European Journal of Cell Biology 100 (2021) 151146

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Research paper

Pathophysiological relationship between hypoxia associated oxidative stress, Epithelial-mesenchymal transition, stemness acquisition and alteration of Shh/ Gli-1 axis during oral sub-mucous fibrosis and oral squamous cell carcinoma

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ARTICLE INFO

Keywords:
Oral sub-mucous fibrosis
Oral squamous cell carcinoma
Oxidative stress
Epithelial-mesenchymal transition
Stemness

ABSTRACT

Oral sub-mucous fibrosis (OSF) is a pathophysiological state of oral cavity or oropharynx having a high chance of conversion to oral squamous cell carcinoma (OSCC). It involves fibrotic transformation of sub-epithelial matrix along with epithelial abnormalities. The present work aims to unveil the mechanistic domain regarding OSF to OSCC conversion exploring the scenario of hypoxia associated oxidative stress, epithelial-mesenchymal transition (EMT), metastasis and stemness acquisition. The study involves histopathological analysis of the diseased condition along with the exploration of oxidative stress status, assessment of mitochondrial condition, immunohistochemical analysis of HIF-1α, E-cadherin, vimentin, ERK, ALDH-1, CD133, Shh, Gli-1 and survivin expressions in the oral epithelial region together with the quantitative approach towards collagen deposition in the sub-epithelial matrix. Oxidative stress was found to be associated with type-II EMT in case of OSF attributing the development of sub-epithelial fibrosis and type-III EMT in case of OSCC favoring malignancy associated metastasis. Moreover, the acquisition of stemness during OSCC can also be correlated with EMT. Alteration of Shh and Gli-1 expression pattern revealed the mechanistic association of hypoxia with the phenotypic plasticity and disease manifestation in case of OSF as well as OSCC. Shh/ Gli-1 signaling can also be correlated with survivin mediated cytoprotective phenomenon under oxidative stress. Overall, the study established the correlative network of hypoxia associated oxidative stress, EMT and manifestation of oral pre-cancerous and cancerous condition in a holistic approach that may throw rays of hope in the therapeutic domain of the concerned diseases.

1. Introduction

Pre-cancers or pre-malignancy refers to certain conditions that have the potential to progress to the cancers (NCI, 2011; Neville and Day, 2002). Pre-cancerous lesions are morphologically atypical tissue that appears abnormal under microscope which is more likely to progress to cancers than the normal tissue (Yardimci et al., 2014a). Oral sub-mucous fibrosis (OSF), a failed wound-healing condition of oral mucosa due to chronic sustained injury, is one of the important potentially-malignant disorders (OPMDs) of oral cavity and oropharynx with a high chance of conversation into oral squamous cell carcinoma (OSCC) (Yardimci et al., 2014b; Banerjee and Chatterjee, 2015). Chewing of arecanut alone or in combination with other tobacco products often triggers the development of such pre-malignant feature (Cox, 2008). Most of the cases of oral pre-cancers and cancers have been recorded in the developing countries where Indian subcontinent shows a

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https://doi.org/10.1016/j.ejcb.2020.151146
Received 6 September 2020; Received in revised form 8 December 2020; Accepted 16 December 2020
Available online 23 December 2020

high rate of incidence (Mortazavi et al., 2014; Dhillon et al., 2018; de Camargo Cancela et al., 2010). The detailed mechanistic aspects of transformation of such OPMDs to the OSCC largely remained elusive till date. The present study holds a mechanistic approach towards understanding the process from the view point of bio-molecular changes.

Abnormal production of reactive oxygen species (ROS) can bring about oxidative stress causing a lot of pathophysiological alterations of normal signaling proteins, macromolecules, nucleic acid etc. which indeed results in the development of a great many human diseases (Li et al., 2016; Schieber and Chandel, 2014). ROS has been reported to promote malignancy through ERK signaling cascade (McCubrey et al., 2007; Jing et al., 2011; Chatterjee and Chatterjee, 2020). Work of Radisky et al. (2005) and Rhyu et al. (2005) also presented ROS as a facilitator of epithelio-mesenchymal transition (EMT) in certain cell types. For more than two decades, the concept of EMT emerged as the major mechanism of "invasion-metastasis cascade" for the carcinoma cells (Fidler, 2003; Thompson and Newgreen, 2005; Shibue and Weinberg, 2017). It includes remarkable morphological alterations from epithelial to mesenchymal phenotype that facilitates enhanced motality and invasion (Sarkar et al., 2017; Brabletz et al., 2018). EMT also promotes cancer stem cell (CSC) phenotype i.e. stemness marker pattern, *in vitro* sphere forming ability, tumor-seeding potency as well as therapeutic resistance (Mani et al., 2008; Morel et al., 2008; Pirozzi et al., 2011; Saxena et al., 2011). Work of Higgins et al. (2007) demonstrated that hypoxia-inducible factor (HIF) signaling plays vital role in the EMT of renal epithelial cells. Copple (2010) also demonstrated the role of HIF-1α in promoting hepatocyte EMT. Hypoxia is associated with the over-production of ROS (Hamanaka and Chandel, 2009) and Zhou et al. (2009) identified mitochondrial ROS as a crucial factor in hypoxia induced EMT of alveolar epithelial cells. Thus there is a clear reflection of the sequential process where hypoxia promotes ROS generation, ROS induces EMT and thereafter, EMT imparts stemness in cancer sub-population. Sonic hedgehog (Shh) signaling has been reported to play crucial role in both promoting EMT and maintaining cancer stem cell (CSC) population (Bhuria et al., 2019). Our recent review work also vividly discussed regarding the association of ROS, EMT and stemness in the perspective of oncogenesis (Chatterjee and Chatterjee, 2020). Apart from cancer cell metastasis, EMT is also reported to be involved in the process of fibrosis. Our previous study depicted the process of EMT as a vital phenomenon behind the progression of OSF (Das et al., 2013). In order to gain deep insights into the detailed intricate mechanistic scenario of OSF and OSCC, the present study made attempt to investigate the role of redox status and associated malignancy related signaling alteration, EMT, development of fibrosis, acquisition of stemness etc. in concerned oral pathophysiologies.

2. Materials and Methods

2.1. Collection of tissue samples

Oral incision biopsy samples from clinically detected OSF and OSCC were collected from Guru Nanak Institute of Dental Science and Research (GNIDSR), Kolkata, India. The patients considered for the study had the habit of smoking, consumption of tobacco products, chewing areca nut, pan masala etc. Each sample of OSF and OSCC was histologically confirmed after routine Haematoxylin and Eosin (H and E) staining by oral-pathologists. Representative clinical images (Fig. 1) were taken, and a proper clinical record (Table 1A, B) was maintained. Ten samples of oral mucosa removed from muco-periosteal buccal flaps obtained from surgical extraction of third molar from healthy individuals having no clinical symptoms of oral pre-cancer and cancer and without any oral habits had been considered as normal samples. Apart from taking biopsy, buccal smears were also collected on slides for Janus green-B staining. All the procedures had been carried out by the approval of Institutional Etical Committee (GNIDSR/IEC/15-1 dt. 05/01/2015) of GNIDSR and abiding by the guidelines of the Indian Medical Association and the World Medical Association.

2.2. Tissue processing

A part of the each biopsy specimen was kept for scanning electron microscopy (SEM) and the rest portion was fixed with 10% phosphate buffered formalin and thereafter subjected to paraffin blocking. Later on the paraffin embedded tissue blocks were subjected to microtomy to

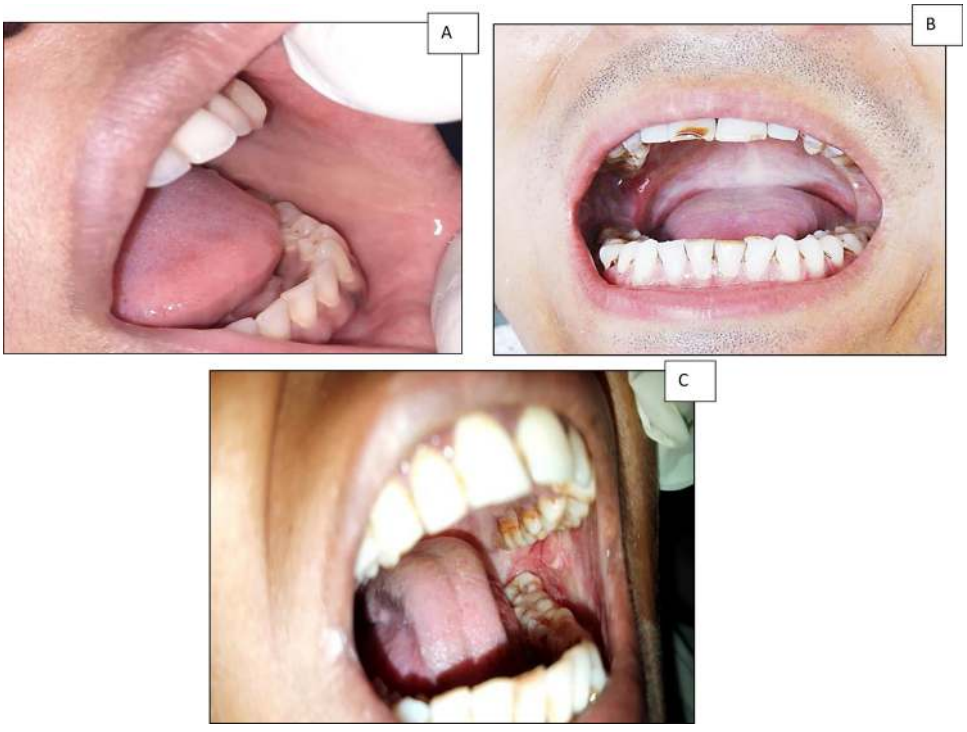

Fig. 1. Representative clinical photographs of Normal oral mucosa (A), Oral sub-mucous Fibrosis (OSF) (B) and Oral Squamous Cell Carcinoma (OSCC) (C).

Table 1
Clinical information on patients.

Table-1A: OSF		
Clinical Features	Number of patients	Percentage (%)
OSF Biopsies used	12	
Age		
Within 20-30 yrs	5	41.66
Within 30-40 yrs	7	58.33
Gender		
Male	8	66.66
Female	4	33.33
Habits		
Areca nut chewing	12	100
Different sites of involvement		
Buccal Mucosa, Labial Mucosa, Retromolar region		

Table-1B: OSCC		
Clinical Features	Number of patients	Percentage (%)
OSCC Biopsies used	14	
Age		
Within 30-40 yrs	6	40
Within 40-50 yrs	8	60
Gender		
Male	5	45
Female	9	55
Habits		
Tobacco product (khaini. Gutkha etc.) chewing	4	33.33
Areca nut chewing	5	41.66
Smoking	3	25
Smoking + tobacco product chewing	2	16.66
Different sites of involvement		
Buccal Mucosa and Gingival region		

obtain 4 μm thick tissue sections which were mounted on albumin and poly-L-lysine coated glass slides for histopathological, immunohistochemical, Fourier transform infrared (FTIR) spectroscopic as well as Atomic force microscopic studies.

2.3. Histopathology

Routine H and E staining was done with the tissue sections of normal, OSF and OSCC oral mucosa to understand the overall structural changes in the tissue level. Mallory's trichrome (MT) staining was done for the detection of altered collagen concentration in the sub-epithelial region of buccal mucosa in normal, OSF and OSCC conditions. Quantitative approach was implied to detect collagen intensity at different sites in sub-epithelium by image analysis using NIH Image J software.

2.4. Immunohistochemistry

Paraffinized tissue sections were baked at 60 °C for one hour followed by de-parafinization with xylene treatment. Re-hydration was done by bringing the sections into water after passing through downgrades of alcohol. Tissue sections were then subjected to antigen-retreval by heating in microwave with Tris EDTA buffer for 20 min using EZ-antigen Retrieval System V2 (BioGenex, USA). Immunostaining of the tissue sections were done using the following antibodies: anti-4-HNE (Abcam, UK), anti-HIF-1α (Abcam, UK), anti-E-cadherin (Abcam, USA), anti-vimentin (Abcam, USA), anti-ALDH-1 (Cell Signaling Technology, USA), anti-CD133 (Cell Signaling Technology, USA), anti-Shh (Cell Signaling Technology, USA), anti-Gli-1 (Cell Signaling Technology, USA), anti-survivin (Abcam, UK). Immuno-detection was done by kit-based chromogenic method (Super Sensitive Polymer-HRP IHC Detection System kit, BioGenex, USA) using HRP-conjugated secondary antibodies and chromogen 3,3′-diaminobenzidine (DAB). Sections were counterstained with Hematoxylin and observed under light microscope (Leica DM750). Microscopic images were analyzed using NIH Image J software to determine the DAB staining intensity.

2.5. FTIR Spectroscopy

Paraffin-embedded tissue sections were thoroughly deparaffinised in xylene, and dehydrated with acetone treatment. The dehydrated tissues were ground with KBr before casting into pellets. The KBr pellets containing the sample were then used for FTIR (Nicolet 6700, Thermo Fisher, USA) in transmission mode. The spectral data [Fig. 2] were collected with a DTGS detector (aperture 8 mm) in the range of 4000-400 cm-1 with a spectral resolution of 4 cm^{-1} by averaging 32 scan per run. A total of N = 3 samples were used for each group for FTIR spectroscopy and analysis. The spectral analysis was performed in Matlab R2019a and Spectragryph 1.2 software. All spectra were cropped in the range of desired bands. For lipid oxidation study the range of 3800-1400 cm^{-1} was used.

2.6. Atomic Force Microscopy

Atomic force microscopy (AFM) of the tissue sections were done following the protocol of Anura et al. 2017 (Anura et al., 2017). In brief, sections were de-parafinized, hydrated and subjected to AFM scanning (Bruker Multimode 8 AFM, Bruker Corporation, USA) at rate of 0.8 Hz with 256 × 256 pixel resolution at room temperature of 24 °C in PFQNM mode. The processing and analysis of the AFM images were done using the NanoScope Analysis 1.5 software (Bruker, USA).

2.7. Scanning Electron Microscopy

Tissue samples kept for SEM were fixed in 2.5% gluteraldehyde for overnight. The tissues were then dehydrated by passing through ascending grades of alcohol. Thereafter, the tissues were dried, coated with gold in vacuum and finally subjected to scanning electron microscopy (Zeiss, Germany).

2.8. Analysis of mitochondrial condition

Mitochondrial status of the oral epithelial cells during normal, OSF and OSCC conditions were studied by Janus Green-B (JGB) staining. It is a supravital staining procedure where the oxidative condition that generally prevails in the mitochondria can be determined by the intensity of the blue-green coloration. JGB staining indirectly determines the mitochondrial dysfunction because any rupture in the mitochondrial membrane results in the release of oxidative content of the mitochondria in the cytosol and increased blue-green staining intensity of the cytosol due to JGB staining correlates to the extent of the mitochondrial membrane rupture. JGB staining was performed with the buccal smears of epithelial cells from normal, OSF and OSCC individuals as per the protocol of Chatterjee et al. (2016). Briefly, the buccal smears were

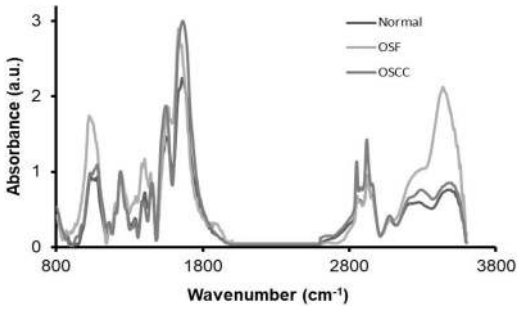

Fig. 2. Representative FTIR spectra from normal, OSF and OSCC oral mucosal samples.

incubated in 0.1 % of JGB solution for 5-7 minutes at 37 °C, washed and observed under light microscope followed by Blue-green staining intensity scoring.

2.9. Statistical analysis

Quantitative data were subjected to Analysis of Variance (ANOVA) followed by Tukey's post hoc test for finding the statistical significance. P-values < 0.05 were considered to be statistically significant. Pearson correlation coefficient (r) was determined for the expression pattern of 4-HNE and HIF-1α as well as Shh and Survivin.

3. Results

3.1. Pathophysiological structural alterations in the epithelial layers of buccal mucosa in OSF and OSCC conditions

Severe histological alterations were found in the oral mucosa during OSF and OSCC as depicted by H and E staining. The epithelial projections (rete pegs) in the underlying connective tissue was clearly visible in case of normal oral mucosa [Fig. 3A] where as flattening of rete pegs was evident in OSF condition [Fig. 3B]. OSCC samples displayed disruption of the general architecture of epithelial layers and migration of epithelial cells in the sub-epithelium to form cellular colonies [Fig. 3C]. Scanning electron microscopy revealed the occurrence of typical "honey-comb" structures [Fig. 3D] with discontinuous parallel micro-ridges and pits on the oral epithelial surface in normal condition whereas OSF epithelium was mostly pitted with holes while the ridges were found to become flat as reported earlier (Nawn et al., 2019). Epithelial disruption and breakage was evident in the case of OSCC surface [Fig. 3E].

3.2. Increased hypoxia associated oxidative stress in OSF and OSCC

Oxidative stress has been regarded as one of the key factor for oncogenesis (Waris and Ahsan, 2006). Lipid peroxidation is a fundamental consequence of oxidative stress and free radical production (Signorini et al., 2013; Taso et al., 2019). Since the quantification of ROS in fixed tissue sample is challenging due to their short half-lives, magnitude of oxidative stress are quantified in biological samples by measuring the oxidation products (Shulaev and Oliver, 2006; Zarkovic, 2003; Erejuwa et al., 2013; Sultana et al., 2013). Oxidation of lipids due to interaction with reactive oxidative species can lead to conversion of unsaturated fats to carbonyl groups (aldehydes and ketones) (Wang et al., 2017). Therefore measuring the ratios of carbonyl stretching (C = O) with an absorbance peak at 1738 cm-1 to Olefinic = CH stretching (for unsaturated lipids) absorbance at 3012 cm^{-1} (Cakmak et al., 2012) can be used as measure to the amount of lipid oxidation. FTIR spectroscopy based evaluation of the absorbance ratios at 1738 cm^{-1} and 3012 cm^{-1} revealed that the each group normal, OSF, and OSCC are significantly different with the lipid oxidation. A gradual increase in the lipid oxidation was noted from normal to OSF to cancerous conditions [Fig. 4A]. Occurrence of oxidative stress in pre-cancerous and cancerous conditions was further validated by measuring the level of 4 Hydroxynonenal (4-HNE). Being a product of lipid peroxidation, 4-HNE is considered as a sensitive biomarker of oxidative stress (Majima et al., 2002; Dalleau et al., 2013; Breitzig et al., 2016). Evaluation of oxidative stress using anti-4-HNE antibody based immunohistochemistry assay is in practice for long time (Uchida et al., 1993; Uchida et al., 1995; Majima et al., 1998; Skrzydlewska et al., 2005; Jo et al., 2011) and we have used the same in the present study as well. Gradual increase of the 4-HNE staining intensity was found in OSF and OSCC as compared to that of normal [Fig. 4B-E] which also hinted towards the increase of oxidative stress in pre-cancerous and cancerous conditions. Hypoxia is often associated with the over-production of ROS. Along with the increased 4-HNE staining, expressional elevation of HIF-1α was found in OSF and OSCC as compared to that of normal [Fig. 4F-I]. Strong correlation (r = 0.9837) between 4-HNE and HIF-1α expressions [Fig. 4J] depicted the association of oxidative stress and hypoxia during OSF and OSCC. Janus Green-B staining showed the increase of the number of oral epithelial cells having moderate to deep blue-green staining intensity in the OSF and OSCC which signifies mitochondrial disruption, a commonly oxidative stress associated process [Fig. 4K]. From all the data a correlative scenario evolved regarding the association of hypoxia and oxidative stress in OSF and OSCC condition [Fig. 4L].

3.3. Oxidative stress associated EMT in OSF and OSCC

Various studies reported the attribution of oxidative stress in the progression of EMT (Chatterjee and Chatterjee, 2020). Phosphorylation of ERK has been regarded as one of the "link-man" between oxidative stress and EMT (Wang et al., 2010; Olea-Flores et al., 2019).

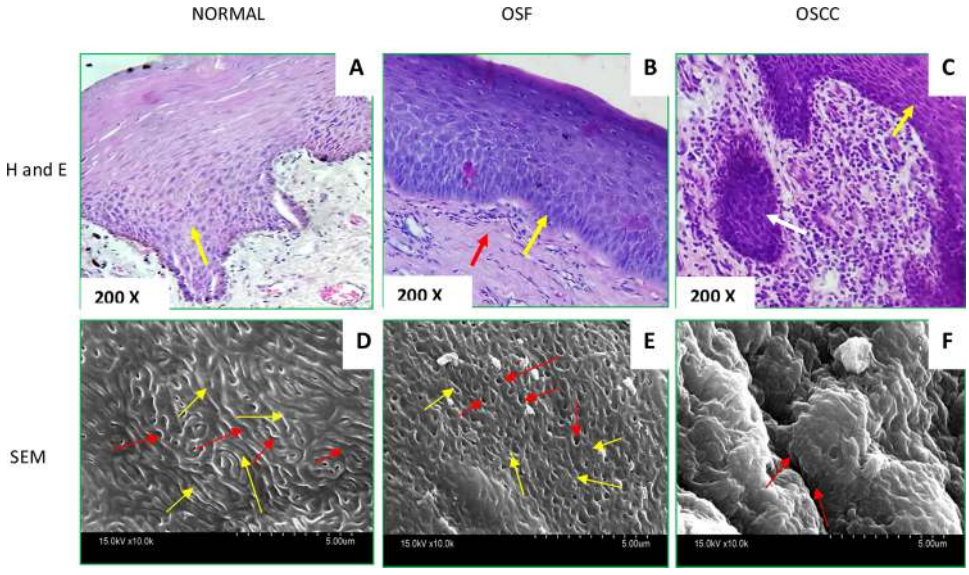

Fig. 3. Morphological alterations in buccal mucosa during OSF and OSCC. Routine H & E staining of the histological sections of oral mucosa showed in normal condition (A) proper rete pegs (marked with yellow arrow) were found in the epithelial region where as flattening of the rete ridges (marked with yellow arrow) were observed in OSF condition (B). Densely packed the sub-epithelial matrix (marked with red arrow) was evidenced in case of OSF. Migration of cells from epithelial region (marked with yellow arrow) and formation of secondary metastatic colony (marked with white arrow) was observed in OSCC sample (C). Scanning electron microscopy of surface epithelia of oral mucosa revealed the occurrence of regular ridges (marked with yellow arrow) and subsequent pits (marked with red arrow) in normal case (D) where as in case of OSF (E), surface was mostly pitted (marked with red arrow) and flattening of ridges (marked with yellow arrow) were found. Structural disruption together with breakage (marked with red arrow) was seen in case of OSCC (F). [Magnification; A-C = 200X, D-F =10 K].

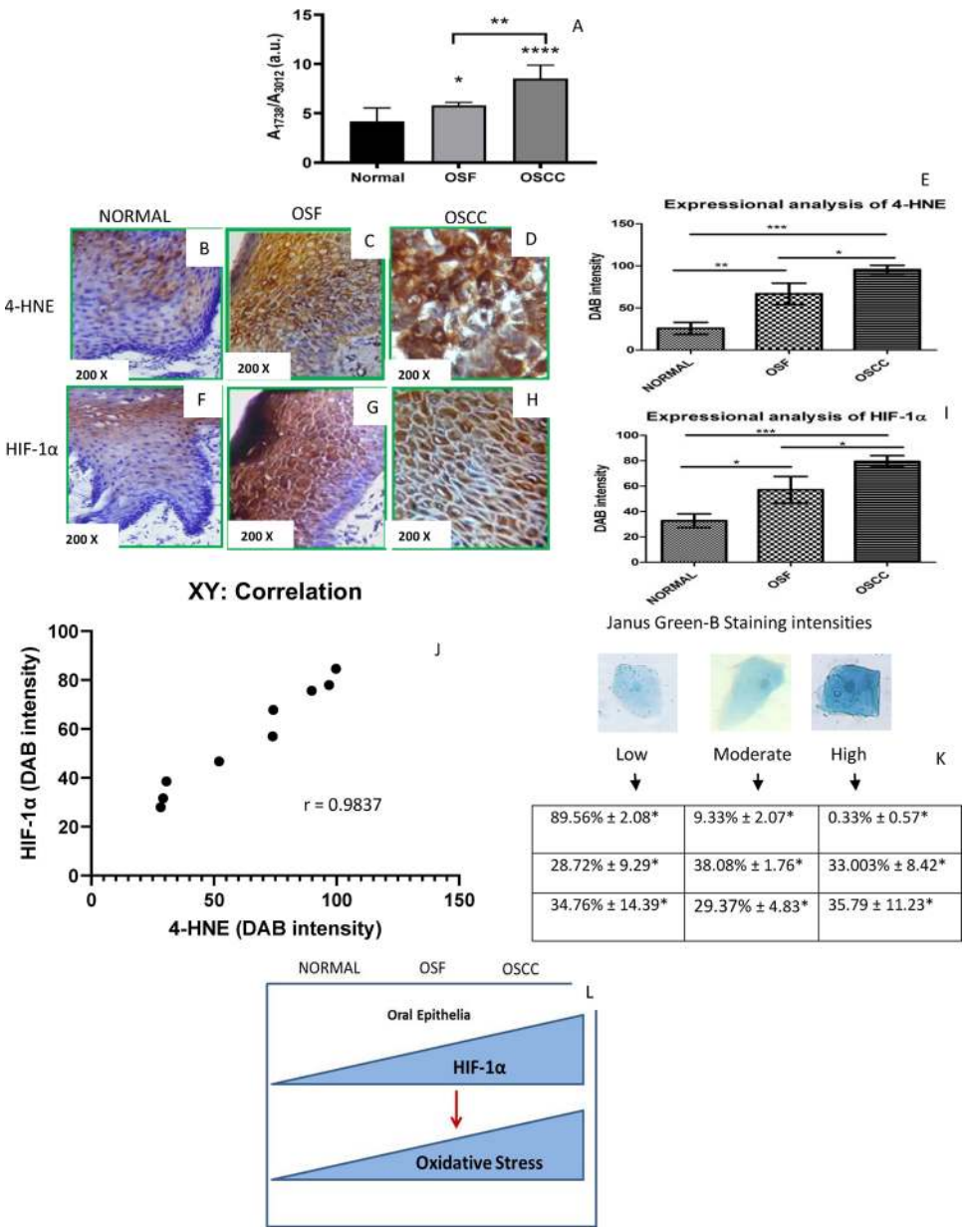

Fig. 4. Hypoxia associated oxidative stress in the buccal mucosa during OSF and OSCC. Graphical representation of the ratio of FTIR peaks corresponding to the amount of oxidation level by conversion of unsaturated lipids to carbonyl groups depicted the scenario of the increased lipid oxidation from normal to OSF to OSCC (A). Immunohistochemistry revealed that compared to normal oral epithelia (B), expression of 4-HNE increased in OSF (C) and OSCC (D). Graphical representation of the variation of 4-HNE expression has been presented in panel-E. Expression of HIF-1α also rises from normal oral epithelia (F) to OSF (G) to OSCC (H) and the variation is represented graphically in panel-I. A Strong expressional correlation (Pearson correlation coefficient; r = 0.9837) was found between the variation of HIF-1α and 4-HNE in different groups (J). Panel-K represents the variation of Janus green-B staining intensity in the buccal epithelial cells from normal, OSF and OSCC subjects. Panel-L consists of the schematic representation of the association of hypoxia and oxidative stress with regard to the progression of oral pre-cancer and cancer. [Magnification; B-D, F-H = 200X, M = 400X]. (P values obtained by ANOVA followed by Tukey's post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001).

Expressional increase of p-ERK was found in the epithelial cells of buccal mucosa in OSF and OSCC conditions as compared to that of normal [Fig. 5A-D]. Cardinal signs of EMT were also evident in OSF and OSCC. Expressional decline as well as gradual cytoplasmic diffusion of epithelial marker E-cadherin was found in case of OSF and OSCC in contrary to deep membrane bound E-cadherin expression in normal [Fig. 5E-H]. On the other hand, expression of mesenchymal marker vimentin was increased in OSF and OSCC cells as compared to that of normal [Fig. 5I-L]. Expressional increase of β-catenin has been linked with the progression of EMT by Zhu et al. (2018). Gradual increase of β-catenin expression was found in OSF and OSCC as compared to that of normal [Fig. 5M-P]. Moreover, more and more cytoplasmic diffusion of β-catenin in case of OSF [Fig. 5N] and nuclear diffusion in case of OSCC [Fig. 5O] were observed which depicted the scenario of gradual malignant conversion from pre-cancerous state to cancerous condition. Thus the overall findings hinted towards oxidative stress associated p-ERK expression in the facilitation of EMT process during OSF and OSCC [Fig. 5Q].

3.4. EMT associated fibrosis in OSF and cellular metastasis in OSCC

Emerging evidences suggests that EMT is one of the vital causes for fibrosis and cellular metastasis (López-Novoa and Nieto, 2009; Heerboth et al., 2015). Mallory`s trichrome staining clearly showed that in comparison with normal oral mucosa [Fig. 6A], considerable increase in the collagen deposition was found OSF condition which was evident by the presence of thick collagen bundles in the sub-epithelial matrix that took blue coloration [Fig. 6B]. In OSCC tissue [Fig. 6C], collagen masses were found to be loosely arranged which is reported to promote cellular migration in malignant condition through the matrix. Quantitative approach by measuring average gray scale intensity from different areas of sub-epithelium of MT stained images minutely depicted the increase of collagen density in OSF and degradation of collagen in OSCC [Fig. 6D]. AFM analysis of the sub-epithelial matrix of oral mucosa also revealed that in contrary, to that of moderate collagen fiber content in normal [Fig. 6E], increased accumulation of collagen bundles occurred in case of OSF [Fig. 6F]. Degradation of the sub-epithelial matrix during OSCC was also depicted by the AFM images [Fig. 6G]. Routine H and E

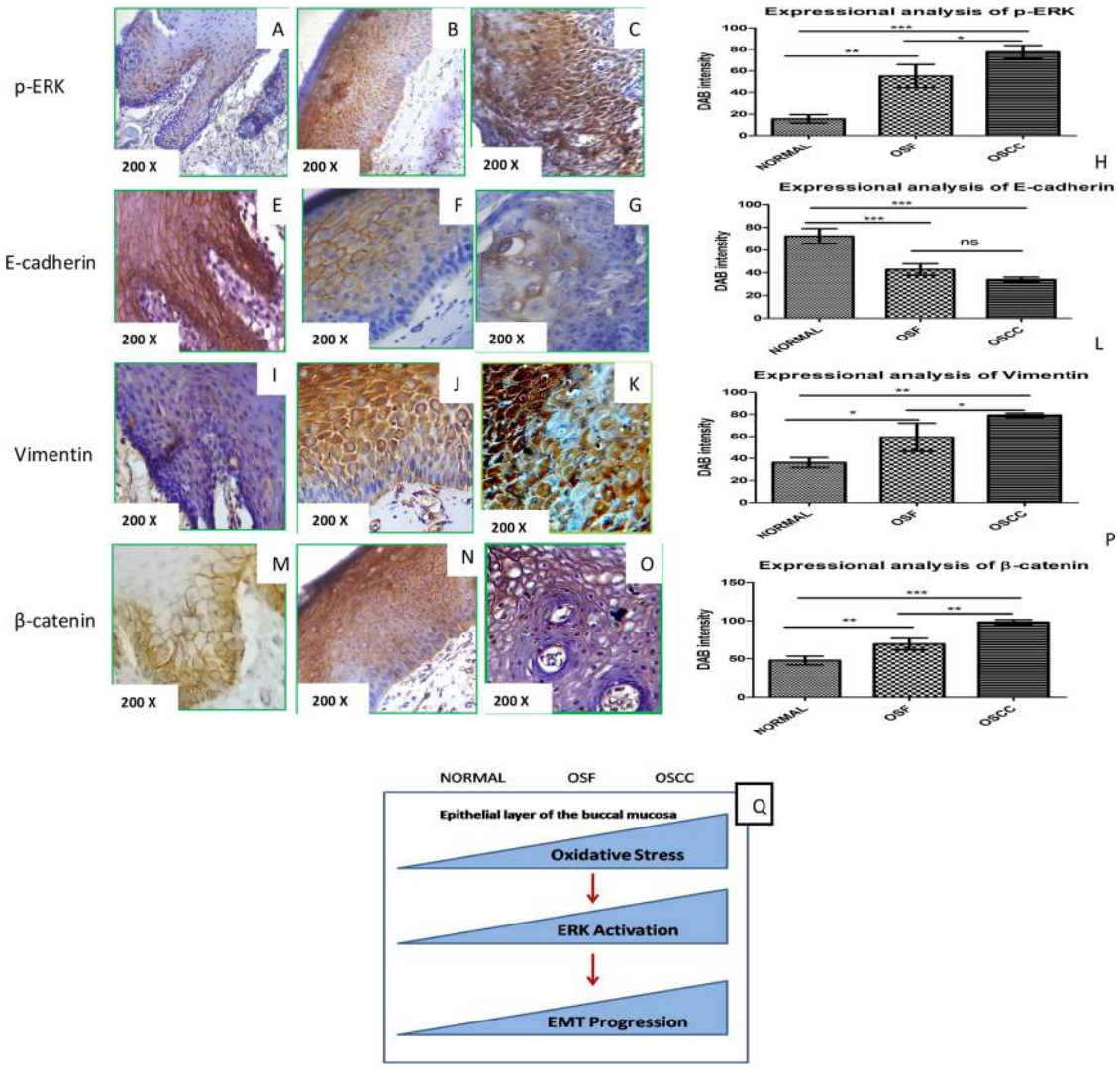

Fig. 5. EMT in buccal mucosa in pre-malignant and malignant conditions. Immunostaining of histological sections showed the increase of phosphor-ERK from normal oral epithelia (A) to OSF (B) to OSCC (C). The variation has been graphically represented in panel-D. E-cadherin expression decreased from normal oral epithelia (E) to OSF (F) to OSCC (G) along with cytoplasmic diffusion. Panel-H presents the variation of E-cadherin expression graphically. Expression of vimentin increased from normal oral epithelia (I) to OSF (J) to OSCC (K). Graphical representation of the variation has been depicted in panel-L. β-catenin expression was found to be increased from normal oral epithelia (M) to OSF (N) to OSCC (O) together with its cytoplasmic diffusion in OSF to cytoplasmic as well as nuclear diffusion in OSCC condition. Panel-P consists of graphical representation of the B-catenin expression among the mentioned conditions. The association of oxidative stress, ERK activation and EMT has been schematically represented in panel-Q. [Magnification; A-C, E-G, I-K, M-O = 200X] (P values obtained by ANOVA followed by Tukey's post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant).

staining further showed that in contrast to normal [Fig. 6H] and OSF condition [Fig. 6I], in case of OSCC, there was cellular migration from primary site to form secondary metastatic knot [Fig. 6J].

3.5. Acquisition of stemness in OSCC

Many studies showed the association of EMT in imparting stemness phenotype as well as drug resistance in cancer cells (Chatterjee and Chatterjee, 2020; Mani et al., 2008; Morel et al., 2008; Pirozzi et al., 2011; Saxena et al., 2011). Population of the cancer stem cells (CSC) is very small among the bulk tumor mass and these cells are reported to be quiescent, drug-resistant acting as "cancer-seeds" (Kaiser, 2015; Nassar and Blanpain, 2016). Liu et al. (2013) used ALDH-1 and CD133 as the potential markers for cancer stem cells (CSCs) during malignant transformation of oral tissue. Immunohistochemical staining showed that in contrast to normal [Fig. 6K] and OSF [Fig. 6L], ALDH-1 positivity was found in OSCC tissues particularly at metastatic knots [Fig. 6M]. Similar type of observation was also documented in case of CD133 expression pattern analysis with normal, OSF and OSCC oral tissues [Fig. 6N-P]. All the findings pointed towards the fact that along with the fibrosis during OSF and metastasis during OSCC, EMT can also be correlated with the acquisition of stemness phenotype in malignant condition [Fig. 5Q].

3.6. Hypoxia associated up-regulation of Shh and Gli-1 in pre-cancerous and cancerous condition

Shh-Gli-1 signaling axis plays vital role in promoting the process of EMT during carcinogenesis (Yoo et al., 2011; Xu et al., 2012; Zhang et al., 2016; Riaz et al., 2019). Various literatures also documented the attribution of Shh-Gli-1 signaling in the acquisition of stemness as well as in the maintenance of CSC population (Syed et al., 2016; Koury et al., 2017; Sari et al., 2018). Works of Bhuria et al. (2019), Liu et al. (2020), Hapke and Haake (2020) showed the interrelationship between the activation of Shh signaling and occurrence of hypoxia. Moreover, Dai et al. (2011), Peterson and Turnbull (2012) emphasized on the cyto-protective role of Shh signaling against oxidative stress. Interaction of

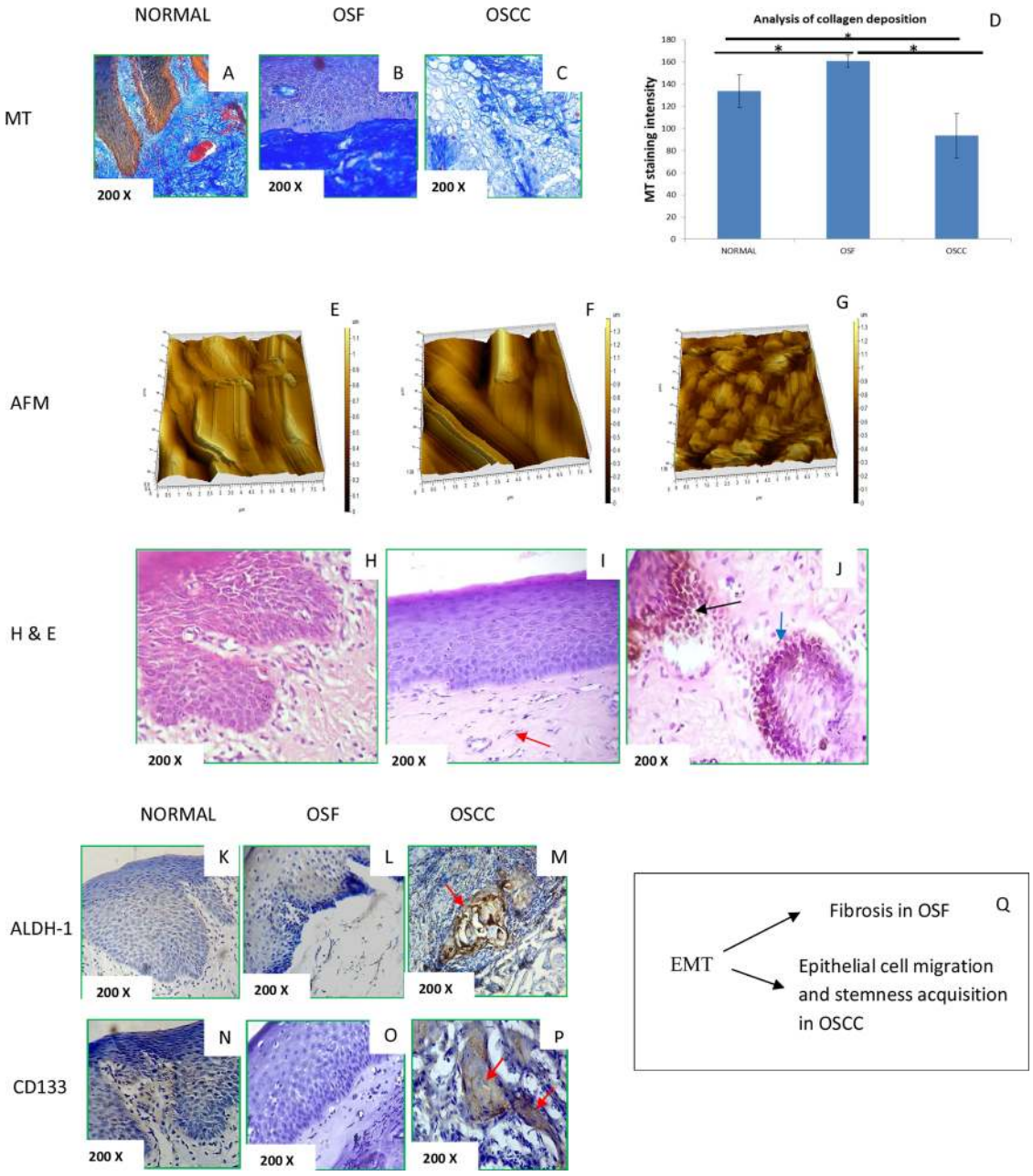

Fig. 6. Attribution of Oxidative stress associated EMT in the pathogenesis of OSF and OSCC. Mallory's trichrome staining depicted moderately arranged collagen fibers in the sub-epithelial matrix of normal oral mucosa (A) that took blue coloration. In case of OSF (B) dense collagen deposition was noticed in the sub-epithelial matrix. Degraded connective tissue arrangement was found in OSCC sample (C). Graphical representation of the differential staining intensity in case of normal, OSF and OSCC oral tissues has been depicted in panel-D.
Atomic force microscopy (AFM) of the sub-epithelial region of oral mucosa also showed that compared to moderate collagen deposition in case of normal (E), OSF sample exhibited densely arranged collagen fibers (F). AFM also depicted matrix degradation in OSCC sample (G). H & E staining showed that in contrary to normal (H) and OSF (I), oral mucosa during OSCC (J) exhibited metastasis of epithelial cells from primary site to form metastatic colony. Immunohistochemical staining exhibited that the oral epithelial cells of normal (K) and OSF (L) samples were negative for the stemness marker ALDH-1 while ALDH-1 positivity was found in the metastatic islands in case of OSCC (M). Similar finding was also documented for another CSC marker CD133. In comparison to normal (N) and OSF (O), metastatic knots in case of OSCC (P) showed positivity for CD133. Panel-Q schematically represents the association of EMT with the manifestation of OSF and OSCC. [Magnification; A-C, H-J, K-P = 200X] (P values obtained by ANOVA followed by Tukey's post hoc test; *P < 0.05)

Shh with specific receptors *viz*, Smo and Patched, leads to the activation and nuclear translocation of Gli-1for transcriptional regulation (Carballo et al., 2018). The present study revealed that as compared to normal [Fig. 7A, D], the expressional increase of Shh was found in oral epithelia in case of OSF [Fig. 7B, D] and OSCC samples [Fig. 7C, D]. Moreover, sequential increase of nuclear expression of Gli-1 was found from normal to OSF to OSCC condition [Fig. 7E-G]. Studies by Parfitt and Driman (2007), Shi et al. (2012), Brun et al. (2015), Vlčková et al. (2016), Hehlgans et al. (2018) established the association between the expression of survivin and Shh-Gli signaling. Survivin is reported to play vital role to prevent oxidative stress associated cellular apoptosis promoting the neoplastic condition of cells (Kan et al., 2013). Expression of survivin was found to be gradually increased in oral epithelia from normal condition to OSF to OSCC [Fig. 7H-K] which depicted the survivin mediated inhibition of tumor-suppressive role of ROS during the process of oral carcinogenesis. A strong correlation (r = 0.9384) was

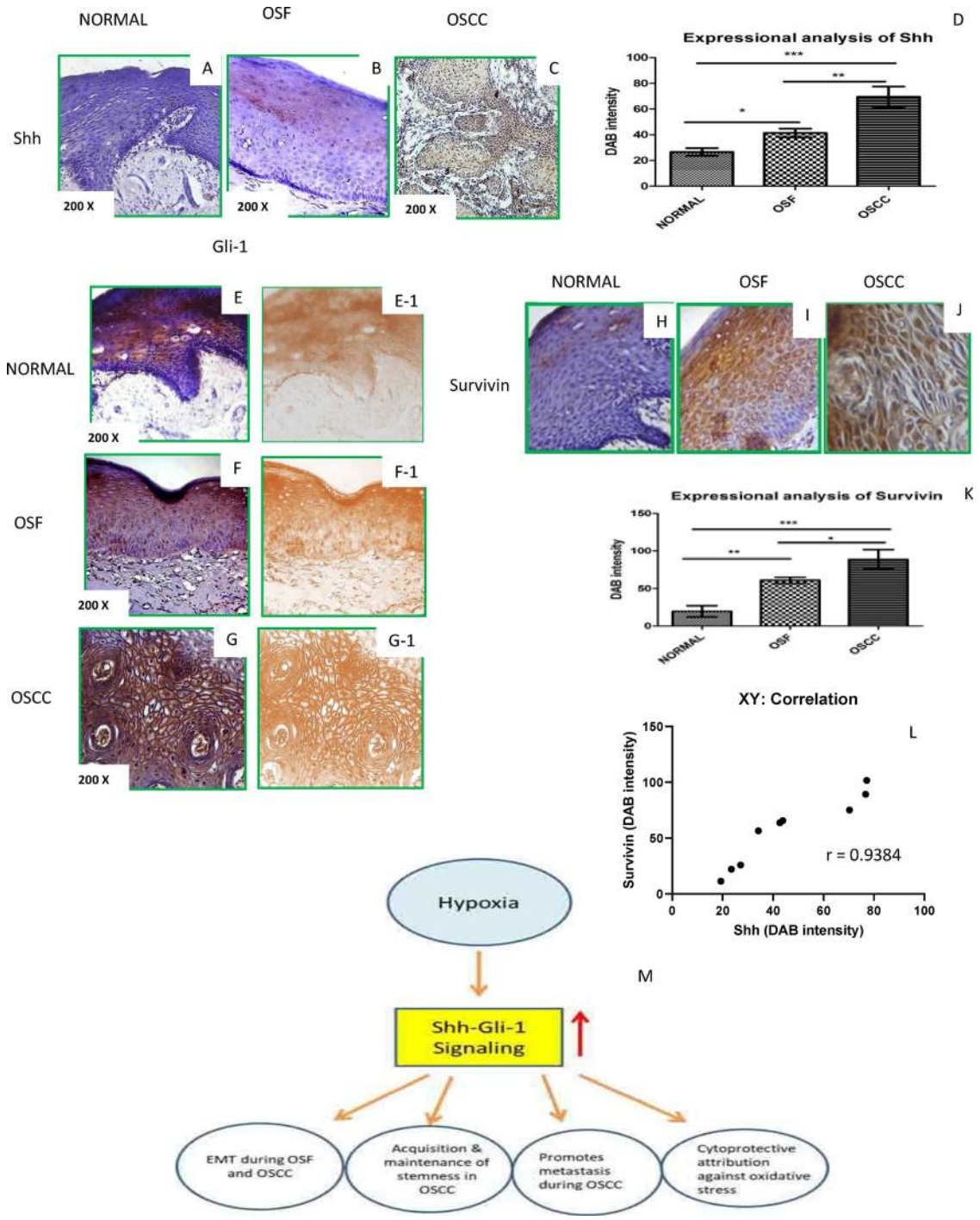

Fig. 7. Alteration of Shh/ Gli-1 axis in OSF and OSCC. Immunohistochemical analysis showed an increase in the expression pattern of Shh from normal (A) to OSF (B) to OSCC (C) samples. This expressional increase of Shh has been graphically represented in panel-D. Images taken for the immunohistochemical study along with the Image-J extracted figures of DAB staining showed that as compared to normal (E, E-1), gradual increase in the nuclear translocation of Gli-1 was evident in case of OSF (F, F-1) and OSCC (G, G-1) samples. Expression of survivin was also found to be increased from normal oral epithelia (H) to OSF (I) to OSCC (J) which has been graphically represented in panel-K. A strong correlation (Pearson correlation coefficient; r = 0.9384) exists between the expression patterns of Shh and survivin (L). Panel-M schematically shows the association of hypoxia and the up-regulation of Shh/ Gli-1 axis which promoted the pathophysiological phenomenon during OSF and OSCC. [Magnification; A-C, E-J = 200X] (P values obtained by ANOVA followed by Tukey's post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001)

found between the expression patterns of Shh and surviving [Fig. 7L]. Thus in the present context of oral oncogenesis, the survivin associated suppression of the tumor suppressive role of oxidative stress can be linked with the up-regulation of the Shh-Gli-1 pathway facilitating the oncogenesis. Moreover, increased expression survivin can further be linked with the EMT associated expressional elevation of β-catenin in pre-malignant and malignant condition as survivin is a common target gene of β-catenin (Kim et al., 2003; Tapia et al., 2006). Simultaneous prevalence of hypoxia along with the activation of the major players of Shh-Gli-1 signaling axis in pre-cancerous and cancerous condition of oral mucosa hinted towards the mechanistic relationship between the said processes in promoting EMT and stemness acquisition, maintaining

CSC phenotype as well as facilitating oxidative stress associated neoplastic transformation [Fig. 7M].

4. Discussion

The present study aims to establish the mechanistic association of oxidative stress and EMT in the oral mucosa during OSF and OSCC. The increase of lipid peroxidation together with mitochondrial disruption depicted the oxidative stress related insults in both the pre-cancerous and cancerous conditions. This was also found to be associated with the development of hypoxic condition, a well discussed phenomenon by Hamanaka and Chandel (2009).

Oxidative stress has been described as a "double-edge sword" with regard to oncogenesis as it can both promote tumor progression as well as suppress tumor formation (Pan et al., 2009; Acharya et al., 2010). One of the tumor suppressive activities mediated by ROS is the induction of cellular apoptosis. Expressional elevation of survivin in buccal epithelia during OSF and OSCC mark the inhibition of redox imbalance associated apoptosis. This was in support of the findings by Kan et al. (2013). Inhibition of the oxidative stress mediated tumor suppression can be linked with the facilitation of malignant conversion in both the pre-cancerous and cancerous lesions of buccal mucosa. Activation of Shh-Gli-1 signaling axis along with the occurrence of hypoxia can be regarded as a vital factor associated with the up-regulation of survivin.

Hypoxia associated with oxidative stress is reported to act as a facilitator of EMT process (Zhou et al., 2009). The hallmarks of EMT viz; expressional decline of E-cadherin, increased expressions of vimentin and β-catenin were found in the oral epithelia in both OSF and OSCC conditions. Moreover, the up-regulation of p-ERK clearly indicated the associative correlation between oxidative stress and EMT in pre-cancerous and cancerous pathologies which is in agreement with the findings of Wang et al. (2010), Olea-Flores et al. (2019).

EMT can contribute in both fibrosis and cellular metastasis (López-Novoa and Nieto, 2009). EMT associated with fibrosis is known as type-2 EMT while metastasis facilitating EMT is called type-3 EMT (Kalluri and Weinberg, 2010). Fibrotic transformation of sub-epithelial matrix in case of OSF samples as depicted by H and E staining, Mallory's trichrome staining as well as AFM imaging indicated the attribution EMT in fibrosis. On the other hand, in case of OSCC, EMT was found to be associated with cellular metastasis (Fig. 8).

Our recent study has focused on the role of EMT in imparting cellular stemness in malignant condition particularly in presence of oxidative stress (Chatterjee and Chatterjee, 2020). Expression of stemness markers in the metastatic colonies during OSCC farther strengthened our views regarding concomitant stemness acquisition with oxidative stress and EMT.

In addition to promoting oxidative stress mediated phenotypic plasticity in oral epithelia, hypoxia was also found to be associated with the expressional activation of Shh and Gli-1 in pre-cancerous and cancerous conditions. Up-regulations of the vital components of Shh-Gli-1 signaling axis can be well correlated with the occurrence of EMT as well as acquisition of stemness during oral carcinogenesis. Moreover, as mentioned earlier, Shh-Gli-1 signaling can also be linked with the cytoprotective phenomenon against oxidative stress.

Taken together all the findings, it can be concluded that the hypoxia associated oxidative stress acts as a promoting factor for EMT in both OSF and OSCC. EMT during OSF is associated with the development of sub-epithelial fibrosis while in case of OSCC it can be correlated with cellular metastasis and acquisition of stemness. Moreover, correlative up-regulations of Shh-Gli-1 signaling axis and prevalence of hypoxic condition added mechanistic clues for both phenotypic plasticity and acquisition of stemness in buccal epithelia during OSF and OSCC. Understanding of the correlation [Fig. 7] between hypoxia associated oxidative stress, EMT together with the manifestation of oral-precancers and cancers may certainly potentiate the development of new therapeutic aspect critically needed in this domain. However, additional studies involving large sample size will be helpful for better understanding of the mechanism.

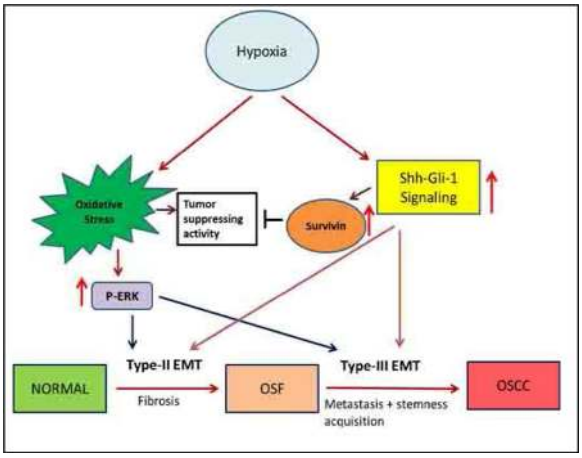

Fig. 8. Study outcome. Hypoxia associated generation of oxidative stress was found to promote type-II EMT in case of OSF and type-III EMT along with the acquisition of stemness in case of OSCC through the up-regulation of p-ERK. Hypoxia can also be related with the aberrant expression of Shh/Gli-1 axis promoting EMT and stemness during oncogenic process along with imparting cytoprotection against oxidative stress via survivin up-regulation.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

Financial support from the DBT-RA Program in Biotechnology and Life Sciences is gratefully acknowledged. The work was also supported by the grant from the Department of Science and Technology, Government of West Bengal, India (151(sanc.)/ST/P/S&T/ 9G-32/2016; Dated. 8.2.2018). Authors are also thankful to Dr. Aditya Jyoti Chowdhury and Dr. Piyali Adhikari of Guru Nanak Institute of Dental Science and Research (GNIDSR), Kolkata, India, for helping in sample collection from patients and keeping all the clinical records and also to Mr. Debnath Das of Indian Institute of Technology Kharagpur for assisting in microtomy process.

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