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Combination Assay for Tumor Markers in Saliva of Potentially Malignant Disorders and Oral Squamous Cell Carcinoma

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Authors' contributions

This work was carried out in collaboration among all authors. Author Jembulingam Sabarathinam performed the analysis, interpretation and wrote the manuscript. Author SD contributed to conception, data design, analysis, interpretation and critically revised the manuscript. Author J. Selvaraj contributed towards the biochemically processing of the samples and critically revised the manuscript. All authors have discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Potentially malignant disorders are "the risk of malignancy being present in a lesion or condition either at the time of initial diagnosis or at a future date" include mainly leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis. Biomarkers were of immense help in diagnosis in recent years but a very few molecular biomarkers have been reported in the literature which are not significantly accurate. The current study aims at quantifying the levels of Transforming growth factor (TGF- β), Platelet derived growth factors (PDGF) and receptor of advanced glycosylation end

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products (RAGE) in saliva of patients with potentially malignant disorders and oral squamous cell carcinoma. Unstimulated saliva of oral squamous cell carcinoma and potentially malignant disorder patients was collected and stored in sub zero temperature. Further biochemical analysis was performed with Raybio ELISA kits. One way ANOVA was performed. The mean concentration of RAGE, PDGF and TGF- β was increased in oral squamous cell carcinoma groups and potentially malignant disorders when compared to healthy controls.p-0.000 ($p<0.05$).These combined assays can be used as potential biomarkers for indicating the prognosis of the disease and can be used as a diagnostic tool for screening and early detection as these combined assays give more reliable and accurate diagnosis when compared to single biomarker assays.

Keywords: RAGE; PDGF; TGF- β; potentially malignant disorders; oral squamous cell carcinoma; diagnosis.

1. INTRODUCTION

Oral cancer, the most destructive disease is characterized by a group of neoplasms affecting any region of the oral cavity including the pharyngeal regions and salivary glands [1]. Moreover, the term is often replaced by Oral squamous cell carcinoma (OSCC) which represents the most frequent of all oral neoplasms. It is estimated that oral squamous cell carcinoma accounts for about 90% of all neoplasms occurring in the oropharyngeal region [2].

Across the globe, Oral squamous cell carcinoma (OSCC) stands as the sixth most common type of carcinoma among all the malignancies [3]. However, higher prevalence of oral squamous cell carcinoma is observed in Pakistan, India and Sri Lanka accounting for more than 40% of all cancer cases [4,5]. Oral squamous cell carcinoma is a complex disease associated with genetic alterations, environmental factors.(habits including tobacco usage in both smoke and smokeless form, alcohol consumption and betel nut chewing) and other risks which interact and thus lead to initiation and progression of the disease [6].

Potentially malignant disorders are groups of disorders of different etiologies, usually characterized by association of tobacco habits and mutation, which could be spontaneous or hereditary alterations of the genetic material of oral epithelial cells with or without clinical and histomorphological alteration that may lead to oral squamous cell carcinoma [7]. The group of disorders include mainly leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis, and other miscellaneous lesions. These lesions of oral mucosa possess an increased risk of malignant transformation when compared to the healthy mucosa [8].

Quantification of specific tumor markers in saliva can be helpful in the detection and diagnosis of oral cancer. They are Transforming growth factor ,Platelet derived growth factors,Receptor of advanced glycosylation end products.

Transforming growth factor (TGF- β) is a regulatory cytokine that is secreted by the tumour and stromal cells in a tumor environment. The members of this family include TGF- β, bone morphogenic proteins inhibins, activins and growth and differentiation factors [9]. TGF- β signaling plays a vital role in cell proliferation and differentiation and is observed to be associated with carcinomas of gastrointestinal tract and liver. TGF- β also functions as a tumour suppressor in normal epithelial cells [10].

Platelet derived growth factors (PDGF) are growth factor cytokines secreted by epithelial and mesenchymal cells.PDGF is produced by platelets and are stored as granules .The PDGF family comprises heterodimers and homodimers which are formed by PDGFA,PDGFB,PDGFC, and PDGFD [11]. Platelet derived growth factors play a pivotal role in carcinoma progression and invasion by facilitating angiogenesis. Overexpression of PDGF is associated with glioma, Neurofibroma, prostate cancer and ovarian cancer [12,13].

The receptor of advanced glycosylation end products (RAGE) often referred to as a pattern of recognition receptor that controls innate immunity belongs to the immunoglobulin superfamily of cell surface molecules with a broad spectrum of ligand specificity [14]. Through interaction with diverse ligands, RAGE hosts many intracellular signalling pathways to control inflammation, apoptosis, proliferation and autophagy [15]. RAGE plays a crucial role in Diabetes, cardiovascular disease and cancer [16,17].

As the incidence and mortality rates due to oral carcinomas have shown enormous rise in the last five decades, more intensified effects are required to safeguard us against the destructive disease. Early diagnosis and preventive measures can help in battling the indestructible disease which have led to several million deaths every year.

Our recent research portfolio in slides numerous articles in reputed journals [18–22]. Based on this experience we planned to pursue our current study which aims at quantifying the levels of Transforming growth factor (TGF- β), Platelet derived growth factors (PDGF) and receptor of advanced glycosylation end products (RAGE) in saliva of patients with potentially malignant disorders and oral squamous cell carcinoma and use them as a effective biomarker for screening and diagnosis of oral squamous cell carcinoma.

2. MATERIALS AND METHODS

This study was conducted among patients attending the outpatient department of a private dental college in Chennai. Newly diagnosed patients with oral submucous fibrosis, Leukoplakia, and oral squamous cell carcinoma, who were not previously treated for the disease were selected for the study. This study was conducted between October 2019 and January 2020.

2.1 Inclusion Criteria

1. Patients clinically and histopathologically diagnosed with OSMF, leukoplakia and Oral squamous cell carcinoma.
2. Patients who had not undergone treatment.
3. Patients who had agreed to participate in the study and signed the informed concern forms.

2.2 Exclusion Criteria

1. Patients below the age of 18 years
2. Patients with any systemic illness which includes respiratory disease, hepatobiliary and gastrointestinal tract disorders and Central nervous system related diseases.
3. Patient with history of treatment for Oral squamous cell carcinoma.

2.3 Sample Collection

Samples were collected specifically in the morning between 8 am to 10 am. Patients were made to sit upright and asked to spit saliva in a sterile container. 5 ml of unstimulated saliva was collected from the patient in the sterile container and stored in sub zero temperature before processing the samples. Simple cluster sampling was done.

2.4 Classification of Groups

Control group - saliva samples of healthy people (n=10)
Study group 1- saliva samples of patients diagnosed with Leukoplakia (n=10)
Study group 2- saliva samples of patients diagnosed with Oral submucous fibrosis (n=10)
Study group 3- saliva samples of patients diagnosed with Oral squamous cell carcinoma (n=10)

2.5 Quantitative Analysis of Human Transforming Growth Factor β (TGF- β)

The quantitative analysis of TGF- β was done using ELISA kit for TGF- β obtained from Shanghai korian biotech co ltd, Shanghai, China. The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Transforming Growth factor β (TGF-β) in samples. 20microliters of the samples were added to monoclonal antibody Enzyme well which is pre-coated with Human Transforming Growth factor β (TGF-β) monoclonal antibody and was incubated for 60 minutes; Then Transforming Growth factor β (TGF-β) antibodies labeled with biotin, and combined with Streptavidin-HRP were added to form immune complex .Then incubation was carried out for 10 minutes. The wells were washed with buffered water to remove the uncombined enzyme. Then Chromogen Solution A, B were added to give colour to the immune complex. The color of the liquid changes into the blue. Finally the reaction was terminated using a stop solution. When the stop solution was added, the color changed to yellow from blue. The chroma of color and the concentration of the Human Substance Transforming Growth factor β (TGF-B) of sample were analysed using a ROBONIX ELISA READER and concentration of samples were tabulated.

2.6 Quantitative Analysis of Platelet Derived Growth Factors (PDGF)

The quantitative analysis of PDGF was done using ELISA kit obtained from Shanghai korian biotech co ltd, Shanghai, China .The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Platelet derived growth factors (PDGF) in samples. The plate has been pre-coated with Human PDGF antibody. 20 microliters of the sample are added and bind to antibodies coated on the wells. And then biotinylated Human PDGF Antibody is added. Then Streptavidin-HRP is added and binds to the Biotinylated PDGF antibody and incubated for 60minutes. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human PDGF and incubated for 10 minutes. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm using a ROBONIX ELISA READER and concentration of samples were tabulated.

2.7 Quantitative Analysis of Receptor of Advanced Glycosylation End Products (RAGE)

The quantitative analysis of RAGE was done using RayBio ELISA kit .The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human receptor of advanced glycosylation end products (RAGE) in samples. Standards and samples are pipetted into the wells and RAGE present in a sample is bound to the wells by the immobilized antibody and incubated for 2.5 hours. The wells are washed and a biotinylated anti human RAGE antibody is added and incubated for 1 hour. After washing away unbound biotinylated antibody, HRP (Horseradish peroxidase) conjugated streptavidin is pipetted to the wells and incubated for 45 minutes. The wells are again washed, a tetramethylbenzidine (TMB) chromogenic substrate solution is added to the wells and color develops in proportion to the amount of RAGE bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm using a ROBONIX ELISA READER and concentration of samples were tabulated.

3. RESULTS

The mean concentrations of Human Transforming Growth factor β (TGF-B) in saliva was 21.05 ng/ml in control group, 32.28 ng/ml in Leukoplakia group, 25.21 ng/ml in Oral submucous fibrosis group and 43.75 ng/ml in Oral squamous cell carcinoma group. There was a significant difference between the groups where *P value was 0.000 (P<0.05)* when one way ANOVA test was performed using SPSS software, version 20 (Fig. 1).

The mean concentrations of Human Platelet derived growth factors (PDGF) in saliva was 32.30 pg/ml in control group, 38.05 ng/ml in Leukoplakia group, 40.10 pg/ml in Oral submucous fibrosis group and 42.25 pg/ml in Oral squamous cell carcinoma group. There was a significant difference between the groups where *P value was 0.000 (P<0.05)* when one way ANOVA test was performed using SPSS software, version 20. (Fig. 2)

The mean concentrations of Human receptor of advanced glycosylation end products (RAGE) in saliva was 14.40 pg/ml in control group, 18.55 pg/ml in Leukoplakia group, 24.45 pg/ml in Oral submucous fibrosis group and 20.40 pg/ml in Oral squamous cell carcinoma group. There was a significant difference between the groups where *P value was 0.000 (P<0.05)* when one way ANOVA test was performed using SPSS software, version 20 (Fig. 3).

4. DISCUSSION

Numerous risk factors or causative agents for Oral Cancer have been described. Tobacco and alcohol, human papillomavirus (HPV), syphilis, oro-dental factors, dietary deficiencies, chronic candidiasis and viruses are significantly associated with causation of Oral Cancer.Among these, usage of tobacco containing products have increased deleterious effects on oral mucosa.It is a well known product which poses the risk of cancer and neoplastic changes.There are a large number of alkaloid present in the betel nut among which arecoline and arecadine are suggested to into induce carcinomas [23].

Numerous studies have been conducted to evaluate the efficiency of tumour biomarkers and have reported that tumour markers are extremely effective in detection and diagnosis of various

types of carcinomas. These tumour markers have predominantly tumour mediated substance. At present most of the authors had measured only a single tumour marker and a very few conducted combination essays [24–30]. It has been documented in literature that only a very few articles had combination essays done with more than two biomarkers being measured simultaneously [31–33] among which there is only one combination assay applicable for diagnosis of oral cancer [34]. In our current study we had performed combination assay for diagnosis of carcinoma among the patients with Potentially malignant disorders were PFDG, TGF beta and RAGE were assessed simultaneously.

4.1 Transforming Growth Factor (TGF- B)

Several cytokines and growth factors play an important role in carcinogenesis and tumour invasion. Predominantly these cytokines are pro-inflammatory (Tumour necrosis factor alpha, Interleukin 6) while other cytokines being affiliated are anti-inflammatory cytokines like Transforming growth factor (TGF- β) [35]. There is very little literature to study the serum and salivary levels of TGF- β among the cancer population. The TGF- β pathway regulates various cellular processes and alteration in the signaling pathway would pave the way for development of carcinoma by initiating carcinogenesis. The anti-proliferating activity of the TGF- β is lost in many tumours [36,37]. Salivary TGF- β levels were estimated to be 24.1ng/ml in oro-pharyneal carcinoma patients while 14.3 ng/ml in carcinoma patients [38]. The results of the previous studies are in favour of our current study as there was a double fold increase in salivary levels of carcinoma patients when compared to health controls. Many studies have revealed an increased expression of TGF beta among oral carcinoma patients and patients with Potentially malignant conditions [39–41]. In the early stages of carcinogenesis, the protein acts as a potent tumor suppressor, later, TGF-beta can function to advance tumor progression [39].

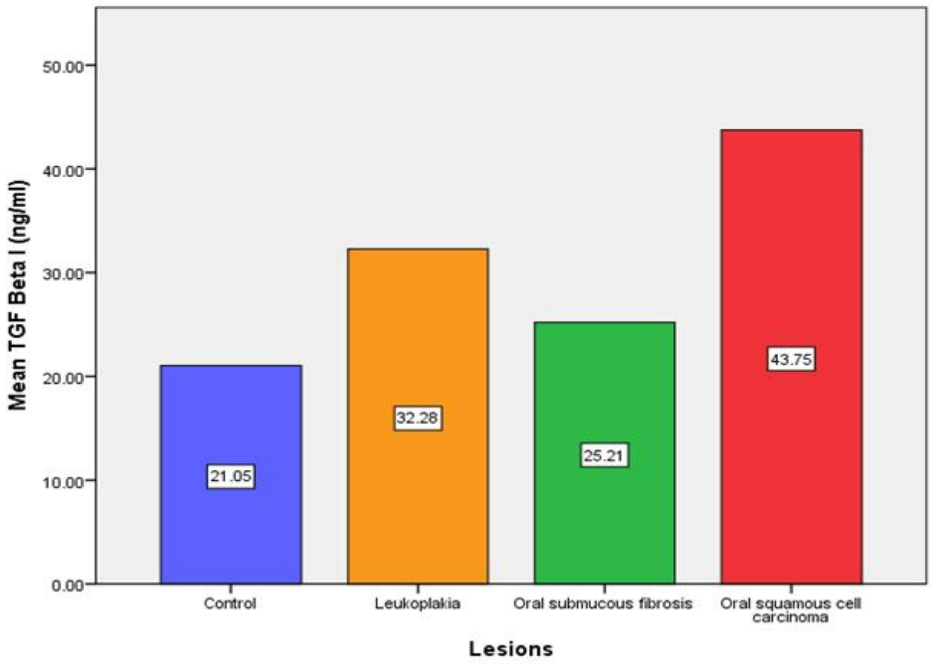

Fig. 1. Bar graph depicts the mean concentration of TGF- β among the study population

X axis denotes the mean concentration of TGF- β in ng/mL, Y axis denotes the study groups which include control (Blue), Leukoplakia (Orange), Oral submucous fibrosis (green) and Oral squamous cell carcinoma (Red). One way ANOVA was performed, P-0.000 (P<0.05) statistically significant proving there is a increased level of TGF- β between control group and the oral squamous cell carcinoma

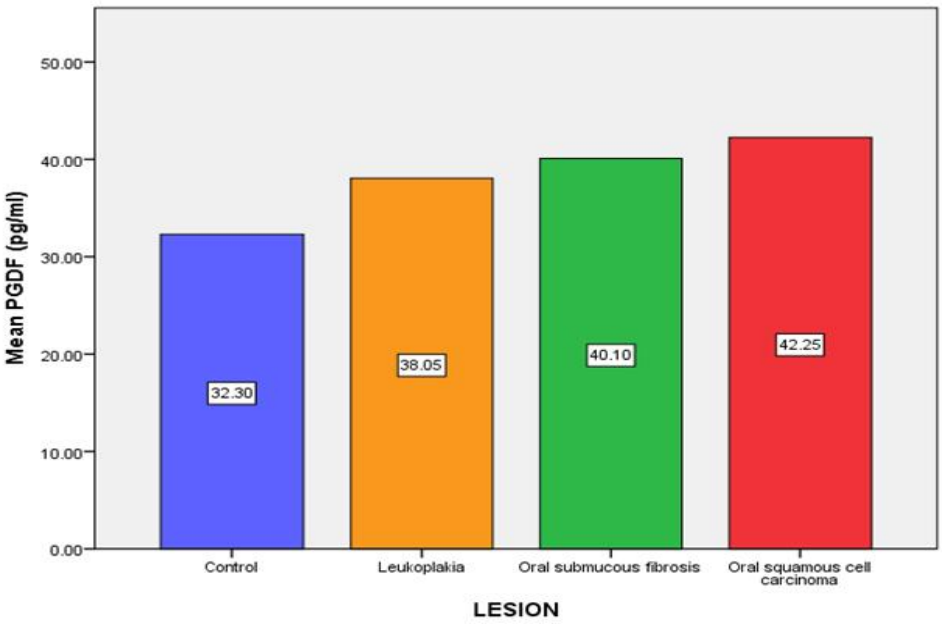

Fig. 2. Bar graph depicts the mean concentration of PDGF among the study population

X axis denotes the mean concentration of PDGF in pg/mL, Y axis denotes the study groups which include control (Blue), Leukoplakia (Orange), Oral submucous fibrosis (green) and Oral squamous cell carcinoma (Red). One way ANOVA was performed, P-0.000 (P<0.05) statistically significant proving there is an increased level of PDGF between control group and the oral squamous cell carcinoma

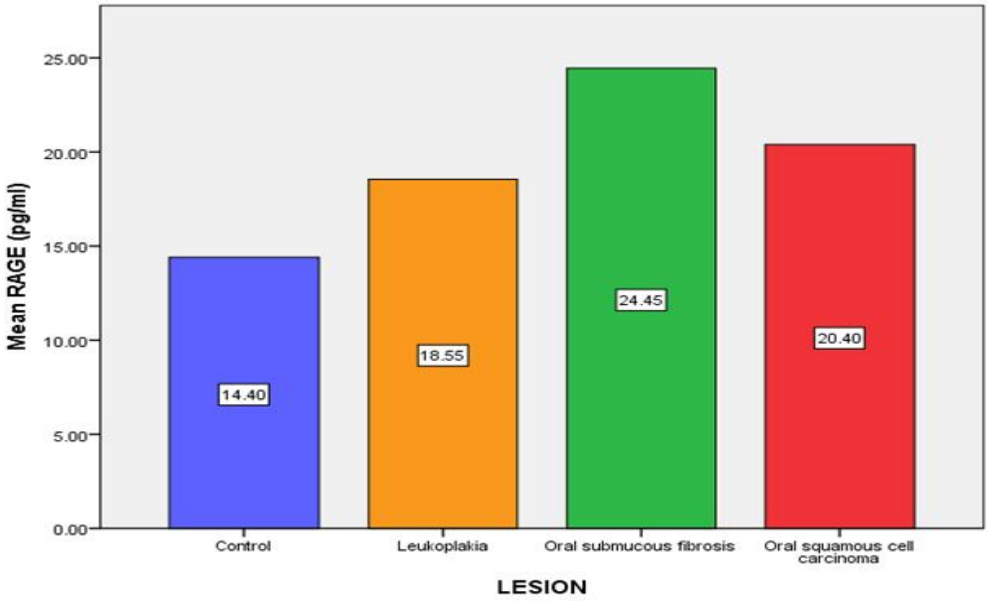

Fig. 3. Bar graph depicts the mean concentration of RAGE among the study population

X axis denotes the mean concentration of RAGE in pg/mL, Y axis denotes the study groups which include control (Blue), Leukoplakia (Orange), Oral submucous fibrosis (Orange) and Oral squamous cell carcinoma (Red). One way ANOVA was performed, P-0.000 (P<0.05) statistically significant proving there is an increased level of RAGE between control group and the oral submucous fibrosis group

4.2 Platelet Derived Growth Factors (PDGF)

Platelet derived growth factors play a pivotal role in carcinoma progression and invasion by facilitating angiogenesis. In tumor formation, angiogenesis is a critical mechanism in which growth factors such as PDGF play a crucial role. For the tumour to survive, the tumour cells need nutritional supply from blood delivered via blood vessels. Increased blood vessels is associated with increased PDGF [11,42]. Many studies reported overexpression of PDGF in oral squamous cell carcinoma [43–45]. Only qualitative analysis had been performed while no attempts to quantify the PGFD had been documented. The current study was the first of its kind which quantified the PGDF levels of various Potentially malignant lesions and oral squamous cell carcinoma.

4.3 Receptor of Advanced Glycosylation End Products (RAGE)

Rage is a multiligand receptor to which ampotercin, advanced glycation end production, HMGB1 and S100 proteins bind [46,47]. This protein receptor is composed of 1.7kb 5'flanking region and 11 exons and 10 introns with 3 UTR [48]. RAGE is observed to be increased in many of the pathological conditions including cancer of various sites in humans. It is also associated with periodontal conditions, liver diseases and cardiovascular abnormalities [16,17]. Through interactions with diversity of ligands, RAGE controls many intercellular signalling pathways such as inflammation, autophagy, and apoptosis [14]. Alteration in these signalling pathways induces carcinoma. Many studies revealed regulation of RAGE in oral carcinoma [15,49–51], However only qualitative analysis had been done while attempts to Quantitatively analyze RAGE has not been documented in literature. The current study was the first of its kind which quantified the RAGE levels of various Potentially malignant lesions and oral squamous cell carcinoma. Further studies have to be done with larger sample sizes of oral submucous fibrosis, oral leukoplakia with different histological and clinical grading to analyze the molecular parameters which cause the intuition and promotion of oral squamous cell carcinoma and Potentially malignant disorders.

5. CONCLUSION

Transforming growth factors (TGF- β), Platelet derived growth factors (PDGF) are found to be increased gradually from Potentially malignant disorders to oral carcinoma. Receptor of advanced glycosylation end products (RAGE) was to be increased in oral submucous fibrosis patients when compared to oral squamous cell carcinoma patients .These combined assays can be used as potential biomarkers for indicating the prognosis of the disease and can be used as a diagnostic tool for screening and early detection as these combined assays give more reliable and accurate diagnosis when compared to single biomarker assays.

CONSENT

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

ETHICAL APPROVAL

The ethical clearance was obtained from the Institutional ethical committee of Saveetha Dental College and Hospitals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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