Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/2033
DOI URL: http://dx.doi.org/10.21474/IJAR01/2033
ISSN NO. 2320-5407

RESEARCH ARTICLE

SALIVA AS DIAGNOSTIC TOOL - EXPRESSION OF GM-CSF IN ORAL POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA PATIENTS.

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Manuscript Info

Manuscript History

Received: 27 September2016
Final Accepted: 12 October 2016
Published: October 2016

Key words:-
Saliva, oral squamous cell carcinoma, granulocyte macrophage-colony stimulating factor (GM-CSF).

Abstract

Oral Squamous Cell Carcinoma (OSCC) is most common malignant tumor of oral cavity with the survival rate of 60-80% if detected during its early stages; however this number drops to 30-40% when the cancer is diagnosed during advanced stages. Oral lesions are in direct contact with saliva, which makes measurement of tumor markers in saliva an attractive, non-invasive, chair-side diagnostic/prognostic aid and alternative to serum testing. It may be used to measure specific salivary macromolecules as well as examining proteomic or genomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratins, mRNAs and DNA transcripts. One of such growth factor is granulocyte macrophage-colony stimulating factor (GM-CSF).

Aim:- To evaluate the change in salivary expression of GM-CSF in Oral Potentially Malignant Disorders(OPMD) and OSCC patients and to evaluate the reliability of salivary GM-CSF as diagnostic/ prognostic marker in OPMD.

material and method:- In this study, we collected salivary samples from OSCC patients (n=25), OPMD patients (n=25) and healthy controls (n=25), and expression of GM-CSF was measured/estimated by quantitative ELISA method.

Result:- The results shown significant difference in expression of salivary GM-CSF in patients with OSCC and OPMD as compared to control group (P<0.001).

Conclusion:- From the result, it was concluded that GM-CSF is an important proinflammatory cytokine, detectable at higher concentration in saliva of patients of OPMD and OSCC and prove to be useful as molecular biomarker for diagnosis and predicting malignant transformation.

Introduction:-

Oral Squamous Cell Carcinoma(OSCC) is most common malignant tumor of oral cavity.[1] The prevalence of OSCC has increased 5.3times for men and 2times increase for women in the past two-decades.[2] The survival rate of oral cancer is 60-80% if detected during its early stages; however if diagnosed during advanced stages, the prognosis becomes poorer with drop in survival rate to 30-40%.[3] In addition, because OSCC has a very high recurrence rate, early identification and detection becomes essential for patient survival. Direct contact between saliva and oral lesion, makes saliva as an attractive, non-invasive, cost-effective, chair-side diagnostic/prognostic tool for oral

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potentially malignant disorders and oralcancer, alternative to serum testing for measurement of tumor markers.[4,5].There is a search for biomarkers in saliva, an easy-to-obtain body fluid, for noninvasive detection of oral cancer. Salivary testing can be an effective modality for diagnosis and prognosis predicting of oral cancer as well as for monitoring the patient's post-therapy status.[6] Saliva may provide a cost-effective and practical approach for the screening of large populations.[7] It may be used to measure specific salivary macromolecules as well as examining proteomic or genomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratins, mRNAs and DNA transcripts.[4]. One of such growth factor is granulocyte macrophage-colony stimulating factor (GM-CSF).

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a proinflammatory cytokine produced by macrophages during phagocytic activity during chronic inflammatory conditions. Head and neck SCC cells are vulnerable to such immune effector cells.[8,9,10] Head and neck SCC cells produces higher level of GM-CSF that contain CD34 natural suppressor cells that inhibits activity of intratumoral T-cells,[10] and produces nitric oxide and TGF-β[11]. This leads to increase metastasis, and chances of recurrence and thereby has poor prognosis[11,12].

The present study was undertaken to evaluate expression of salivary biomarker GM-CSF for development of possible marker in oral potentially malignant disorders and oral squamous cell carcinoma and to evaluate the reliability of salivary GM-CSF as diagnostic/ prognostic marker in potentially malignant oral disorders (OPMD), to develop a cost-effective chair side salivary prognostic marker of oral squamous cell carcinoma (OSCC).

Materials and methods:-

The study was conducted at Department of Oral Medicine and Radiology, Maulana Azad Institute of Dental Sciences and Department of Biochemistry, GovindBallabh Pant Institute of Postgraduate Medical Education And Research. The study protocol was approved by the Institutional Ethical Committee.Totally 75 cases were included under the study. The cases were divided into three groups consisting of 25 cases each. G**roup A** comprised of patients with clinically diagnosed and histopathologically proven OSCC. **Group B** included the patients with clinically diagnosed and histopathologically proven OPMD (oral submucousfibrosis(n=10), oral lichen planus(n=10), leukoplakia(n=4), discoid lupus erythematosus(n=1)). Clinically proven cases of OPMD and OSCC with additional confirmation by biopsy of age group ranging from 20 to 70 years, both males and females were included in the study. **Group C** comprised of age and sex matched healthy individuals with matched periodontal conditions. Patients who are already undergoing some treatment for existing OPMD and patients with severe systemic diseases, pregnant and lactating females were excluded**.**

Informed written consent was obtained from all the patients selected for the study. The clinically suspected cases of OPMD includes leukoplakia, oral submucous fibrosis, oral lichen planus, discoid lupus erythematosis and the patients with non-healing ulcer/ ulcero-proliferative growth with or without lymphadenopathy as OSCC. Incisional biopsy was done for all the suspected cases and diagnosis was established based on clinical and histopathological findings, except for healthy controls. All the cases selected under the three groups were age and sex matched. The periodontal status of all the cases (n=75) was matched using community periodontal index (CPI) as per WHO guidelines. [13]

Saliva samples were collected by simple drooling method. The saliva samples were used for the study only when the histopathological results confirmed the presence of either OPMD or OSCC. The participants were refrained from eating and drinking for at least one hour before sample collection. Unstimulated whole saliva was collected by requesting the subject to swallow first, tilt their head forward and expectorate all the saliva into a sterile wide mouthed vaccuntainer for 5 minutes without swallowing. There, the saliva samples were centrifuged at 3000 rpm for 10 minutes. Then the supernatants were carefully drawn using micropipettes and transferred to eppendorf tubes. The supernatants were then stored at --80˚C in the deep freezer until analysis.

Elisa method:-

The concentration of GM-CSF present in the saliva samples was determined by using quantitative ELISA technique. Standard human GM-CSF ELISA kit (Diaclone, France) was used for analysis with the help of ELISA reader (TECON). And Based on data information and collected, results were analysed using SPSS software(14^{th} version) and Mann-Whitney statistical tests was applied.

Result:-

Following observations were made:-

Mean Distribution of GM-CSF According To Sex - There were 21 (84%) males and 4 (16%) females in Group A compared to 16 (64%) males and 9 (36%) females in Group B. The p-value for sex distribution between groups using chi-square test was found to be statistically significant (<0.05). The mean value for GM-CSF was found highest (2.17) in males and lowest (1.81) in females.

Mean Distribution of GM-CSF According To habit distribution- The mean value for GM-CSF was found highest (3.09) in patients with habit of smokeless form of tobacco intake, followed by GM-CSF mean value (1.69) in patients with habit of smoking, and lowest (0.41) in patients with no habit.

Salivary GM-CSF levels (pg/ml) in patients and control group:-

	GROUP-A (n=25)	GROUP-B (n=25)	GROUP-C (n=25)
MEAN	5.7	1.6	0.0
STANDARD DEVIATION	5.25	3.87	0.00
MEDIAN (MIN-MAX)	5.1 (0-28)	0.0 (0-18)	0.00 (0-0)

Mann-Whitney test for salivary GM-CSF:-

COMPARISON GROUPS	GM-CSF p-value
GROUP-A AND GROUP-C (OSCC AND CONTROL)	0.001
GROUP-A AND GROUP-C (OSCC AND CONTROL)	0.01
GROUP-A AND GROUP-C (OSCC AND CONTROL)	0.001

p-value<0.05 is significant

AS shown in table, the mean value for GM-CSF was highest (5.70±5.25) in group-A, followed by group-B (1.60±3.87) and lowest (0.0±0.00) in group-C. With MANN-WHITNEY test, the difference in mean values between groups was found statistically significant with p-value <0.05.

ROC curves and cut-off values for GM-CSF:-

Group A (OSCC) and group C (CONTROL): As shown in graph, the area under curve was 0.92 and mean value of GM-CSF in OSCC patients and control were 5.7 and 0.0 respectively. As the mean of GM-CSF in group C (CONTROL) is 0pg/ml, so cut-off value cannot be obtained.

Group B (OPMD) and group C (CONTROL): As shown in graph, the area under curve was 0.620 and mean value of GM-CSF in OPMD patients and CONTROL were 1.62 and 0.0 respectively. As the mean of GM-CSF in group C (CONTROL) is 0pg/ml, so cut-off value cannot be obtained.

Group A (OSCC) and group B (OPMD): As shown in graph, the area under curve was 0.862 and mean value of GM-CSF in OSCC patients and control were 5.7 and 1.6 respectively. The possible cut-off value derived from the ROC curve for GM-CSF was 4.35pg/ml. At this value, sensitivity was 84% and specificity was 84%. If this cut-off value was lowered to 2.15 pg/ml(close to mean value of OPMD group i.e 1.6pg/ml), the sensitivity remained same but specificity reduced to 76%.

Discussion:-

In spite of the various diagnostic modalities available today, considerable number of oral cancer patients are diagnosed only at advanced stages with poor survival rate. This necessitates the early detection of potentially malignant disorders. Saliva as a sample has many advantages over serum and tissue. Saliva is a non-invasive specimen which is relatively easy to collect in sufficient quantities for analysis even in small clinics or laboratory.[14]

Measurement of molecular markers in saliva could potentially aid in development of a practical screening tool.[15] It is well recognized that numerous cytokines have various roles in the diseases of oral cavity. In this study we aimed at comparing the level of salivary GM-CSF in patients with OSCC, OPMD and healthy control subjects and to find out whether GM-CSF can serve as a molecular marker for detecting OSCC and OPMD. And we found, significant increase in the concentration of salivary GM-CSF in both OSCC and OPMD patients than the healthy controls. Also, there is significant increase in GM-CSF in OSCC than in OPMD patients. We also found increased GM-CSF levels in males than females. This finding was also found consistent with observations of Dr. S R Prabhu.[16] With respect to habit, the levels of salivary GM-CSF were found highest in patients with habit of smokeless intake of tobacco. This was consistent with observation of Dr. S RPrabhu.[16] Habit of smokeless tobacco was found more prevalent than smoking. Also because it takes longer exposure with smoke to develop lesion (OPMD or OSCC) while it develops sooner due to local irritation for longer duration caused by placement of quid or chewing.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important hematopoietic growth factor and immune modulator. GM-CSF also has profound effects on the functional activities of various circulating leukocytes. It is produced by a variety of cell types including T cells, macrophages, endothelial cells and fibroblasts upon receiving immune stimuli. GMCSF can be produced by a wide variety of tissue types, including fibroblasts, endothelial cells, T cells, macrophages, mesothelial cells, epithelial cells and many types of tumor cells.[17] In these cells, bacterial endotoxins and inflammatory cytokines, such as IL-1, IL-6, and TNF-α, are potent inducers of GM-CSF.[17]Head and neck SCC cells produces higherlevel of GM-CSF that contain CD34 natural suppressor cells that inhibits activity ofintratumoral T-cells,[8] and produces nitric oxide and TGF-β[25]. This leads to increase metastasis, and chances of recurrence and thereby has poor prognosis.[8,9,10]GM-CSF are proangiogenic, proinflammatory cytokines, are elevated in the saliva of patients with OSSC and OPMD as compared to controls, which may have diagnostic and/or prognostic significance.[11,12] The fact that the same cytokines are significantly elevated both in OPMD and OSCC, these may serve as diagnostic marker of malignant transformation of oral potentially malignant disorders.[11]

In our study, the concentration of salivary GM-CSF was observed to be higher inpatients with OSCC than in patients with OPMD and healthy controls. This was inaccordance with the studies of Hamad AWR et al.[18] Although lot of work has been done on estimation of GM-CSF in serum and tissue specimen of OSCC patients, but very few studies have been conducted on salivary GM-CSF of OSCC patients.[10,12] On the other hand, not much work has been reported in serum / tissue /salivary GM-CSF in patients with OPMD.[19] In the present study, when the concentration of salivary GM-CSF was compared between the OSCC, OPMD and healthy control, it was found to be highest in OSCC, followed by OPMD and healthy control. The difference in the concentration of GM-CSF was found to be statistically significant (p-value <0.05).These results suggest that this proangiogenic, proinflammatory cytokineis elevated in the saliva of patients with OSCC and OPMD as compared to controls which may have diagnostic and/or prognostic significance.

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