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Comparative evaluation of e2f family of transcription factors in normal oral mucosa, oral submucous fibrosis and oral squamous cell carcinoma

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Abstract

Oral Submucous Fibrosis(OSF), a disease entity described since ancient times , presents a diverse clinicopathological features in the macro and microscopic arena. It's much debated malignant potentiality has been studied by various researchers in the light of molecular genomics, among which the analysis of transcription factors is one of the virgin fields to explore. The present study involves the analysis of E2F family of transcription factors in oral cancer and precancer (OSF) with a view to explore the malignant potentiality of OSF.

Keywords: genomics, submucous fibrosis, transcription

Introduction

OSF, the disease entity that dates back to the ages of Susruta, has been comprehensively defined by Pindborg and Sisrat (1966) in modern times as-" an insidious chronic disease affecting any part of the oral cavity and

sometimes the pharynx. Although occasionally presented by and or associated with vesicle formation it is always associated with juxtaepithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelail atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat."(1) Widely prevalent among young and middle aged individuals, it is closely related to the use of betel quid containing alkaloid arecholine (causing nitrosation and toxicity)(2), that coupled with other factors renders the collagen metabolic pathway go astray- the initial role being played by the inflammatory mediator namely TGFβ (3,4) (Fig1), causing transcriptional activation of the procollagen genes, mediated by activator and repressor factors- the E2F being such a family that decides in favour of or against proliferation (Fig 2,3) in the pathogenesis of various diseases including cancer as has already been established in gastric and other visceral malignancies(5,6). Growth fraction of cells can also be studied by ki67 expression analysis(7). Therefore in light of the molecular biology of oral carcinogenesis, a humble effort has been made to relate the transcriptional and proliferational status of OSF, cancer and normal oral mucosa through a comparative study to assess the malignant potentiality of OSF..

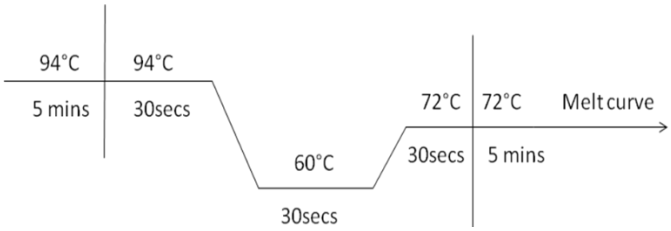
Subjects And Methods

The research study was conducted in the Department of Oral And Maxillofacial Pathology, Guru Nanak Institute Of Dental Science And Research, Kolkata in collaboration with the Department of Biochemistry, University of Calcutta, during June 2013 to July 2014.

2500 OPD patients of GNIDSR were thoroughly screened for the presence of OSF, OSCC and OSF associated with OSCC as stated by Neville et al (8) (Fig 6 to 10); subjected to medical examination as laid down by Skully et al (9) and eventually 16 OSF, 8 OSCC and 5 clinically normal cases were chosen for the study.

Incisional biopsies were performed , each sample was divided into two parts one for molecular and other for histopathological evaluation according to guidelines laid down by Bancroft et al (10). Samples preserved in the RNA Later were subjected to expression analysis for E2F1(classical cell cycle activator), E2F5(cell cycle repressor) and Ki67 genes (cellular proliferation index).

The qRTPCR protocols followed were as



The Cycle Threshold (Ct) values of target gene concentration in PCR were obtained and the delta Ct(δCt) calculated

ΔCt = (Ct of target mRNA – Ct of endogenous control) in the same tissue

ΔΔCt = ΔCt of diseased tissue- ΔCt of normal tissue ;

Fold change = $2^{(-\Delta\Delta Ct)}$

Results

The histopathological evaluation revealed OSF samples showing epithelial hypertrophy to maximum showing epithelial atrophy with varying degrees of epithelial dysplasia. The subepithelial connective tissue revealed varying degrees of fibrosis with focal hyaline degenerations leading to homogenized appearance, with nonspecific inflammatory changes (Fig 11 to 14). Carcinoma samples revealed heterogeneity in terms of invasion and cellular arrangement and histopathologically varied from well (Fig 14) to moderately differentiated SCC (Fig15).

Relative expression levels of E2F1, E2F5, Ki67 genes were represented as follows

Type of sample	Number	Relative level of gene expression (Δct)¥		
		E2F1	E2F5	Ki-67
Normal	05	20.4986 ±1.41	16.6944 3±2.32	16.21669± 2.74
OSMF	11	19.2485 9±2.32	15.5419 8±1.27	16.83215± 3.07
OSCC	08	18.1926 7±2.74	17.4630 6±1.12	15.96753± 2.79

Thus the fold changes among expression of these three genes in the control, OSF and OSCC were graphically represented and the significant (P) values were calculated in the Mann Whittney analysis using the SPSS software (Fig17, Fig 18, Fig 19)

Discussion

In the Indian scenario, OSF is encountered in day to day clinical practice posing challenges to the clinician in proper management. Recent past has seen evidences of its malignant potentiality, pioneered by Paymaster in 1956 and others; but yet no breakthrough has been achieved(11). The field of molecular genomics has opened a new arena but with paucity of work in the field of oral cancer- precancer.

Of the molecules TGFβ demands special mention, regulating vital processes like proliferation, differentiation, ECM elaboration, hematopoiesis, angiogenesis, immune responses and cell death; playing a dual role in tumor inhibition and progression(2,12). It directly influences OSF pathology acting as a ligand, the signals being transduced to nucleus via SMAD pathway, influencing intramolecular events like repression or activation of transcription by families like E2F(13). Ki67 bears testimony to the proliferation status.

With the above biological essence the E2F and Ki67 gene study was done.

E2F1,though a classical cell cycle activator, has revealed variable expression, being increased in ovarian, urothelial cancers(14) and antitumor effects in gastric, colorectal, hepatocellular malignancies(15) (Fig 3 to 5). **ΔCt** values for E2F1in our studies did not depict a significant increase, OSF occupying an intermediate position.

E2F5, a classical repressor showed worst prognosis in prostate, ovarian, esophageal cancers(16); our study revealed maximum expression in OSF.

Ki67, a cell cycle associated nuclear protein, though with theoritical limitations, depicts the proliferative potentiality of cells(7, 17). Our case showed upregulation in OSCC, with less than normal expression in OSF, possibly due to atrophic epithelium.

Expression of E2F1 and E2F5 were consistent with literature, with OSF occupying intermediate position in terms of E2F1, and maximum repression status in terms of E2F5. Activation in terms of E2F1 might suggest malignant transformation.

The varied results in our study could be attributed to the sample heterogeneity (clinicoanatomical and histopathological); therefore a larger sample size in future has an immense potentiality in assessing carcinogenicity of OSF in light of cell cycle transcription. Thus, markers of cell cycle regulators and proliferative potential can provide a link between OPMDS, OSF (in our case) to that of the complex process of carcinogenesis. This field therefore demands substantial research to anticipate the role of E2Fs in carcinogenesis.

Summary And Conclusion

Analysis of transcriptional factor family is emerging as a promising approach in the field of cancer genomics. Extensive research has been undertaken in recent past in gastrointestinal, prostate, lung, pancreatic malignancies

with promising results. But exploratory studies on the role played by transcription factors in the field of oral precancer and cancer are in their budding stage; so are to be undertaken on a wider scale to give the research a new dimension.

E2F family of transcription factors with its multiple subtypes go about a complex interplay amongst themselves, in determining the proliferative status of a disease in terms of cell cycle progression and or repression. E2F1, E2F2, E2F3A have been regarded as activators while E2F3B, E2F4, E2F5, E2F6 and E2F7 are regarded as repressors of the cell cycle, but classical presentations have frequently seen to deviate. In the field of oral cancer and Oral Potentially Malignant Disorders there is paucity of exploration in terms of transcription factor analysis. With these facts in mind, the present study was carried out with eleven OSF, eight cancer cases and five normal individuals; along with study of proliferation index of the tissue components assessed in terms of Ki67 analysis, in the Department of Oral And Maxillofacial Pathology, Guru Nanak Institute Of Dental Sciences And Research between November 2012 and July 2014, with a view to assess the role of these factors in the malignant potentiality of the disease process.

The study subjects were clinicopathologically evaluated and observations recorded carefully. Incisional biopsies were performed following written consent by the patients. Biopsized specimens were divided into two halves, one evaluated histopathologically and the other used for molecular study for E2F and Ki67 expression. E2F1 being a classical activator and E2F5 being a classical repressor were assessed along with Ki67 expression. The data were statistically analyzed. Significant difference could not be deciphered between the samples in terms of E2F1 and Ki67 expression. However, E2F5 was seen to increase in OSF compared to normal while Ki67 expression was decreased as compared to normal in OSF. Interestingly, Ki67 was found increased in malignancy with respect to normal and OSF samples.

Carcinogenesis is a complex, multifactorial disease influenced by an array of pathways acting synchronously, resulting in uncontrolled and abnormal proliferation. Markers of this proliferative potential and cell cycle regulators can provide a link between Oral Potentially Malignant Disorders, Oral submucous Fibrosis, in the present study, to that of cancer. However, in view of the observations made in the present study relating to the role played by E2F1, E2F5 and Ki67, in the pathogenesis of malignant progression in oral submucous fibrosis, further in depth study is advocated to unravel the mystery involved.

Acknowledgement

A virgin study demands lot of able guidance and enthusiasm to curb it to fulfilment. My heartiest thanks to the Department of Oral and Maxillofacial Pathology, GNIDSR for providing the appropriate platform and guidance and to the Department of Biochemistry, Calcutta University for rendering the platform for molecular work. My thanks to my guide Prof Dr R.R. Paul, coguides Prof Dr M. Pal, Prof Dr S.Sengupta for their critical guidance and appreciation; and my thanks to the Departmental faculty for their constant encouragement and valuable inputs. Heartiest thanks to the laboratory staff at GNIDSR for technical assistance at the histopathology work. Thanks to my PhD scholar friends at the C.U. especially Miss Amrapali Ghosh (project assistant) for her timeless efforts. And above all my family members whose sacrifices have allowed me to dream of my aspirations.

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Legend Figures

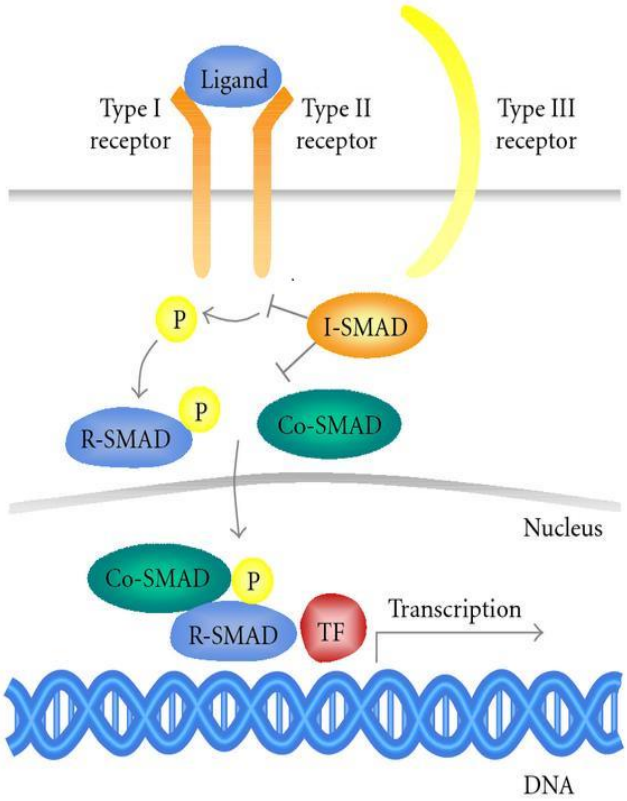

Fig. 1: TGF- β signaling cascade: TGF-β Superfamily Receptors—Targets for Antiangiogenic Therapy? Jasmin Otten, Carsten Bokemeyer, and Walter Fiedler.

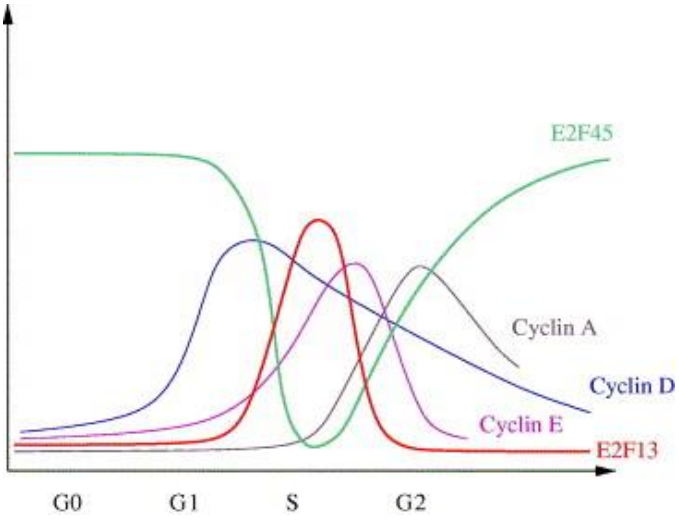

Fig. 2: The E2Fs and cell cycle progression. The orderly progression through the cell phases is orchestrated by the cyclins, cyclin-dependent kinases (CDKs), their inhibitors (CDKIs) and the members of the E2F family. Switching from "repressive" to "activating" E2Fs allows G_1 to S-phase transition

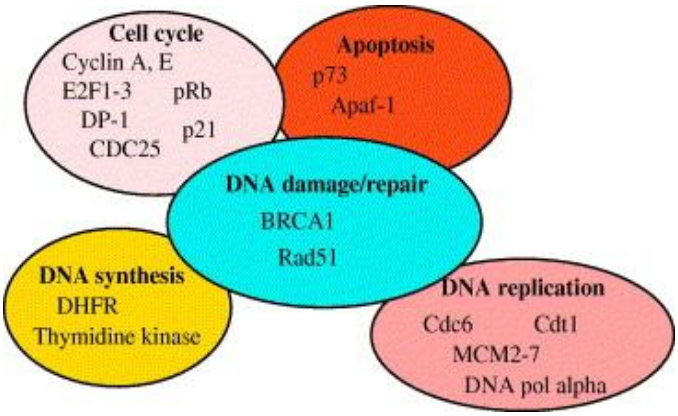

Fig. 3: The E2F target genes: Involvement of E2F transcription factor family in cancer: P.J. TSANTOULIS, V.G.GORGOULIS.

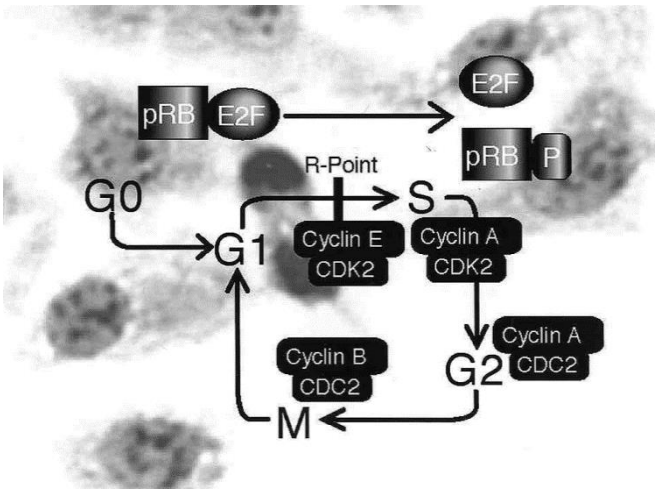

Fig. 4: Regulation of the G1 to S phase transition by the retinoblastoma suppressor protein (pRB). G0, G1, S, G2, and M refer to the quiescence, first gap, DNA synthesis, second gap, and mitosis phases of the cell cycle. CDK refers to cyclin-dependent kinases. CDC-2refers to cell cycle control-2. Phosphorylated pRB is represented aspRB-P. Briefly, pRB-E2F transcriptional repression of genes regulating DNA synthesis is released by phosphorylation of pRB, allowing for cell cycle progression from G1 to the S phase.

Cell Cycle Dysregulation in Oral Cancer: R. Todd, P.W. Hinds, K. Munger, A.K. Rustgi, O.G. Opitz, Y. Suliman and D.T. Wong; *CROBM* 2002 13: 51

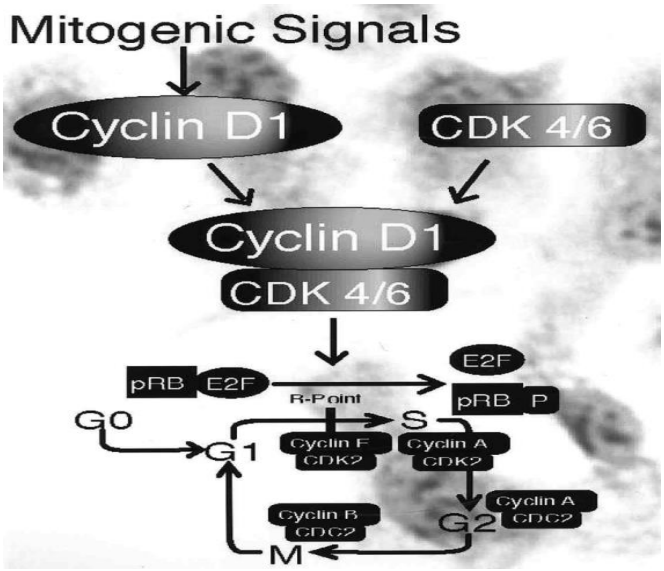

Fig. 5: Mitogen-activated G1 transition to the S phase. Mitogen stimulation leads to cyclin D1 synthesis. Together with its catalytic partners CDK4 and CDK6, cyclin D1 accelerates G1 progression by phosphorylating pRB. **Cell Cycle Dysregulation in Oral Cancer:** R. Todd, P.W. Hinds, K. Munger, A.K. Rustgi, O.G. Opitz, Y. Suliman and D.T. Wong; *CROBM* 2002 13: 51

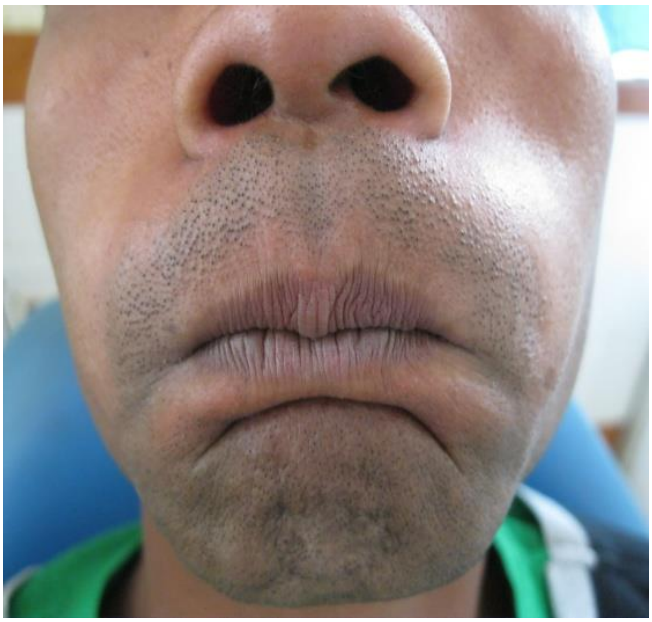

Fig 6: Extraoral Clinical Photograph Showing Reduced Buccal Fullness In Osf Patient.

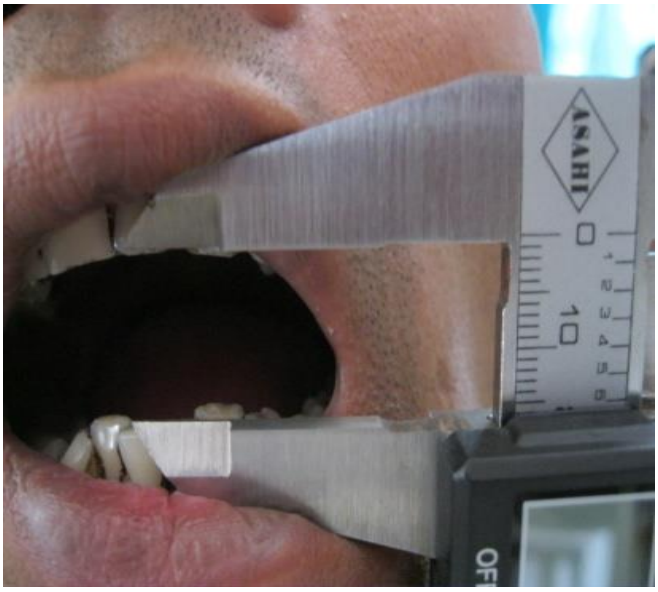

Fig 7: Extraoral Clinical Photograph Showing Reduced Mouth Opening In Osf Patient

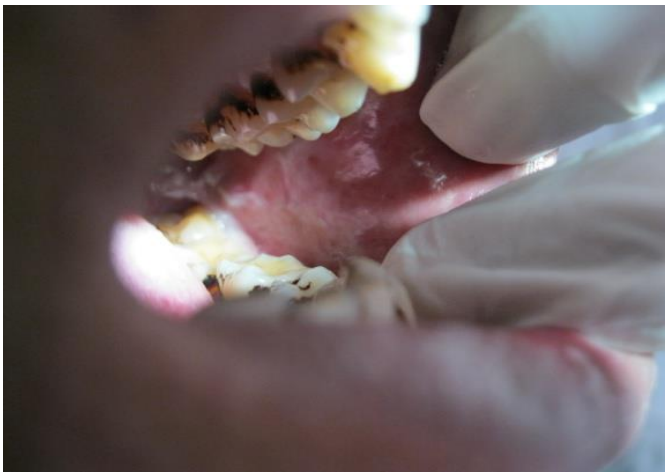

Fig 8: Intraoral Photograph Showing Coarse, Blanched Buccal Mucosa

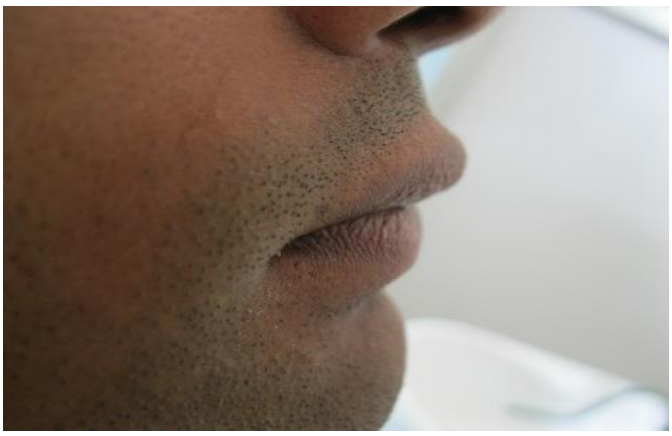

Fig 9: Extraoral Photograph Showing Intruded Lower Lip.

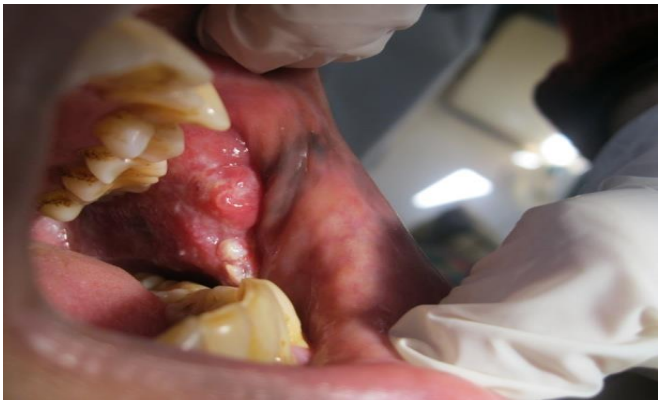

Fig 10: Intraoral Photograph Showing Ulcer proliferative Growth Involving Left Buccal Mucosa

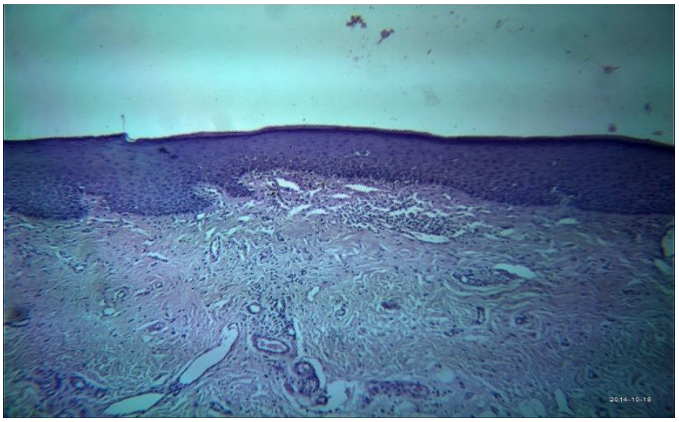

Fig 11: Photomicrograph Showing Features of Oral Submucous Fibrosis with Atrophic Epithelium (10x H&E Staining)

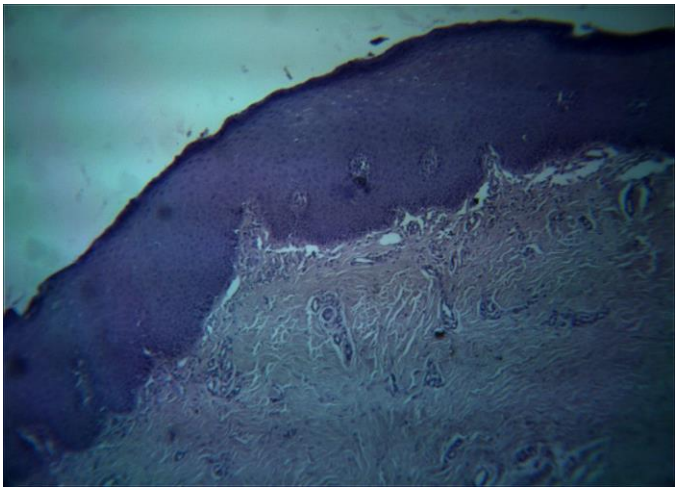

Fig 12: Photomicrograph Showing Features of Oral Submucous Fibrosis with Hyperplastic Epithelium And Connective Tissue Hyalinization At Places (10x H&E Staining)

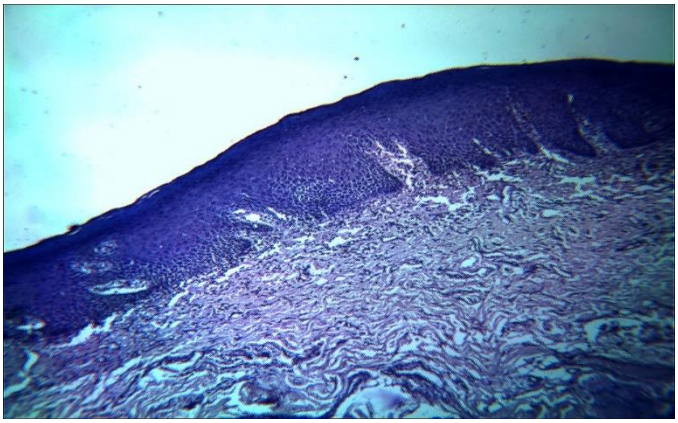

Fig 13: Photomicrograph Showing Features of Oral Submucous Fibrosis with Epithelial Dysplasia (10x H&E Staining)

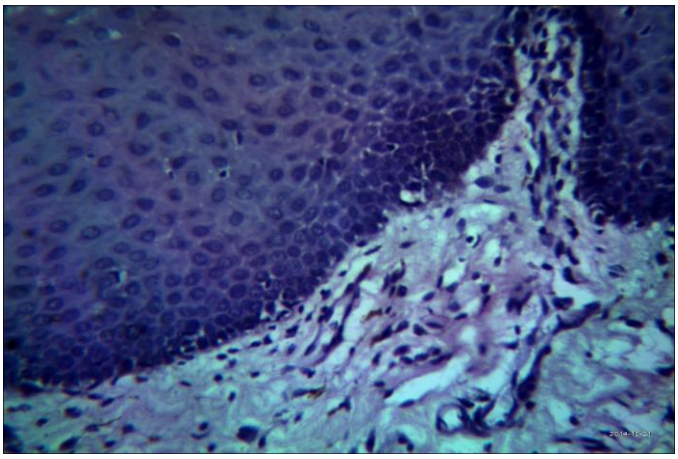

Fig 14: Photomicrograph Showing Features of Oral Submucous Fibrosis With Epithelial Dysplasia (40x H&E Staining)

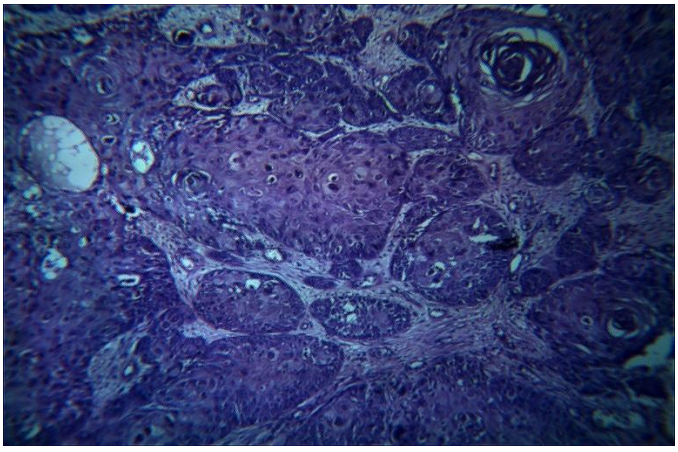

Fig 15: Photomicrograph Showing Features Of Well Differentiated Squamous Cell Carcinoma (10x H&E Staining)

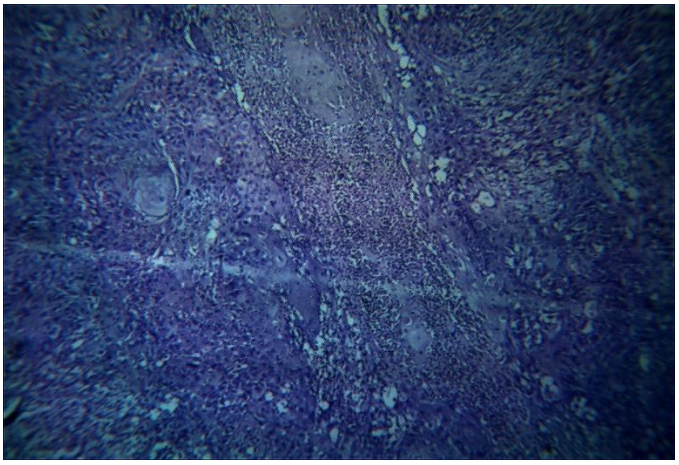

Fig. 16: Photomicrograph Showing Features of Moderately Differentiated Squamous Cell Carcinoma (10x H&E Staining)

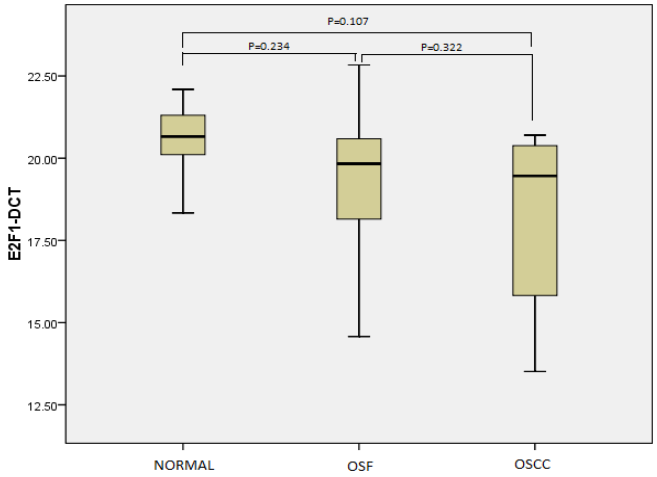

Fig. 17: Boxplot of E2f1

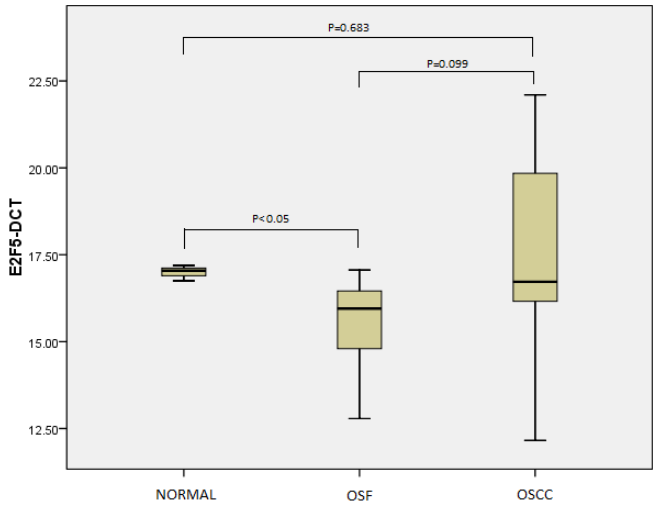

Fig 18: Boxplot of E2F5

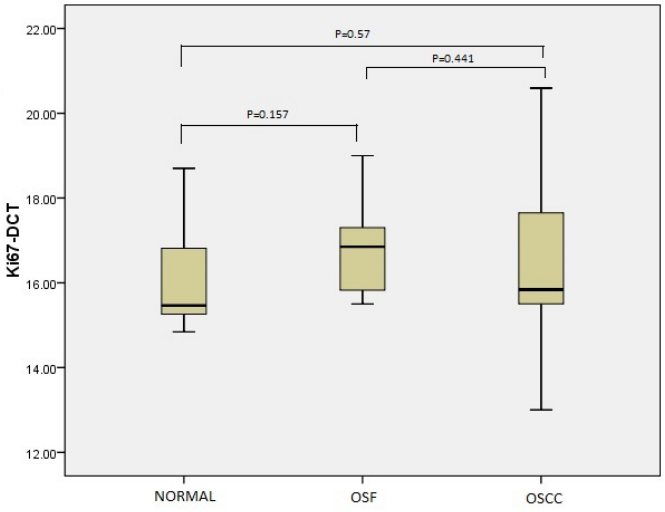

Fig. 19: Boxplot of KI67