(International Print/Online Journal)

SJIF IMPACT FACTOR: 5.565
PUBMED-National Library of Medicine ID-101739732

ISSN (Print): 2209-2870
ISSN (Online): 2209-2862

International Journal of Medical Science and Current Research (IJMSCR)
Available online at: www.ijmscr.com
Volume 4, Issue 4, Page No: 1272-1279
July-August 2021

IJMSCR

Role of chemerin in alteration of extracellular microenvironment and malignant transformation of Oral Submucous Fibrosis

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Type of Publication: Original Research Paper
Conflicts of Interest: Nil

Abstract

Context: Oral submucous fibrosis (OSMF) is a chronic disease affecting any part of the oral cavity leading to stiffness of oral mucosa. Due to a high rate of malignant transformation the condition has been described as a "potentially malignant disorder".The association of obesity and malignancies are documented. Extra fat in the body secretes number of chemicals called as adipokines which are multifunctional peptide hormones causing pleiotropic effects. Chemerin is one such adipokines which regulates tumor modulation, promotion and invasion.
Aims: This study was planned to assess the role and levels of chemerin in non-obese patients with different grades of OSMF and in OSMF patients with malignant changes.
Methods and materials: This is a cross sectional study with sample size of 42 non-obese OSMF cases. Patients were divided into Grade I to IV as laid by Andrade and Khanna. 5 subjects each in different grades of OSMF and 12 cases of OSMF with malignant changes were included. Body mass index (BMI) of all the subjects was measured. Age and sex matched 10 healthy controls without any tobacco habits or systemic diseases were studied.
Results: Chemerin values were least in controls 0.06 ± 0.01. It showed a high in grade I OSMF at 0.90 ± 0.39 and thereafter a drop to 0.57 ± 0.18 in grade II, 0.43 ± 0.19 in grade III and then a rise to 0.67 ± 0.41 in grade IV OSMF cases. However, the highest values were recorded for OSMF with malignant change cases at 1.07 ± 0.76.
Conclusion: Chemerin in blood decreases as OSMF progresses from early to advanced stage and then the levels suddenly increase as OSMF undergoes malignant transformation. Thus, levels of serum chemerin in OSMF might help in predicting the progression of disease and its susceptibility to undergo malignant transformation.

Keywords: Adipocytokines, Cancer promotion, Chemerin, Oral submucous fibrosis, Tumor angiogenesis

INTRODUCTION

Sirsat and Pindborg in 1966 described Oral submucous fibrosis (OSMF) as "an insidious chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although, not always preceded by vesicle formation, it is usually associated with juxta-epithelial inflammatory reaction followed by fibro-elastic change of the lamina propria with epithelial atrophy leading to stiffness of oral mucosa, causing trismus and inability to eat".[1]Due to a high rate of malignant transformation in cases of OSMF, the condition has aptly been described as a "potentially malignant disorder".[2] Malignant transformation of a lesion is multi-stage, multi-step process which requires several

molecular and cellular events to convert a normal cell into a neoplastic one.[3]

Further, the association of obesity and malignancies are quite largely being documented in literature. Some of the commonly associated cancers with obesity are breast (after menopause), bowel, womb, oesophageal, pancreatic, kidney, liver, upper stomach, gallbladder, ovarian, thyroid cancers, myeloma, and meningioma.Extra fat in the body doesn't just sits passively but acts as an active endocrine organ that secretes number of chemicals called as adipocytokines e.g. leptin, tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), angiotensin I and II, chemerin etc. These adipokines are biologically active multifunctional peptide hormones causing pleiotropic effects. They perform essential regulatory functions related to energy balance, immunity, tissue metabolism, inflammation, differentiation, tumor angiogenesis, progression and invasion. For the past few years these adipocytokines have been a center of appreciation and interest.

Chemerin is one such adipokines which has widely been studied in terms of tumor modulation, promotion and cellular invasion. Thus, a study was planned in non-obese patients, to investigate the role of cytokine (chemerin) andextracellular cellular matrix, in malignant transformation of OSMF - a prevalent oral precancerous condition inIndian subcontinent. The aim of the study was to compare the levels of chemerin in patients with different grades of OSMF and in OSMF cases with malignant changes.

METHODOLOGY:

The study was approved by the institutional ethical committee.The sample comprised of patients who were clinicaly and histopathologicaly diagnosed of OSMF and OSMF with early malignant changes.A detail information leaflet of the study was given to each participants and consent was obtained from all of them. The subjects were asked to complete a medical and dental questionnaire.

The OSMF patients were divided clinically into Grade I to IV as per criteria laid down by Andrade and Khanna.[4] 5 subjects each in different grades of OSMF from grade I to grade IV and 12 subjects of OSMF withmalignant changes were enrolled in the study. Body mass index (BMI) of all the subjects were measured based on World Health Organization guidelines and only non-obese cases were included in the study.Age and gender matched 10 healthy individuals,without any habit of chewing areca nut or tobacco and underlying systemic diseases, subjected for hematological investigations prior to minor dental surgical procedures were taken as controls.

Inclusion criteria

Clinical and histopathological confirmed cases of OSMF and OSMF with malignant changes. Patients with habit of chewing areca nut or one of its commercial preparations with presence of burning sensation, stiffness of buccal mucosa, vesicle formation, ulceration and blanching of oral mucosa were included in OSMF group.OSMF patients with malignant change who had recently been diagnosed with primary disease and had not received any prior treatment in the form of chemotherapy, radiotherapy, surgery or alternative remedies were included.

Exclusion criteria

Patients with any systemic disease, pre-obese (BMI 25-29) and obese (with BMI 30-40) were excluded. Carrying and lactating mothers were also excluded.

Sample collection

5 ml of peripheral blood was taken by standard venipuncture from ante cubital fossa of patients and controls. Samples were allowed to clot for 30-40 mins at room temperature. Subsequently it was centrifuged at 1500 rpm for 15 mins. Clear supernatant serum was pippetted and stored in aliquots at -20 degree centigrade until assayed. Haemolyzed samples were discarded. Repeated thawing and freezing was avoided.

Detection of chemerin level in serum samples

The serum levels of chemerin were measured using Cat No E1435Hu Human Chemerin Elisa which is a sandwich enzyme immunoassay for the quantitative measurement of human chemerin. The kit was provided by Insta Biotech, China. In this kit, the reagents provided were Standard solution, Standard diluents, pre-coated ELISA plates, Streptavidin-HRP conjugate, Biotin-conjugate anti-human chemerin antibody, Substrate solution and Stop solution. All reagents were brought to room temperature and prepared before use. The assay was performed at room temperature.

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Standard was added to standard wells and samples were added to pre-coated wells with chemerin monoclonal antibody. Biotin conjugated anti-human chemerin antibody was added that binds to the human chemerin. After 60 mins of incubation at room temperature, unbound biotin-conjugated anti-human chemerin antibody was washed away using freshly prepared PBS. Streptavidin-HRP conjugate was added that binds to the biotin-conjugated anti-human chemerin antibody. After incubation unbound Streptavidin-HRP was washed away with another wash of PBS. Substrate solution was then added and incubated for 10 mins at 37 degrees centigrade in a dark and cool place. Yellow coloration develops in the wells in proportion to concentration of human chemerin present in it.

The reaction was stopped by addition of acidic solution. Absorbance of resulting yellow product was measured at 450 nm filter. The absorbance was directly proportional to the concentration of chemerin in the sample. A standard curve was constructed by plotting absorbance values against standard chemerin concentration. Concentration of unknown samples was determined using the standard curve. Chemerin levels were determined as nanograms per ml of serum.

Data Analysis

Data collected was transferred into electronic format. IBMSPSS Statistics for Windows Version 24 was used for data analysis. Data was checked for normality using Shapiro Wilk Test. Since the data did not follow the assumptions of Normal Distribution, Non-Parametric tests were used. Chemerin values were compared between different groups using Kruskal Wallis ANOVA test. Individuals' groups were compared using Mann Whitney Test. Alpha error was set as 0.05 and p value less than 0.05 was considered statistically significant.

RESULTS:

The study population consisted of a total 42 subjects; with a mean age of 41.29 ± 13.28 yrs. Among them 13 (31%) were females and 29 (69%) were males.

Chemerin values were least in Control group 0.06 ± 0.01. It showed a high in grade 1 OSMF at 0.90 ± 0.39 and thereafter a drop to 0.57 ± 0.18 in grade 2 OSMF, 0.43 ± 0.19 in grade 3 OSMF and then a rise to 0.67 ± 0.41 in grade 4 OSMF cases. However, the highest values of chemerin were recorded for OSMF with malignant change cases at 1.07 ± 0.76. Kruskal Wallis ANOVA test showed a statistically significant difference between the mean chemerin values among different grades of OSF($p < 0.001$) as shown in Figure 1 (Graph) & Table 1.

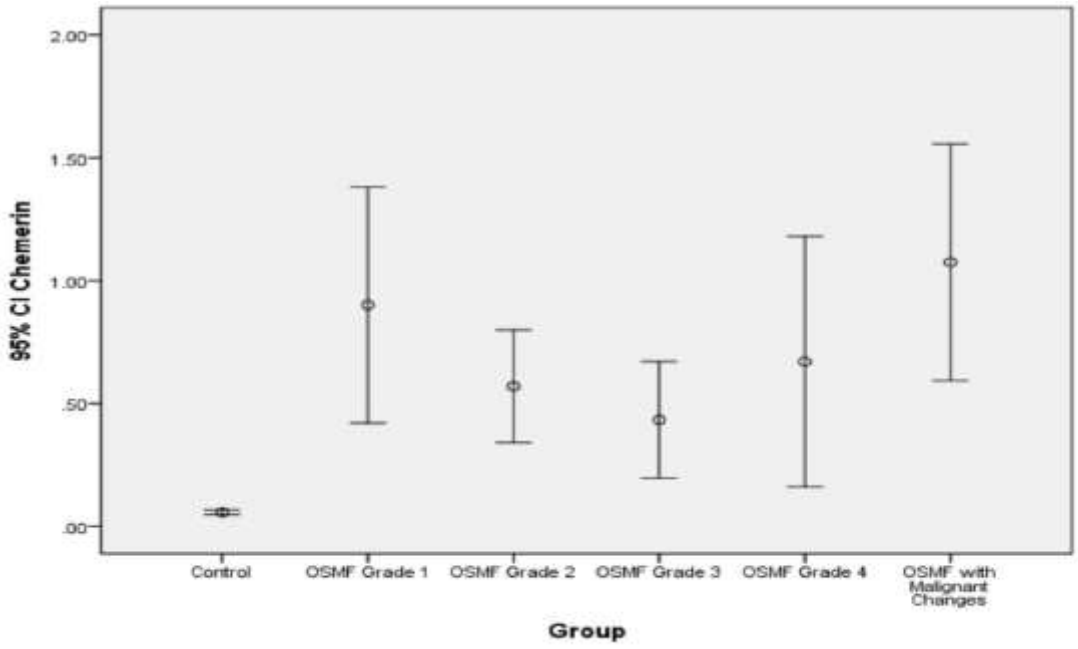

Figure-1: Graph showing levels of Chemerin and patients ofdifferent grades of OSMF and OSMF with malignant changes

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TABLES:

Table 1: Mean Chemerin value among different groups

	N	Mean	SD	Minimum	Maximum
Control	10	0.06	0.01	0.04	0.08
OSMF Grade 1	5	0.90	0.39	0.49	1.49
OSMF Grade 2	5	0.57	0.18	0.37	0.87
OSMF Grade 3	5	0.43	0.19	0.29	0.77
OSMF Grade 4	5	0.67	0.41	0.42	1.4
OSMF with Malignant Changes	12	1.07	0.76	0.31	2.28
Total	42	0.63	0.59	0.04	2.28
Kruskal Wallis ANOVA – p <0.001 ; Significant					

Intergroup comparison was done using Mann Whitney Test. The mean chemerin values of the control group was lowest and statistical significantly different from OSMF grade and OSMF with malignant change patients. The mean Chemerin levels were statistically similar to OSMF groups. There are a statistical difference in mean chemerin levels between OSMF Grade 1 and Grade 3 as shown in Table 2.

Table 2: Intergroup comparison of mean Chemerin values using Mann Whitney Test

	Control	OSMF Grade 1	OSMF Grade 2	OSMF Grade 3	OSMF Grade 4	OSMF with Malignant Change
Control		Sig	Sig	Sig	Sig	Sig
OSMF Grade 1	p = 0.002		Not Sig	Sig	Not Sig	Not Sig
OSMF Grade 2	p = 0.002	p = 0.117		Not Sig	Not Sig	Not Sig
OSMF Grade 3	p = 0.002	p = 0.028	p = 0.117		Not Sig	Not Sig
OSMF Grade 4	p = 0.002	p = 0.175	p = 0.917	p = 0.076		Not Sig
OSMF with Malignant Change	p < 0.001	p = 0.916	p = 0.673	p = 0.058	p = 0.673	

DISCUSSION:

OSMF carries a high risk of transition to oral cancer. In an epidemiological study over a period of 17 years in India, malignant transformation rate of 7.6% to 12% were evaluated for this precancerous condition.[3]Pathogenesis of OSMF is related to collagen-disorder which is induced by constant chewing of betel quid with nuts (Areca catechu). The alkaloids and tannins released from Areca nuts bring about proliferation of phenotypically altered fibroblasts resulting in qualitative alteration and quantitative accumulation of collagen[5]which undergoes decreased degradation. Further these chemicals also induceincreased cytokine productions in lamina propria which disrupts the equilibrium

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between matrix metalloproteinase (MMP) and tissue inhibitors of matrix metalloproteinase (TIMP) promoting increased and continuous deposition of extracellular matrix.[6]

A tumor needs constant support from its microenvironment to progress towards malignancy. The stromal cells and the extracellular matrix chiefly comprise this microenvironment. Stromal cell types are mainly inflammatory and immune cells, endothelial cells, pericytes and fibroblast cell lineage while the extracellular matrix (ECM) is mainly made up of collagen, proteoglycans and glycoproteins. It forms a physical and biochemical template for regulating organ development, tissue homeostasis, inflammation and disease progression. The ECM provides a critical tumor cellular ecosystem, which drives tumor cell growth, invasion, survival, as well as metastasis. Further, abnormal collagen deposition in tissue is a well-recognized ECM alteration in cancers. During tumor progression, matrix metalloproteinase (MMPs) are crucial for cleaving collagen, degrading and organizing the ECM that facilitates tumor cell migration.[7] Thus the study of tumor microenvironment acts as a key player in the development, progression and metastasis of tumor and is recently considered to be as important as the study of tumor cells per se.

Tumor angiogenesis is a fundamental property of cancer which is vital for tumor progression and metastasis. It is regulated by the production of angiogenic stimulators eg. fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) etc. Formation of new blood vessels withintumor is essential for providing nutrients to maintain tumor growth.

Besides this, new vessels also provide a route for cancer cells to exit the tumor and enter the circulation to be metastasized.Angiogenesisis measured by assessment of parameters like mean vasculature density (MVD) which in turn is assessed using important marker for tissue vascularisation e.g. CD34, CD31, CD105, VEGF, bFGF etc. In recent years, finding molecular markers using body fluid, such as saliva, urine, blood and others for detecting cancer, predicting prognosis, and monitoring disease progression has been over emphasized. A novel marker for angiogenesis which has hit the interest of researchers is serum levels of Chemerin protein, an adipocytokine.

Adipocytokines are cell signaling proteins that are usually secreted by adipose tissues. Leptin was the first adipokine discovered in 1994. Several other adipokines have been discovered in human viz: adiponectin, apelin, chemerin, interleukins, visfatin, retinol binding protein (RBP- 4), plasminogen activator inhibitor (PAI-1). Adipokines are usually either inflammatory mediators (e.g interleukins 6, interleukins 8), angiogenic proteins (e.g VEGF) and/or metabolic regulators (e.g. adiponectin, leptin).

Chemerin, also known as retinoic acid reactor responder protein-2(RARRES 2) is a recently discovered adipokine that has multiple roles in obesity and related complications. It helps in lipid metabolism, insulin signaling, inflammation, regulation of angiogenesis, cell proliferation and migration through its binding to ChemR23 receptor.[8] It is secreted as an inactive precursor protein pro-chemerin, which in turn is activated by an extracellular serine protease cleavage removing a C-terminal hexapeptide to liberate a 157 amino acid (16kDa) active form chemerin, found in blood. In humans, it is reported to be present at 3.4nM concentration in plasma and at 4.4 nM concentration in serum.[9]

The prevailing concept about vascularity in OSMF is that, as the disease advances, vascularity decreases resulting in epithelial atrophy because of lack of perfusion. Pandiar et al confirmed this theory by assessing the mean vasculature density (MVD) using endothelial markers such as CD34 and bFGF. They observed a statistically significant stepwise decrease in MVD as OSMF advanced from stage I to stage IV.[10] These findings were in accordance with the observations of Fang *et al.* and Singh *et al*. who did morphometric analysis of blood vessels in mucosa of OSMF patients and found that there is microvessel hyperplasia in early stages of OSMF but vascularity decreases markedly in stage III and stage IV.[11,12]

A highly vascular primary tumor will have a higher propensity for malignant transformation and metastasis as compared to a poorly vascular tumor. Thus, the vascular density is used as a prognostic indicator of metastatic potential of many tumors.[13]

Our findings were also consistent with these data. The chemerin levels in serum of OSMF patient decreased

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as the severity of the disease increased, suggestive of an overall decreased angiogenic state. Significant difference was found in chemerin concentration of normal control and in patients with increasing grades of OSMF.

Interestingly, in contradiction to this theory,in OSMF, as disease progresses, the blood supply/ angiogenesis decreases, yet this mucosal lesion has a high rate of malignant transformation. This suggest that some altered pathway of tumorogenesis involving some different chemical mediators- causing several cellular and molecular events, would be playing a role to suddenly change the previously benign and unsupportive microenvironment into an ECM that supports and promotes angiogenesis and tumor progression, so as to help proceed the condition to malignancy.

In support of this hypothesis it was observed that as OSMF turns dysplastic/ malignant, neoangiogenesis occurs. A significant increase in MVD was found by CD34 expression in cases of OSMF with dysplasia and cases of OSMF turning malignant.[10] The chemerin levels in present study were also found to be significantly increased in serum of OSMF patients with malignant changes.The sudden increased expression of chemerin may have occurred due to induction of altered cytokine milieu, critical for the kinetic and synthetic stimulation of activated ECM, supporting tumor progression.

The decreased vascularity seen in advanced stages of OSMF leads to transient ischemia/hypoxia that stimulates release of certain growth factors such as hypoxia inducible factor (HIF), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and basic fibroblastic growth factor (bFGF) which contributes in maintaining the vascularity of underlying connective tissue of OSMF. These growth factors help in angiogenesis by promoting proliferation and organization of endothelial cells into tube like structures.

bFGF has angiogenic properties and is highly mitogenic for a variety of cells. Bishen et al found that bFGF expression in ECM increases as OSMF progresses. bFGF may either directly stimulate endothelial cell proliferation or facilitate VEGF-endothelial cell interaction through the modulation of endothelial cell integrin or VEGF-receptor expression.[5]

The hypoxic microenvironment seen in advanced OSMF brings about cellular adaptations such as reflex angiogenesis, neovascularisation and increased glycolysis – all of which are correlated with tumour invasion and metastasis. The adaptation of cells to hypoxia appears to be mediated via hypoxia inducible factor-1α (HIF-1α). HIF-1α is said to be associated with malignant transformation of epithelium in OSMF patients.[3] The functional HIF-1 helps in cell survival under hypoxia and thus helps cell proliferation promoting tumorigenesis. However, a significant rise in the expression of HIF-1α was found in the basal and suprabasal layers of OSMF epithelium. This indicates the role of hypoxia in malignant transformation of OSMF. Thus, the up regulation of HIF-1α is an early event of carcinogenesis in OSMF patients.[3]

It has been proposed that hypoxia, in addition to promoting cell proliferation and tumorigenesis, also activates chemerin expression by inducing TNF-alpha expression which in turn induces early angiogenesis.[14]The overexpression of chemerin is significantly associated with tumor angiogenesis, poor tumour differentiation, lymph node metastasis, high clinical stage of disease and poor clinical outcomes for patients.[15]Ghallab et al found that in patients with OSCC, both serum and salivary levels of chemerin were significantly higher than chemerin levels in patients with oral premalignant lesions (OPMLs) and control group.[16]He considered chemerin as appropriate salivary diagnostic biomarkers for OPMLs, early detection of OSCC and also for detecting early cancerization in OPMLs.

In vitro studies of renal fibrosis have shown that hypoxia stimulates fibroblast proliferation. Hypoxia due to transient ischemia and decreased vascularity in advanced stages of OSMF may play a role in the progression of fibrosis in OSMF by the increased production of extracellular matrix (ECM).[17] The dense fibrosis and decreased vascularity of the corium, in presence of altered cytokine activity, creates a unique environment for carcinogens from tobacco and areca nut to act on the epithelium turning it dysplastic.[6]

As the epithelium acquires dysplastic features and invades the underlying connective tissue, the

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neoplastic cells create an environment that would favour liberation of angiogenic factors (e.g. VEGF, bFGF, chemerin etc.), neoangiogenesis and thus proliferation of tumor cells. This hypothesis possibly explains an increased serum chemerin level in patients with advanced OSMF, who revealed early invasive changes at sites undergoing dysplastic transformation.

CONCLUSION:

The results of our study indicated that chemerin levels in blood decrease as OSMF progresses from early to advanced stage and then the chemerin levels suddenly increase as OSMF undergoes malignant transformation. Thus, Chemerin in serum of OSMF patients might help in predicting the progression of disease and its susceptibility to undergo malignant transformation.

In order to establish standard serum chemerin level range for any such transformation, additional studies are proposed herewith with more number of patients of either gender in different grades of OSMF, OSMF with dysplasia and in OSMF with malignant transformation. Besides, looking into the role of adipocytokines in varied form of disease progression and its association with various cancers, further study can be looked forward with other adipokine than chemerin and its association with disease and/or cancers. These studies may offer novel options for diagnosis and timely therapeutic intervention.

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