the protein when compared to normal individuals. When concluded, IKKB and Nf Kb levels are higher in patients with oral squamous cell carcinoma.

CONCLUSION:

In conclusion, NF – κB and IKKB expression is altered in OSCC when compared to normal individuals and there was no significant association between the levels of the proteins.
It is concluded that our results showed an increase in the levels of salivary IKKB and NfkB levels in patients with oral squamous cell carcinoma(OSCC) with different grades of cancer when compared with the levels of protein in an healthy individual.

CONFLICT OF INTEREST:
The authors would like to declare no conflict of interest in the present study.

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CONFLICT OF INTEREST

The author declares that there were no conflicts of interests in the present study.

AUTHORSHIP CRITERIA

Khushi, Dr. Selvaraj , DR. R.Priyadharshini and Dr. Palati Sinduja framed the Concept, design of study or acquisition of data or analysis and interpretation of data
Drafting the article or revising was done by Dr.R.Priyadharshini and Dr.Palati sinduja

Final approval of the version to be published was done by Dr. R.Priyadharshini and Dr. Palati Sinduja
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