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Research Article

Expression of Carbonic Anhydrase -IX in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma

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Abstract

Objective: Carbonic anhydrase IX (CAIX) is a protein that is associated with low oxygen levels and used as an indicator of the biological behaviour of oral squamous cell carcinoma (OSCC). However, the predictive significance of this has not yet been confirmed. Hence, the present study was conducted to evaluate IHC expression of carbonic anhydrase IX in Oral squamous cell carcinoma, oral submucous fibrosis, and normal oral mucosa obtained from healthy volunteers

Material and Method: A cross-sectional study was conducted, which involved a total of 46 formalin-fixed paraffin-embedded tissue blocks that comprised the case group. Based on histopathology, 46 tissue blocks were divided into two groups: 23 cases of oral submucous fibrosis and 23 cases of OSCC. The control group was made up of tissue blocks from 23 healthy volunteers that had been fixed in formalin and embedded in paraffin. The IHC expression of CAIX was evaluated using the H score.

Results: The mean histoscore of CA IX in OSMF was 31.74±29.49 (mean ± SD), and the mean histoscore in OSCC was 113.04 ± 64.98 (mean ± SD). The NOM did not show any immunohistochemical staining for carbonic anhydrase IX. The ANOVA, when performed between the groups, showed a significant difference between the groups with a p value of 0.0001. Pair-wise comparison of three groups (OSMF, OSCC, and Normal Oral Mucosa) with mean H score using Tukey's multiple posthoc procedures showed a significant difference between the OSMF and OSCC ($p = 0.0001$) groups, the OSMF and NOM ($p = 0.0296$) groups, and the OSCC and NOM ($p = 0.0001$) groups.

Conclusion: The study revealed that Carbonic Anhydrase IX (CA9) increased its average H score as the illness progressed from OSMF to OSCC, successfully distinguishing OSMF from OSCC.

Key Words: Carbonic Anhydrase IX, Oral Squamous Cell Carcinoma, Oral Submucous Fibrosis, and Normal Oral Mucosa

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Introduction

Oral cancer is the tenth most common malignancy among males globally. Oral cancer, among malignant neoplasms, has a relatively poor overall survival rate, but the 5-year survival rate has improved over the previous 20 years (with the end-stage survival rate

being less than 20%). To effectively manage this lethal cancer type, it is essential to create new biomarkers that can predict the likelihood of cancer development.[1]

A group of zinc metalloenzymes known as carbonic anhydrases (CAs) catalyzes the pH-maintaining, reversible hydration of CO2 and water to bicarbonate ions and protons. These CAs' enzymatic activity is responsible for regulating various physiological processes, such as transfer of solutes and CO2 and the acidity of the surrounding tissue, which can alter the malignant phenotype of tumors. CAIX is made up of intracellular tails (IC), transmembrane regions (TM), CA domains, proteoglycan-like region (PG), and signal peptides (SP) eliminated during protein maturation. Many solid tumors, such as renal cell carcinoma,non-small cell lung, ovarian, esophageal, breast, gastric, and cervical malignancies, promote the expression of CAIX in hypoxic settings. Hypoxia induces the expression of CAIX by binding the hypoxia-inducible factor (HIF)-1 to a hypoxia-response region in the basal promoter of the CA9 gene. This subsequently initiates transcription.. CAIX delivers bicarbonate ions for intracellular pH neutralisation and protons for extracellular microenvironment acidification, thereby enhancing cell survival and proliferation, diminishing cell adhesion, activating proteases that degrade the extracellular matrix (ECM), and facilitating cell migration and invasion.[1]

Oral submucous fibrosism(OSMF) is a chronic, persistent, precancerous disorder with a significant risk of malignant transformation. Multiple biological processes contribute to the pathophysiology of submucous fibrosis and its progression to cancer. Detailed molecular mechanisms need investigation. Some stages of OSF show inflammation, which may contribute to disease progression and malignant transformation. It is characterized by a gradual fibrosis of submucosal tissues [2] and linked to a juxta-epithelial inflammatory reaction, fibroelastic alterations in lamina propria layer, and epithelial atrophy, which causes oral mucosa to become rigid and eventually cause trismus and difficulties opening the mouth.[3]

Approximately 80–90% of all malignant neoplasms in the oral cavity are oral squamous cell carcinoma (OSCC), the most common kind of oral cancer. [4]The prevalence of oral cancer varies significantly worldwide; nonetheless, it is well recognised that oral cancer ranks as the sixth–ninth most prevalent malignancy, mostly influenced by the patient's gender and geographic location, including particular regions. The primary risk factors for OSCC include alcohol intake, tobacco use, and ultraviolet radiation. Additional variables include dietary inadequacies, genetic susceptibility, and infections caused by human papillomavirus (HPV) and Candida. The main sign of OSCC, which is more common in adults and older people, is an ulcerated lesion with a raised, rolling border around a completely necrotic centre. [5]

Normally, CAIX, a protein found in certain parts of the body, like the choroid plexus, ovaries, testicles, biliary systems, pancreatic ducts, and gastrointestinal mucosa, is often overexpressed in solid tumors, including oral cancer. Research has shown that lower CAIX and HIF-1α levels are linked to better prognosis in people with oral squamous cell carcinoma (OSCC). Higher stromal CAIX expression is linked to nodal metastases and lower disease-specific survival over five years. Increased plasma CAIX levels are correlated with the advanced TNM stage, while heightened CAIX expression in tissue is linked to the advanced clinical stage, lymph node metastases, and diminished overall survival. [1] The function and molecular mechanism of CAIX in oral cancer cells remain unclear. Research on the role of CAIX in oral potentially malignant disorders is limited, and especially, the significant role of CAIX in OSMF is unclear. Hence, The present study aimed to examine the expression of CAIX as a prognostic indicator for the malignant progression of OSMF.

MATERIALS AND METHODOLOGY

The study population consisted of 46 tissue sections obtained from archival specimens of histopathologically confirmed cases of oral submucous fibrosis (Group I (n=23)) and oral squamous cell carcinoma (OSCC) (Group II (n=23)) from the Department of Oral & Maxillofacial Pathology and Oral Microbiology at Teerthanker Mahaveer Dental College & Research Centre, forming the case group of the case-control study. The control group (Group III (n=23)) consisted of healthy oral tissue samples taken from 23 volunteers who were undergoing removal of an impacted third molar. These samples were collected after the volunteers gave their written consent.

Inclusion criteria:

Group I:

1. Only Tissue sections obtained from histopathologically confirmed cases of Oral Submucous fibrosis with sufficient available tissue and the availability of minimum five-year follow-up data with history of tobacco consumption (smoke or smokeless) or Arecanut were included in the group.
2. If there were multiple paraffin-embedded tissue specimens of oral Submucosis fibrosis belonging to multiple biopsys taken over a period of follow-up with no malignant transformation in follow up biopsies, only the paraffin-embedded tissue specimens from the initial biopsy were selected for study.

Group II:

1. Only Tissue sections obtained from histopathologically confirmed cases of primary OSCC with history of tobacco usage (smoke or smokeless) were included in study group.

Group III:

1. Only Tissue sections obtained from histopathologically confirmed normal oral mucosa at site of an impacted third molar without any histological changes of inflammation collected from healthy individuals who visited dental clinic were included in control group.

Exclusion criteria:

Group I:

1. Samples with insufficient tissue were excluded.
2. Samples with incomplete minimum follow-up data were excluded.

3. Samples that demonstrated histological evidence of positive tissue margins on the subsequent excision were excluded.

Group II:

1. The tissue samples of patients who have had a relapse of oral squamous cell carcinoma, have any other systemic malignancy or illness, or currently receiving therapy for primary OSCC were excluded.

Group III:

1. The tissue samples of healthy volunteers with any other systemic malignancy or illness were excluded.
2. The tissue samples of healthy volunteers who failed to give informed consent were excluded.

Immunohistochemical technique:

The immunohistochemical technique for staining of CA IX was performed according to the manufacturer's protocol (BioSB). About 3 µm thick sections from tissue blocks of samples and control (lung carcinoma) were obtained and mounted on APES-coated slides. These slides were incubated at 37°C overnight on the previous day of staining and at 60°C for 1 h on the day of staining. The slides were deparaffinised using three changes of xylene, each of 5 min duration, and were then hydrated through decreasing grades of isopropyl alcohol (100%, 90%, and 70%) and then in distilled water. The tissues were then incubated with a peroxide block for 10 min at room temperature to block endogenous peroxide activity and washed in distilled water and Tris buffer for 5 min. The slides were then subjected to antigen retrieval using Tris EDTA buffer in a pressure cooker at 150°C for 55 min. After the retrieval, slides were allowed to cool down to room temperature. The slide sections were subjected to two washes of Tris buffer for 10 min each and were subsequently incubated for 15 min with protein block (Polyexcel Protein Block, supplied with the kit) to eliminate background staining. The sections were then incubated with rabbit monoclonal antibody carbonic anhydrase IX (BioSB, Lot no. 7471DHE12) for 60 minutes in a humid chamber. The sections were washed with Tris buffer twice for 5 min each. Subsequently, the slides were covered with Poly Excel Target Binder and incubated at room temperature for 10 minutes. The sections were covered with PolyExcel PolyHRP and incubated for 15-20 minutes at room temperature. The slides were then washed as before and incubated with fresh 3,3'-diaminobenzidine (DAB) chromogen for 2 min. The slides were then washed in water to stop the chromogen reaction and excess DAB and counterstained with Mayer's Haematoxylin for 2 min. The slides were then dehydrated through graded isopropyl alcohol (70%, 90%, and 100%) cleared using xylene and mounted with DPX . For the negative control slide of lung carcinoma, the tissue sections were not treated with the primary carbonic anhydrase IX antibody.

Identifying Carbonic Anhydrase IX expression: The cells that displayed a brown colour were deemed to be Carbonic Anhydrase IX positive (Figure1 A (Positive control), Figure 2A (Oral Submucous Fibrosis), Figure 2B (Oral Squamous cell Carcinoma)). If there was no such display by cells were considered as negative (Figure1 B (Negative control), Figure 2C (Normal oral mucosa))

Quantification of Carbonic Anhydrase IX IHC expression: Quantification of Immunohistochemistry of Carbonic Anhydrase IX was performed according to technique Allred etal. The intensity of Immuno histochemistry expression was determined using the Intensity Score Criteria as described by Allred et al [6]. And the score were interpreted as 0 – No positive cells, 1+ - Mild Intensity, 2+ -Moderate Intensity, 3+ -Strong Intensity. For semi quantitative analysis of immunohistochemistry, H score as described by Hinsch et al [7] was used. The H-score is determined by the formula H-score = 0 × P0 + 1 × P1 + 2 × P2 + 3 × P3+, where P0 represents the percentage of negative (0) cells, P1+ denotes the percentage of 1+ cells, P2+ indicates the percentage of 2+ cells, and P3+ signifies the percentage of 3+ cells, all expressed as a percentage (0% to 100%). This establishes an analytical range for the H-score from 0 to 300.

RESULTS

The clinical data analysed in archives indicated that the average ages of participants in groups I, II, and III were 35 years, 45 years, and 28 years, respectively, as shown in Table 1. There were 39% (09) females and 61% (14) males in group-I, whereas 35% (08) females and 65% (15) males were in group-II, and 30% (07) females and 70% (16) males were in group-III, as indicated in Table 2. The mean histoscore of CA IX in Group I was 31.74 ± 29.49 (mean ± SD), and the mean histoscore in Group II was 113.04 ± 69.98 (mean ± SD). Group III did not show any immunohistochemical stains for carbonic anhydrase IX, as indicated in Table 3.The Kolmogorov-Smirnov test showed a normal distribution. Therefore, parametric one-way ANOVA and Tukey's multiple post hoc procedures were performed. One-way ANOVA showed a significant difference between and within groups, with a p-value of 0.0001 as indicated in Table 4 and Graph 1. A pairwise comparison of three groups (OSMF, OSCC & Normal Oral Mucosa) with mean H score using Tukey's multiple post hoc procedures showed a significant difference between group 1 and group 2 (p=0.0001), group 1 and group 3 (p=0.0296), and group 2 and group 3 (p=0.0001), as indicated in Table 5. The study's results showed that the mean CA IX score was significantly higher in OSCC than in OSMF.

TABLE 1:- Distribution of mean age amongst three groups

GROUP	MEAN AGE(YEAR)
GROUP I (n=23)	35
GROUP II (n=23)	45
GROUP III(n=23)	28

GROUP I- ORAL SUBMUCOUS FIBROSIS, GROUP II- OSCC, GROUP III- NORMAL ORAL MUCOSA

TABLE 2:-Distribution of male and female amongst the study groups

GROUP	FEMALE % (n)	MALE % (n)
GROUP I (n=23)	39	61
GROUP II (n=23)	35	65
GROUP III(n=23)	30	70

GROUP I- ORAL SUBMUCOUS FIBROSIS, GROUP II- OSCC, GROUP III- NORMAL ORAL MUCOSA

Table 3: Indicating mean histo score (H. score) among the study groups

Group	Mean H Score (Mean ± S.D.)
GROUP I (n=23)	31.74± 29.49
GROUP II (n=23)	113.04± 69.98
GROUP III(n=23)	0.00± 0.00

GROUP I- ORAL SUBMUCOUS FIBROSIS, GROUP II- OSCC, GROUP III- NORMAL ORAL MUCOSA

Table 4: Comparison among three groups (OSMF, OSCC, Normal) with mean H-scores by one way ANOVA

Source of variation	DOF (Degree of Freedom)	Sum of squares	Mean sum of squares	p-value
Between groups	2	156373.91	78186.96	0.0001*
Within groups	66	112017.39	1697.23	
Total	68	268391.30		

Graph 1: Comparison of three groups (OSMF, OSCC, Normal) with mean H-scores

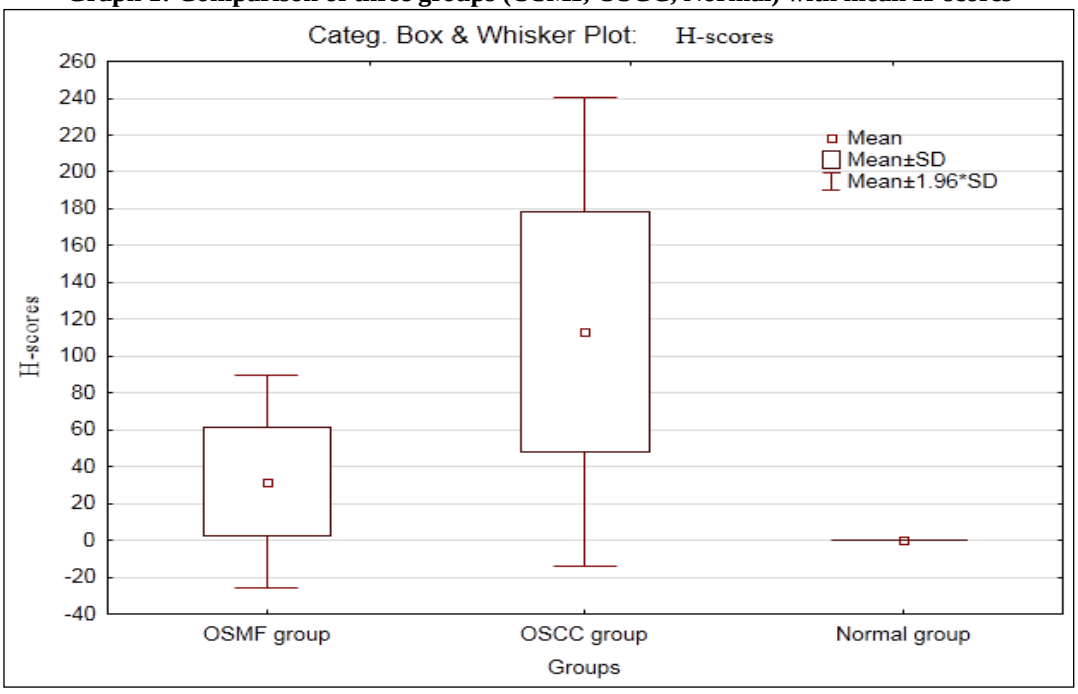

Table 5: Pair- wise comparison of three groups (OSMF, OSCC, Normal) with mean H-scores by Tukeys multiple posthoc procedures

Group	GROUP I (n=23)	GROUP II (n=23)	GROUP III(n=23)
Mean	31.74	113.04	0.00
SD	29.49	64.98	
GROUP I (n=23)	-	p=0.0001*	p=0.0296*
GROUP II (n=23)	p=0.0001*	-	p=0.0001*
GROUP III(n=23)	p=0.0296*	p=0.0001*	-

GROUP I- ORAL SUBMUCOUS FIBROSIS, GROUP II- OSCC, GROUP III- NORMAL ORAL MUCOSA

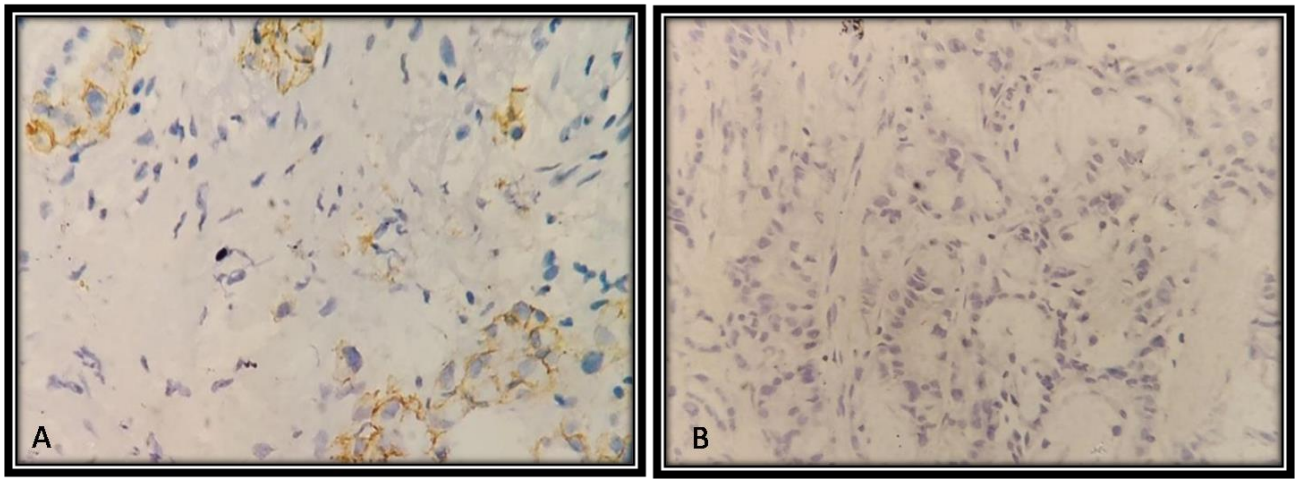

Fig 1 - A : IHC expression of Carbonic Anhydrase IX in Positive control (Lung carcinoma) at 10 X, B : No IHC expression of Carbonic Anhydrase IX in negative control (Lung carcinoma) at 10X

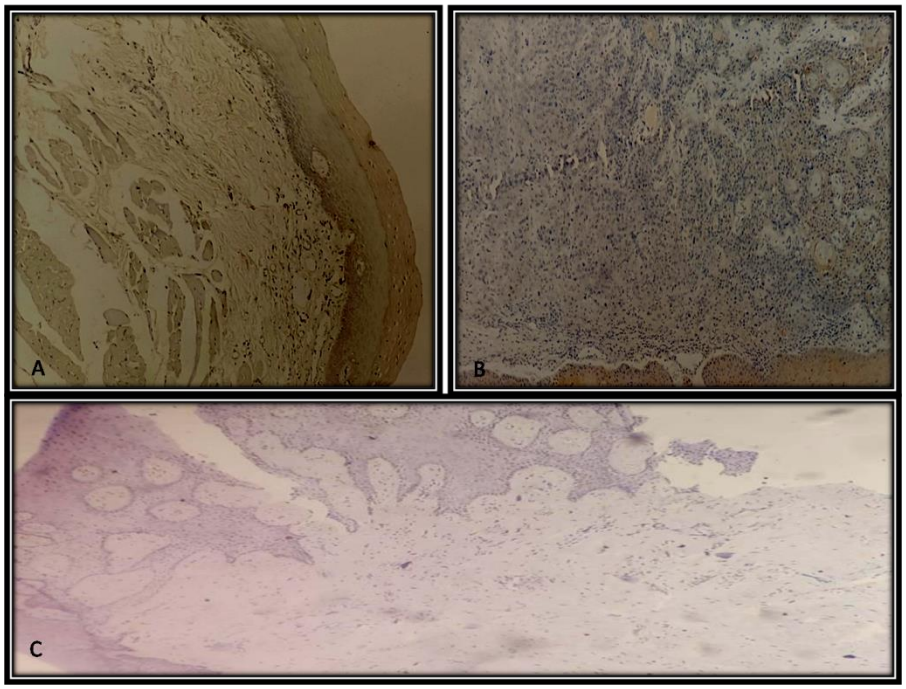

Fig 2 - A : IHC expression of Carbonic Anhydrase IX in OSMF at 10 X, B : IHC expression of Carbonic Anhydrase IX in Oral Submucous Fibrosis at 10X, C: No IHC expression of Carbonic Anhydrase IX in oral squamous cell carcinoma.

Discussion

Studies have shown that the prevalence of OPMDs in the Indian subcontinent ranges from 0.6 per 1,000 to 30.2 per 1,000. In 1870, Sir James Paget first documented the malignant development of an oral lesion into tongue cancer. The predominant potentially malignant disorders are erythroplakia, oral leukoplakia, oral lichen planus, and oral submucous fibrosis. Tissue inflammation is thought to significantly contribute to the development of tissue fibrosis. Oral submucous fibrosis (OSF) and lichen planus are potentially malignant conditions in which immune inflammatory mechanisms are involved in their development and malignant progression[8]. A biopsy is the preferred method for evaluating the possibility of malignant change. Despite the fact that histopathological diagnosis is subjective and insensitive, the World Health Organisation has proposed histological criteria to assess cellular and tissue changes. Furthermore, molecular anomalies precede the histological alterations that characterise epithelial dysplasia[9]. Therefore, the detection of malignant transformations in potentially malignant oral disorders relies on early molecular events.

In oral cancer, tissue inflammation and hypoxia are inextricably linked, forming a microenvironment that promotes tumour growth and progression by improving cancer cell survival, invasion, and therapeutic resistance . Inflammatory cells can induce hypoxia through increased oxygen consumption, while hypoxia can elicit inflammatory responses, perpetuating a detrimental cycle within the tumour tissue.

Hypoxia is a prevalent characteristic of human solid tumours [10]. Tumour hypoxia induces adaptive alterations in tumour cells, facilitating their survival and proliferation [11]. Hypoxic tumours are associated with aggressive growth, metastasis, and treatment failure in a number of human solid tumours [12]. By identifying a marker for hypoxic tumours, it might be possible to figure out how biologically aggressive some tumours are, which would help tailor treatments.

CA9 was first discovered in HeLa cells, where its expression was associated with cell density. The studies demonstrated that CA9 was expressed in many human carcinomas but lacking in the corresponding normal tissues, proposing that CA9 may play a role in cell proliferation and transformation. Certain factors, including the transcription factor HIF-1 (hypoxia-inducible factor-1) and the activation of the MAPK/ERK pathway [13,14], can change the amount of CA IX that is expressed. HIF-1 has a role in regulating several stages of carcinogenesis [15] . It comprises two subunits: O_2 labile HIF-1α and constitutively expressed HIF-1β[16] . Under hypoxic circumstances, the deficiency or reduced concentration of O_2 leads to the stabilisation of HIF-1α and its dimerisation with HIF-1β [17]. After that, the HIF-1 dimer attaches to DNA at the hypoxia response element (HRE) and turns on genes, such as the CA9 gene, that play a role in changing metabolism, genetic instability, and cancer [18]. The MAPK pathway is very important for sending signals outside of cells that are caused by different mitogenic and microenvironmental factors. Hypoxia stimulates ERKs by phosphorylation and nuclear translocation, enhancing the trans-activation capability of HIF-1 and facilitating the transcription of HIF-1-regulated genes [19]. CA IX is proposed to serve as a prognostic indicator, diagnostic marker, and therapeutic target in several cancer types[20,21]. This protein is mostly absent in most normal tissues, being prevalent only in the stomach and gallbladder. On the other hand, tumours such as those in the colon, breast, lung, ovary, brain, and so on frequently exhibit this protein, usually in their more aggressive forms. CA IX expression is associated with worse prognosis in several cancer types [22,23].

The results of our study showed that CA9 expression was common in oral SCC, with a mean histoscore of 113.04 ± 69.98 (mean ± SD). CA9 expression was also expressed in OSMF with a mean histoscore of 31.74 ± 29.49 (mean ± SD) and no expression in normal oral mucosa. The mean histoscore of carbonic anhydrase IX showed a substantial distinction between OSMF and OSCC. According to our results and the data from the literature, the intensity of CAIX expression is mainly strong, which may be related to the high accumulation of this protein in OSCC. This upregulation of CA9 in OSCC is probably because OSCC is frequently accompanied by necrosis, and CAIX is expressed predominantly around necrotic regions. This peri-necrotic expression reflects the association of tissue tumour CAIX with hypoxia [24]. The present study is in accordance with studies that also demonstrated a higher expression of CAIX in vulvar squamous cell carcinoma (VSCC) [25], esophageal squamous cell carcinoma (ESCC) [26], penile squamous cell carcinoma [27] , and oral squamous cell carcinoma [1].

The expression of Ca9 in OSMF is in accordance with the study conducted by Yang JS et al. [1], and the expression identifies with its abilities of malignant transformation. This is due to the fact that earlier studies have found an association of HIF-1α expression being significantly upregulated in areca quid chewing-associated oral cancer, mediated by arecoline [28]. HIF-1α regulates CA9 gene expression by binding to the hypoxia-response element (HRE) within the basal promoter of CA9 [29]. The study of Yang JS et al.,[1] has indicated an increase in mRNA and protein levels of CAIX in buccal mucosal fibroblasts treated with arecoline. Since arecoline is a cause of OSMF, it's possible that the production of arecoline from chewing areca nuts is linked to higher Ca9 levels in OSMF. This research posits that Ca9 levels progressively rise as the illness advances from OSMF to OSCC. The research demonstrated the pathogenic significance of direct CAIX detection in surgical specimens. This research is limited by its failure to examine plasma levels of Ca9, non consideration of various stages of OSMF and grades of OSCC.Further research is necessary to determine whether Ca9 can serve as a

potential biomarker to predict malignant transition from OSMF to OSCC with relatively larger sample size and study designs to overcome the limitation of this study before considering the Ca9 as a potential biomarker for malignant transformation.

Conclusion

Carbonic Anhydrase IX (CA9), a protein linked to low oxygen levels in tumour microenvironment (TME), showed increase in its average H score as the disease progressed from OSMF to OSCC. This could be due to the heightened hypoxic conditions in OSCC. The study found that average histo score of CA9 effectively differentiated OSMF from OSCC.

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