Single nucleotide polymorphisms and serologic levels of hypoxia-inducible factor1 α and vascular endothelial growth factor are associated with increased risk of oral submucous fibrosis in gutka users among a North Indian population

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Objectives. Tissue hypoxia in oral submucous fibrosis (OSMF) induces hypoxia-inducible factor (HIF)-1 α and vascular endothelial growth factor (VEGF), causing angiogenesis. Single nucleotide polymorphisms (SNPs) may predict susceptibility to environmental carcinogens and to development of OSMF, as well as its severity and malignant transformation. This study aimed to determine the serologic levels and frequencies of SNPs of HIF-1 α and VEGF in OSMF.

Study Design. In this prospective pilot study, the frequencies of SNPs of HIF-1 α (C1772 T, G1790 A); VEGF-A 936 C/T; and VEGF-C (rs7664413, rs1485766) in patients with OSMF or oral squamous cell carcinoma (OSCC) and in healthy controls were determined by using polymerase chain reaction (PCR) (n = 100 each), and serologic levels were determined by using enzyme-linked immunosorbent assay (ELISA (n = 50 each), in a North Indian population.

Results. Heterozygous forms of HIF-1 α C1772 T (*CT:* odds ratio [OR] 5.0; 95% confidence interval [CI] 2.24−11.16; $P < .001$); HIF-1 α G1790 A (*GA:* OR 2.8; 95% CI 1.62−5.16; $P < .001$); and VEGF-C rs1485766 (*AC:* OR 2.18; 95% CI 1.19−3.99; $P < .05$) were associated with OSMF. The mean serologic levels of HIF-1 α, VEGF-A, and VEGF-C were significantly raised in patients with OSMF compared with healthy controls ($P < .001$).

Conclusions. The SNPs of HIF-1 α, VEGF-A, and VEGF-C and their serologic levels can act as prognostic biomarkers and aid in the development of specialized anti-HIF-1 α or anti-VEGF drugs for the management and prevention of OSCC in patients with OSMF.
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Oral submucous fibrosis (OSMF) is considered a high-risk precancerous condition.[1] Its prevalence in India has been steadily increasing over the past 4 decades, from 0.03% to 6.42%.[2] OSMF is a chronic insidious disease characterized by rigidity and submucosal fibrosis of the oral mucosa and sometimes the oropharynx, leading to progressive and irreversible trismus, burning sensation, and dysphagia.[3] The malignant transformation rate of OSMF into oral squamous cell carcinoma (OSCC) in Indian patients has been reported to be as high as 7.6% in a 17-year follow-up study.[4] OSCC is the most common oral cancer in the Indian population, and the concomitant presence of OSMF has been reported to be as high as 25.77%, indicating that the malignant transformation rate of OSMF has been significantly underestimated.[5] Patients with OSMF have to be under regular follow-up because there is currently no reliable biomarker to predict the malignant potential of OSMF, and there is a lifelong risk of malignant transformation.

OSMF is associated with habitual consumption of various forms of areca nut and tobacco. According to the recent Global Adult Tobacco Survey-2 (GATS-2) (2016−2017) the consumption of areca nut (5%−8%) and tobacco products (28.6%) is very high in the Indian population. India is the largest consumer of areca nut and areca nut−containing products (variously referred to as *pan masala, mawa, gutkha, supari, kharra, misri*, or *gudakhu*, as well as mouth fresheners, and betel quid with/without tobacco/slaked lime/catechu) in the world.[6] Gutka, a commercial betel quid substitute, a flavored and sweetened dry mixture of areca nut, catechu, slaked lime, and tobacco, is very popular among the Indian population because of its ready availability. The low cost, long shelf life, and colorful attractive

Statement of Clinical Relevance

Raised serologic levels and increased frequencies of some single nucleotide polymorphisms of hypoxia-inducible factor-1 α and vascular endothelial growth factor (VEGF)-C and VEGF-A, increase susceptibility to the development of oral submucous fibrosis in gutka users of North Indian ethnicity.

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packaging of the product has caused an increase in its consumption, especially among children and young adults. The increased prevalence of OSMF in the Indian population and the high rate of malignant transformation may be attributed to the synergistic effect of the carcinogens in tobacco and areca nut that are present in gutka.[2] The areca nut constituents (the alkaloids, polyphenols, and tannins) have carcinogenic potential and are responsible for the changes in the oral mucosa in patients with OSMF. The most abundant and widely studied alkaloid is arecoline, which has been shown to be cytotoxic, genotoxic, and mutagenic, and it induces inflammation and affects the epithelial cells, fibroblasts, and endothelial cells in a time- and dose-dependent manner, causing fibrosis and malignant transformation in OSMF. The underlying submucosal fibrosis in OSMF causes obliteration of the blood vessels and hypoxia of the tissues, leading to the induction of hypoxia inducible factor -1 α(HIF-1 α) and angiogenesis through upregulation of vascular endothelial growth factor (VEGF).[7,8]

HIF-1 α is a transcriptional factor that regulates oxygen homeostasis and is induced in hypoxic cells with oxygen concentration less than 6%. HIF-1 α plays an important role in tumor development, tumor susceptibility, tumor size, progression, lymph node metastasis, and prognosis by upregulating genes that are involved in cancer development and those that allow metabolic adaptation to hypoxia, such as VEGF.[9] HIF-1 α activates genes that are involved in glucose uptake and metabolism, angiogenesis, cell proliferation, differentiation, and apoptosis and is upregulated in OSMF and OSCC.[10-12]

VEGF, an angiogenic/lymphangiogenic factor with high expression levels in tumor tissues, plays a crucial role in tumor angiogenesis by increasing blood vessel permeability and endothelial cell growth, proliferation, migration, and differentiation. It may also facilitate extravasation of tumor cells and thereby the formation of metastases by degrading the tumor marginal extracellular matrix via activation of proteolytic enzymes. VEGF is upregulated in OSMF and in OSCC, where it is associated with tumor stage, lymph node metastasis, and a poor prognosis.[13-16]

The development of OSMF and OSCC is a multistep process that involves not only environmental factors, such as areca nut, tobacco, and alcohol, but also intrinsic factors, such as host genetic susceptibility. Observational studies have shown that many individuals do not develop OSMF/OSCC despite their areca nut and tobacco habits, that not all OSMF transform into OSCC, and that malignant transformation can occur several years after stopping the habit, and this could be attributed to genetic variation and ethnicity.[4,9,17]

Single nucleotide polymorphisms (SNPs) are one of the most common types of genetic variation that can alter protein expression, and this could be biologically significant and could account for heterogeneity in disease risk and outcome. Several SNPs of HIF-1 α (C1772 T and G1790 A), VEGF-A 936 C/T, and the *VEGF-C* gene (rs7664413, rs2046463) have been implicated in increased susceptibility to OSCC, poor survival rates, vascular invasion, and increased tumor size.[11,13,18-20] The results have shown differences in European and Asian populations, indicating that SNPs and OSCC risk modifiers are affected by ethnicity. There is only 1 reported study in OSMF related to VEGF-A 460 CT in Indian patients. The association of SNPs of HIF-1 α (C1772 T and G1790 A), VEGF-A 936 C/T, and the *VEGF-C* gene (rs7664413, rs2046463) with OSMF has not been reported before.

MATERIALS AND METHODS

A prospective case-controlled pilot study was conducted over a period of 2 years with the objective to determine the frequencies of SNPs of HIF-1 α (C1772 T and G1790 A), VEGF-A 936 C/T, and the *VEGF-C* gene (rs7664413, rs2046463) and the serologic levels of HIF-1 α, VEGF-A, and VEGF-C in OSMF. Adult patients of North Indian ethnicity, with clinically and histologically confirmed diagnosis of OSMF (n = 100) and OSCC (n = 100) were consecutively recruited from the Oral Medicine and Radiology outpatient department. Age- and gender-matched healthy controls (n = 100) were also recruited from among patients reporting to the outpatient department for other routine dental problems but not any oral potentially malignant disorders (OPMDs)[21] or OSCC. Exclusion criteria were a history of significant and serious uncontrolled systemic disease, a history of any malignant disease, pregnancy, and conditions known to have elevated levels of HIF-1 α and VEGF, such as rheumatoid arthritis, diabetic retinopathy, age-related macular degeneration, endometriosis, ovarian hyperstimulation syndrome, psoriasis, cardiovascular ischemia, recent trauma, or major surgery. The demographic characteristics, risk factors, clinical characteristics, and staging/grading of OSMF[22] and OSCC (according to the tumor−node−metastasis [TNM] classification) were recorded as per the prepared proforma for each group. Routine blood investigations were done before incisional biopsy and histopathologic confirmation of the diagnosis. The hematoxylin and eosin−stained histopathologic features were recorded and grading done for each study group per the standard World Health Organization criteria. For both study and control groups, 3 mL of peripheral blood was collected in EDTA (ethylenediaminetetraacetic acid) and plain tubes, respectively, and coded before analysis. Ethical clearance from Institute Ethics Committee and informed written consent were obtained before recruitment of study and control

patients and STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines were adhered to in reporting of this study.

Polymerase chain reaction analysis for SNPs

For the polymerase chain reaction (PCR) analysis, 3 mL blood was diluted with phosphate-buffered saline in 1:1 ratio and then layered on Ficoll gradient histopaque in a 2:1 ratio. Peripheral blood mononuclear cells were isolated by using Ficoll-Hypaque density gradient centrifugation method. Genomic DNA were extracted from peripheral blood mononuclear cells using DNA isolation kit (Quiagen, Germantown, MD), based on the phenol chloroform/isoamyl method. DNA was then quantified by using the Nano Drop spectrophotometer, and purity was determined by the 260:280 and 260:230 ratios. For the analysis, primers were designed by using the Primer3 software. To determine the specificity of selected primers, basic local alignment search tool was done to align the primers with the genome sequence in the database, and the specificity of the sequences was checked. Annealing temperature of the primers was standardized by putting up gradient PCR at temperatures 5 degrees higher and 5 degrees lower than the melting temperature of the primers, and a 20 μL reaction was set up for PCR analysis. The thermal cycling conditions for initial denaturing was 1 cycle at 95˚C for 5 minutes followed by 35 cycles at 95˚C for 30 seconds for denaturing; this was followed by 35 cycles at 56˚C to -62˚C for 30 seconds for annealing and then 35 cycles at 72˚C for 30 seconds for extension. Then 2% agarose gel electrophoresis was performed to check for product formation and to select the annealing temperature giving maximum intensity of the desired product size. After determining the annealing temperature by gradient PCR, similar reactions were put in all the study patients for all the genes. The protocol and the conditions were same as those used for gradient PCR. The PCR products were subjected to digestion by restriction endonucleases specific for each polymorphism. The reaction was incubated at 37˚C overnight and then run on 1.2% to 2% agarose gel to determine the polymorphism haplotype. Allele-specific PCR was performed for 1 polymorphism in which PCR conditions were similar as in PCR, but 2 sets of primers were used, one set corresponding to wild type allele and other specific for mutant type allele.

Enzyme-linked immunosorbent assay for serum levels

Serum was collected by centrifuging the blood collected in plain uncoated vial at 3000 rpm for 10 minutes. High-sensitivity commercially available enzyme-linked immunosorbent assay (ELISA) kits (Ray Biotech, Peachtree Corners, GA) were used for determining the levels of HIF-1 α, VEGF-A, and VEGF-C in serum samples of the study patients by using the standard protocol. The color development was terminated by adding acid, and absorbance was measured at 450 nm. A reference curve was obtained by plotting the different concentrations of standard samples versus absorbance, and levels of the antigens in the tested samples were calculated by the standard plot.

Statistical analysis

Mann-Whitney, Kruskal-Wallis, and Pearson's χ^2 tests were used to determine the differences in patient demographic, clinical, and histologic characteristics and SNP frequencies between the groups. Fisher's exact test was applied, if necessary. All P values were calculated at significance levels set at .05. Multinomial logistic regression analyses were done to determine the odds ratio at 95% confidence intervals to identify the association of the polymorphisms with the risk of OSMF and OSCC. STATA software version 12 (StataCorp, College Station, TX) was used to analyze the data.

RESULTS

The comparison of demographic characteristics, risk factors, and clinical and histologic characteristics of the 3 groups is shown in Table I. There were no significant differences in mean age and gender distribution among the 3 groups. The patients in all the 3 groups had a history of harmful habits in some form or other, only 8% in the control group reported having no harmful habits. The most common habit in patients with OSMF was use of gutka (72%), whereas the most common habit in patients with OSCC was tobacco use (52%). Upon comparing the duration of exposure to various habits between the OSMF and control groups, it was found that there was no significant difference, except for the use of gutka. There was significant difference between OSMF and OSCC group in the duration of exposure to gutka, bidi, and alcohol. The most common habit in patients with OSCC with OSMF was use of smokeless tobacco, (59%), use of gutka (56%), and smoking tobacco (34%), whereas the most common habit in patients with OSCC but without OSMF was smoking tobacco (51%), use of smokeless tobacco (47%), and alcohol consumption (23%). Interestingly, the gutka habit was seen in only 20% of the patients in this group. OSMF was present in 32% of patient with OSCC, with functional grading of "moderate" (16%) or "advanced" (11%). In OSMF, the functional grading was mostly "early" (57%) and "moderate" (30%).

The frequencies of SNPs of HIF-1 α VEGF-A, and VEGF-C are listed in Table II. There were significant differences in the frequencies of SNPs of HIF-1 α C1772 T and HIF-1 α G1790 A between

Table I. Comparison of demographic characteristics, risk factors, clinical, and histologic characteristics in patients with oral submucous fibrosis or oral squamous cell carcinoma and controls

Variable	*Patients with OSMF (N = 100)*	*Patients with OSCC (N = 100)*	*Controls (N = 100)*
Age (years) (mean, range)	36.08 (19−71)	47.49 (26−74)	38.26 (18−72)
Gender			
Male	80	87	84
Female	20	13	16
Habits			
Tobacco	42	52	31
Gutka	72	32	24
Areca nut	18	8	10
Betel quid	21	16	16
Bidi	9	35	8
Cigarette	17	12	16
Alcohol	8	23	6
Other OPMDs			
Oral leukoplakia	22	12	–
Oral lichen planus	8	0	–
Erythroplakia	1	1	–
OSMF	100	32	–
OSMF functional grading[22] (M)			
M1	20	0	–
M2	37	5	–
M3	30	16	–
M4	13	11	–
Histologic features in OSMF			
Mild dysplasia	12	–	–
Moderate dysplasia	3	–	–
TNM Staging in OSCC			
Stage I	–	3	–
Stage II	–	16	–
Stage III	–	23	–
Stage IVA	–	47	–
Stage IVB	–	11	–
Histologic features in OSCC			
Well differentiated	–	25	–
Moderately differentiated	–	56	–
Poorly differentiated	–	19	–

OSMF, oral submucous fibrosis; *OSCC,* oral squamous cell carcinoma; *N,* total number of patients; *OPMDs,* oral potentially malignant disorders; *M,* interincisal mouth opening; *M1,* ≥ 35 mm; *M2,* 25−34 mm; *M3,* 15−25 mm; *M4,* < 15 mm.

the OSMF and control groups and the OSCC and control groups ($P < .001$). OSCC and OSMF showed increased frequency of the heterozygous allele of HIF-1 α C1772 T (CT) compared with controls. The homozygous mutant allele (TT) was found in only 8 of the study patients. Similarly, heterozygous allele of HIF-1 α G1790 A SNP (GA) was found to be associated significantly ($P < 0.0001$) with OSMF and OSCC with relative risk of 2.8 and 3.88, respectively, compared with controls.

There was significant difference in the frequencies of SNPs of VEGF-A 936 C/T between the OSCC and control groups ($P < .01$) but no significant differences were found between the OSMF and OSCC or control group. The heterozygous form of VEGF-A 936 C/T (CT) was significantly increased in OSCC in comparison with controls, with the relative risk of 3.05 ($P < .01$). The heterozygous allele of VEGF-A 936 C/T (CT) was also increased in OSMF with respect to controls, but the result was nonsignificant. The homozygous mutant allele (TT) was found in only 3 of the study patients.

There were significant differences in the frequencies of SNPs of VEGF-C rs1485766 between the control group and the OSMF and OSCC groups ($P < .001$) but not between the OSMF and OSCC groups. Both heterozygous and homozygous mutant alleles (AC, CC) in OSMF showed relative risk of 2.18 and 6.62, respectively, whereas in OSCC, the relative risk was observed to be 2.87 and 11.25, respectively.

VEGF-C rs7664413 SNP was studied by using allele-specific PCR, but not PCR-restriction fragment length polymorphism because restriction enzymes were not present at this polymorphism. With use of

Table II. Frequencies of genotype of single nucleotide polymorphisms of HIF-1 α, VEGF-A, and VEGF-C in patients with oral submucous fibrosis or oral squamous cell carcinoma and controls

SNP	*Groups*	*Genotype*	*Frequency (%)*	*Odds ratio*	*P value*	*95% confidence interval*
HIF-1 α C1772 T	OSMF	CC	66			
		CT	33	5.0	< .001	2.24−11.16
		TT	1			
	OSCC	CC	59			
		CT	34	5.78	< .001	2.58−12.89
		TT	7			
	Controls	CC	91			
		CT	9			
		TT	0			
HIF-1 α G1790 A	OSMF	GG	42			
		GA	58	2.80	< .001	1.62−5.16
		AA	0			
	OSCC	GG	35			
		GA	65	3.88	< .001	2.16−7.0
		AA	0			
	Controls	GG	68			
		GA	32			
		AA	0			
VEGF-A 936 C/T	OSMF	CC	80			
		CT	19	2.11	NS[d]	0.93−4.81
		TT	1			
	OSCC	CC	73			
		CT	25	3.05	< .01	1.38−6.76
		TT	2			
	Controls	CC	90			
		CT	10			
		TT	0			
VEGF-C rs1485766	OSMF	AA	31			
		AC	50	2.18	< .05	1.19−3.99
		CC	19	6.62	< .01	2.25−19.48
	OSCC	AA	24			
		AC	51	2.87	< .01	1.32−8.44
		CC	25	11.25	< .001	3.84−32.92
	Controls	AA	55			
		AC	40			
		CC	5			
VEGF-C rs7664413	OSMF	CC	100			
		CT	0			
		TT	0			
	OSCC	CC	100			
		CT	0			
		TT	0			
	Controls	CC	100			
		CT	0			
		TT	0			

OSMF, oral submucous fibrosis; *NS,* not significant; *OSCC,* oral squamous cell carcinoma; *SNP,* single nucleotide polymorphisms.

allele-specific PCR, this SNP was not found in any of the study patients. All the patients were positive for the homozygous wild-type allele (CC) only.

The SNPs of HIF-1 α, VEGF-A, and VEGF-C were not associated with TNM staging, histologic grading in OSCC, or functional grading of OSMF in the OSCC group. There was also no association of SNPs with functional grading or presence of dysplasia in the OSMF group.

The serologic levels of HIF-1 α, VEGF-A, and VEGF-C in the OSMF, OSCC, and control groups (n = 50 each) are shown in Table III. The mean serologic levels of HIF-1 α, VEGF-A, and VEGF-C were found to be the highest in the OSCC group, intermediate in the OSMF group, and lowest in controls. The serologic levels of HIF-1 α in the OSMF and OSCC groups were found to be significantly higher than in controls ($P <$ .0001). The serologic levels of VEGF-A were also observed to be significantly higher ($P <$ .0001) in the OSMF and OSCC groups in comparison with controls. Similarly, VEGF-C levels were found to be significantly higher in the OSCC group ($P <$ .0001) and the OSMF

Table III. Comparison of serologic levels of HIF-1 α, VEGF-A, and VEGF-C in patients with oral submucous fibrosis or oral squamous cell carcinoma and controls

Study patients (N)	*HIF-1 α (ng/mL) Median (range)*	*VEGF-A (pg/mL) Median (range)*	*VEGF-C (pg/mL) Median (range)*
OSMF (50)	58.86 (17.09−119.61)	109.5 (10−398)	431.80 (151.26−675.32)
OSCC (50)	144.17 (24.41−289.27)	283 (29−1006)	504.04 (226.71−940.06)
Controls (50)	34.81 (2.44−86.66)	65 (10−169)	285.2 (148.29−522.52)
Significance (P value)			
OSCC vs C	< .0001	< .0001	< .0001
OSMF vs C	< .0001	< .0001	< .001
OSCC vs OSMF	< .0001	< .0001	< .001

N, total number of patients; *ng/mL,* nanogram/milliliter (1 ng/mL = 1000 pg/mL); *OSCC,* oral squamous cell carcinoma; *OSMF,* oral submucous fibrosis; *pg/mL,* picogram/milliliter.

group ($P < 0.001$) compared with controls. There was significant difference in the mean levels of HIF-1 α, VEGF-A, and VEGF-C in patients who had some form of a harmful habit compared with those who did not have any habits. There was also no association of the serologic levels with functional grading or the presence of dysplasia in the OSMF group or TNM staging in the OSCC group.

In OSCC with concomitant OSMF, mean VEGF-C levels were found to be significantly low ($P < .001$), and the frequencies of VEGF-C SNP (rs1485766) were significantly less ($P < .01$) compared with OSCC without OSMF.

DISCUSSION

The *HIF-1 α* gene is located on chromosome 14 q21-24, and the 2 important SNPs in exon 12 of the gene—HIF-1 α C1772 T and HIF-1 α G1790 A—lead to amino acid substitution of proline to serine at position 582 and alanine to threonine at position 588 of the protein, respectively. These 2 SNPs are the most important functionally because they give stability and cause increased transcription and expression of HIF-1 α in OSCC.[9-11,18,23] HIF-1 α C1772 T has been found to be associated with higher cancer risk, especially in Asians, and with increased transcriptional activity in OSCC, especially in association with the TT genotype.[9,23,24] HIF-1 α G1790 A was found to be associated with increased transcriptional activity, lymph node metastases, susceptibility to OSCC, and a poor prognosis even in the early stages (T1/T2).[10,18] The G/A genotype and the A allelotype were found to be more frequent in T3/T4 OSCC, and the A allelotype was found to be associated with disease relapse and shorter disease-free survival.[11,18] HIF-1 α is upregulated in OSMF and related to the severity of epithelial dysplasia.[12] We found the heterozygous genotype (CT) of HIF-1 α C1772 T to have significant association with OSMF and OSCC and the TT genotype to be very rare in contrast to the findings of Zhou et al.[10] The heterozygous genotype of HIF-1 α G1790 A was found to be significantly associated with both OSMF and OSCC, similar to the findings of Shieh et al.,[11] although we did not find any association with tumor size or lymph node metastases in OSCC. We also found increased transcriptional activity of these SNPs, as reflected by the significantly high serologic levels of HIF-1 α in the OSMF and OSCC groups compared with controls but not related to the presence of dysplasia.

The *VEGF* gene is located in chromosome 6 p21.3 and 936 C/T SNP in the 3'−untranslated region is associated with variations in the production of the VEGF protein. Several studies and meta- analyses related to the association of VEGF-A 936 C/T with OSCC have yielded contradictory results. Some have reported no association,[25,26] whereas others have found the T allele to be associated with increased risk of OSCC, early stages of OSCC, and positive family history of cancer.[19,27-29] VEGF-A 936 C/T has also been found to be associated with decreased plasma levels of VEGF-A and increased vascular invasion in OSCC.[26] VEGF-A 936 C allele has been found to be associated with decreased risk of OSCC but the CC genotype is associated with increased nodal metastases.[26,30] VEGF-A expression has been found to be increased in OSCC and correlates with increased microvessel density, high serum levels of VEGF-A, lymph node metastases, staging, and prognosis.[19,25] Circulating VEGF-A levels correlate well with VEGF-A expression in OSCC and OPMDs and can be used a reliable biomarker and target for chemopreventive and chemotherapeutic interventions.[16] Increased VEGF-A expression in OSMF has been linked to malignant transformation in OSMF.[14] We also found the VEGF-A 936 C/T genotype to be significantly increased in OSCC, but no correlation with TNM staging was seen. In OSMF, the CT genotype was increased in comparison with that in controls but not significantly. The CC genotype was the most frequent among all the 3 groups, but had no significant difference or correlation with TNM staging in OSCC or OSMF grading. The VEGF-A serologic levels showed significant differences in the OSMF and OSCC groups compared with controls.

VEGF-C is a lymphangiogenic and angiogenic factor that is implicated in tumor growth, invasion, and metastasis. The SNPs of VEGF-C may interact with environmental factors, such as smoking and betel quid chewing. The polymorphic genotypes of VEGF-C rs7664413 TT might increase the risk of OSCC, and the haplotype of rs1485766 might aid in predicting susceptibility to OSCC.[13] In this study we did not find the VEGF-C rs7664413 CT/TT genotype in any of our patients in contrast to the findings in a study performed in Taiwan. However, the AC and CC genotypes of VEGF-C rs1485766 were significantly associated with both OSMF and OSCC.

Interestingly, we found OSCC arising in a background of moderate and severe OSMF in 32% of cases, indicating that the rate of malignant transformation in OSMF is underestimated, as also found by Chourasia et al.[5] Such OSCCs are found to be clinicopathologically distinct and more common in younger males and have a better prognosis because they have better grade of tumor differentiation, less nodal metastases, and less extracapsular spread.[31] We found that the frequencies of VEGF-C rs7664413 SNP and VEGF-C levels were significantly lower in these patients with OSCC (mean age 44.34 years; male-to-female ratio 29:3). The tumors were mostly well to moderately differentiated (81%), and nodal staging was N0/N1 (65%), similar to the findings of Chaturvedi et al.[31] The low levels of VEGF-C, a lymphangiogenic factor, could be related to less lymphatic spread to regional lymph nodes, as was seen in these patients compared with patients with OSCC without OSMF.

The study patients were of North Indian origin, and the findings are different from studies reported in Caucasians and Southeast Asians, and this can be attributed to differences in ethnicity and in exposure to harmful habits. Most of the patients in the control group had the same harmful habits but did not have the clinical features of OSMF or any other OPMD. The low association with the SNPs of HIF-1 α, VEGF-A, and VEGF-C and the low serologic levels of the associated proteins could account for this difference in outcome despite exposure to the same risk factors. Other clinical risk factors to be considered are prolonged exposure to the gutka habit, which increases the risk of OSMF, and moderate to severe OSMF, which increases the risk of OSCC. This could be attributed to the harmful effects of arecoline in gutka related to dose and duration of exposure. With increasing severity of OSMF, such factors as trismus, poor oral hygiene, chronic irritation, and nutritional deficiencies could also play a role in malignant transformation of OSMF. The results were derived from a small sample of patients and need to be corroborated by findings in studies with larger sample sizes in different ethnicities from the southern and northeastern regions of India.

CONCLUSIONS

The heterozygous forms of HIF-1 α C1772 T, HIF-1 α G1790 A, VEGF-A 936 C/T, and VEGF-C rs1485766 and the homozygous form of VEGF-C rs1485766 were associated with OSMF and OSCC. These SNPs were related to increased transcription of the HIF-1 α, VEGF-A, and VEGF-C proteins in the serum of patients with OSMF and OSCC. These SNPs interact with harmful environmental factors, such as gutka and tobacco, in North Indian patients and increase their susceptibility to OSMF and its malignant transformation into OSCC. These SNPs can, therefore, be used as prognostic biomarkers and aid in the development of specialized anti−HIF-1 α/VEGF drugs in the management and prevention of OSMF and its malignant transformation into OSCC.

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PRESENTATION

The interim results of this study were presented at the 2017 World Congress of Preventive Dentistry, New Delhi, India, and at the 2018 American Academy of Oral Medicine Conference, San Antonio, TX, USA, and the abstract was published in the *Journal of Dental Research* and *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology.*

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