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Original Research Article

Molecular Detection of Association of Vascular Endothelial Growth Factor (VEGF) Gene in Oral Sub Mucosal Fibrosis (OSF) Cancer

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ABSTRACT

Oral submucous fibrosis (OSMF or OSF) is a chronic, complex, premalignant (1% transformation risk) condition of the oral cavity, characterized by juxta-epithelial inflammatory reaction and progressive fibrosis of the sub mucosal tissues. A recent study shows strong association of VEGF gene in Oral Sub mucosal Fibrosis (OSF) Cancer. To know the association of VEGF gene polymorphism in patients with OSMF and to compare the same among healthy subjects. The study included a total of 50 subjects from patients reporting to the Out-patient Department of Dentistry, ENT of which 30 were diagnosed to have Oral submucous fibrosis and 20 were healthy controls (without habits and free from any lesions). Isolated deoxyribonucleic acid (DNA) samples from both were subjected polymerase chain reaction- based restriction analysis was carried out for VEGF gene. VEGF gene has to be used as a specific biomarker for the Oral submucous fibrosis Cancer (OSMF or OSF).

Key words: Oral submucous fibrosis Cancer (OSMF or OSF); Polymerase Chain Reaction; VEGF Gene.

INTRODUCTION

Oral submucous fibrosis (OSMF or OSF) is a chronic, complex, premalignant (1%Transformation risk) condition of the oral cavity, characterized by juxta-epithelial inflammatory reaction and progressive fibrosis of the sub mucosal tissues. Oral Sqamous Cell Carcinoma (OSCC) is one of the 6th leading causes 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% in females [3]. The diverse etiological factors included tobacco (smoking or chewing forms) in combination with areca nut or alone, alcohol [4, 5].

Oral cancer two to three times more prevalent in men than women in most ethnic groups the risk for developing oral cancer is 3 times higher in smokers compared with nonsmokers [6]. Angiogenesis is a fundamental prerequisite for the development, growth and progression of solid malignant tumors, such as oral sqamous cell carcinoma (OSCC) [7]. The cytokine vascular endothelial growth factor (VEGF) is the most important regulator of tumor angiogenesis, is over expressed in several tumor tissues and appears to be associated with the prognosis and outcome of various cancers [8]. Some of the studies revealed that 405GG & 1154GG were associated with higher levels of VEGF gene

in OSCC [9]. VEGF mRNA gene expression showed more than 50- fold increase in PMOLs and OSCC [10] Serum VEGF levels were significantly higher in oral cancer patients.

The present study aims to know the association of VEGF polymorphism in patients with OSMF in comparison with healthy subjects, to establish the role of VEGF gene polymorphism as a prognostic marker.

1. Procedure

A total of 50 subjects from patients reporting to the Out-patient Department of Dentistry, ENT of our hospital, 30 were diagnosed to have Oral sub mucous fibrosis and 20 were healthy controls (without habits and free from any lesions). All the subjects were interviewed with the help of a structured, pretested questionnaire. This was followed by clinical examination and relevant laboratory investigations. Ethical clearance was obtained from Institutional Review Board. An informed written consent was obtained from all the participants before blood samples would be taken.

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.

Sample collection

Consent was obtained from the subjects, who were enrolled in the study. After taking consent, 1 ml peripheral blood samples were collected in the EDTA coated vacutainers (BD367863) and stored at 4°C. Along with the patient blood samples, healthy control blood samples were also collected.

DNA extraction, polymerase chain reaction amplification, and molecular analysis

Genomic DNA was isolated from 300 µl of peripheral blood samples using a commercial DNA isolation kit (Bangalore Genei, India). All the isolated DNA samples were quantified using biophotometer (Eppendorf, Germany) and Nanodrop (Quawell). Exon-specific intronic primers were designed (Table.1) to cover full length of exon (Reference sequence: NG_011951.2), keeping the amplicon size appropriate for genetic analyzer (ABI 3500 × L) using primer-3 (Bioinformatics tool). Primers got synthesized by commercial oligo synthesizer (MWG Biotech, India). (Figure 1)

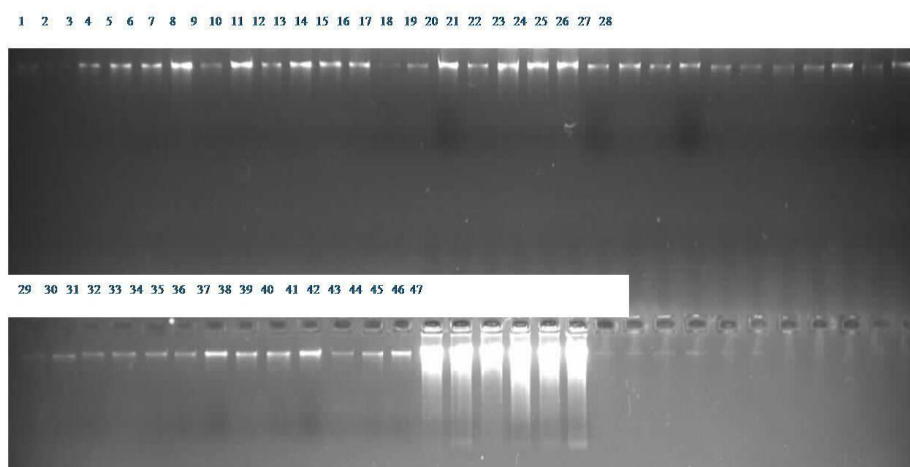

Fig 1: Genomic DNA Samples

Table 1. Details of the primer sequences and annealing temperatures used for the amplification of exon of VEGF gene.

Name of the primer	Sequence	Amplicon Size (Base Pairs)	Annealing Temperature
VEGF F	TGTGCGTGTGGGGTTGAGCG	**175**	**70.3**

PCR amplification was carried out in a 20µl reaction volume containing 0.5 µl of genomic DNA (75ng/µl to 150 ng/µl), 0.5µl of each primer (5pmol), 0.4µl of dNTP (10pmol), 0.2µl Taq DNA polymerases (3units/ µl), 4 µlTaq Buffer (5X) (BioRad, USA) and total volume was adjusted to 20µl using molecular biology grade water. Amplification was carried out in Master cycler gradient (Eppendorf, Germany) under the following conditions: an initial denaturation at 98^0C for 10sec, followed by 35 cycles at 98^0C for 10sec (cycle denaturation). Primer annealing temperature was set depending on the annealing temperature of each primer (Table-1) for 10sec 72^0C for 15sec (primer extension) and a final extension at 72^0C for 5 min. PCR products were confirmed for their respective amplicon size by gel electrophoresis with standard 100bp ladder. The PCR cycling conditions were as follows Initial Denaturation is 98^0C for 10 sec, Denaturation is 98^0C for 10 sec, Annealing is primer dependent for 10 sec, Elongation 72^0C for 5min & Hold at 4 ^{0}C. PCR products were confirmed for the irrespective amplicon size by gel electrophoresis with standard 100-bp ladder.

The PCR product of 175 bp as shown in table no 1 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 10 units 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 bands were observed under the gel documentation system (Figure 2)

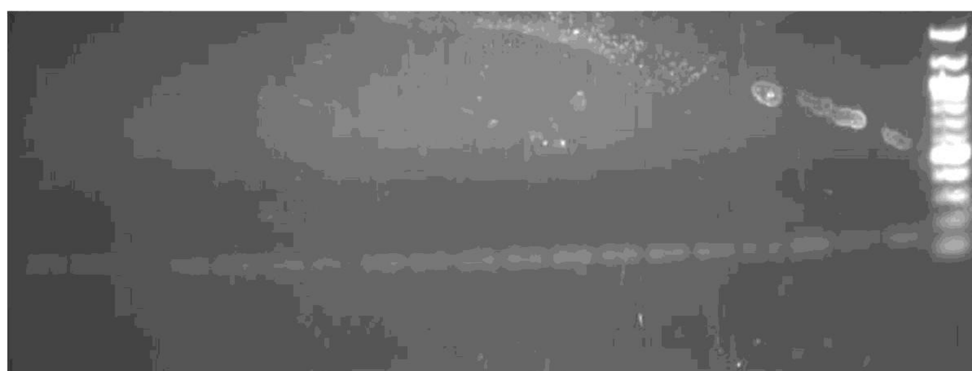

Fig 2: PCR Products with amplicon size 175

2. Data, Value and Validation

We have received total 50 samples, out of which 30 were diagnosed to have Oral submucous fibrosis and 20 were healthy controls (without habits and free from any lesions).

Out of 50 cases we received males were 90% and females were 10%. Out of 20 normal cases (control) males were 80% and females were 20%. Table no 2 shows the percentage of male and female cases. We have recorded the age of patients in table no 3. The maximum and minimum age of

patients from 63 and 28 in which the frequency of 20-39 age groups is 50%, 40-59 age group is 40% & 60-79 age group is 10%. In control the maximum and minimum age of infants are 63 and 28 in which the frequency of 20-39 age group is 60%, 40-59 age group is 30% and 60-79 age group is 10%.

Table2: Distribution of patients according to Gender.

UBJECTS	CASES		CONTROL		p value
	Frequency	Percent	Frequency	Percent	
MALE	27	90	16	80	
FEMALE	3	10	04	20	
TOTAL	30	100	20	100	0.308

Table 3: Distribution of patients according to Age

AGE Group	CASES		CONTROL		p value
	Frequency	Percent	Frequency	Percent	
20-39	15	50	12	60	0.757
40-59	12	40	06	30	
60-79	03	10	2	10	

Table 4: Distribution of Polymorphism in Cases & Controls

Polymorphism type	Cases (%)	Controls (%)	p value
CC	25(83.3)	18(90)	
CT	1(3.33%)	2(10)	0.365
TT	4(13.33%)	0	

There was no correlation between cases & sex of the patients. The age of the patients of our study ranges from 28-65 yrs. VEGF in OSMF group was observed CT polymorphism in 1 (3.33%) case and 4 cases (13.33%) TT polymorphism (Table.4). By looking at our sample size, since p value < 0.05, findings are considered statistically significant.24% of OSMF group cases associated with T allele in the current sample implying a statistically significant finding.

Our result analysis shows 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 period of habits and polymorphism revealed that consumption of tobacco related products for a period ranging from 5-10 yrs did show CT and TT polymorphisms, 35.5% and 62.1%, 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.

Oral Cancer is also known as mouth cancer is a type of head and neck cancer and is any cancerous tissue growth located in the oral cavity. It will affect both men & women. It occurs due to consumption of tobacco that may be chewing or smoking. Genetic factors also plays very important role in oral cancer [11].

According to epidemiological data of 2015 revealed that oral cancer resulted in 146,000 deaths up from 84,000 deaths in 1990 [12] Five-year survival rates in the United States are 63% [13]. The term Oral potentially malignant disorders (OPMDs) were introduced in the year 2007 to collectively refer to a group of disorders which are at a risk of turning into malignancies in future [14].

Areca-nut which is strongly associated with the disease is now considered as a group one carcinogen [15] the condition is thought to be multi factorial in origin with a high incidence in people who chew areca-nut with or without tobacco [16].

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. Chaturvedi et al [17] have proposed that oral cancers with OSMF constitute a clinico pathologically distinct disease.

Sarode SC et al [18] 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.

Liquid biopsies plays vital role in the treatment of cancer and even it will help to screen for tumors [19].

The innovative identification revealed that strong association of VEGF in tumor progression. Most of the studies have shown VEGF protein expression 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 [20].

Nayak S et al [10] in their studied indication of serum levels of VEGF-A may serve as alternate for tissue expression.

Our aim of the study to know 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.

Incident rate OSMF seen in males i.e. 27 (90%) in our study. This is in comparison with results of Hazarey et al [21] and Wahi et al [22] reporting male predominance of condition. This is due to 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.

In our study none of the cases were below 20 yrs of age, all of the cases were above 25yrs of age. 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 [23].

According to Ku et al [24] their study revealed that ‘‘TT’’ homozygote genotype is risk factor for oral cancer, as it was the most common genotype found in patients with oral cancer. They also conclude their results by stating 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 VEGF460 region [25].

Our study with exclusively gutkha chewing habits- 1(10%) each were having CT and 4 (13.33%) were having TT polymorphism, which shows only gutkha chewers are at a greater risk of OSMF and greater polymorphism rate and hence rate of malignant transformation is high.

Our study also shows there was no strong association between the type of duration of habits, tobacco chewing and the type of polymorphism. Important fact is span of chewing habits increased above 10 years, the severity of the disease also increased with maximum number of cases having stage III and stage IV OSMF.

CONCLUSION

Oral Submucous Fibrosis (OSMF) is a malignant disorder of the oral mucosa. Most of the studies have been done to identify the important aspects in malignant transformation of OSMF. Within the limitations of our 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 see the association among different stages of OSMF to gain deeper insights into the field for better predicting of outcomes.

Conflict of Interest: Declared nil

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REFERENCES

1. J.O. Boyle, E.W. Strong, Oral cavity cancer, Cancer of the Head and Neck (ACS Atlas of Clinical Oncology) (2011) London B C Decker, pp 100.
2. R. Radhakrishnan, S. Kabekkodu and K. Satyamoorthy, DNA hypermethylation as an epigenetic mark for oral cancer diagnosis, J Oral Pathol Med. 40 (2011) 665–676.
3. World Health Organization - Cancer Country Profiles. [home page of WHO] Cancer country profiles [ONLINE] 2014 [cited in 2014] available from: http://www.who.int/cancer/ country-profiles/ind_en.pdf?ua=1.
4. V. Kadashetti, M. Chaudhary, Patil et al., Analysis of various risk factors affecting potentially malignant disorders and oral cancer patients of Central India, J Can Res Ther. 11 (2015) 280- 286.
5. K.L. Kumaraswamy, M. Vidhya, Human papilloma virus and oral infections: An update, J Can Res Ther. 7 (2011) 120-127.
6. S. Gandini, E. Botteri, S. Iodice et al., Tobacco smoking and cancer: a meta-analysis, Int J Cancer. 122(2008) 155–164.
7. W. Renner, S. Kotschan, C. Hoffmann et al., A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels, J Vasc Res. 37 (2000) 443–448.
8. S. Johnstone, R.M. Logan, Expression of vascular endothelial growth factor (VEGF) in normal oral mucosa, oral dysplasia and oral sqamous cell carcinoma, Int J Oral Maxillofac Surg. 36 (2007) 263–266.
9. R. Gunesacar, E. Erken, H.T. Ozer et al., Analysis of vascular endothelial growth factor gene 936 C/T polymorphism in patients with familial Mediterranean fever, Int J Immunogenet.35 (2008) 33-6.
10. S. Nayak, M.M. Goel, S. Chandra et al., VEGF- A immune Histochemical and mRNA expression in tissues and its serum levels in potentially malignant oral lesions and oral squamous cell carcinomas, Oral Oncology. 48 (2012) 233-239.
11. R. Mishra, Biomarkers of oral premalignant epithelial lesions for clinical application, Oral Oncol. 48 (2012) (48) 578-584.
12. GBD 2013 Mortality Causes of Death Collaborators (2015). "Global, regional, and national age–sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study. The Lancet 2013;(385) :(9963)
13. “SEER Stat Fact Sheets . Oral Cavity and Pharynx Cancer".2014 NCI. Retrieved.
14. S. Warnakulasuriya, N.W. Johnson, I. Van der Waal, Nomenclature and classification of potentially malignant disorders of the oral mucosa, J Oral Pathol Med. 36 (2007) 575-580.
15. F. Guo, X.C. Jian, S.H. Zhou et al., A retrospective study of oral squamous cell carcinomas originated from oral submucous fibrosis, Zhonghua Kou Qiang Yi Xue ZaZhi. 46 (2011) 494- 497.
16. I.R. Kramer, J.J. Pindborg, V. Bezroukov et al., Guide to epidemiology and diagnosis of oral mucosal diseases and conditions. World health organization, Community Dent Oral Epidemio. 8 (1980) 1–26.
17. P. Chaturvedi, S.S. Vaishampayan, S. Nair, D. Nair et al., Oral squamous cell carcinoma arising in background of oral sub mucous fibrosis: a clinic pathologically distinct disease, Head Neck. 35 (2013) 1404-1409.
18. S.C. Sarode, G.S. Sarode, Better grade of tumor differentiation of oral squamous cell carcinoma arising in background of oral sub mucous fibrosis, Med Hypotheses. 81 (2013) 540- 543.
19. N. Karachaliou, C. Mayo-de-Las-Casas, M.A. Molina-Vila et al., Real-time liquid biopsies become a reality in cancer treatment, Ann Transl Med 3 (2015) 36.
20. A.Z. Dudek, H. Mahaseth, Circulating angiogenic cytokines in patients with advanced non- small cell lung cancer: correlation with treatment response and survival, Cancer Invest 23 (2005) 193–200.
21. V.K. Hazarey, D.M. Erlewad, K.A. Mundhe et al., Oral sub mucous fibrosis:

study of 1000 cases from central India, J Oral Pathology Med. 36(2007) 12–17.

22. P.N. Wahi, V.L. Kapoor, U.K. Luthra et al., Submucous Fibrosis of the oral cavity: 2. Studies on Epidemiology, Bull WHO. 35 (1966) 793-799.
23. S.D. Rajiv, G.S. Mamatha, J.K. Musarrat et al., Immuno Histochemical Expression of Vascular Endothelial Growth Factor (VEGF) and its Possible Role in Tumour Progression during Malignant Transformation of Atrophic Epithelium in Oral Submucous Fibrosis, Current Angiogenesis. 4 (2015) 347-353.
24. K. Ku, L. Wan, H. Peng et al., Vascular endothelial growth factor gene-460 C/T polymorphism is a biomarker for oral cancer, Oral Oncology. 41(2005) 497–502.
25. S. Sirsat, V. Khanolkar, The effect of arecoline on the palatal and buccal mucosa of the Wistar rat: An optical and electron microscope study, Indian Journal of Medical Sciences. 16 (1962) 198-202.

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