Original Article

Qualitative expression of hypoxia-inducible factor-1α in malignant transformation of oral submucous fibrosis: An immunohistochemical study

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Abstract

Background: Oral submucous fibrosis (OSF) is a precancerous condition predominantly seen in people of Asian descent. About 7%–12% of OSF patients develop oral squamous cell carcinoma. Morphological features of OSF, especially fibrosis, suggest a possibility of hypoxic environment in diseased tissues. Neovascularization and increased glycolysis, represent adaptations to a hypoxic microenvironment that are correlated with tumor invasion and metastasis. The adaptation of cells to hypoxia appears to be mediated through hypoxia-inducible factor-1α (HIF-1α). HIF-1α is said to be associated with malignant transformation of epithelium in other sites.

Aim: The aim of this study was to investigate the relationship between the expression of HIF-1α in OSMF and its role in malignant transformation.

Materials and Methods: A retrospective study which included 20 histopathologically diagnosed cases of OSF was conducted. A qualitative evaluation of HIF-1α was performed. Statistical analysis was carried out using the IBM Statistical Package for Social Sciences 20.0 version (IBM Corporation, Armonk, NY, USA).

Results: Results showed an increased expression of HIF-1α in OSF.

Conclusion: HIF-1α appears to play a role in malignant transformation of OSF.

Keywords: Epithelial dysplasia, hypoxia, hypoxia-inducible factor-1α, malignant dysplasia, oral submucous fibrosis

INTRODUCTION

Oral and pharyngeal cancers (OPC) are considered an important part of the global cancer burden. Oral cancer is the sixth most common malignancy. More than 90% of all oral cancers are squamous cell carcinoma (oral squamous cell carcinoma [OSCC]). The risk of developing OSCC is increased by the highly prevalent habits of chewing tobacco, betel quid and areca nut in the Indian subcontinent. Survival rates for OPC have not significantly improved over the past three decades.[1]

The concept of stepwise development of cancer in the oral mucosa, i.e., the initial presence of a precursor lesion subsequently developing into cancer is well

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established.[2] Many OSCC has been documented to be associated with or preceded by a precancerous lesion, especially leukoplakia.[3] It is observed that the malignant transformation potential increases with the severity of epithelial dysplasia with a malignant transformation rate ranging from 3% to 33%.[4]

Hypoxia is a common feature of many cancers and contributes to local and systematic cancer progression.[5] It reflects the imbalance between oxygen consumption by the rapidly proliferating cancer cells and the insufficient oxygen delivery due to poor vascularization and blood supply and is a common feature of solid tumors, including head and neck squamous cell carcinoma (HNSCC).[6]

Oral submucous fibrosis (OSF) is a chronic, complex, premalignant condition of the oral cavity, characterized by juxta-epithelial inflammatory reaction and progressive fibrosis of the submucosal tissues. The molecules involved in the biological pathways of the fibrotic process appear to be either down or upregulated at different stages of the disease.

Hypoxia causes cell death if it is severe or prolonged. However, cancer cells acclimatize to this hostile environment.[7] Protection against hypoxia in solid tumors is an important step in tumor development and progression. One system in hypoxia protection of tumor cells is represented by the hypoxia-inducible factor-1α (HIF-1α) under hypoxic conditions, especially in angiogenesis and carcinogenesis. HIF-1α is a heterodimeric protein consisting of an alpha and beta subunit, in which the HIF-1α subunit mediates HIF-1α function as a transcription factor.[8] In human solid tumors, where hypoxia is found, it serves as a selective environment for survival of aggressive cancer cells and as protection from anticancer therapies.[9] The expression of HIF-1α has been demonstrated in a variety of cancers, including renal, bladder, colorectal, breast, ovarian, endometrial, cervical and head and neck carcinomas.[5] Morphological features of OSF, especially fibrosis, suggest a possibility of the hypoxic environment in diseased tissues. The adaptation of cells to hypoxia appears to be mediated through HIF-1α.

The purpose of this study was to investigate the expression on HIF1-α in OSF through its effect on the staining intensity by analyzing the mean blood vessel and mean fibroblast density. The study also aimed at finding out a correlation between the staging of the disease, the grading of epithelial dysplasia and the staining intensity based on epithelial dysplasia, if any.

MATERIALS AND METHODS

This retrospective study was carried out in the Department of Oral and Maxillofacial Pathology, D. Y. Patil Dental College, Navi Mumbai, India. Relevant history of each individual was obtained from the archives. Twenty blocks of formalin-fixed and paraffin-embedded tissues were randomly selected from the archives. The five subjects for the control group were selected from age- and sex-matched individuals. These individuals were ruled out for the presence of any systemic illness. Further, these individuals did not have any oral tissue abuse habits or any obvious oral lesions. Individuals who had a history of oral tissue abuse habits such as tobacco chewing, smoking, areca nut chewing and alcohol consumption were included in the study. Relevant history of each individual was checked thoroughly to rule out any systemic illness or any prior therapy. Individuals who had taken antibiotics in the previous month for any tooth infection, those on anticoagulant therapy, chemotherapy, radiation therapy were excluded from the study. The study was carried out over a period of 6 months. Written informed consent and institutional ethical committee clearance were obtained for the study.

Clinical staging of severity of fibrosis was carried out based on the study conducted by Lai *et al*.[10]

The paraffin blocks of the study individuals were sectioned with a semi-automatic microtome (Leica, Germany) into two tissue sections of 3 μm thickness. One section was stained with hematoxylin and eosin (H & E).

Further, the H & E stained sections were histopathological graded using the criteria given by Utsunomiya *et al*.,[11] which is based on the concept of the Pindborg and Sirsat grading system.

Immunohistochemistry

The other 3 μm section of the tissue was cut and transferred to APES (Sigma-Aldrich Chemical Co., USA) coated slides and incubated overnight at room temperature. After warming in a slide warmer for 15 min, the sections were deparaffinized in three changes of fresh xylene each for 5 min followed by dehydration in a series of absolute alcohol each for 5 min. Endogenous peroxidases were blocked with peroxide block (BioGenex Life sciences Pvt., Ltd, CA, USA) for 15 min at room temperature and washed in distilled water followed by citrate buffer (pH 6.0) wash for 10 min. Antigen retrieval was undertaken with a help of BioGenex antigen retrieval system. The sections were immersed in citrate buffer solution and placed into the

BioGenex antigen retrieval system and heated for 15 min. The system was allowed to cool to room temperature by placing it under running tap water, and later, the slides were washed with distilled water for 5 min. With an intention to block endogenous biotin, the sections were incubated with a blocking agent (BioGenex Life sciences Pvt., Ltd., CA, USA) for 15 min. Excess power block solution was drained, and the sections were incubated with primary monoclonal antibody of HIF-1α (BioGenex Life Sciences Pvt., Ltd, CA, USA) for 1 h and later thoroughly washed with citrate buffer. For further enhancement of the staining, the sections were then incubated with the anti-mouse secondary antibody (super-enhancer) for 30 min followed by two consecutive buffer washes; each for 5 min. Horseradish peroxidase (BioGenex Life Sciences Pvt., Ltd., CA, USA) was added to the sections and incubated for 30 min. The chromogen diaminobenzidine was prepared just prior to use by mixing one drop of chromogen to 1 ml of the buffer in a mixing vial and later added over the sections. After 5 min, the sections were washed in buffer followed by water and counterstained with Harris hematoxylin, air dried, cleared and mounted with dibutyl phthalate xylene.

Assessment of the hypoxia-inducible factor-1α staining intensity

Cells positive for HIF-1α were regarded as fibroblasts and blood vessels. Fibroblasts and blood vessels were counted using a Leica research microscope with provision for photomicrograph (Model no. DM1000 LED, Germany). The stained sections were first screened at low power (×10) to determine the areas of most intense staining (Hot spot method). Fibroblasts and blood vessel counting was then performed under high power (×40) magnification. Five high-power fields (HPFs) with the highest number of these cells were chosen. The representative areas were carefully scanned from left to right of every slide to avoid recounting of same areas. The cells for each case was the average of the number in these five chosen HPFs and expressed as the number of fibroblasts or blood vessels per HPF. The mean of 10 values was calculated and expressed as mean (standard deviation). All immunohistochemistry (IHC)-stained slides along with the corresponding H & E slides were evaluated by two qualified observers to minimize the subjective bias.

The section was considered either as negative or positive according to the absence or presence of brown staining in epithelial or stromal cells. The positive cases were graded into three categories depending on the intensity of staining. Homogeneous dark brown staining was considered as strong (+++) and light faint staining was considered as mild (+), and cases in between the two extremes were graded into the moderate (++) category.

Statistical analysis

Descriptive and inferential statistical analyses were carried out in the present study. Results on continuous measurements were presented on mean ± standard deviation, and results on categorical measurement were presented in number (%). The level of significance was fixed at $P = 0.05$ and any value ≤0.05 was considered to be statistically significant.

Analysis of variance (ANOVA) was used to find the significance of study parameters between the groups (intergroup analysis). Further *post hoc* analysis was carried out if the values of ANOVA test were significant.

The Statistical software IBM Statistical Package for Social Sciences statistics 20.0 (IBM Corporation, Armonk, NY, USA) was used for the analyses of data.

OBSERVATIONS AND RESULTS

The study comprised of 20 OSF (study group) and five healthy controls (control). The study population comprised of 16 males and four females with a mean age of 29 years, and the control group comprised of three males and two females. The study group as well as control group showed a male predominance. Clinical staging of the patients was made depending upon the mouth opening as suggested by Lai *et al*. There were seven cases in Stage 1, five cases in Stage 2, seven cases in Stage 3 and one case in Stage 4. We observed that the majority of the study group falls in Stage 1 and Stage 3. We further did a comparison of staining intensity with grades of epithelial dysplasia ($P = 0.199$), comparison of epithelial dysplasia with clinical staging ($P = 0.283$) and a comparison of epithelial dysplasia with histopathological grading ($P = 0.283$). All the above values were statistically insignificant.

The collected data were then statically analyzed to find a significance of the blood vessels in correlation to the different grades of staining intensity. Among the 20 cases with respect to staining intensity, nine cases showed mild staining intensity with a mean of 7.13 (4.267), six cases showed moderate staining intensity with a mean of 6.50 (3.779) and five cases showed a severe staining intensity with a mean of 7.49 (6.316). The $P = 0.594$ [Table 1 and Figure 1]. When the data were analyzed to find the significance of the fibroblasts in co-relation to the different grades of staining intensity it was observed that among the 20 cases with respect to staining intensity,
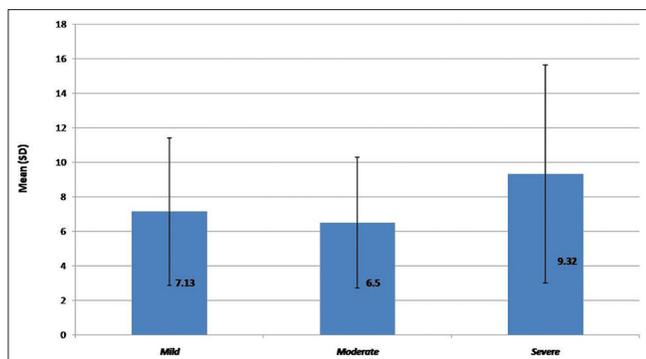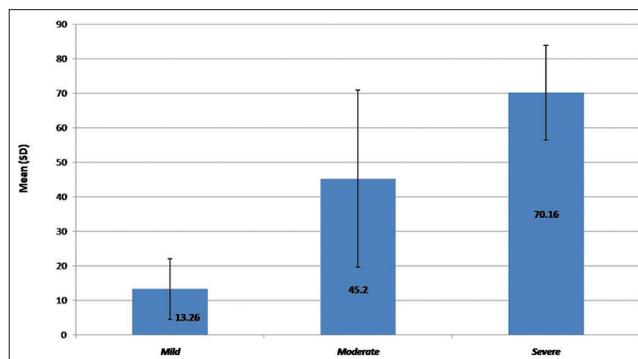
Figure 1: Comparison of blood vessels in terms of (mean [standard deviation]) among different grades of dysplasia using analysis of variance test

Figure 2: Comparison of fibroblast in terms of (mean [standard deviation]) among different grades of dysplasia using analysis of variance test

nine cases showed mild staining intensity with a mean of 13.26 (8.792), six cases shows moderate staining intensity with a means of 45.20 (25.652) and five cases showed a severe staining intensity with a mean of 70.16 (13.723). The $P < 0.001$ which was highly significant [Table 2 and Figure 2].

DISCUSSION

HNSCC is the sixth most common malignancy worldwide. OSCC remains a significant health burden across the globe.[12] It usually arises from a preexisting malignant disorder, and occasionally *de novo*; but in either case from within a field of precancerized epithelium. Important risk factors related to the carcinoma itself that are associated with a poor prognosis include large size of the tumor at the time of diagnosis, the presence of metastases in regional lymph nodes, and a deep invasive front of the tumor.[13] Despite advances in therapeutic approaches, percentages of morbidity of OSCC and mortality have not improved significantly during the past 30 years.[14] Oral cancers arise from sustained, stepwise accumulations of mutations resulting in the transition of normal mucosa to dysplasia to invasive carcinoma over time.[15]

Furthermore, many OSCCs are believed to develop from oral premalignant disorders (OPMD) and early detection and diagnosis of these premalignant lesions should be possible. Identification of high-risk OPMD and intervention at premalignant stages could constitute one of the keys to reducing mortality, morbidity and cost of treatment associated with SCC. There are several OPMD that precede the development of OSCC. The most commonly encountered are erythroplakia, leukoplakia and OSF.[12] Several potential markers of molecular changes in oral premalignant lesions and conditions have been studied, and interest in the genetic changes associated with these lesions is increasing. Progression from benign to malignant

Table 1: Comparison of blood vessels in terms of (mean±standard deviation) among different grades of staining intensity using analysis of variance test

Staining intensity	n	Mean±SD	F	P
Mild	9	7.13±4.267	0.536	0.594
Moderate	6	6.50±3.779		
Severe	5	9.32±6.316		
Total	20	7.49±4.590		

SD: Standard deviation

Table 2: Comparison of fibroblast in terms of (mean±standard deviation) among different grades of staining intensity using analysis of variance test

Staining intensity	n	Mean±SD	F	P
Mild	9	13.26±8.792	20.002	<0.001**
Moderate	6	45.20±25.652		
Severe	5	70.16±13.723		
Total	20	37.07±28.684		

**P<0.001: Highly significant. SD: Standard deviation

disease is a genetic process that later becomes evident at the cellular level and ultimately at the clinical level. The use of molecular markers allows diagnosis and staging of tissue change before changes in cell morphology occurs and certainly before tissue changes become clinically visible. Ultimately, the use of molecular markers in diagnosis may lead to survival and less treatment-associated morbidity through early recognition of and intervention for at-risk oral lesions.[16]

Hypoxia is a common feature and contributes to local and systemic cancer progression, resistance to therapy and poor outcome.[17] Oxygen concentrations are markedly reduced in many human cancers compared with normal tissue, and major mechanism mediating adaptive responses to reduced oxygen availability (hypoxia) is the regulation of transcription by HIF-1α.[18] Hypoxia is a state of reduced oxygen availability or decreased oxygen partial pressures below critical thresholds, and it restricts or even abolishes the function of organs, tissues or cells. In solid tumors,

oxygen delivery to the respiring neoplastic and stromal cells is frequently reduced or even abolished by deteriorating diffusion geometry, severe structural abnormalities of tumor microvessels and disturbed microcirculation. Neovascularization and increased glycolysis represent adaptations to a hypoxic microenvironment that are correlated with tumor invasion and metastasis.[19]

The development of hypoxic microenvironment is caused by the imbalance between oxygen consumption and oxygen delivery. The rapidly proliferating HNSCC has insufficient vascularization with poor blood supply. The hypoxic stress stimulates solid tumors to upregulate the expression of a variety of oncogenes such as HIF and endothelial growth factor which enhances irregular vascular endothelial cell proliferation and differentiation. The adaptation of cells to hypoxia appears to be mediated through HIF-1α which is said to be associated with malignant transformation of epithelium in other sides. It appears that HIF-1α plays a significant role in both prostate and cervical carcinogenesis at early stages.[20]

Little is known about the role of HIF-1α in early events of carcinogenesis in the oral cavity. Therefore, the aim of our study was to evaluate the expression of HIF-1α in OSF by IHC.

Hypoxia is a common environmental stress factor and is associated with physiological and pathological conditions related to cancer invasion and metastasis.[21] The process of tumor cell invasion involves degradation of extracellular matrix and matrix metalloproteinases which play an important role in invasion in OSCC.[22] The higher incidence of metastasis could also be explained by the fact that the glycolytic products lactase and acid induce secretion of matrix-degrading hyaluronidase and metalloproteinase by tumor-associated fibroblasts create a tumor microenvironment favorable for tumor cell migration.[23]

OSF is histopathologically characterized by fibrosis of subepithelial connective tissue. Collagens are the major structural component of extracellular matrix; hence, precise regulation of collagen metabolism is essential to maintain the normal integrity of connective tissue.[24]

With the progression of the disease process of OSF, the production of collagen type 1 is increased, and the degradation of collagen is reduced by up to 75%. Extensive fibrosis of the connective tissue causes a reduction of vascularity, resulting in subsequent hypoxia in both fibroblasts and surface epithelium. Hypoxia causes atrophy and ulceration of the epithelium by inducing apoptosis. In addition, the overexpression of HIF-1α is seen in OSF, which indicates changes in cell proliferation, maturation and metabolic adaptation, increasing the possibility of malignant transformation.[6] On degradation, HIF-1 becomes inactive and the cells lacking oxygen supply are unable to survive and undergo apoptosis. However, under hypoxic conditions as in OSF, HIF-1α is stabilized and on stabilization, it translocates to the nucleus where it forms the functional transcription factor HIF-1. The functional HIF-1α helps in cell survival under hypoxia and thus helps cell proliferation promoting tumorigenesis.[25] HIF-1α is rarely expressed in the normal oral mucosa. However, a significant HIF-1α expression was found in OSF in the basal and suprabasal layers of epithelium. This indicates the role of hypoxia in malignant transformation of OSF. Thus, the upregulation of HIF-1α is an early event in carcinogenesis.[26]

Dunkel *et al*.[27] found that the CD44 low HIF-1α high signature was associated with poorer disease-free survival. Uehara *et al*.[7] found expression of HIF-1α in OSCC is likely to be of great value in treatment planning and to predict the prognosis of OSCC. Lee *et al*.[28] found that HIF-1a expression is significantly upregulated in areca quid chewing-associated OSCC. Zheng *et al*.[29] in their reverse transcription-polymerase chain reaction study measured mRNA levels of HIF-1α and found HIF-1α mRNA levels were significantly increased in carcinoma of the tongue, and a positive correlation was observed with pathological differentiation grade. On the contrary, dos Santos *et al*.[9] showed a significant relationship between strong HIF-1α protein expression and lower local disease relapse ($P = 0.002$) and increased local disease-free survival ($P = 0.013$). In a recent study on prostate carcinogenesis, it was reported that the upregulation of HIF-1α is an early event.[30] They showed a gradual increase in the expression of HIF-1α from benign prostatic hyperplasia, prostatic intraepithelial hyperplasia to prostatic cancer compared with normal prostatic epithelium.

In the present study conducted, we hypothesized a possible role of HIF-1α in progression and malignant transformation of OSF and to investigate the role of hypoxia in OSF and its relationship to epithelial dysplasia. We have demonstrated the evidence of marked upregulation of HIF-1α in a reasonably large number of OSF samples by conducting IHC and then by using the hot-spot method, the counting of the stained blood vessels and fibroblast was done. Although this finding was not consistent as in few cases, staining was restricted to the dysplastic zone only. In the past literature, increased

expression of HIF-1α was accompanied by an increase in the number of blood vessels in OSF cases. However, in the present study, it was seen that the number of fibroblasts was more as compared to the number of blood vessels which supported our hypothesis that the process of OSF is due to increased fibrosis which is initiated by the fibroblast and thus there is increased amount of hypoxia leading to an increased expression of HIF-1α. It has also been reported that some of the molecules which have a direct relationship to carcinogenesis exhibit a correlation with HIF-1α levels. One of the most important molecules in this regard is vascular endothelial growth factor which is responsible for angiogenesis, a key event in carcinogenesis.[31-33]

CONCLUSION

Hypoxia is a common characteristic of locally advanced solid tumors that has been associated with diminished therapeutic response and more recently with malignant progression, that is, an increasing probability of recurrence, locoregional spread and distant metastasis. Emerging evidence indicates that the effect of hypoxia on malignant progression is mediated by a series of hypoxia-induced proteomic and genomic changes activating angiogenesis, anaerobic metabolism and other processes that enable tumor cells to survive or escape their oxygen-deficient environment. Thus, hypoxia should be considered an independent adverse prognostic factor in patients with head and neck cancers.[34] Since this is a preliminary study, a larger sample size is a must. In addition, there should be approximately equal number of samples in each histopathological group. In conclusion, our data indicate that HIF-1α appears to play a role in the malignant transformation of OSF. Further, over-expression of HIF-1α may at least be partly responsible for the progression of fibrosis. Finally, our data suggest that it may be possible to use HIF-1α as a marker for malignant transformation of OSF.

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Conflicts of interest
There are no conflicts of interest.

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