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Vascular endothelial growth factor (VEGF) gene polymorphism in oral submucous fibrosis subjects - A preliminary study

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ABSTRACT

Background: Oral Submucous Fibrosis is one of the oral potentially malignant disorders presenting with progressive restriction in mouth opening. The condition having a high malignant transformation rate necessitates identification of biomarkers to be employed for early detection of malignant change. This will influence the prognosis in addition to adding better quality of life to patients. **Aims and Objectives**: To relate the association of VEGF -460C/T polymorphism in patients with OSMF and to compare the same among healthy subjects. **Materials and Methods:** Thirty patients with Oral submucous fibrosis and 20 controls free from habits and any form of lesions were included in the study. The polymorphism of VEGF gene was detected by polymerase chain reaction-based restriction analysis. **Results:** Sixty-four percent of the population in the study was in the age group of 21-40 yrs suggesting the prominence of disease in younger individuals with male predominance. With reference to polymorphism, 6.67% of the subjects from OSMF group showed CT polymorphism and 16.67% showed TT polymorphism. There were no statistically significant differences in the polymorphism between the study group and controls. However the frequency of T allele in the patient group 12 (20%) was greater than that in the control group 1 (2.5%), which was a significant finding. There was no association between the habits, frequency of habits, duration of quid placement, site of quid placement and style of chewing with the nature of polymorphism. **Conclusion:** VEGF 460C/T has the potential to be used as a prognostic marker in predicting the malignant transformation of OSMF.

Key words: VEGF gene polymorphism, OSMF, Oral cancer, Malignant transformation, Polymerase chain reaction

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) or oral cancer is considered to be the 6th leading cause of death in western world.[1] Among Southeast Asian countries, it accounts for about 1–4% of malignancies.[2] In India alone the mortality rate due to oral cancer accounts to 18.3% in males and 6.8% among females.[3] The diverse etiological factors include tobacco (smoking or chewing forms) in combination with areca nut or alone, alcohol, and viral association mainly, human papillomavirus (HPV).[4,5] Oral cancer involving a multistep process of carcinogenesis is frequently preceded by development of, 'oral potentially malignant disorders'.[6] Though the use of this term may not imply that all disorders described under this term may transform into cancer but the chances of malignant transformation is high.[6] Oneof the projected reasons for high mortality and morbidity rates associated with oral cancer is late diagnosis.[7] The challenge to overcome this is identification of newer techniques to diagnose the disease at the earliest stage. This is facilitated

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by advances in research, which highlights the emerging molecular and biochemical markers to be employed for early detection. These markers will provide the advantage for early detection of malignant transformation, and may also act as prognostic indicators for treatment outcomes.[8]

Basic fibroblast growth factor, placental growth factor, transforming Growth Factor and Vascular Endothelial Growth factor (VEGF) are few among the various tumor growth factors assessed cancer biomarkers to detect early changes.[9]

Adequate blood supply undoubtedly is an important part for the growth of solid tumors and growth factors regulating this have been studied extensively in various tumors.[10] Vascular endothelial cells are ordinarily dormant in adult humans and divide less than once per decade. During the growth phase of tumors, hypoxia ensues when the size reaches around 0.2-2 mm in diameter. In order to continue to increase in size, tumors undergo an angiogenic switch where in the action of pro-angiogenic factors predominate, resulting in angiogenesis and tumor progression. Following up-regulation of the hypoxia-inducible transcription factor, there is increased production of vascular endothelial growth factor (VEGF).[11] which is one of the critical angiogenic cytokines involved in the development of blood supply to neoplastic cells. Also there is a proven significant increase in vascularity, in the process of transition from normal oral mucosa, to degrees of dysplasia and to oral squamous cell carcinoma.[10] The possible role of VEGF has been investigated not only in angiogenesis in squamous cell carcinoma but also in disease progression in oral potentially malignant disorders and has also been linked to metastasis of oral cancer.[12]

Oral submucous fibrosis (OSMF) is one of the most debilitating oral potentially malignant disorders. The condition involves progressive restriction in mouth opening and progression to oral cancer.[13] OSMF is notoriously known for 7.5-10% of malignant transformation.[14] This undoubtedly necessitates identification of molecular markers for early detection of malignant transformation. The present study aims to link the association of VEGF -460C/T polymorphism in patients with OSMF in comparison with healthy subjects, to establish the role of VEGF gene polymorphism as a prognostic marker.

MATERIAL AND METHODS

The study included a total of 50 subjects from patients reporting to the Out-patient Department of Oral Medicine, of which 30 were diagnosed to have Oral submucous fibrosis and 20 were healthy controls (without habits and free from any lesions). Ethical clearance was obtained from Institutional Review Board. An informed written consent was obtained from all the participants before blood samples were drawn.

Inclusion criteria

Subjects willing to participate in the study with informed consent and diagnosed with clinical features of Oral submucous fibrosis like restriction in mouth opening, presence of palpable fibrous bands, in addition to burning sensation in the mouth on intake of spicy foods or normal food. In addition a detailed history in a questionnaire format was recorded regarding the duration of habits, frequency and type i.e. only tobacco chewing, areca nut or in combination, placement of quid in the mouth.

For clinical grading of the condition the classification proposed by More C B et al[15] was followed.

Control group included patients who were free from any adverse habits and lesions and willing to participate in the study with informed consent.

A detailed history was recorded and all subjects were screened for the presence of Oral sub mucous fibrosis. All the subjects consenting to participate in the study were subjected to punch biopsy for histopathological confirmation.

Exclusion criteria

Patients unwilling to consent for providing blood samples.

Patients already under treatment for oral submucous fibrosis.

Standardization of DNA extraction method

Prior to the actual assessment, standardization of the method of DNA extraction was done to determine the final sample size by collecting samples from 2 individuals (1 OSMF group and 1 control group). Peripheral blood of 15 µl was collected in an EDTA coated vacutainer. Genomic DNA was isolated from the samples using standard DNA isolation kit (QIAGEN DNeasy Blood and Tissue Kit, Cat. No.69506 QIAGEN USA) after standardizing the protocol to the laboratory conditions. Primers for VEGF gene were based on reports by Ku K T[16] et al. Polymerase chain reaction (PCR) mixture of 25 µl containing 0.2 µM forward and reverse primer each was prepared with 200 µM of dNTPs (NEB #NO447L), 1X Taq Buffer, 1 unit Taq enzyme (NEB #MO273L) and 1000 ng of DNA made with nuclease free water.

After the PCR amplification, the amplicons were run through 4% agarose gel electrophoresis and the DNA bands were observed in gel documentation (Figure 1).

The PCR product of 175 bp was mixed with 2U of reaction buffer according to the manufacturer's protocol. Restriction site located at – 460 bp upstream of exon 1 (C to T); Two fragments measuring 155bp and 20bp were the product for digestion. The reaction was incubated for 2 hr at 37°C and then checked for polymorphism. Restriction enzyme digestion was carried out by incubating at 37°C for 15 minutes All PCR products (10µl) were digested with 10units of BstUI (NEB # R0518)/Tth111I (NEB # R0185) restriction endonuclease enzyme. The restriction enzyme digestion was subjected to agarose gel electrophoresis (4% agarose was prepared in 1X TAE buffer of 300 ml). To this 30 µl of ethidium bromide was added. 5 µl of restriction enzyme digest product was mixed with 3 µl of loading dye bromophenol blue. The electrophoresis was conducted at a constant voltage of 100V for 60 minutes. The observed bands were analyzed under the gel documentation chamber (Figure 2).

Statistical analysis

The data was analyzed using chi-square test. The polymorphic nature of VEGF -460C/T gene was compared in both OSMF group and healthy controls.

RESULTS

The study group comprised of 28 males (93.33%) and 2 females (6.67%). There was no statistical significance in the distribution of cases and controls by sex. The age of the patients in the present study ranged between 15-70 yrs.

In the present study, 64% of the population was in the age group of 21-40 yrs with 4 (14%) of the cases below 20 yrs.

CT polymorphism of VEGF in OSMF group was observed in 2 (6.67%) subjects and TT polymorphism in 5 subjects (16.67%) - Table 1. In view of the current sample size, since p value < 0.05, findings are considered statistically significant.

The frequency of T allele in the patient group 12 (20%) was greater than that in the control group 1 (2.5%) (Table 2).

In the OSMF group 20% of cases associated with T allele in the current sample implying a statistically significant finding.

There was no statistically significant association found between the type of tobacco chewing i.e. tobacco alone, tobacco with betel quid, gutkha chewing or combination of these products and the type of polymorphism.

The duration of habits and polymorphism revealed that consumption of tobacco related products for a period ranging from 0-5 yrs did show CT and TT polymorphisms, 36.5% and 63.6%, respectively in the OSMF group. It was also strongly evident that the polymorphism was more significant in the advanced stages of OSMF than in the earlier stages. However, there was no significant association seen in between the association of habits, frequency of habits, duration of quid placement, site of quid placement and style of chewing with the nature of polymorphism.

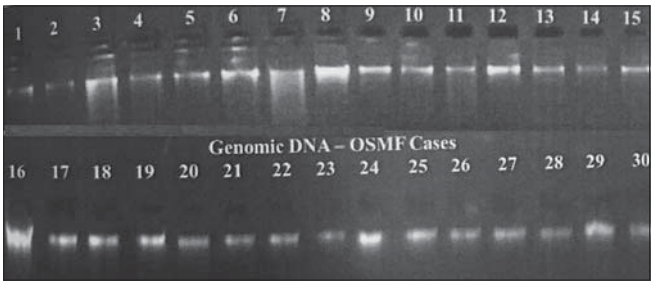

Figure 1: 1% agarose gel image of PCR products of OSMF samples. Line M – DNA ladder of 100bp. Line 1-14 – OSMF sample 1 to 14

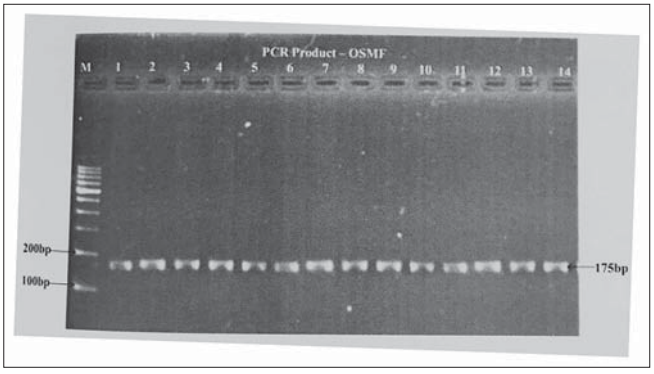

Figure 2: 4% agarose gel image of Restriction digestion PCR products with BstUI enzyme

DISCUSSION

Oral cancer is defined as a malignant neoplasm on the lip or in the mouth presenting more often in men than in

Table 1: Comparison of polymorphism among OSMF and control group

Polymorphism type	Cases (%)	Controls (%)
CC	23 (76.67)	19 (95)
CT	2 (6.67)	1 (5)
TT	5 (16.67)	0
Total	30 (100)	20 (100)

Chi square-5.6392, p-0.0596*

Table 2: Frequency of distribution of the alleles in OSMF and control group

Group	C allele (%)	T allele (%)
Control	39 (97.5)	1 (2.5)
OSMF	48 (80)	12 (20)

women. Tobacco use with or without betel leaf and areca nut in addition to other factors like genetic factors is a known etiological cause for oral cancer.[8]

The term Oral potentially malignant disorders (OPMDs) was introduced in the year 2007 to collectively refer to a group of disorders which are at a risk of turning into malignancies in future.[6] It has been emphasized that the role of dentists in early detection of these conditions with the use of modern diagnostic techniques is very crucial.[7]

Oral submucous fibrosis (OSMF) is one such OPMDs presenting as a chronic progressive disorder with limitation in mouth opening.[17] It was described as early as first described in the early1950s.[18] It is predominant in countries where the practice of habitual of areca nut chewing is common.[17] The main aetiological agent causing the disease is confirmed as arecoline in arecanut.

Areca-nut which is strongly associated with the disease is now considered as a group one carcinogen.[19] The condition is thought to be multifactorial in origin with a high incidence in people who chew areca-nut with our without tobacco.[20]

Of more concern is the fact, that there has been evidence of more invasive OSCC originating from OSMF which exhibits higher metastasis and recurrence rate than OSCC, which did not originate from OSMF.[19] Chaturvedi P et al[21] have proposed that oral cancers with OSMF constitute a clinico pathologically distinct disease.

Sarode SC et al[22] have hypothesized that in OSMF, the malignant transformed epithelial cells may retain the genetic memory of faster differentiation and maturation resulting in better grade of tumor differentiation therefore the well differentiated OSCC has good prognosis, better survival rate and less chances of recurrence of regional and distant metastasis if detected at an early stage. They also have suggested that studies are needed to explore the biomarkers or molecular markers associated with carcinogenesis like genetic instability, oncogenes, tumor suppressor genes and angiogenesis in OSCC associated with OSMF.

Specifically in conditions like OSMF obtaining a tissue biopsy is quite challenging owing to, pain and limited access due to restriction in mouth opening. The emerging trends to tackle the aforementioned hurdles include liquid biopsy[23] which is in the form of assessing various bodily fluids, such as saliva and plasma. Blood connects each human body part and carries different products/metabolites. The detection of circulating antigens may provide information regarding any disease that is present, including OSCC. Among the various markers which can be assessed; circulatory VEGF has been reported to be an important indicator for early detection of malignancy.[24]

Liquid biopsies could be used to guide cancer treatment decisions and perhaps even screen for tumors that are not yet visible on imaging.[25]

The identification of strong association of VEGF in tumor progression has paved a new way to explore the horizon. Numerous studies have confirmed that an altered VEGF protein expression and/or an altered VEGF related function may be important factor for development, invasiveness, and metastasis rate and treatment response of cancer in preclinical and clinical settings.[26-30]

Nayak S et al[31] in their study aimed to assess whether circulating VEGF A levels can be used as surrogate of VEGF- A expression in tissues in oral premalignant tissues. Their results indicate the serum levels of VEGF-A may serve as alternate for tissue expression.

The present study assessed the polymorphic nature of VEGF -460C/T gene in subjects with Oral submucous fibrosis in an attempt to identify the progression to malignancy at an early stage.

OSMF was seen at a higher preponderance in males i.e. 19 (93.33%) in the present study. This is in accordance with the results of Hazarey V K et al[32] andWahi et al[33] reporting male predominance of condition. This probably reflects, increased frequency of tobacco related habits in males than females in society. All the female subjects in the present study with OSMF were addicted to areca nut or betel quid, and none being addicted to gutkha. These findings are in accordance with the observations in the study conducted by Hazarey V K et al.[32]

The age range of participants in the present study was between 15-70 yrs and 64% (19) of the subjects were in the age group of 21-40 yrs suggesting that OSMF is more commonly seen in younger age group.

Strikingly, 14% of the cases in the present study were below 20 yrs of age, this could be attributed to the changing lifestyle of the younger generation, who are easily fall in to adverse habits unknowingly or due to peer group influence.

Agarwal S et al[34] have reported that the serum VEGF levels were significantly higher in oral cancer patients as compared to normal controls that further showed an increasing trend with clinical stage and lymph node involvement. It was significantly up regulated in tumor tissues and in OSCC cell lines. The study emphasized that serum VEGF levels is a reliable biomarker and a potential target for development

of chemopreventive and chemotherapeutic strategies for patients with tobacco-related oral carcinoma.

Angiogenesis being crucial for development and metastasis of tumors and VEGF is a key mediator of this process. Selected VEGF single Nucleotide Polymorphisms appear to be associated with risk of different types of cancer. Various studies have confirmed the expression of VEGF in tissues based on immunohistochemistry (IHC) in OSMF and its role in malignant transformation.[31,35]

Literature review suggests that the present study is first of its kind to assess the polymorphic nature of VEGF -460C/T gene from the blood samples of OSMF patients.

23 (76.67%) cases and 19 (95%) controls show CC genotype which indicate that most of them have a C/C homozygote in their VEGF -460 regions. However among the OSMF subjects, 2 (6.67 %) of them showed CT polymorphism and 5 (16.67%) subjects showed TT polymorphism. Subjects with TT homozygote in the VEGF 460 region are at a higher risk of developing Oral submucous fibrosis and can have a higher rate of malignant transformation.

Ku K T et al[16], highlighted that the "TT" homozygote genotype seems to be a significant risk factor for oral cancer, as it was the most common genotype found in patients with oral cancer. They also concluded that there was a much higher risk in developing oral cancer, especially in the presence of tobacco related habits and alcohol abuse for patients with T/T homozygote in their VEGF 460 region.[16]

The frequency of the "T" allele in the study group (20%) was greater than control group (2.5%). 20% of OSMF cases showed the presence of T allele in the study group which is a statistically significant finding. The risk of malignant transformation is higher in subjects with "T" allele. This is in accordance with the study conducted by Ku K T et al.[16]

In an attempt to relate the type of habits and polymorphism, it was observed in the present study that in patients who had mixed habits of chewing gutkha as well as betel quid and pan masala have only CC genotypes. The possible explanation for this could be that the commercially available gutkha products are concentrated; freeze-dried and have higher dry weight concentration of pathology causing irritants in comparison to the traditionally prepared products like panmasala.[36-38]

On the other hand the rich beta carotene content of betel leaf quench the free radicals that are known to be mutagenic which counteracts irritants.[39,40] In the present study, subjects with exclusively gutkha chewing habits- 2(10%) each were having CT and 2(10%) were having TT polymorphism, which indicates that only gutkha chewers are at a higher risk of OSMF and higher polymorphism rate and hence higher malignant transformation rate.

There was no significant association between the type of tobacco chewing, duration of habits and the type of polymorphism. Also noted was the fact that, as the span of chewing habits increased above 10 years, the severity of the disease also increased with maximum number of cases having grade III and grade IV OSMF.

In accordance to the proposed new staging of OSMF,[15] CT polymorphism was noted in grades S2M2 (14.3%), S3M2 (50%) and TT polymorphism was seen in grades S2M3 (50%), S3M3(42.9), S4M3(33.3%). Thus suggesting that polymorphism is evident at the later stages of the condition.

However further research is required to understand the possible role of VEGF-460C/T gene in malignant transformation of OSMF with a larger sample size and a long term follow up of these patients has to be done in order to determine the significance of VEGF as a prognostic marker.

CONCLUSION

Oral submucous fibrosis (OSMF) is a well-recognized potentially malignant disorder of the oral mucosa. Various studies had been conducted so far in order to identify the important aspects in malignant transformation of OSMF. Within the limitations of the present study it can be concluded that there is a strong correlation of VEGF gene polymorphism and OSMF. Future studies are essential with a larger sample size to reconfirm the association among different stages of OSMF to gain deeper insights into the field for better predicting of outcomes.

Limitations of the study- the present study being a preliminary study included a small sample of subjects. Future studies with a larger sample size or mutilcentric design may provide more insights into the disease process.

Inputs from the study – the present study is first of its kind with regards to assessment of VEGF gene polymorphism in OSMF subjects to be assessed from blood. The fact that polymorphism was noted in later stages supports the role VEGF as a biomarker for malignant transformation and also in the progress of OSMF stages.

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Authors Contribution:
KSG and DVR - Concept and design of the study, Manuscript preparation, Drafting and Revision of manuscript, Data Acquisition, Data Analysis, Editing and Final Approval; **BBK -** Data Acquisition, Data Analysis, Revision of Manuscript, Editing and Final Approval; **KNB and SH -** Concept and design of the study Editing and Final Approval.

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