TO STUDY THE EXPRESSION OF PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IN ORAL SUB MUCOUS FIBROSIS (OSMF).

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INTRODUCTION

Cell proliferation is a biological process that is essential to all living organisms due to its role in the growth and maintenance of the tissue homeostasis. The control of this important process is completely deregulated in some types of neoplasia, and the assessment of cell proliferation activity in tumors has become a common tool used by histopathologists to provide useful information for assessing diagnosis, clinical behavior, and therapy. The cell cycle consists of a series of phases, during which there are changes that lead to cell division[1,2]

Proliferation markers refers to specific proteins or other factors whose presence in actively growing and dividing cells serves as an indicator for such cells. Today, the most common method for determining proliferative activity is the use of immunohistochemical techniques, which are increasingly being applied in routine pathology[1]. Biomarkers are now used to characterize different aspects of development of premalignant and malignant lesions and conditions[3]

Proliferating Cell Nuclear Antigen (PCNA) expression pattern can be helpful in the comprehension of the process of tumorigenesis, since there is a gradual increase in terms of the number of PCNA-positive cells with the progress from normal epithelium to malignancy. Furthermore, the distribution of PCNA-positive cells in the basal and parabasal layers in the normal epithelium and benign lesions, and the distribution of PCNA-positive cells in the suprabasal layers in dysplasias, carcinomas in situ, and invasive carcinomas demonstrate that early dysplastic alterations may occur without the histopathological signs seen by optical microscopy through conventional histochemical methods[3,4,5,6,7].

Proliferating Cell Nuclear Antigen (PCNA), which is also known as **cyclin**, is an intranuclear polypeptide of 36 kDa that shows peak synthesis during the S-phase of cell cycle. PCNA is an auxiliary component of DNA polymerase and appears in all proliferating cells. It plays an important role in DNA synthesis, DNA repair, cell cycle progression and cell proliferation. PCNA is a marker of proliferation and may prove to be a useful objective indicator of the biological behavior of various tumors. Increased PCNA expression is observed as tissue progresses from normal epithelium to hyperplasia, to dysplasia and to squamous cell carcinoma.

The aim of the present study was to study the Expression of Proliferating Cell Nuclear Antigen in Normal Mucosa, Oral Sub Mucous Fibrosis

MATERIAL AND METHODS

This study was undertaken in order to detect the presence of Proliferating Cell Nuclear Antigen (PCNA) in Normal oral mucosa and oral submucous fibrosis under light microscope. These samples were taken from the archive of the department of oral pathology and microbiology, D.J College of dental sciences and research, Modinagar. The study comprised of control group and study group. Samples were divided into four different groups: Group I was a control group (NOM); Group II was further divided into 3 subgroups: Group IIA includes 15 patients with oral submucous fibrosis

Group I (Control group) included only non-smokers with no history of areca/betel quid chewing. Out of the 10 control subjects, 8 were men and 2 were women and their age range was from 18 to 30 years. In group IIA, the male: female ratio was 9:1 and age ranges was from 20 to 62 years. These cases were diagnosed histopathologically as moderate to severe grades of OSMF. In group IIB and IIC, all patients were men and their age ranges from 25 to 68 years.

The diagnosis for each oral lesion was combined with histological examination of hematoxylin and eosin stained sections..

Immunohistochemical staining procedure for PCNA

The demonstration of antigens by immunohisto chemistry is a two-step process involving first, the binding of a primary antibody to the antigen of interest, and second, the detection of bound antibody by a chromogen.

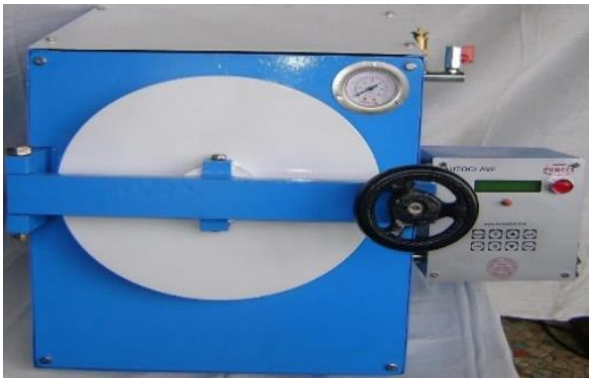
Reagent used for IHC staining

Super Sensitive Polymer-HRP IHC Detection System kit was used. Contents: Hydrogen peroxidase (6 ml vial, 3% H2 O2 in water); Power Block™ Reagent HK083-5K: One vial (6 ml) of a highly effective universal protein blocking reagent. (Contains casein and proprietary additives in PBS with 0.09% sodium azide); Secondary antibody (Biotinylated anti-rabbit, antimouse and anti-goat immunoglobulin in phosphate buffered saline containing carrier protein on 15mM azide); Streptavidin peroxidase (Streptavidin conjugated horse radish peroxidase in phosphate buffered saline containing carrier protein and antimicrobial agent); Buffered substrate (Buffered substrate

solution, pH 7.2, containing hydrogen peroxide and a preservative); DAB chromogen (3,3- diamino benzidine chromogen solution) [25]. Anti PCNA (prediluted, BIOGENEX Corporation, CA, USA); Phosphate buffered saline (PBS); Xylene; Absolute alcohol; 95% alcohol; Distilled water; Mayers Haematoxylin; 1% acid alcohol; Mounting medium (DPX). The following reagents were prepared prior to staining. Phosphate buffered saline (PBS) (Wash solution, buffer bath) 0.05 M/L, 0.1mol /L Nacl (pH 7.2-7.6). Citrate buffer: 10m mol/L citrate (pH 6.0). Substrate chromogen solution (4-7 sections): Transfer 1ml aliquot of buffered substrate into the calibrated test tube. To this add one drop (approximately 20 µl) of DAB chromogen.

PHOTOGRAPH 1. CHEMECALS USED FOR H & E STAINING

PHOTOGRAPH 2. MICROTOME USED FOR SECTIONING



PHOTOGRAPH 3. AUTOCLAVE USED FOR ANTIGEN

PHOTOGRAPH 4. SUPER SENSITIVE POLYMER HRP- IHC DETECTION KIT

Procedure:

Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on silane-coated slides. The slides were incubated in a pre-heated incubator at 37ºc for overnight. Slides were treated with two changes of Xylene for 5 minutes each for deparaffinization and hydrated by treating with one change each of 100% alcohol followed by graded alcohol 90%, 70%, 50%, for 5min each. The slides were rinsed with the running tap water. Antigen retrieval was done using heat induced epitopes retrieval (HIER) microwave oven method. The slides were kept in a coupling jar filled with PBS buffer (pH 6) and placed in a microwave oven. Slides were given two cycle at high (80ºC) mode of microwave for 15 minutes, one cycle at medium high (60ºC) for 5 minutes and one cycle at low (40ºC) mode for 5 minutes. After every cycle buffer was added to fill the coupling jar. The sections were then allowed to cool to room temperature and then rinsed with distill water for 1 minutes and then washed with PBS buffer thrice for 5 minutes each. All the incubations were carried out at room temperature, with a humidifying chamber. At no time, the tissue sections were permitted to dry during the staining procedure: Step 1: Blocking endogenous peroxidases: Endogenous peroxidase activity was blocked by incubating the slides with peroxide block (3% hydrogen peroxide in water) for 12-15 minutes. Sections were again washed with wash buffer thrice for 5 minutes. Step 2: Sections were treated with Power Block (A highly effective universal protein blocking reagent. Contains casein and proprietary additives in PBS with 15m sodium azide) for 12-15 minutes. No washing was done after the incubation in Power Block. Step 3: Application of primary antibody: Sections were incubated with mouse anti-rat monoclonal PCNA (Bio Genex) for 1 hour in a humidifying chamber at 37ºC. Sections were then washed thrice with wash buffer for 3 minutes each. Step 4: The sections were incubated with Super Enhancer (A reagent that enhances the signal and is

used after the primary antibody incubation) for 25 minutes and were again washed thrice with wash buffer for 5 minutes each. Step 5: Secondary antibody application: Slides were then incubated with Super sensitive poly HRP (anti-mouse anti-rabbit IgG labeled with enzyme polymer in phosphate buffered saline with stabalizers, carrier protein and 0.1% Proclin 300) secondary antibody for 30 minutes in a humidifying chamber and were washed thrice with wash buffer for 5 minutes each. Step 6: Slides were then incubated with 3,3' – diaminobenzidine tetrahydrochloride (DAB) chromogen for 1-5 minutes. Chromogens was freshly prepared by adding 1 ml stable DAB with 1 drop chromogen and 5 microliter of 30%H2O2. Step 7: Slides were rinsed with distilled water and counterstained with Harris hematoxylin for 20 seconds followed by bluing in tap water for 10 minutes. The Slides were then dehydrated through graded alcohol, cleared in xylene, and mounted with DPX.

Assessment of PCNA expression:

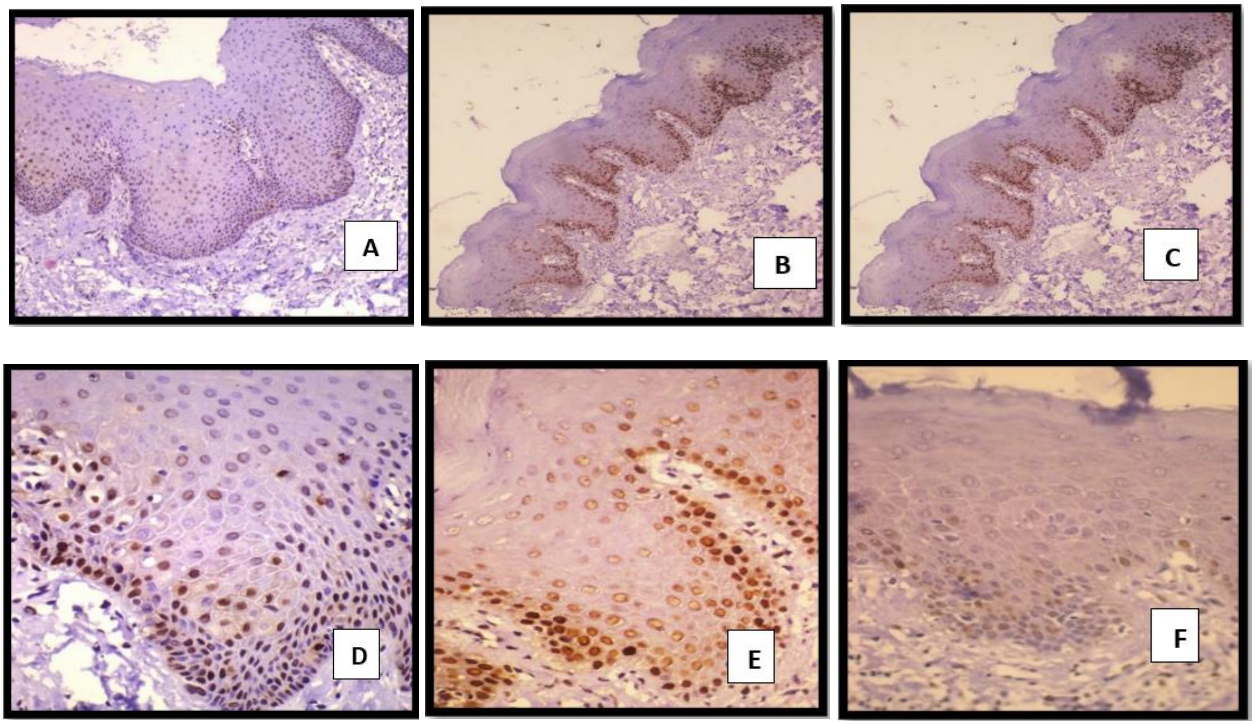
The nuclear expression of PCNA was assessed at 10x and then 40x magnification and manual counting was done using manual counter. Presence of brown colored end product at the site of target antigen was indicative of positive reactivity. The negative control tissue demonstrated absence of specific staining. For qualitative assessment the expression of PCNA was observed in basal, suprabasal and spinous layers on NOM and epithelial dysplasia. The basal layer- nuclei positive in a single layer just above the basement membrane; The suprabasal layer- nuclei positive 2 - 3 layers above the basal layer. The spinous layer- nuclei positive in the superficial layer above the suprabasal layer. Total number of positive cells in each layer i.e. basal, suprabasal and spinous was visually counted using light microscope in epithelial dysplasia and NOM. The percentage of positively stained nuclei for PCNA was counted in three non -overlapping X400 fields by three different observers and then average of the three was calculated.

Statistical analysis:

Data obtained in the present study was subjected to the statistical analysis using SPSS 18 software for one-way ANOVA and independent t-test. One way analysis (ANOVA) test is used to compare the mean scores between more than two groups. Descriptive statistical analysis has been carried out in the present study. Results on continuous measurements are presented on Mean ± SD (Min Max) and results on categorical measurements are presented in Number (%). Significance is assessed at 5 % level of significance.

Results:

All specimens of OSMF showed positive staining for PCNA. Staining was observed in oral epithelium and in infiltrating inflammatory cells. Staining for PCNA was localized to the nucleus and exhibited a differential pattern of expression in OSMF



DISCUSSION

It is well understood that the transition of the normal oral epithelium to dysplasia to malignancy is featured by increased cell proliferation. Discovery of various proliferation markers has enabled the detection of the hyperactive state of the epithelium and has been suggested to be of prognostic significance. The basal layer is the only proliferative compartment for normal oral epithelium, whereas in the rest of the epithelial layers, cellular maturation takes place without any proliferative activity. Hence, any sign of proliferative cellular activity beyond the basal layer should be considered as a warning sign[10].

The term "proliferation markers" refers to specific proteins or other factors whose presence in actively growing and dividing cells serve as an indicator for such cells, for example, PCNA and Ki-67[9]. It has been reported that the proliferating cell growth fractions in oral potentially malignant disorders were significantly higher than those in normal mucosa and suggested that these growth fractions correlated with the degree of severity of dysplasia which linked to the increasing probability in the development of malignancy[10]. This study and other recent studies have shown that nuclear staining for PCNA in dysplastic oral epithelium involved the basal, parabasal and spinous cell layer as compared to normal and hyperplastic epithelium where the nuclear PCNA staining was only in the basal area.

Our study shows that the expression of PCNA in group I i.e. leukoplakia with dysplasia was maximum in basal layer followed by suprabasal and least in the spinous layer in all the four groups including normal oral mucosa. The percentage of PCNA positive cells increases with increasing grades of epithelial dysplasia. The results of the present study showed that PCNA expression was seen in all cases of normal oral mucosa, oral submucous fibrosis and leukoplakia with and without dysplasia. The overall percentage of PCNA positive cells increases gradually from normal oral mucosa, through leukoplakia without dysplasia to leukoplakia with dysplasia with significantly higher expression in severe dysplasia. These results are in accordance with previous studies which have shown an increased expression of PCNA as the tissue progresses from normal state to severe dysplastic stage[12,13]. These results confirm that an increase cell proliferation is a characteristic feature of malignancy. Higher the cell proliferation rate the higher is the risk of cells suffering mutations and effect of carcinogens during mitosis which could result in malignant phenotype[14].

In the normal oral mucosa PCNA positive cells were observed chiefly in the basal and parabasal layer with no statistical difference in the PCNA expression in any of the layer. This is in accordance with the previous studies conducted by **Kaur et al (2011)[2]** and **Azzawi LM (2014)[9]** which also shows the same result.

Our study shows a significant statistically difference between non-dysplastic and dysplastic lesions which provide a strong correlation between the expression of PCNA and degree of dysplasia which is also in accordance with the previous studies conducted by **Zain RB et al (1995)[15]** and **Lan HA et al (1996)[16]** which states that as the tissue progresses from the normal to the dysplastic stage, there is gradual increase in the expression of PCNA from basal to the superficial layers of the epithelium

Another important finding obtained by PCNA immunohistochemistry used in the present study is the loss of polarity of PCNA positive cells, particularly in dysplasia and cancer lesions. In normal squamous epithelium, PCNA-positive cells were well localized in the basal layer, but the normal was more or less distributed in hyperplasia and mild dysplasia and remarkably distributed in higher grades of dysplasia. These findings indicates the presence of disturbed cell differentiation and proliferation in the dysplastic epithelium which in later stages may undergoes malignant transformation.

Conclusion

PCNA can play an important role in objectively grading dysplasia in certain oral premalignant disorders such as leukoplakia. Further studies with a higher sample size will be essential to prove the suitability of PCNA in the different layers of the epithelium as a marker for determining the degree of dysplasia

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