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Estimation of Clinocopathological Correlation and Comparison of Salivary TNF-α among Normal and Post Radiotherapy Patients of Oral cancer-A Cross-Sectional Study

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ABSTRACT

Introduction: Tumour Necrosis Factor-Alpha (TNF-α) is a member of the TNF receptor (TNF/TNFR) cytokine family. TNF-α is mainly involved in the maintenance of the source and homeostasis of the basic immune system, inflammation, and host defence. TNF- α is a multifunctional cytokine that plays an important role in diverse and a variety of cellular events such as cell survival, proliferation, differentiation, and death. As a major proinflammatory cytokine, TNF-α can act as an endogenous tumor promoter to bridge inflammation and carcinogenesis.

Aim: The aim of the present study is to evaluate the efficacy of TNF- α as a salivary biomarker in oral cancer.

Materials and Methods: An invivo cross-sectional study was conducted on saliva samples of Well differentiated and Moderately differentiated Squamous Cell Carcinoma (WDSCC and MDSCC) patients. Unstimulated saliva was collected from patients and ELISA was run on the samples to determine the TNF-α levels. Statistical analysis was performed using SPSS software. The standard curve was plotted using SPSS software version 23. Independent t-test was done to compare the results.

Results: The salivary TNF-α levels in Moderately Differentiated Squamous Cell Carcinoma (MDSCC) patients was found to be 12.31 ± 2.5 and the level in Well Differentiated Squamous Cell Carcinoma (WDSCC) patients was found to be 8.023 ± 1.642. The difference was found to be significant (P<0.05).

Conclusion: Salivary TNF-α in Moderately Differentiated Squamous Cell Carcinoma (MDSCC) patients was considered higher when compared to Well Differentiated Squamous Cell Carcinoma (WDSCC). Hence salivary TNF-α can be used as a biomarker for oral cancer.

Key words: Salivary TNF-α, Oral cancer, Post radiotherapy, Moderately differentiated squamous cell carcinoma, Widely differentiated squamous cell carcinoma, Innovative technique

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INTRODUCTION

Tumour Necrosis Factor - Alpha (TNF-α) is a member of the TNF/TNFR cytokine family [1]. TNF-α is mainly involved in the maintenance of the source and homeostasis of the basic immune system, inflammation, and host defence [2]. It is a type II transmembrane protein with an intracellular N terminus. It has a particular signalling potential both as a membrane-integrated protein and as a soluble cytokine released after proteolytic cleavage [2–5]. TNF-α has regulatory soluble functions on various crucial physiological processes such as synaptic plasticity, learning and memory and astrocyte-induced total synaptic strengthening TNF-α is a multifunctional cytokine that plays an important role in diverse and a variety of cellular events such as cell survival, proliferation, differentiation, and death [6]. As a particular pro-inflammatory cytokine family, Tumor necrosis factor is secreted by the inflammatory cells, which may be involved in inflammation and leads to carcinogenesis.

Oral cancer is the type of cancer that develops in the tissues of the oral cavity or throat. It belongs to a larger group of cancers called head and neck cancers [7]. Most of these develop in the squamous cells found in the oral cavity, tongue, and the lips. One of the biggest risk factors or the main cause for oral cancer is tobacco use [8]. The causes include smoking cigarettes, cigars, and pipes, as well as chewing tobacco. People who consume or take up

large amounts of alcohol and tobacco are at an even greater risk, especially when both products are used on a regular basis [9].

As a major proinflammatory cytokine, TNF-α can act as an endogenous tumor promoter to bridge inflammation and carcinogenesis. Regarding cancer, TNF-α plays a double role [10,11]. On one hand, TNF-α could be an endogenous tumor promoter, because TNF stimulates cancer cells' growth, proliferation, invasion, and metastasis, and tumor angiogenesis. On the other hand, TNF-α could be a cancer killer [10–12]. The property of TNF-α in inducing the cancer cell death makes it a potential cancer therapeutic, although much effort is needed to decrease its toxicity for systematic TNF-α administration. It is also aimed on sensitizing cancer cells to TNF-α induced apoptosis by inhibiting survival signals such as NF-κB, by combined therapy [13]. Our team has extensive knowledge and research experience that has translate into high quality publications [14–33]. The aim of the present study is to evaluate the efficacy of TNF-α as a salivary biomarker in oral cancer.

MATERIALS AND METHODS

An in vivo cross-sectional study on saliva samples of various oral cancer patients of normal and post radiotherapy conditions for the estimation of salivary TNF-α levels. The study was non-invasive and easy to perform without much inconvenience to patients. However, the sample size was limited. Prior to the initiation of the study, clearance was obtained by the Scientific Review Board with Ethical approval number IHEC/SDC/BDS/1977/01. The samples were obtained from patients who came to the clinics of Saveetha Dental College and Hospitals. The number of samples collected were 10 (n=10). The samples were collected in an unbiased manner using randomized sampling. Validation was done by an expert pathologist.

Criteria for selection of study subjects

Patients with oral cancer were included in the study. It was also ensured that patients with systemic comorbidities or terminally ill patients were not taken up for the study.

All the subjects included in the study belonged to the same ethnic group of South India (Dravidian population). Informed consent was obtained from the study subjects for inclusion in the study and it was also ensured that the subject anonymity was maintained. All the participants completed a questionnaire covering medical, residential, and occupational history.

Sample collection

10 saliva samples were collected from normal patients and patients with post radiotherapy of oral cancer. Unstimulated saliva from the patients was collected in Eppendorf's for a volume of 1ml. Then it was stored in -200 Celsius. During the procedure it was thawed and centrifuged. Samples were collected during the time frame between November 2020 to January 2021.

Principle of the test

ELISA is based on the competitive binding technique in which the TNF-α present in the sample competes with a fixed amount of horseradish peroxide (HRP) - labelled TNF-α on a human monoclonal antibody. Standards and samples are pipetted into the wells and TNF-α present in a sample is bound to the wells by the immobilized antibody. The wells are washed and a biotinylated anti-human TNF-α antibody was added.

After washing away unbound biotinylated antibody, HRP conjugated streptavidin is pipetted to the wells. The wells were washed again, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Reagent preparation

All reagents and samples were brought to room temperature (18-25°C) before use. Also Assay Diluent B (Item E) should be diluted to 5-fold with deionized or distilled water before use. For dilution of sample Assay Diluent, A (Item D) should be used for dilution of serum and plasma samples.

The suggested dilution for normal serum/plasma is 2-20-fold. For preparation of standard a vial of Item C was briefly spun. 400 µL of Assay Diluent A (for serum/ plasma samples) was added into Item C vial to prepare 50ng/ml standard. The powder was dissolved thoroughly by a gentle mix.

15 µL Ghrelin standard (50 ng/ml) was added from the vial of Item C, into a tube with 485 µL Assay Diluent A or 1X Assay Diluent B to prepare a 1,500 pg/ml standard solution. 400 µL Assay Diluent A or 1X Assay Diluent B was pipetted into each tube. 1,500 pg/ml standard solution was used to produce a dilution series (shown below). Each tube was mixed thoroughly before the next transfer. Assay Diluent A or 1X Assay Diluent B served as the zero standard (0 pg/ml). If the Wash Concentrate (20X) (Item B) contained visible crystals, it was warmed to room temperature and mixed gently until they dissolved.

20 ml of Wash Buffer Concentrate was diluted into deionized or distilled water to yield 400 ml of 1X Wash Buffer. Detection Antibody vial (Item F) was briefly spun before use. 100 µL of 1X Assay Diluent B (Item E) was added into the vial to prepare a detection antibody concentrate.
This was then pipetted up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B (Item E) and used in relevant prior steps. The HRP-Streptavidin concentrate vial (Item G) was briefly spun and pipetted up and down to mix gently before use, as precipitates may form during storage. HRP- Streptavidin concentrate should be diluted 200-fold with 1X Assay Diluent B (Item E).

Assay procedure

All reagents and samples were brought to room temperature (18-25°C) before use. It is recommended that all standards and samples be run at least in duplicate. Removable 8-well strips were labelled as appropriate for the experiment. 100 µL of each standard and sample was added into appropriate wells. These wells were then covered and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded and washed 4 times with 1X wash solution. Each well was filled and washed with Wash Buffer (300 µl) using a Pipette. Complete removal of liquid at each step is essential for good performance. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted with clean paper towels. 100 µl of 1X prepared biotinylated antibody was added to each well. This was then incubated for 1 hour with gentle shaking. The solution was discarded, and the wash was repeated. 100 µl of prepared Streptavidin solution was added to each well. This was then incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded, and the wash repeated. 100 µL of TMB One-Step Substrate Reagent (Item H) was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. 50 µl of Stop Solution (Item I) was added to each well and read at 450 nm immediately.

Calculation of results

The mean absorbance was calculated for each set of duplicate standards, controls and samples, and the average zero standard optical density was subtracted. The standard curve was plotted using SPSS software version 23, with standard concentration on the x-axis and absorbance on the y- axis. The best-fit straight line was drawn through the standard points.

Typical data

A standard curve was run with each assay.

Sensitivity

The minimum detectable dose of Human TNF-α was determined to be 3pg/ml. The minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluents buffer).

Statistical analysis

Statistical analysis was performed using SPSS software. Independent t-test was done to compare the results. Dependent variables include oral cancer while independent variables include age and sex.

RESULTS

Demographic data

The study included a total of n=10 participants who were divided into two groups. Group I consists of Moderately Differentiated Squamous Cell Carcinoma (MDSCC) n=5, Group II consists of Well Differentiated Squamous Cell Carcinoma (WDSCC) n=5.

Statistics

Statistical analysis was performed using SPSS (IBM SPSS statistics for Windows Version 23.0, Armonk, NY: IBM corp. Released 2015).

Independent t-test was done to compare the groups and $p<0.05$ was considered significant and $p>0.05$ was considered not significant.

Prevalence of salivary TNF-α levels among the groups

Salivary TNF-α in patients with well differentiated squamous cell carcinoma (WDSCC)

Among the 5 samples analysed, all 5 showed presence of TNF-α in salivary samples. The prevalence of salivary TNF-α among Group I was 100% with a minimum value of 0.068ng/dl and maximum value of 0.072ng/dl.

Salivary TNF-α in patients with moderately differentiated squamous cell carcinoma (MDSCC)

Among the 5 samples analysed, all 5 showed presence of TNF-α in salivary samples. The prevalence of salivary TNF-α among Group II was 100% with a minimum value of 0.062ng/dl and a maximum value of 0.121ng/dl.

Comparison of prevalence of TNF-α among salivary samples of Moderately differentiated squamous cell carcinoma (MDSCC) and well differentiated squamous cell carcinoma (WDSCC)

The comparison between the groups shows statistical significance, a higher prevalence of salivary TNF-α was found among Moderately Differentiated Squamous Cell Carcinoma (MDSCC) patients when compared to those of Well Differentiated Squamous Cell Carcinoma (WDSCC) patients (Table 1 and Figure 1).

Table 1: Table showing mean of three observations significance at the levels of p<0.05, WDSCC-Well differentiated squamous cell carcinoma, MDSCC-Moderately differentiated squamous cell carcinoma.

Group	Mean	Std. Deviation	Std. Error	P Value
WDSCC (pg/dl)	8.023	1.642	0.8211	<0.05
MDSCC (pg/dl)	12.31	2.5	1.25	

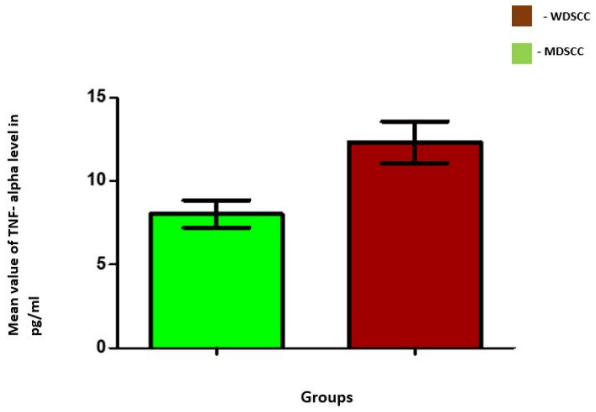

Figure 1: Bar graph showing salivary TNF -alpha levels in Well differentiated Squamous Cell Carcinoma (WDSCC) and Moderately Differentiated Squamous Cell Carcinoma (MDSCC). X axis represents type of squamous cell carcinoma and Y axis represents concentrations of salivary TNF alpha. Green denotes Well Differentiated Squamous Cell Carcinoma (8.023 ng/ml) and red denotes Moderately Differentiated Squamous Cell Carcinoma (12.31 pg/dl).

DISCUSSION

On comparing the TNF-α levels in saliva in normal and post radiotherapy patients, it was found that TNF-α was increased in Moderately Differentiated Squamous Cell Carcinoma (MDSCC) patients. The difference was found to be significant as $p<0.05$.

Salivary TNF-α is a proinflammatory cytokine which is released by macrophages. This type of pro-inflammatory cytokine has a special role in the regulation of the immune response and has a prognostic significance. Enhanced expression of salivary TNF-α has been found in patients with rheumatoid arthritis, chain smokers and chronic obstructive pulmonary disease [34]. And also, the most of the salivary cytokines are majorly being correlated with the periodontal status and oral inflammatory issues in recent days [35]. On the other hand, systemic inflammation also influences salivary inflammatory diseases. Mostly in the anti tumor therapy may lead to changes in the oral mucosa and certain changes in salivary cytokines levels.

Salivary diagnostics is a dynamic and emerging research field utilising molecular technology and diagnostics to aid in the diagnosis of oral and other systemic diseases. Saliva sample collection is easily done and is stored for the early detection of diseases as it contains specific biological markers [36,37]. These days, the saliva sample collection is majorly preferred as it is easily taken without any invasive procedures, the need for blood draw would become unnecessary. TNF-α also activates the transcription of NFκB factor, which helps in the stimulation of cell proliferation, and mainly increases the secretion of inflammatory cytokines. TNF-α is usually not detectable in normal healthy individuals but increases in the serum and tissue levels are found in inflammatory and infectious conditions and the serum levels well correlate with the severity of the infection. A recent survey done, reported that dentists and the medical professionals accepted that screening for medical conditions is important, and they were ready to participate when the sample collection was saliva rather than a finger prick [38–40].

In recent days, higher levels of baseline TNF-α is associated with more aggressive behaviour of disease and poor survival in patients with immunotherapy resistance [41]. A lower TNF-α in saliva may be related to a significantly higher TNF-α levels in serum in a similar group in (Acute Lymphoblastic Leukemia) ALL children. In the present study, the salivary TNF-α levels were increased in case of moderately differentiated squamous cell carcinoma (MDSCC). In contrast to the present study, the study conducted by Deepti G et al [42], the author concludes that the salivary TNF-α levels were majorly increased in the oral leukoplakia and Oral Squamous Cell Carcinoma (OSCC) as the OSCC patients were majorly having tobacco/ smoking habits.

In the previous studies done, there was a significant correlation between IL-lβ and sTNF-RI. The mean values of SCC were also elevated at a particular concentration [38]. IL-6 and sTNF-RI were the most sensitive parameters in early stages of cancer and may be used as an additional biomarker in oral cancer [43]. In the present study, the levels of TNF-α were considered higher in Moderately Differentiated Squamous Cell Carcinoma (MDSCC). The limitation of the present study includes limited sample size. Also, the treatment modality of diabetes mellitus patients was not considered, i.e., whether they take insulin tablets or shots or any other mode of treatment. With further research and greater sample size, salivary TNF-α can be used as a very good biomarker of oral cancer.

CONCLUSION

Within the limitations of the present study, salivary TNF-α levels in Moderately Differentiated Squamous Cell Carcinoma (MDSCC) patients were increased when compared to Well Differentiated Squamous Cell Carcinoma (WDSCC) patients. This difference was found to be significant. With further research, salivary TNF-α can be used as a biomarker of oral cancer.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest in the present study.

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