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UNRAVELING ABERRANT MICROENVIRONMENT IN ORAL SUBMUCOUS FIBROSIS

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Abstract

Objectives:. The objective of the present study was to assess the clinical correlation with biochemical changes in serum with histological findings in the tissue and serum of oral submuous fibrosis subjects and to explore the possibilities of blood-based biomarkers for the disease leading to oxidative stress

Methods: This study was conducted on serum and tissue sample of Oral Submucous fibrosis (OSF) (n = 20) compared with the healthy group (n = 20). Tissues were stained with special histochemical stains for carbohydrates [Periodic acid Schiff (PAS)], Lipids [Sudan IV], Collagen [Van Gieson's (VG Stain)] for histochemical feature analysis. The expression of glucose metabolism-related proteins glucose transporter 1 and hexokinase 2 in tissue was validated by western blot technique. A commercially available ELISA Kit quantified glucose transporter1(GLUT1)and hexokinase2(HK2) molecule in serum.

Results: Histochemical analysis by special stains supported the evidence of altered metabolic activity in tissues. Detail study of metabolic protein by western blot showed that the glucose transporter 1 and hexokinase 2 expression in oral submucous fibrosis tissues were significantly higher compared to normal. Further in serum elevated level of glucose transporter 1 and hexokinase showed that the serum expression followed a trend similar to the expression pattern observed and in tissues.

Conclusion: Histochemical analysis showed aberrant expressions of carbohydrate, lipid, and protein in the tissue. Finally, the study reports altered expression status of glucose transporter 1 and hexokinase 2 molecule. These intermediate molecules are precursors, as well as significant molecules of metabolic pathways which may increase oxidative damage and it can be used as a minimal invasive prognostic indicator of the disease.

Keywords: Glucose transporter 1; Hexokinase 2; Oral submucous fibrosis; Oxidative stress

1. Introduction

Oral Submucous fibrosis (OSF) is considered as a chronic inflammatory disease of unknown etiology with a high rate of malignant transformation. Diagnosis of oral submucous fibrosis currently consists of clinical examination, followed by biopsy (Rai, Mukherjee, Ghosh, Routray, & Chakraborty, 2017). There is a requirement for minimal invasive, markers for the disease that are specific for risk assessment. To achieve this goal better understanding of complex molecular events that regulate the progression of this disease is required (Varela-Centelles et al., 2017). Early detection not only decreases the incidence and mortality rate but also improves the survival of oral pre cancer (Epstein, Zhang, & Rosin, 2002). One of the main difference between biochemical characteristics of malignant cells compared to normal cells is altered metabolic pathway while the progression of disease towards carcinogenesis (Rai, Bose, Saha, & Chakraborty, 2019). Metabolic alteration takes place in three stages all of which lead to reprogramming in cancer metabolism (Rai, Bose, Saha, Kumar, & Chakraborty, 2019). First stage changes which occur in the cell is involvement of nutrient uptake. The main nutrient uptake in cells takes place through glucose transporter 1, a protein that mediates glucose transport through the plasma membrane and the glycolytic enzyme hexokinase2 which initiates the glycolysis pathway (Kato et al., 2002; Rai, Mukherjee, et al., 2018; Wolf et al., 2011).
In contrast, many studies have reported overexpression of glucose transporter 1 and hexokinase in various types of cancers, like lung, esophageal, breast, uterine, pancreatic, and oral squamous cell carcinoma (Cho, Lee, Kim, Chung, & Kim, 2013; Kunkel et al., 2003; Li, Yang, Wang, & Ran, 2013; Rai et al., 2017; Wang et al., 2017). Moreover, the results of the pilot study conducted by us in the previously published paper also showed the alteration of these metabolic profiles in the tissue of Oral submucous fibrosis (Rai, Bose, Mukherjee, Sarbajna, & Chakraborty, 2018). The present study aims to identify the altered metabolic pathways and correlate altered metabolites with histopathological and serum biochemical features for identification of potential diagnostic markers related to Oral submucous fibrosis. The identification of metabolic markers can significantly improve diagnosis by focusing on the onset and progression of tumor development.

2. Material & Methods

Ethical clearance was obtained from the Institute [No IIT/SRIC/SAO/2015]. Biopsy specimens and serum samples of same patients were collected from dental OPD in Bidhan Chandra Roy Technology Hospital, I.I.T Kharagpur and Barasat cancer research hospital, Kolkata.

2.2.1 Histochemical Analysis

Paraffin-fixed tissues were processed, and 4-µm ribbons were stained with special stains for Carbohydrates [Periodic acid Schiff stain], Collagen [Van Gieson's Stain] frozen sections were used for lipids [Sudan IV stain], Different areas of the stained slides from each were selected to analyze the expression intensity. All the specimens were fixed with 10 % neutral buffered formalin for 24 hrs. 5-6 µm thick tissue sections were obtained from formaldehyde fixed, paraffin-embedded tissue using Microtome. Staining was followed by deparaffinization in xylene and hydrated through 100% ethanol and washed thoroughly in deionized water for 10 minutes. The sections will be then stained according to the methods specific for Periodic acid Schiff and Van Gieson staining respectively. Frozen, fixed tissues were used for sudan IV staining. periodic acid Schiff stain was used for staining carbohydrate. In periodic acid Schiff stain periodic acid reacts with the aldehyde group of carbohydrates without over-oxidation. The product formed reacts with Schiff reagents with the product developed. A periodic acid Schiff positive slide picks up magenta to purple-red. Van Gieson Staining is used to demonstrate the increase of collagen production in diseases state. Van Gieson Stain is composed of Picric Acid and Acid Fuchsin. The tissue was sectioned and labeled for further procedure. The thickness and color of collagen fibers were observed in the red color. Sudan IV was used for staining lipids. Some of the tissue specimens were made of unfixed frozen sections for Sudan IV staining. As fat is soluble in alcohol, thus it is not possible to use sections cut from paraffin-embedded blocks. Frozen sections after a short rinse in distilled water were put in in 50% ethanol for 1-2 min. Then the sections were transferred in a saturated Sudan IV solution for 5 min at 37°C or room temperature.

Then the sections were dipped in 50% ethanol and distilled water, and counterstain Hematoxylin and eosin stain (H & E) was used. The lipids were observed in red color (Coleman, 2011).

2.2.5 Microscopic imaging

Images were captured by LEICA DM750 Microscope (Leica Microsystems, Germany) under 10x (0.25 N.A., with a pixel resolution of 0.63 μm x 0.63 μm and final magnification 100x) The images were captured under bright field microscopy with a LEICA ICC50E camera.

2.2.6 Western Blot Analysis

The expression of glucose transporter 1 and hexokinase 2 in tissue was validated by western blot technique. Frozen tissue specimen was homogenized in lysis buffer. Several times tissue lysates were sonicated and centrifuged. The supernatants were collected, and the protein concentration of each lysate was determined using the Bradford method. Further, Equal amount of protein extracted from the tissue of both the groups (oral submucous fibrosis with dysplasia) and Control was loaded and separated by SDS-polyacrylamide gel electrophoresis.The separated proteins were electroblotted onto a Polyvinylidene difluoride membrane. The nonspecific binding sites were blocked, and further, the layers were washed with Tris buffered saline with Tween-20 and incubated overnight at 4˚C with rabbit polyclonal antibody against gluocuse transporter 1. The blots were then washed with Tris buffered saline with Tween-20 , incubated at room temperature with, horseradish peroxidase-linked goat anti-rabbit IgG. After further washing, the proteins were revealed using 3,3′-Diaminobenzidine as a substrate. GAPDH was used as a control. The protein bands were quantified by densitometric analysis and were normalized to the intensity of GAPDH bands (Eissa & Seada, 1998).

2.2.7 Estimation of GLUT 1 and HK2 in serum by ELISA

The serum concentrations of glucose transporter 1 and Hexokinase in serum sample of same patients was measured using human SLC2A1 (Solute carrier family 2, facilitated glucose transporter member 1) (Immunotag, Geno Technology Inc., USA) and human HK2 (hexokinase 2) ELISA kit, (Immunotag, Geno Technology Inc., USA). All analyses and calibrations were performed in duplicate. Data were analyzed concerning the standard curve prepared of respective molecules.

2.2.8 Statistical analysis

An independent sample *t*-test was performed for comparison between the two groups, oral submucous fibrosis and normal. Data was analyzed using SPSS software (version 11; SPSS, Inc., Chicago, IL) and compared using Student's *t*-test at 5% significance ($p < 0.005$). The receiver operating characteristic (MedCalc software) was applied to investigate the level in serum that discriminates between two groups. The sensitivity and specificity with 95% confidence intervals (95% CI) were calculated of the markers.

3. Results

3.1 Histochemical features analysis:

All the stained sections revealed atrophic and dysplastic changes in the epithelium in comparison to normal. In periodic acid Schiff stained slide, the deviations from normal were result from an alteration in carbohydrate and glycogen content within the tissue sections were visible in representative periodic acid Schiff sections (Fig. 1A, 1B). In oral submucous fibrosis with dysplasia group epithelium there was the presence of glycogen expression in the suprabasal region, which was not present in the control group. In oral submucous fibrosis with dysplasia , glycogen expression was visible in magenta color according to the characteristics of stain. In Van Gieson stained oral submucous fibrosis with dysplasia cases (Fig. 1C and 1D), increased collagen deposition was noticed in red color in the sub-epithelial region in comparison to control, and literature report that fibrosis not only contributes in the development of trismus but also has a role in stiffening of connective tissue. In sudan IV stained

slides (Fig 1E and 1F) showed the presence of altered lipid status in oral submucous fibrosis with dysplasia in comparison to control group. The lipid accumulation expression was visible in red color in the epithelium region and subepithelial region of oral submucous fibrosis with dysplasia group. The above observation supported the evidence of altered carbohydrate, lipid, and protein status in tissue samples.

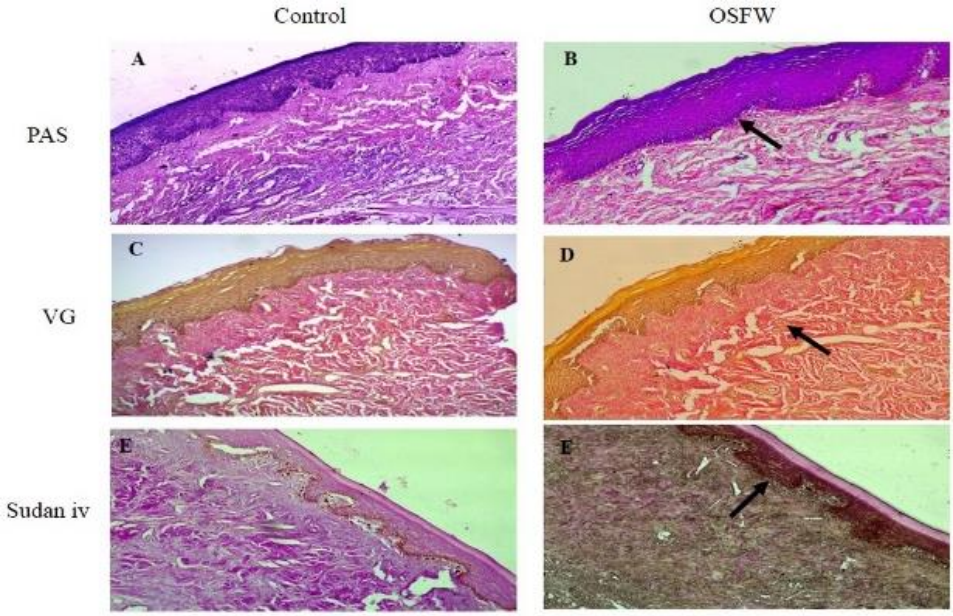

Figure 1: Depicted histochemical features of the normal oral mucosa (NOM) and OSFW conditions, i.e., OSF with dysplasia (OSFWT). The A, B, C, D, E represented for, PAS, VG, SUDAN IV staining respectively [Lens magnification 10X].

3.2 Validation of GLUT1 and HK2 by Western blot observation

We validated the expression of Glucose transporters and hexokinase 2 in tissue by using western blot analysis based on the above results. Our results by western blot showed that the glucose transporters 1and hexokinase 2 expression in oral submucous fibrosis with dysplasia tissues was significantly higher than compared to normal (Figure 2). Densitometry analysis also confirmed that glucose transporters 1 and hexokinase expression at the protein level was elevated in with comparing with the normal group (Figure 2). From, this we can predict that both the proteins have got a key role in oral precancer tumor progression and prompted further analysis.

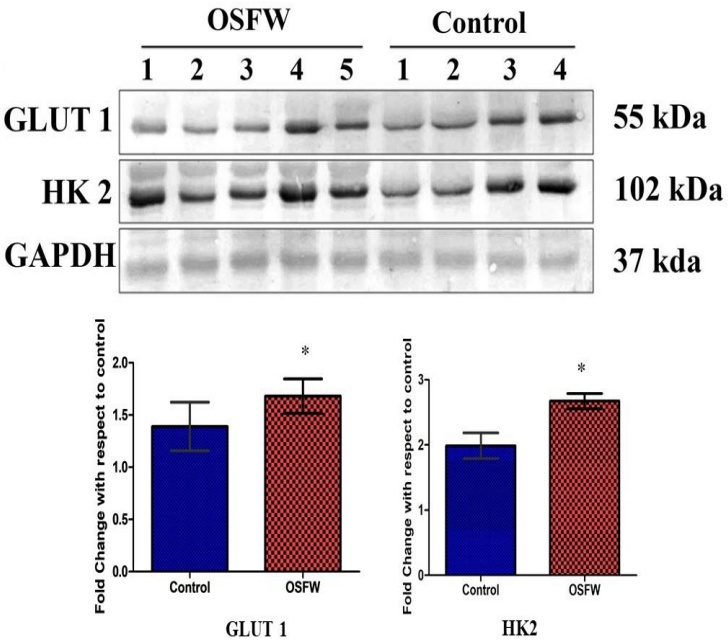

Figure 2 depicts validation and densitometric analysis of results done by western blot analysis of GLUT 1 and HK2 in the OSF and control group tissue. Data were presented as mean ± SEM, *P < 0.05

3.3 Quantification of GLUT 1 and HK2 by ELISA in serum

Figure 3 shows that the serum expression followed a trend similar to the expression pattern observed and in tissues reported above. glucose transporters 1 and hexokinase 2 being higher in oral submucous fibrosis with dysplasia compared to control group. The receiver operating characteristic curves and the plots of sensitivity vs. one − specificity were made for each. Based on the data the area under the receiver operating characteristic curve was calculated to analyze the efficacy of each marker for detecting oral submucous fibrosis with dysplasia. The score has the closest distance to the point with both maximum sensitivity and specificity was considered. Based on this the sensitivity and specificity values were calculated, and the receiver operating characteristic (Sensitivity/True positive rate vs. (1-Specificity)/ false positive rate) graphs of glucose transporters 1 and hexokinase 2 was obtained. (Figure 3A and 3B, Table 1).

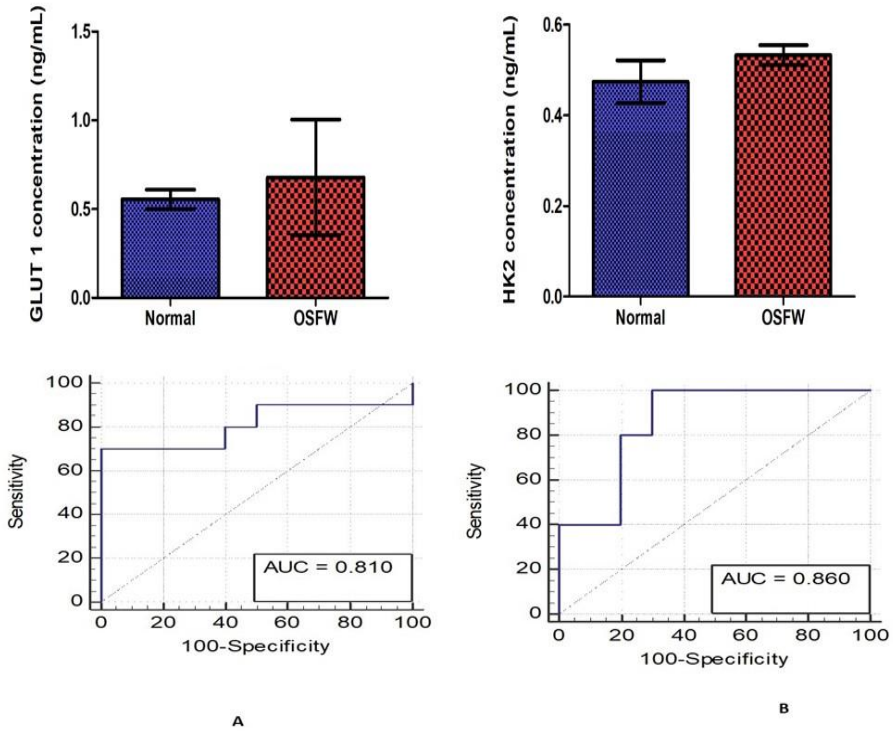

Figure 3 Serum concentrations and ROC curve analysis of (3A) GLUT1 (3B) HK2

Receiver operating characteristic (ROC curve) analysis of glucose transporters 1 >1.530 ng/ml in oral submucous fibrosis revealed significant area under the curve (0.81) of receiver operating characteristic with a sensitivity of 70% and specificity of 90% (Figure 3A, Table 1). Whereas hexokinase > 0.5163, ng/ml also has a significant area under the curve (0.86) of receiver operating characteristic curve sensitivity of 80% and specificity of 70% (Figure 3B, Table 1). Hence receiver operating characteristic analyses suggested accurate discrimination between the disease and the control group.

Table 1 Diagnostic efficacy of the studied markers

Markers	**Cut-off**	**AUC**	**Sensitivity**	**Specificity**	**P value**
GLUT1 ng/ml	>1.530	0.815	70%	90%	0.01
HK2 ng/ml	> 0.5163	0.710	90%	70%	0.00

4. Discussion

To our knowledge, the current analysis was the first to investigate and correlate glucouse transporters and hexokinase 2 analysis in tissue and serum of the same patients. The high energy level is required to malignant cells to proliferate and divide. glucouse transporters manages the influx of glucose in cells and provides energy for metabolic reactions (Li et al., 2013). Whereas the glycolytic enzyme hexokinase 2 initiate the first step of the glycolytic pathway (Wolf et al., 2011). Previous study report overexpression of both the markers in many types of cancer including oral cancer (Gatenby & Gillies, 2007; Wang et al., 2017; Wolf et al., 2011). Since the identification of serum markers would be minimal invasive methods, as compared to biopsy the present paper reports serum markers that could contribute in early prediction of disease. Our results are in accordance with the reported results earlier. Moreover, we observed that the expression level in tissue and serum was the same in both groups. However, from the results, it can be concluded that the biochemical changes taking place in serum reflect the changes in tissue. Our results suggest that assessment of this serum marker might provide clinically useful prognostic information. This study provides evidence of an altered metabolic profile

between oral submucous fibrosis with dysplasia and normal. The altered metabolic molecules may be useful as indicators of the initial changes occurring in neoplastic cells.
However, the correlation of these key metabolites and their underlying mechanisms in the pathogenesis of the disease is still unclear and require further investigation. Cellular energy homeostasis requires a good coordination of mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis. Normal cells rely mostly on glycolysis to generate adenosine triphosphate (ATP) for energy production. Changes in the metabolic state and oxidative stress is control by GLUT-1 and HK2 expression . GLUT-1-mediated induction of ROS. Induction of ROS is implicated in the dysregulation of apoptosis and can damage both DNA and protein in tissues (Higuchi M et.al,1998.

Competing financial interests
The authors declare no competing financial interests

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