IMMUNOHISTOCHEMICAL EVALUATION OF PROLIFERATING CELL NUCLEAR ANTIGEN IN ODONTOGENIC KERATOCYST, DENTIGEROUSCYST, RADICULAR CYST AND AMELOBLASTOMA

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CERTIFICATE

This is to certify that this dissertation titled "**IMMUNOHISTOCHEMICAL EVALUATION OF PROLIFERATING CELL NUCLEAR ANTIGEN IN ODONTOGENIC KERATOCYST, DENTIGEROUS CYST, RADICULAR CYST AND AMELOBLASTOMA"** is a bonafide dissertation performed by **SHAHELA TANVEER** under our guidance during the postgraduate period 2008-2011.

This dissertation is submitted to THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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CONTENTS

Odontogenic tumors are lesions derived from epithelium and mesenchymal elements of tooth forming apparatus and therefore exclusively found in jaw bones.[1] Odontogenic tumors compromises a heterogenous group of lesions that ranges from hamartomatous to benign and malignant neoplasms of variying aggressiveness. Odontogenic tumors are very infrequent lesions compared to other pathological process of oral and maxillofacial regions. These tumors represent between 0.8% and 3.7% of all specimens sent to oral pathology laboratories.[2]Ameloblastoma is one of the most frequently encountered tumor arising from odontogenic epithelium characterized as benign, but exhibits locally invasive behavior with high tendency to recur.[3]

Odontogenic cysts have varifying origins and these multiple origins represent multiple sources of lining epithelium that is from Cell rests of Malassez , cell rests of Serre.[3,4] The most commonly occurring odontogenic cysts are radicular, dentigerous and odontogenic keratocyst. Radicular cysts are the most common cysts (65%) and they arise from the epithelial cell rests of Malassez in the periodontal ligament as a sequelae of inflammation which usually follows the death of a dental pulp and represent more than a half of all odontogenic cysts. Dentigerous cysts are the most common developmental cysts (24%). They usually enclose the crown of an unerupted tooth and are attached to its neck. It develops by

pericoronal or intraepithelial accumulation of fluid surrounded by reduced enamel epithelium.[3,4]

Odontogenic keratocyst (OKC) is considered to be arising from derivatives from embryonic dental lamina. It compromises approximately 11% of all cyst of the jaws. Some cysts have the potential for aggressive behavior and local recurrence. It is documented that in comparison with parakeratotic type of keratocyst the orthokeratinised type is less aggressive and have lower rate of recurrence[3]. In some situations there is a bud like proliferation of lining epithelium of OKC into the connective tissue capsule of the cyst and is mistaken for as ameloblastoma.[5] Biological behaviour of few OKCs are as aggressive as benign neoplasms such as ameloblastoma. OKC is now designated by WHO as a Keratocystic odontogenic tumor (KCOT). The clinically aggressive behavior is a result of properties of the lining epithelial cells and connective tissue capsule.[6] There is also greater proliferative activity in the epithelial cells of inflamed KCOT which could be associated with the disruption of the typical structure of the cystic linings.[5] Neviod basal cell carcinoma syndrome is usually associated with multiple OKCs.[3] Identification of the proliferating activity in such tumors is useful to predict the biological behavior of different lesions.[7]

. Proliferating Cell Nuclear Antigen (PCNA) is a 36KDa acidic non histone nuclear protein important for DNA synthesis. It has been used for evaluation of proliferative ability of many lesions. In the presence of replicating factor C, a multi sub unit complex, PCNA allows DNA polymerase δ to initiate leading strand for DNA synthesis[7]. Quiescent and senescent cells have a very low level of PCNA mRNA. PCNA expression may be used as a marker of cell proliferation because cells remain longer time in G1 – S phase when proliferating. Also this protein has essential role in nucleic acid metabolism as a component of DNA replication and repair mechanism. An increase in PCNA levels may be induced by growth factors or as a result of DNA damage in absence of cell cycle.[8]

Immunostaining with monoclonal antibody (PC10) against this antigen has been shown to demonstrate the proliferative compartment of normal tissue.[9]

Determination of epithelial proliferative activity in these cysts and tumors is a potentially useful means of investigating differences in their biological behavior.

Thus this study was done to evaluate the expression of PCNA in odontogenic cysts and ameloblastoma.

To evaluate and compare PCNA labelling index in

1. Radicular cyst
2. Odontogenic keratocyst
3. Dentigerous cyst
4. Ameloblastoma

Hypothesis

1. There is increased expression of PCNA in odontogenic keratocyst when compared to radicular and dentigerous cyst.

2. There is no difference in the expression of PCNA when compared between odontogenic keratocyst and ameloblastoma.

Study setting

- The study was conducted in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using paraffin embedded tissues.

A retrospective study was done to evaluate the expression of PCNA using immunohistochemistry in formalin fixed, paraffin embedded tissue specimens (archival tissues) of odontogenic cyst (Radicular, Dentigerous cyst, Odontogenic Keratocyst and Ameloblastoma) using immunohistochemistry in formalin fixed, paraffin embedded archival tissue specimens.

Study sample size

The study material comprised of 60 formalin fixed, paraffin embedded tissue specimens archival blocks randomly selected, with adequate clinical details.

- 15 histopathologically confirmed tissue dentigerous specimens.
- 15 histopathologically confirmed odontogenic keratocyst tissue specimens.
- 15 histopathologically confirmed radicular cyst tissue specimens.
- 15 histopathologically confirmed ameloblastoma tissue specimens.

Study subject

The study comprised of 4 groups:

This project was approved by Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai.

Group 1

15 clinically, radiographically and histologically confirmed cases of dentigerous cyst

Histological criteria:

The epithelial lining consist of two to four layers of flattened nonkeratinizing squamous cells and a fibrous connective tissue. The connective tissue wall is frequently composed of very loose fibrous connective tissue or sparsely collagenized myxomatous tissue. Rushton bodies within lining epithelium is seen in the inflamed cyst.

Group 2

15 clinically, radiographically and histologically confirmed cases of keratinizing cystic odontogenic tumor.

Histological criteria:

Keratinizing cystic odontogenic tumor is characterized by a thin fibrous connective tissue capsule and a lining of corrugated parakeratinized stratified squamous epithelium usually about five to eight cell layers in thickness. Basal layer exhibits a palisaded pattern.

Group 3

15 clinically, radiographically and histologically confirmed cases of radicular cyst.

Histological criteria:

Radicular cyst is lined by stratified squamous epithelium and consist of a dense fibrous connective tissue capsule with an inflammatory infiltrate containing lymphocytes, neutrophils plasma cells and histocytes. Abundant fibroblasts, lymphocytes and plasma cells can be identified within cystic wall is seen in the long standing (chronic) cases.

Group 4

15 clinically, radiographically and hisologically confirmed cases of ameloblastoma

Histological criteria:

Ameloblastoma is characterized by sheets and islands of tumor cells in the connective tissue stroma, showing an outer rim of columnar ameloblast with nuclei polarized away from the basement membrane. The centre of these nests is composed of stellate shaped epithelial cells that mimic the stellate reticulum.

Methodology

From the paraffin embedded blocks 4 micron thick, sections were cut and used for routine hematoxylin and eosin (H & E) and immunohistochemical (IHC)staining.

HEMATOXYLIN & EOSIN STAINING

Reagents

- Harry's hematoxylin
- 1% acid alcohol
- Eosin

Procedure

- The slides were dewaxed in xylene and hydrated through graded alcohol to water.
- The sections on the slides were flooded with Harry's hematoxylin for 5 minutes.
- The slides were washed in running tap water for 5 minutes.
- The slides were differentiated in 1% acid alcohol for 5 minutes.
- The slides were washed well in running tap water for 5 minutes.
- The tissue sections on the slides were then stained in eosin for 30 seconds.
- The slides were washed in running tap water for 1 minute.
- The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).(40x,10x)

IMMUNOHISTOCHEMISTRY (IHC)

Armamentarium (Fig. 1)

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminium foil
- Micro-pipettes
- Toothed forceps
- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

Reagents used

1. Conc. HCl
2. Laxbro solution
3. APES (3 amino propyl tri ethoxy silane)

4. Acetone
5. Citrate buffer
6. Phospho Buffer Saline (PBS)
7. 3% Hydrogen peroxide
8. Deionized distilled water
9. Haematoxylin
10. Absolute alcohol
11. Xylene

Antibodies used

1. Primary antibody-Mouse Monoclonal antibody diluted in phosphate buffered saline, pH 7.6 (Anti Human PCNA , PC10 Prediluted in PBS (Biogenex,USA)
2. Secondary antibody-Super Sensitive Polymer/ HRP/ DAB

IHC Procedure

Pretreatment of the slides

- The slides were first washed in tap water for few minutes
- The slides were then soaked in detergent solution for 1 hour
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water.
- The slides were washed in autoclaved distilled water.
- The slides were immersed in 1 N HCl (100 ml HCl in 900 ml distilled water) overnight.

- The following day slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES (3 Amino propyl tri ethoxy silane) coating

Slides first dipped in couplin jar containing acetone for 2 minutes

↓

Dipped in APES for 5 minutes

↓

Dipped in two changes of distilled water for 2 minutes each

↓

Slides left to dry

Preparation of paraffin sections

After the slides were dried, tissue section of 4 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labeled as positive (P) and the other negative (N) sections

Procedure

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put through descending grades of alcohol and then rehydrated with water. Slides were then treated with peroxidase for 10 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. Then the slides were dipped in 3 changes of distilled water for 5 minutes each. Circles were drawn using diamond pencil around the tissues, so that the antibodies added later on do not spread and are restricted to the circle.The tissues were incubated in protein blocking for 15 minutes in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The primary antibody, rabbit polyclonal antibody, prediluted was added to P tissue on the slide and then to the N, PBS was added. The slides were incubated overnight. Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. Then a drop of biotin conjugated secondary antibody was added on both the sections and the slides were incubated for 30 minutes. Later slides were washed in three changes of cold PBS for 5 minutes in each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of avidin biotin enzyme reagent was added

on both the sections and the slides were incubated for 30 minutes. The sections were washed in 3 changes of cold PBS for 5 minutes in each. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of freshly prepared DAB (3' – Diaminobenzidine Tetra Hydrochloride - a substrate chromogen) was added on both sections. Slides were then washed in distilled water to remove excess DAB and counter stained with hematoxylin. The slides were placed in a tray with tap water for bluing. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with DPX. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

Positive Control

A known case of oral squamous cell carcinoma specimen tissue were fixed, processed, embedded, sectioned and stained in same manner and used as positive control. One positive control tissue slide was included for each batch of staining

IHC procedure for PCNA:

APES coated slides with2 paraffin embedded tissue

↓

Placed in xylene I (5 min)

↓

Placed in xylene II (5 min)

↓

Placed in xylene III (5 min)

↓

Placed in 100% isopropanol (5min)

↓

Placed in 70% isopropanol (5min)

↓

Washed in distilled water thrice (5 min each)

↓

Kept in citrate buffer, autoclaved and allowed to cool

↓

Blot dry with blotting paper

↓

Add one drop of peroxidase (10min)

↓

Washed in PBS thrice (5 min each)

↓

Add one drop of power block(15min)

↓

Blot dry with blotting paper

↓

Primary antibody(diluted) added and incubated

↓

Washed in PBS thrice (5 min each)

↓

Add superenhancer (20min)

↓

Washed in PBS thrice (5 min each)

↓

Add SS label(30 min)

↓

Washed thrice in PBS (5 min each)

↓

DAB (diaminobenzidene tetrahydrochloride) chromogen

↓

Washed in PBS thrice (5 min each)

↓

Stained with haematoxylin

↓

Washed in tap water (5 min)

↓

Placed in 70% isopropanol (1 min)

↓

Placed in 100% isopropanol (1 min)

↓

Placed in xylene (1 dip)

↓

Slides were mounted using DPX

↓

Slides were observed under the microscope

↓

(Staining in the cells evaluated under 10x and 40x magnifications using light microscope)

Criteria for evaluation of PCNA staining

The following parameters were used to evaluate PCNA staining. Tissue localization of stain – PCNA staining was present either in the epithelium/connective tissue or were present in both. Localization in basal layer or suprabasal layer of the epithelium were assessed.

Cellular localization of stain – Nucleus[7]

Labelling index (LI)

PCNA positivity was calculated by dividing the number of positive cells by the total number of cells counted in the slide and expressed as percentage. Nuclei with brown colour regardless of staining intensity were regarded as positive for PCNA. The percentage of PCNA was evaluated from a minimum of 1000 nuclei per case. A minimum of thousand cells was counted for each slide.[7]

LI = Number of positive cells /Total number of cells counted x100.

Statistical analysis was done using SPSS ^{TM} software (version 10.0.5). p value less than 0.05 was considered to be statistically significant.

- Pearson's Chi-square test was done to compare mean age, the distribution of gender and site, tissue localization of stain, cellular location, nature of stain, and the percentage of cells stained among the four study groups.
- To compare the mean labeling index between the groups, Mann Whitney U test was used.

PCNA -INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is a 36 KDa acidic non-histone nuclear protein which functions as an auxiliary protein for DNA polymerase delta and is an absolute requirement for DNA synthesis.[10] It plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery[7]. In 1978 Miyachi et al initially identified an auto-antigen in patients with systemic lupus erythematosus, which they named PCNA, because the protein was observed in the nucleus of dividing cells.[7,9]. In 1980, Bravo and Celis used two dimensional gel electrophoresis to identify a protein which was synthesized during the S-phase of the cell cycle which they named cyclin. Mathews et al in1984 demonstrated that PCNA and cyclin are the same 29 kDa protein.[8]

Gene encoding PCNA has been isolated from several eukaryocytes archebacteria amino acid sequence which has been found to be highly conserved. 70% of the Drosophila protein is identical to the rat and human proteins. Furthermore, while the sequence of yeast PCNA shows only 35% homology at the DNA level with humans while the molecules are functionally interchangeable.[11]

STRUCTURE

Crystallographic studies of PCNA structure revealed it is composed of six repeating domains and exhibits six-fold symmetry. The resulting ring has two nonequivalent surfaces, an outside

surface composed of beta-sheets; and a layer of alpha-helices rich in basic residues lining the innerside of the hole, which are positioned perpendicularly to the phosphate backbone of DNA. Owing to this unique structure, PCNA is topologically linked to the double helix, encircling it. It is able to freely slide along the DNA lattice by virtue of the alpha-helices lining the inner channel. Thus, PCNA and its homologs increase the processivity of a polymerase by engaging in protein-protein interactions with its outer surface and tethering it to the DNA. This property of PCNA prevents the polymerase from dissociating while advancing along the template DNA and is the reason for the name - sliding clamp[8,9]

FUNCTIONS

PCNA plays important roles in

- DNA replication
- DNA excision repair
- Cell cycle control[8,9,10,11]

In *DNA replication,* polymerase-alpha/primase synthesizes the first RNA/DNA primer on the leading strand. Then polymerase delta, together with its processivity factor PCNA, performs continuous leading strand synthesis. Completion of Okazaki fragment synthesis, however, requires the processive *pol delta*, owing to its toroidal structure. The PCNA trimer has to be opened and then closed around the nascent DNA strand by the enzymatic

activity of the clamp loader RF-C(replication factor C).[8,10] Synthesis of an Okazaki fragment is terminated when the *poldelta* or *pol epsilon* holoenzyme meets the 5' end of the RNA portion of the previously synthesized fragment and performs strand displacement synthesis.[8]

Immunohistochemical studies using PCNA antibodies to study the distribution of the protein within the cell suggested a role for PCNA in DNA repair, the nucleus stained strongly with anti-PCNA antibodies following DNA damage by ultravoilet irradiation although the cells were not in S-phase and in non-cycling normal human keratinocytes *in vivo* after mild UV exposure[8,10]. This observation suggested the involvement of the protein in DNA repair processes polymerase epsilon plays a major role in DNA repair while interaction with polymerase delta may be important only during chromosomal DNA replication.[8]

PCNA forms complexes with all these CDK–cyclin complexes as well as with critical checkpoint proteins, transducing both positive and negative signals. DNA damage, senescence or differentiation of cells through either p53-dependent or independent pathways induces the expression of the p21 protein, which blocks progression from G1 to S phase of the cell cycle. p21 binds to CDKs through its N-terminal region and to PCNA through its C-terminal region p21 prevents PCNA from binding to the cell cycle or DNA replication machinery, thus targeting the protein for destruction.

Indeed, p21 can form a stable complex with PCNA on DNA, preventing further interaction with the replication proteins RF-C and polymerase delta .[9]

REGULATION OF PCNA

PCNA expression is regulated at both the transcriptional and post-transcriptional leve1. There appears to be differences between the regulation in quiescent cells and those which are continuously cycling. In the latter, there is very little variation in protein or mRNA levels during the cell cycle. Regulation of PCNA expression by post-transcriptional mechanisms appears to be very important *in vitro*. Many cells continuously make PCNA mRNA (at least at low levels) but rapidly degrade this without translation.[9]

Under the correct circumstances cells are able to rapidly respond to growth factors by inducing PCNA production by stabilizing PCNA m-rna.[8] Half-life of the PCNA is 20 hours. Support for this interpretation comes from the work of Scott *et* al who reported that in tumour xenografts with a defined growth fraction and a cell cycle time of about 20 hours the number of PCNA immunoreactive cells was nearly 100%. Thus, there may be situations where cells that have recently left the cell cycle also express PCNA.[10]

ANTIBODIES TO PCNA

Monoclonal antibodies that recognize PCNA have been reported to be of value in assessing cell proliferation using immunohistological methods. Monoclonal antibodies were generated to genetically engineered rat PCNA using conventional methods. Eleven clones generated with anti PCNA specificity, six were found to react with formalin fixed histological material, and one clone designated PC l0 was chosen for further detailed study because of its highest avidity in an ELISA assay. PC 10 immunostaining can demonstrate the proliferative compartment of conventionally fixed and processed normal tissues.[9,10] Commercially available reagents include the monoclonal antibodies 19A2, 19F4, PC10. Different antibodies may have very different properties even though they recognize the same protein. (For instance PC9 raised to recombinant PCNA only recognizes PCNA when in the nucleolus. This particular epitope is not seen anywhere else in the cells). Differences may exist in the data generated by different anti-PCNA antibodies and the effect of technical factors, including fixation, on patterns of PCNA staining should be taken into consideration.[10]

DISTRIBUTION OF PCNA IMMUNOREACTIVITY IN TUMORS.

PCl0 staining is almost entirely confined to the nucleus and may show a diffuse or granular pattern or a mixture of both. Rarely cytoplasmic staining is observed, the nature of this is unclear but it may represent cytoplasmic synthesis or breakdown. The distribution

of nuclear PCl0 staining in non-neoplastic histological material is entirely consistent with PCNA being associated with cell proliferation. Staining is seen in those tissues known to be actively proliferating cells like in germinal centres and scattered cells in the paracortex of lymphoid tissue; it is present in the basal layer of stratified squamous epithelia and in the majority of cells in the hair bulb. Those tissues known to be non-proliferative show only low turnover, PCNA immunoreactivity is minimal. For example staining is not seen in the adult central or peripheral nervous system, nor in skeletal, smooth nor cardiac muscle, nor is it presenting normal hepatocytes, although rare Kupffer cells show nuclear immunoreactivity.[9] Oncogenes may participate in the regulation of PCNA mRNA level and it is conceivable that alterations of oncogene expression in neoplasia may lead to the deregulation of PCNA expression.[8,9] In histopathologically normal tissues adjacent to tumours there is in some, but not all cases, a dramatic increase in immunohistologically detectable PCNA continence. Autocrine and paracrine growth factor mediated regulation of PCNA expression also explains the excess of PCNA immunoreactive cells seen in certain tumours. PCNA immunoreactivity (as detected by PC10) can occur without cell proliferation in association with neoplasia and that this may be mediated by growth factors *in vivo*. It would seem that expression of PCNA is a necessary but not sufficient requirement for proliferation. [10,11]

Tie-Jun Li,Motoo Kinaano, *et al* in 1997 has reviewed and summarized different studies on the biological process governing the growth among OKC, Radicular and Dentigerous cyst and concluded characteristic predominant suprabasal location of proliferating cells in OKC in contrast to that in dentigerous and radicular cyst. Cell proliferation in epithelial linings was assessed by mitosis and autoradiographic studies. Mitotic figures per centimeter length of basement membrane in OKC lining ranged from 0-19 with mean of 8.0. This figure was similar to Ameloblastoma (7.0) and the dental lamina (8.4),and was higher compared to radicular cyst (4.5). Immunohistochemical studies later done using monoclonal antibodies to PCNA and Ki67. In odontogenic kerayocyst, the higher level of labeling was seen predominantly in the suprabasal cell layer in comparison to dentigerous and radicular cyst. Another quantitative study of p53 immunoreactivity demonstrated a significantly higher level of p53 labelling and a predominant suprabasal reactivity in OKC linings as compared to that in dentigerous and radicular cyst.[12]

Li T-J. Browne RM. Matthews JB, *et al* in **1994** investigated the reactivity of the epithelial linings of the three odontogenic cysts. PCNA expression was studied in 31 cases of odontogenic cyst and 10 cases of normal oral epithelium. The PCNA positive cells were present in all cystic linings, their number and distribution varied between cyst types. Odontogenic keratocyst

epithelium contained highest number of PCNA positive cells most of which were located in the suprabasal layers with less than 5% detected in the basal layers. Total PCNA positive cell counts in OKC epithelia were significantly higher ($P < 0.005$) than those in dentigerous cyst and radicular cyst Distribution of PCNA positive cells within OKC linings differed significantly from that in other odontogenic cyst and oral epithelium, with greater than 95% of stained cells located in suprabasal layers this could also be explained by differences in epithelial turnover or cell cycle times.It might also indicate that lateral, rather than vertical migration of cells is greater in OKC linings. This latter possibility would explain the characteristic regular, thin epithelium of the OKC and contribute significantly to cyst growth. By contrast, the apparent indolence of the epithelial linings of the majority of radicular and dentigerous cysts suggests that factors other than epithelial proliferation are of greater importance in their enlargement. [13]

Murtadi, Grehan *et al* in 1996 studied and analyzed the relatively aggressive behavior of the OKC's might be expected to correlate with the degree of proliferation of cyst epithelium. They performed immunohistochemistry using PC 10 monoclonal antibody in n=21 sporaidic OKCs and n=20 of syndrome associated OKCs. The staining was present in all the cyst predominantly in the suprabasal layer, and the mean PCNA count was statistically significantly higher($p<0.05$) than in nonsyndrome. This correlates

with the differing clinical behavior of these two groups suggest the aggressive behavior in the latter is not simply due to quantitative increase in jaw epithelium but due to higher proliferation rate in epithelial lining.[14]

Alfredo, Batista de Paula, *et al* in 2000 studied the effect of inflammation on the lining epithelium of OKCs. The existence of greater proliferative activity in the epithelial cells of inflamed odontogenic keratocysts, which may be associated with increase in the number of epithelial cells in cycle and suggests that increased epithelial cell proliferation is associated with the disruption of the typical structure of odontogenic keratocyst linings. Growth factors and cytokines released by the inflammatory infiltrate present in the fibrous tissue capsule of OKCs may be responsible for greater proliferative activity in inflamed lesions. An immunohistochemical expression of PCNA and Ki-67 proteins and AgNORs were studied in 20 odontogenic keratocyst.Among them 10 cases were non-inflammed and 10 cases were inflamed. The total cell number and PCNA and Ki-67 cell counts in inflamed OKC epithelium were significantly higher than those in non-inflamed OKC (P=0.01). Cell counts were significantly higher in the suprabasal layer than in the basal layer, both for inflammatory and non-inflammatory OKC (P=0.01).[15]

Piattelli A, Fironi M, *et al* in (1998) assessed the proliferative activity among three odontogenic cysts. He studied 12 cases of OKC, 12 cases, dentigerous cyst and 8 cases of radicular cyst were evualated. The PCNA positive cells were present in all cyst types but their number and distribution varied between cyst types. OKC contained the highest number of PCNA positive cells when compared to those in DC and RC and the location of PCNA positive cells were located in suprabasal layer.[7]

Mervyn Shear, *et al* in 2002 reviewed and summarized various studies and demonstrated qualitative and quantitative differences in nuclear reactivity for PCNA linings of the three major odontogenic cyst and the predominant suprabasal position of PCNA positive cells in OKC. In one study 12 cases of OKC, 12 cases of dentigerous cyst and 12 cases of radicular cyst were incubated with CM-1, a rabbit polyclonal antibody raised against whole p53 protien. The presence of p53 protein in the nucleus was identified by positve staining. Further staining of OKCs was done using the monoclonal antibody PC10 which recognizes proliferating cell nuclear antigen (PCNA). To assess the rate of cell division the number of suprabasal mitosis was counted in a randomly selected area of 1500 epithelial cells in each OKC in the sample. All OKCs were positive for PCNA. Positivity was present in all basal cells and most parabasal cells. Another study using the same monoclonal antibody PC10 to PCNA, investigated the PCNA activity among the

three cysts in 10 cases of OKC, in 10 cases of DC and 10 cases of RC. To quantify PCNA activity after staining, PCNA positive cells were counted manually and related to the length of basement membrane (mm) and epithelial area (mm^2) using TV image analysis. Cells counts were expressed as total or PCNA positive cells per mm of basement membrane (mm) and epithelial area (mm^2). The epithelial lining of OKC (n=11) contained the highest number of PCNA positive cells, most of which were in suprabasal layers and fewer than 5% in the basal layer. PCNA count in OKC had mean value of 94.4±22.7 cells/mm. This was significantly higher ($p<0.005$) than dentigerous cyst and the radicular cyst.[16]

Mervyn Shear, et al in 2002 reviewed and summarized that inflamed OKCs showed significantly higher numbers of PCNA positive cells than in the non-inflamed samples. An IHC study using antibodies to PCNA, Ki-67 and Ag NORs was done. The sample size was taken as 10 cases of inflamed OKC and 10 cases of non-inflamed OKC. The PCNA positive cell counts were significantly higher in the suprabasal than in the basal layer in both inflamed and uninflammed OKCs. Furthermore inflamed OKCs showed significantly higher numbers of PCNA and Ki-67 positive cells in the basal and suprabasal layers than in the non-inflamed sample($P=0.01$), The mean number of AgNORs per nucleus was significantly higher in the inflamed than in the uninflamed sample($P=0.01$).[16]

Kaplan and Hirshberg *et al* in 2004 study was to investigate the effect of inflammation on Ki-67 and PCNA labeling indices of odontogenic keratocysts. The study included 45 cases of OKC. In 10 high power fields, the epithelial lining was recorded separately for each field either as metaplastic squamous or classic parakeratinized. Labeling indices for Ki-67 and PCNA and the inflammatory infiltrate density in the depth of one high power field adjacent to the basement membrane were recorded. Parameters were compared between fields, and for each case the average inflammatory score and average labeling indices were calculated, and cases compared. No inflammation was observed in 24.5% of cases, mild in 30.5% and moderate to severe in 45%. Foci of metaplastic non-keratinizing epithelium were observed in 64% of cases, which were twice as common in inflamed cysts (90%) than in noninflamed cysts (44%). The average labeling indices for PCNA and Ki-67 yielded no significant differences between inflamed and non-inflamed cysts. When compared between high power fields there was an increase in the Ki-67 labeling index in metaplastic epithelium in areas with moderate to severe inflammation score (p=0.036). PCNA labeling index did not significantly change between areas with low and high inflammation. No differences in labeling indices were observed between areas of classic and metaplastic epithelium with equal inflammation density[17].

Kichi E, EnokiyaY, Muramatsu T, *et al* in 2005 studied and analysed correlation between the cellular proliferation, cell death and expression of apoptosis related protein in the lining cells of OKCs and dentigerous cyst and concluded that OKCs are observed as cystic lesions but not as tumour masses. An IHC and TUNEL study was conducted in OKC (n=20) and dentigerous cyst (n=20). In OKCs the TUNEL positive cells were about 25% in the lining cells of surface layer and ratio in dentigerous cyst was about five times less than OKC (5.5%). Using ki-67 as a proliferative marker the results were that the cells constituting the intermediate and suprabasal layer possess the highest proliferative activity in OKC and relatively high levels of proliferation in the basal layer of dentigerous cyst. Similar findings were observed with Bcl2 immunoreactivity in OKC, the positive ratio was 96% in basal cells and only 3% in dentigerous cyst and staining was restricted to basal layer. These results suggest that apoptosis does not occur in the basal cells of the lining epithelium of OKC. The results that bcl-2 is an apoptosis inhibiting protein and positive cells detected exclusively in the basal layer, and that TUNEL positive cells were found only in the surface layers of both cyst types are reasonable as bcl2 inhibits apoptosis to facilitate cellular proliferation in the basal layer, whereas apoptosis maintains the homeostasis of the thickness of lining epithelium and allows large amounts of synthesis of keratin in the surface layer of OKCs. This ingenious system to keep balance between cellular proliferation and cell death can be

detected in the lining epithelium in dentigerous cyst, but its degree is lower than that found in OKCs. [19]

Zdenek K, Marie G, Jan B, *et al,* in 2006 studied the usefulness of detecting important apoptosis and proliferation markers in assessing the biological potential of odontogenic keratocysts (OKC). The expressions of p53 phosphorylated, Bcl-2, Bax, p21 Waf1, p27Kip1, c-erbB-2/HER2/neu and proliferation antigens PCNA and Ki-67 were determined by indirect immunohistochemistry in 10 samples of dentigerous cyst, 29 cases of radicular cyst, 11 cases non-specified odontogenic cysts, 39 cases of sporiadic OKC and 18 cases of syndrome associated OKC. Separated sporadic cysts contained only a higher number of Bax (cytoplasmic staining) and PCNA (suprabasal layer). Sporadic keratocysts showed against radicular cysts differences only in higher expression of Bcl-2 and PCNA in suprabasal, and lower expression of PCNA in basal cells. Dentigerous cyst revealed a lower level of basal cell proliferative activity measured by expression of PCNA than odontogenic cyst.[20]

Marcia G, Lauxen I, *et al* in 2008 conducted a study to analyze p53 and proliferating cell nuclear antigen expression in 4 different odontogenic cyst (radicular, dentigerous, odontogenic keratocysts, and calcifying odontogenic cysts) and concluded that the patterns of p53 and PCNA expression in DC and RC were similar although the two lesions are of different origin. PCNA and

p53 expression was found in all the odontogenic cyst samples. Mean percentages of p53 positive cells showed that radicular cyst had the greatest number of positive cells. However, a significant difference between layers was found only in keratocyst which had greater values in supra basal layer whereas in the radicular cyst greater values were found in the basal layer. In radicular cyst the basal layer staining is explained that it may result from a response to cell stress generated by the inflammatory stimulus. Growth factors and cytokines (IL-1, IL-6, TNF) are released during inflammatory events, inflammatory stimuli increase cell proliferation, and inflammatory cytokines may also cause cell stress, the inflammatory stimulus may originate from the continuous agression caused by bacterial contamination of root canal, which makes epithelial cells, particularly those in basal layer, increase and maintain proliferation, therefore high expression of p53 and PCNA in radicular cyst reflects both cell stress and cell proliferation caused by inflammatory stimuli, which may inhibit degeneration of the p53 protein and increases the level of PCNA even in the absence of cell cycling. In dentigerous cyst the rate of proliferation is slower than that of radicular cysts, and the expression of markers may be more closely associated with responses to inflammatory stimuli that may be the result of the eruptive process,may make the cell to proliferate, but may be inconstant and present only for short periods of time.This may explain the lower PCNA and p53 percentages found in dentigerous cyst. [21]

Baghaei F, Eslami M, *et al* 2004 aim of the present study was to investigate the expression of P53 and Ki-67 markers in two types of OKCs parakeratotic and orthokeratotic variants. A total of 20 cases of 10 cases of parakeratotic and 10 cases of orthokeratotic OKC were stained immunohistochemically. The average number of positively stained cells for Ki-67 in parakeratotic OKC was 29.90±4.90 cells/mm basement membrane and in orthokeratotic OKCs was 29.90 ± 4.90 cells per mm of basement membrane ($P<0.05$). Positive cells for Ki-67 were dominantly located in parabasal layer. Mean stained cells for P53 were 4.30± 2.21cells/mm basement membrane and in parakeratinized variant and 4.80±1.75 cells/mm basement membrane in orthokeratotic types that was not statistically significant ($P<0.58$).[18]

Devi Charan Shetty, Aadithya B *et al* in (2010) conducted a study to evaluate the expression of p53 in KCOT and Ameloblastoma, to correlate with the aggressiveness of these lesions and to establish OKC as an aggressive lesion. An IHC staining was performed using anti-p53 antibody in eighteen cases each of OKC and ameloblastoma. Total p53 count was significantly higher in ameloblastoma as compared to OKC. The intensely stained p53 cell count showed no statistically significant difference between the two lesions. The mean p53 count in basal and suprabasal cells in OKC was 21.3±38.30 and 27.56±8.35 respectively. p53 expression was significantly higher in suprabasal

cells than the basal layer ($p<0.05$) and in ameloblastoma was 24.78±11.123 and 24.83 ±4.878. No significant difference could be found using nonparametric test ($p>0.05$). Thus dense p53 expression in both OKC and ameloblastoma were similar, intense p53 expression correlates with higher proliferative activity of the cells. It also explains the molecular basis of the proliferative advantage gained by the cell due to p53 dysfunction, p53 mutation and accumulation of this stable mutated p53 protein leading to intense p53 expression immunohistochemically in KCOT.[6]

Piattelli A, Fironi M, et al in (1998) evaluated and compared the proliferative activity between OKC and ameloblastoma. An immunohistochemistry study was done using antibodies to PCNA. The study included OKC (n=12) and ameloblastomas (n=22). The percentage of PCNA positive nuclei was in range of 20.30±27.10 for OKC and 24.70-58.30 for Ameloblastomas. All histological variants of ameloblastoma showed a higher PCNA count than the OKC ($P<0.00001$). Statistical analysis showed that ameloblastoma had higher PCNA positive cell counts than OKC.[7]

Jin Kim, Jong In Yook *et al* (1994) The aim of this study was to evaluate the proliferating activity of ameloblastoma and its correlation to the biological behavior and localisation of staining pattern of each histological type. The labelled cells were counted from 8.6 to 21.3 cells per 2000 epithelial cells in the positive cases. In solid ameloblastomas, there was no significant difference in the

proliferative labeling indices. All showed less than 100 positive cells per 2000 tumour cells in each histological type (follicular, plexiform, acanthomatous, granular cell and basal cells. But localization of the staining pattern differed in between the follicular and the plexiform type. In follicular ameloblastomas, PCNA positivity was found mainly in the peripheral layered cells and in the plexiform type the positive cells were found not only in the peripheral layer, but also in the central areas. [22]

Funaoka K, Arisue M, I, Kobayashi, *et al* in (1996) conducted an immunohistochemical study in 23 cases of ameloblastoma to evaluate the correlation between the positive index of PCNA and the clinical and histological character. Higher rates of the positive index of PCNA was seen in the follicular type compared to plexiform ($P< 0.10$), suggesting that the follicular type of ameloblastoma may grow more quickly than the plexiform type. The cystic type showed low rate for the positive index of PCNA. In plexiform patterns the cells positive for PCNA were mainly basal cells or suprabasal cells of the tumour. Scarcely positive inner stellate cells of the tumour island were seen.The more positive inner cells there were, the higher was the PI of PCNA. In addition, relatively many positive cells were observed in the area showing squamous metaplasia.[23]

Takahashi H, Fugita, *et al* (1998) studied PCNA expression in OKC and ameloblastoma. The study included 15 cases of

odontogenic keratocysts 46 cases and ameloblastomas. The percentage of mean index for PCNA-positive cells determined by point counting and was significantly lower in the ameloblastomas 9.4± 11.0 than in odontogenic keratocysts where the mean index was 29.9± 24.0. In contrast, the odontogenic keratocyst exhibited a mean percentage of PCNA-positive cells which was statistically higher than that in other histological elements of ameloblastomas. The present study suggests that odontogenic keratocyst is regarded as a benign odontogenic tumour.[24]

Sandra F, Mitsuyasu T, Nakamura N, et al (2001) conducted an IHC study, thirty-two ameloblastoma tissues were evaluated and analyzed in relation to the WHO classification, cytological pattern of the outer layer cell, clinical appearance, tumor location, radiographic appearance and patient's age. Most of the tissue sections did not contain positive inner layer cells, and outer layer cells are known to reflect the growth activity of ameloblastoma and only outer layered cells were evaluated. Cytological pattern of the outer layer cells, the basal cell type had significantly higher PCNA and Ki-67 ($P<0.05$) labeling indices than the cuboidal cell type, the columnar cell type was in the middle and also, in the mixed type, basal cells had the highest PCNA and Ki-67 labeling indices. It may be reasonable to assume that the basal cells are the most actively proliferating and, accordingly, the most immature cells in the ameloblastoma. The solid type had

significantly higher PCNA and Ki-67 ($P<0.05$) labeling indices than the cystic and the mixed type the inner layers of epithelial component in acanthomatous ameloblastoma were negative, and only outer layers were stained positively. This suggested that inner layer cells of acanthomatous ameloblastoma were in a mature stage and not actively proliferating.In acanthomatous ameloblastoma showed positive staining for both PCNA and Ki-67 and its labeling indices were higher than those of desmoplastic and unicystic ameloblastomas.[25]

Takahashi H, Fugita S, *et al* (1998) studied PCNA expression in 15 cases of odontogenic cysts and 46 cases of ameloblastomas. In ameloblastomas, the mean percentage of PCNA-positive cells was lowest in the acanthomatous pattern and highest in plexiform pattern. The mean percentage of PCNA-positive cells in plexiform pattern was non-significantly higher than that in follicular pattern. The mean percentage of PCNA-positive cells in plexiform and follicular patterns was significantly higher than that in cystic and acanthomatous patterns. The frequency of PCNA positive cells was significantly higher in the peripheral cells of follicular and plexiform patterns than in the central cells of both patterns ($p < 0.01$). Therefore, peripheral cells were regarded as reserve cell of central cells. The mean percentage of PCNA-positive cells in the epithelial lining of odontogenic keratocyst was not

significantly different from those in the peripheral cells of follicular and plexiform patterns of ameloblastoma..[24]

Tracy R, Antony P M, *et al* (2001) the aim of this study was to evaluate the proliferative capacity of the cystic tumors (n=10) and compare to solid ameloblastomas (n=10) and dentigerous cyst (n=10) through assessment of stains for cell cycle associated protein Ki-67. The distribution of Ki-67 positive cells were in basal and suprabasal layer. The proliferative index of 4.3% in cystic type and in solid ameloblastomas positivity was detected in an average of 2.8% of the tumour cells and in dentigerous cyst 6.6% of the epithelial lining cells, suggesting that the biological aggressiveness of cystic ameloblastomas is less likely related to increased cellular proliferation than to other factors. The literature suggests that cystic ameloblastoma follows a biologically low grade course, with limited recurrence potential even when treated with simple curettage, and therefore should have a better prognosis.[26]

Piattelli A, Giovanna L, *et al* in 2002 conducted an IHC study with the hypothesis that an ameloblastoma arising from a dentigerous cyst has a similar biological behavior to the unicystic ameloblastoma and should be considered as merely a histologic variant.An immunohistochemical evaluation of Ki-67 in dentigerous cysts, unicystic ameloblastomas, and ameloblastomas arising in dentigerous cysts were done. The values of Ki-67 positivity were 3.14 for the dentigerous cyst, between 5.32 and 16.56 for unicystic

ameloblastoma, and 11.77 for ameloblastoma arising in a dentigerous cyst. Statistically significant difference were found between the dentigerous cyst and the unicystic ameloblastoma and between the dentigerous cyst and the ameloblastoma arising from a dentigerous cyst. No statistically significant difference was present between unicystic ameloblastoma and ameloblastoma arising from dentigerous cyst.[27]

Shabnum Meer, *et al* in 2003 performed study to determine the *in situ* proliferative activity of solid and multicystic ameloblastomas and of unicystic ameloblastomas by using proliferating cell nuclear antigen (PCNA) and Ki67 to provide a sound scientific basis for any differences in reported biologic behavior that might exist between these 2 groups of lesions. Ten cases of the solid and multicystic ameloblastoma and 10 cases of the unicystic variant were selected. The unicystic ameloblastoma comprised an even spread of 3 recognized histologic types, namely, the simple unicystic type, the intraluminal type, and intramural type. Representative fields were selected. These areas included only labeled tumor epithelial areas, including cyst linings, intraluminal nodules, and invading tumor islands. PCNA positive nuclei were found predominantly in the basal cell layers with fewer positive cells in the superficial layers or in the center of tumor island unicystic ameloblastomas demonstrating higher PCNA labeling indices than the solid and multicystic ameloblastomas (P <0.002).

Ameloblastoma had a statistically significantly greater PCNA labeling index than the mural follicles of this type, as well as the simple unicystic ameloblastoma. They concluded unicystic ameloblastomas showed statistically significant higher PCNA and Ki67 labeling indices than the solid variants. Within the unicystic group, highest mean labeling index being shown for the lining of the intraluminal and intramural unicystic ameloblastoma types. However, because the number of cases of each subtype was small, it was reluctant to draw any definite conclusions from these data.[28]

Thosaporn W, Iamaroon A *et al* (2004) The proliferation index, employing a novel cell proliferation marker IPO-38, was studied by the immunohistochemical technique in 10 OKC, seven orthokeratinized OKC, eight DC and 10 ameloblastomas. The ameloblastoma had no higher labeling index (LI) of IPO-38 than the OKC ($P = 0.910$) but had higher LI than the orthokeratinized OKC ($P = 0.001$) and DC ($P = 0.000$); the OKC had higher LI than the orthokeratinized OKC ($P = 0.002$) and DC ($P = 0.000$); and the orthokeratinized OKC had higher LI than the DC ($P = 0.011$). IPO-38-positive cells in the OKC and orthokeratinized OKC were located principally in the suprabasal cell layers while the ameloblastomas were found in the peripheral portion in particularly, the follicular and plexiform types. These findings support previous studies that the proliferation indices are useful in predicting the different biological behavior of the odontogenic lesions and the

OKC should be regarded as a benign tumor rather than simply an odontogenic cyst. [29]

Carlos A, Galvo B et *al* in (2005) studied the expression of PCNA and p53 protein expression. Total 16 cases of ameloblastoma (n=7 follicular, n=4 plexiform, n=3 follicular and acanthomatous , n=2 basal cell) and 8 cases of AOT was assessed by immunohistochemistry. The quantitative analysis for PCNA and p53 expressions were undertaken. Using the index positivity (IP) was calculated. All examined cases were positive for the antibodies used. In cases of Ameloblastoma, the peripheral cells of the islets presented, in general strong PCNA labelling index and moderate to weak p53 index. Some of the cells in the central area of the follicles resembling stellate reticulum of enamel organ also present positive labeling index. PCNA histochemical expression revealed stronger quantitative labeling index for follicular ameloblastomas. However results showed no statistically significant difference ($p>0.05$). For p53 the strongest quantitative labeling index was detected in the in the plexiform type. Considering the different histological patterns of ameloblastoma, plexiform had strongest labeling index (46.0%) followed by follicular subtype (42.4%). [30]

PATIENT CHARACTERISTICS:

Fifteen cases of (Group I) Dentigerous cyst, 15 cases of OKC (GroupII), 15 cases of (Group III) Radicular cyst and 15 cases of (GroupIV) Ameloblastoma were analyzed for immunoreactivity of PCNA cell cycle protein.

The patients age ranged from 12 to 47 years (mean 35.5 ± 7.8) in group I, from 30 to 51 years (mean 39 ± 7) in group II, from 12 to 43 years (mean 31.4 ± 8) in group III and from 24 to55 years (mean 40.2 ± 8.55) in group IV. There was no significant difference in the age distribution between the different age groups (p=0.160). **(TABLE 1, GRAPH 1)**

The study group comprised of 46 males and 14 females In group I, 73.3 % (n=11) were men and 26.6% (n=4) were women. In group II 80% (n=12) were men and 20% (n=3) were women, in group III 66.6, % (n=10) were men and 33.3% (n= 5) were women and in Group IV 86.6% (n=13) were men and 13.3% (n=2) were women. There was no significant difference between the gender (p=0.601). **(TABLE 2, GRAPH 2).**

Of the 15 patients in group I, the site of the lesion was 13.3% (n=2) in the posterior mandible and 55.3% (n=8) in the posterior maxilla, in group II 66.6% (n=10) were in the posterior mandible,13.3% (n=2) in the anterior mandible and 20% (n=3) in the posterior maxilla, in group III 33.3% (n=5) in the anterior

mandible, 40% (n=6) in the posterior mandible, 13.3% (n=2) in the posterior maxilla and 20% (n=3) in the anterior maxilla..In group IV all the cases 100% (n=15) were in the posterior mandible.There was significant difference between the different habits of study group (p=0.017). **(TABLE 3, GRAPH 3)**

DISTRIBUTION OF PCNA STAINING AMONG 4 GROUPS IN THE EPITHELIUM

PCNA revealed positivity in all the groups I,II, III and IV. In Group I 66.6% (n=10) showed positivity and (n=5) 33.3% showed negative staining. In Group III 80% (n=12) showed positive staining in the epithelium, and Group ll and Group IV, the cases showed 100% staining. There was significant difference in the staining pattern. **(TABLE 4, GRAPH 4)**

Distribution of expression in the basal layer and suprabasal layers among the cyst.

PCNA staining was present in the basal layer among three study groups . 100% (n=15) of cases showed staining in both basal and suprabasal layer in group II . In group II and III none of the cases have shown suprabasal positivity.There was no significant difference in the basal and suprabasal layers among the study groups. (p=0.131). **(TABLE 5, GRAPH 5).**

Labelling index among the groups in the epithelium

In group I, of the 15 cases examined 66.6% (n=10) showed positive staining in epithelium whereas 40% (n=5) showed absence of staining. Of the positive stained cases, the labelling indices were 3.3%, 0.6%, 4.7%, 3.7%, 0.95, 4.5%, 1.0%, 2.5%, 0.9% and 1.0% respectively. The median value is 1.75

Of the 15 cases examined in group II, 100% (n=15) cases showed positive staining in odontogenic epithelium. The labelling index of these cases which expressed 9.3%, 12.5%, 7%, 15%, 9.7%, 10.8%, 11.0%, 12.5%, 1.8%, 6.2%, 6.5%, 8%, 4.7%, 13.7%, 20%and 8.7% respectively. The median value is 9.30

Of the 15 cases examined in group III, 80% (n=12) cases showed positive staining in epithelium, of the positive staining cases in this the labelling index of the 12 cases were 1.8%, 1.9%, 5.5%, 3.7%, 4%, 2.7 % ,11.3%,3.6%,7.3%,5.1% ,9.5% ,2.9 % and 1.9% respectively. The median value is 3.80

Of the 15 cases examined in group IV, 100% (n=15) cases showed positive staining in epithelium. The labeling indices were 40%, 28.2% ,16.5% ,4.3% ,14% ,3.5% ,2%, 1.9% ,3.7% ,5% 2.7%, 27.5%,4%, 2.9% and 27.5% respectively. The median value is 4.30

COMPARISON OF MEAN LABELLING INDEX AMONG THE STUDY GROUPS

In group I, 10/15 cases showed nuclear staining and their mean labelling index were 2.3±1.6 . In group II, 15/15 cases showed nuclear staining which had the mean labelling index of 9.5±4.4. In group III, 12/15 case showed nuclear staining and its mean labeling index is 3.9± 1.8. The mean labelling index for ameloblastoma were 12.2±12.6 respectively. There was significant difference in the mean labeling index among the groups.(p=0.00). (**TABLE 6, GRAPH 6**)

Comparison of mean labeling index between histological variants of ameloblastomas

In group IV 100% (n=15) showed PCNA positivity. Follicular variant (n=5) showed mean labelling index of 22.6±13.7, with median of 27.5. Plexiform variant (n=2) showed mean labeling index of 22.35±8.27,with median of 22.35. Acanthomatous variant (n=1) showed mean labellling index of 3.50. Unicystic variant (n=5) showed mean labeling index of 2.6±0.73.with median as 2.70. Basaloid variant (n=2) showed mean labeling index of 4.50±0.71 with median value of 4.5. There was significant difference among these sub groups.(p=0.002)

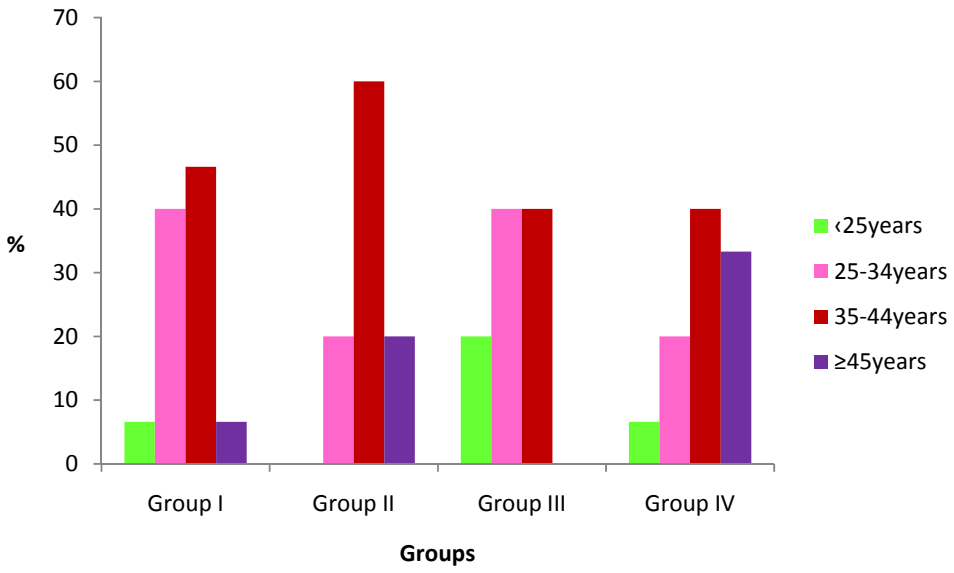
Comparison of mean labeling index between unicystic ameloblastoma and other variants of ameloblastomas

The mean labeling index of unicystic ameloblastoma (n=5) is 2.6±0.7 with median of 2.7, other variants (n=10) the mean labeling index is 17.0±13.0 with a median of 15.2.There was significant difference among these groups. (p=0.003)

TABLE 1: AGE OF THE SUBJECTS IN THE STUDY GROUPS

Age (in years)	Group I n=15		Group II n=15		Group III n=15		Group IV n=15		p-value
	n	%	n	%	n	%	n	%	
<25	1	6.6	0	0	3	20	1	6.6	0.160
25-34	6	40	3	20	6	40	3	20	
35-44	7	46.6	9	60	6	40	6	40	
≥45	1	6.6	3	20	0	0	5	33.3	

GRAPH-1 :AGE DISTRIBUTION AMONG STUDY GROUPS



Group I - Dentigerous cyst

Group II - Odontogenic keratocyst

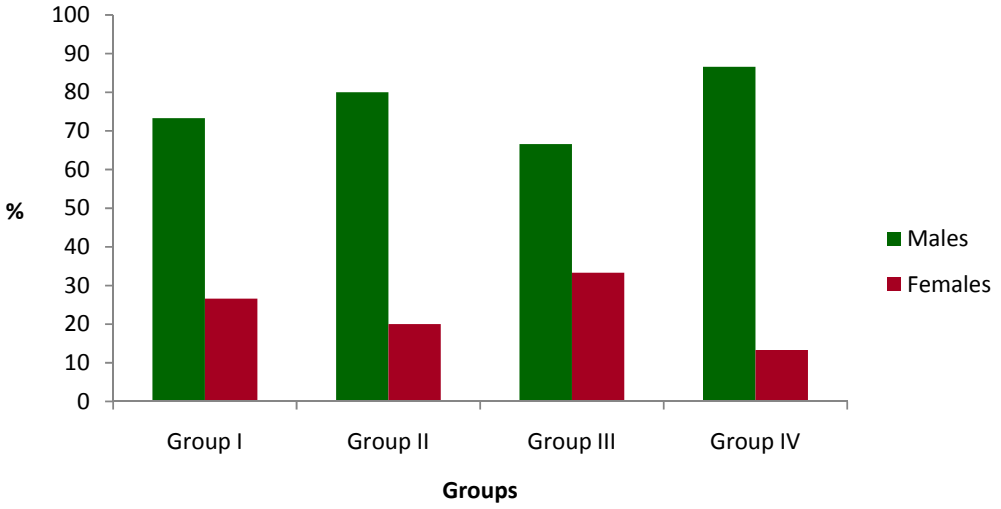
Group III - Radicular cyst

Group IV - Ameloblastoma

TABLE 2: GENDER DISTRIBUTION AMONG STUDY GROUPS

Groups	Males		Females		p-value
	n	%	n	%	
Group I (n=15)	11	73.3	4	26.6	0.601
Group II (n=15)	12	80	3	20	
Group III (n=15)	10	66.6	5	33.3	
Group IV (n=15)	13	86.6	2	13.3	

GRAPH 2: GENDER DISTRIBUTION AMONG STUDY GROUPS



Group I - Dentigerous cyst

Group II - Odontogenic keratocyst

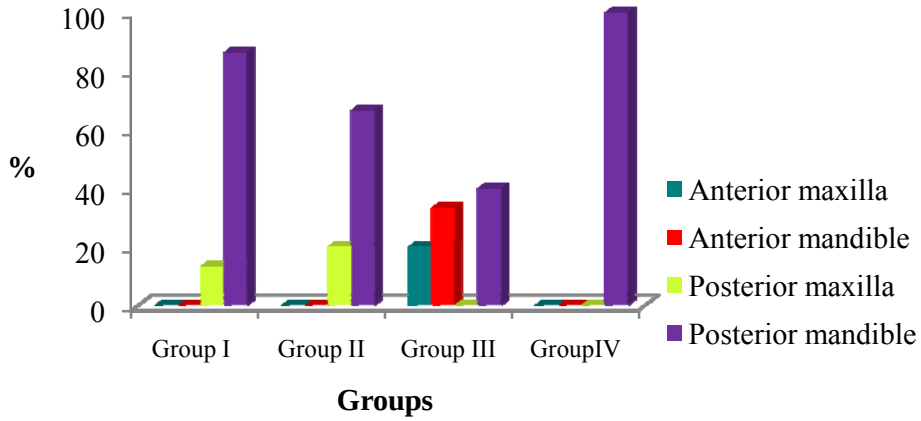
Group III - Radicular cyst

Group IV - Ameloblastoma

TABLE 3: SITE DISTRIBUTION AMONG STUDY GROUPS

Site	Group I n=15		Group II n=15		Group III n=15		Group IV n=15		p-value
Maxilla	n	%	n	%	n	%	n	%	0.017
Anterior	0	0	0	0	3	20	0	0	
Posterior	2	13.3	3	20	2	13.3	0	0	
Mandible	n	%	n	%	n	%	n	%	
Anterior	0	0	2	13.3	5	33.3	0	0	
Posterior	13	86.6	10	66.6	6	40	15	100	

GRAPH 3: SITE INVOLVED IN STUDY GROUPS



Group I - Dentigerous cyst

Group II - Odontogenic keratocyst

Group III - Radicular cyst

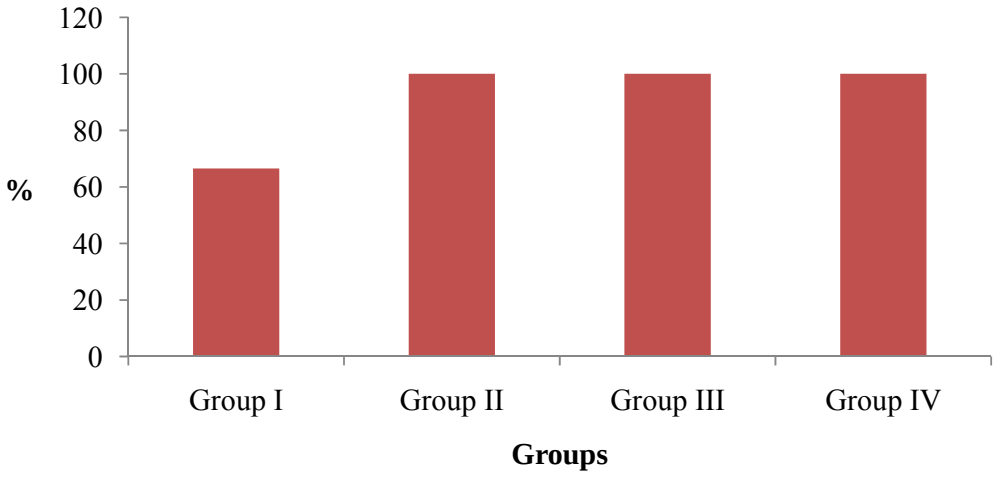
Group IV - Ameloblastoma

TABLE 4: PCNA POSITIVE STAINING AMONG STUDY GROUPS

Group I n= 15		Group II n= 15		Group III n= 15		Group IV n= 15		p-value
n	%	n	%	n	%	n	%	0.001*
10	66.6	15	100	15	100	15	100	

* p value $\leq$ 0.05 was consider to be statistically significant

GRAPH 4:POSITIVITY OF PCNA AMONG STUDY GROUPS



Group I - Dentigerous cyst

Group II - Odontogenic keratocyst

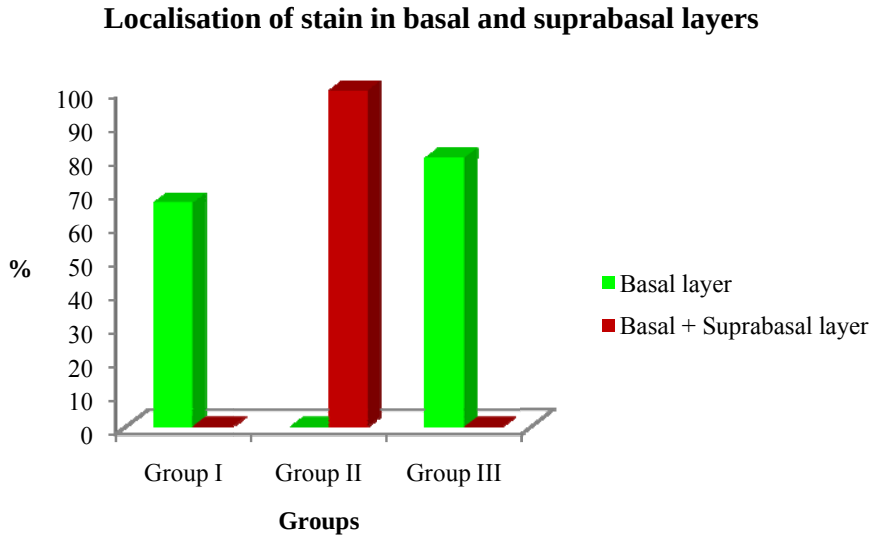
Group III - Radicular cyst

Group IV - Ameloblastoma

TABLE 5: LOCALIZATION OF STAIN IN BASAL AND SUPRABASAL LAYERS AMONG ODONTOGENIC CYSTS

Groups	Localization						p-value
	Basal Layer		Basal and Supra-basal		No stain		
	n	%	n	%	n	%	
Group I	10	66.6	0	0	5	33.3	0.131
Group II	0	0	15	100	0	0	
Group III	12	80	0	0	3	20	

GRAPH 5: LOCALIZATION OF STAIN IN BASAL AND SUPRABASAL LAYERS IN ODONTOGENIC CYSTS



Group I - Dentigerous cyst

Group II - Odontogenic keratocyst

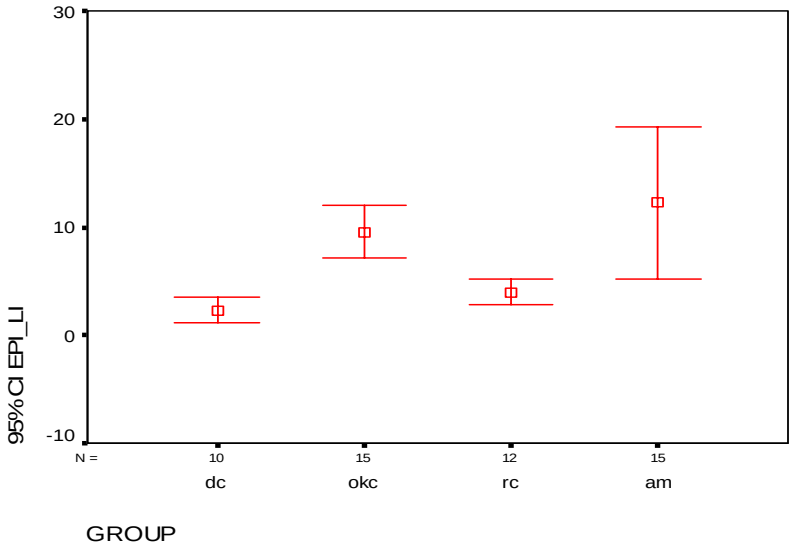
Group III - Radicular cyst

TABLE 6: PCNA LABELLING INDEX IN THE ODONTOGENIC EPITHELIUM AMONG THE STUDY GROUPS

Groups	Labelling index			p- value
	Mean	SD	Median	
Group I (n=10)	2.31	1.63	1.75	0.000*
Group II (n=15)	9.58	4.42	9.30	
Group III (n=12)	3.99	1.86	3.80	
Group IV (n=15)	12.25	12.64	4.30	

* p value $\leq$ 0.05 was consider to be statistically significant

GRAPH 6: PCNA LABELLING INDEX IN THE ODONTOGENIC EPITHELIUM AMONG THE STUDY GROUPS



Group I - Dentigerous cyst

Group II - Odontogenic keratocyst

Group III - Radicular cyst

Group IV - Ameloblastoma

Figure 1: Armamentarium

Figure 2: Antibody Kit

PCNA STAINING IN ORAL SQUAMOUS CELL CARCINOMA

Figure 3 : H & E; 10 x

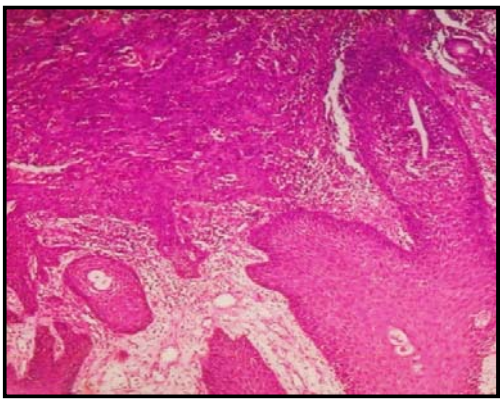


Figure 4: Negative control; 10 x

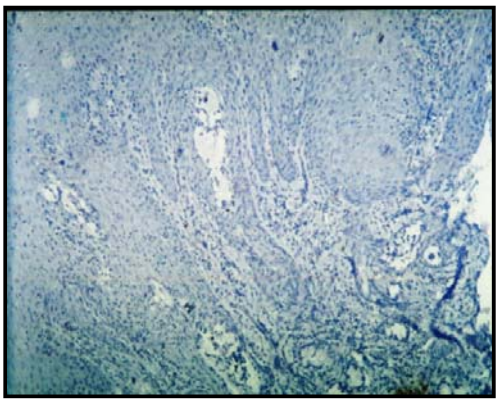


Figure 5: PCNA stain; 10 x

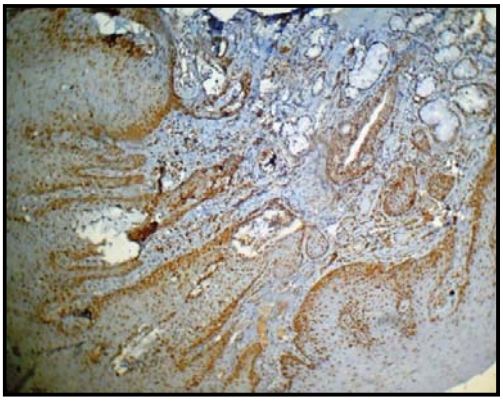
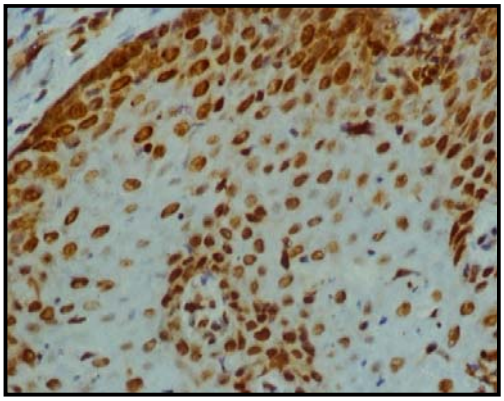


Figure 6: PCNA stain; 40 x



PCNA STAINING IN DENTIGEROUS CYST

Figure 7: H & E; 10 x

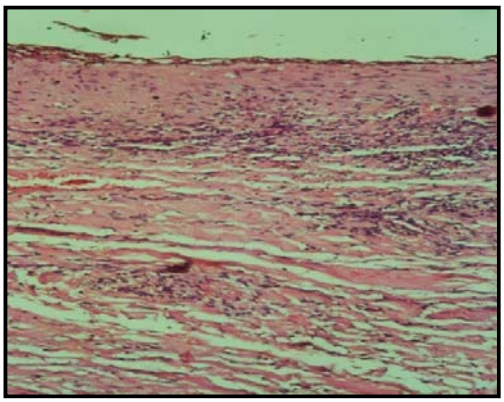


Figure 8: Negative control; 10x

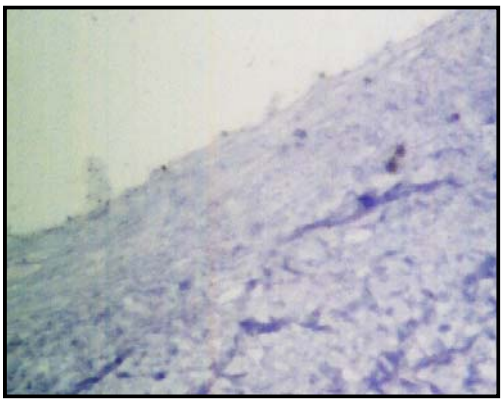


Figure 9: PCNA stain; 10 x

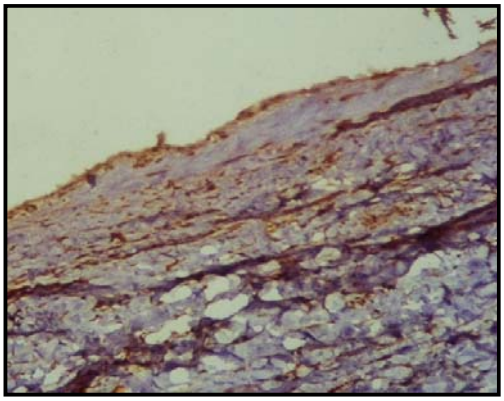
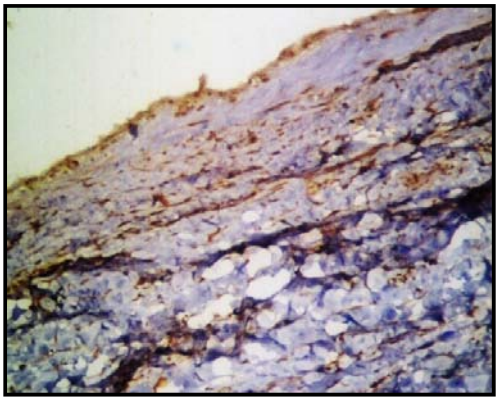


Figure 10: PCNA stain; 40 x



PCNA STAINING IN RADICULAR CYST

Figure 11: H & E; 10 x

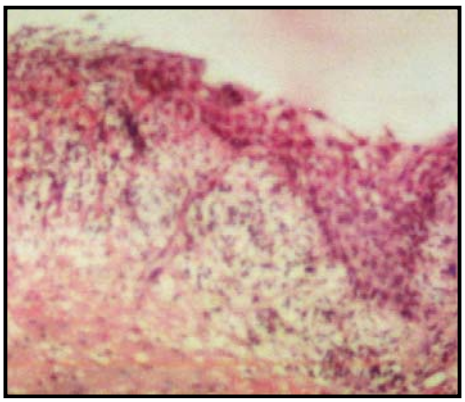


Figure 12: Negative control; 10x

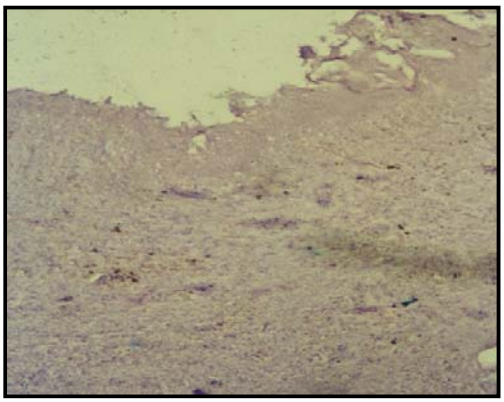


Figure 13: PCNA stain; 10 x

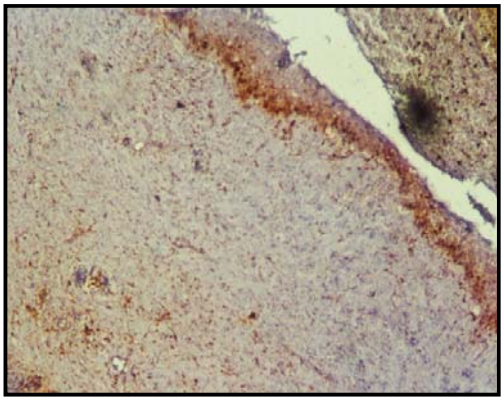
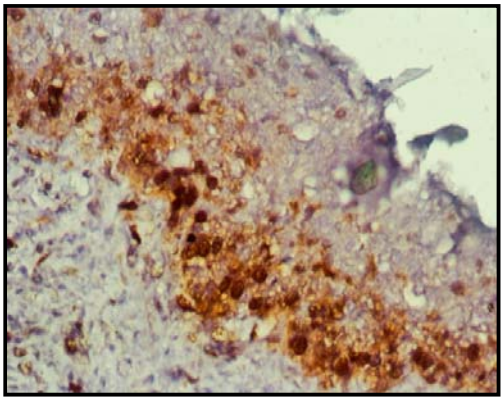


Figure 14: PCNA stain; 40 x



PCNA STAINING IN ODONTOGENIC KERATOCYST

Figure 15: H & E; 10 x

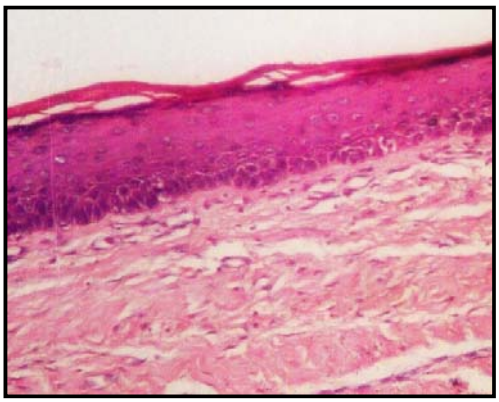


Figure 16 : Negative control; 10x

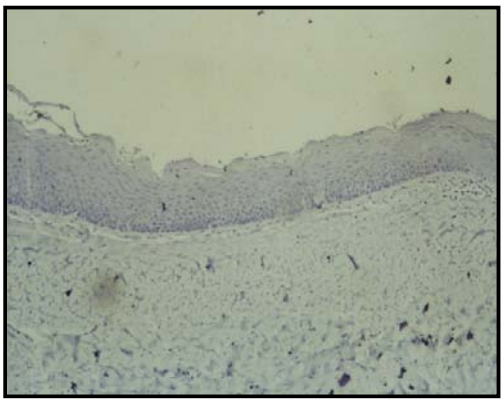


Figure 17: PCNA stain; 10 x

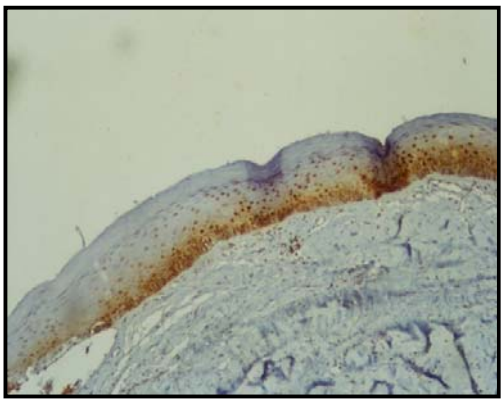
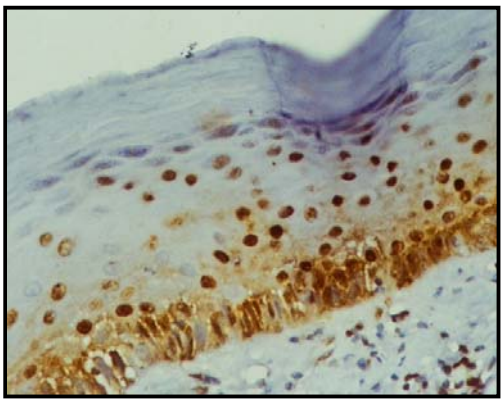


Figure 18: PCNA stain; 40 x



PCNA STAINING IN STAINING IN AMELOBLASTOMA

Figure 19: H & E; 10 x

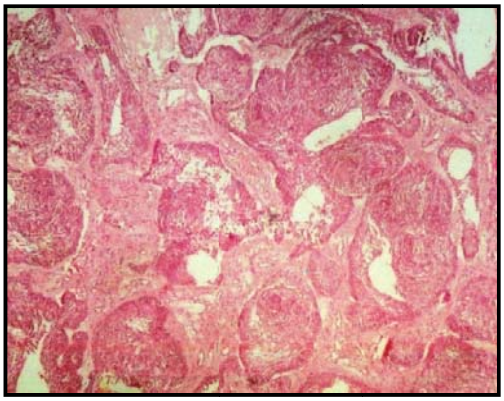


Figure 20: Negative control; 10x

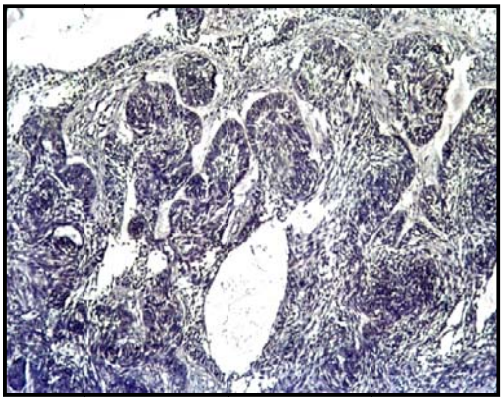


Figure 21: PCNA stain; 10 x

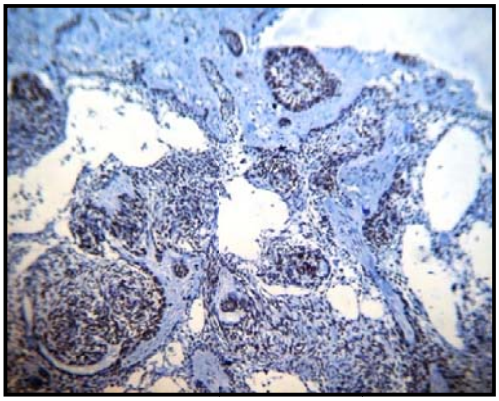
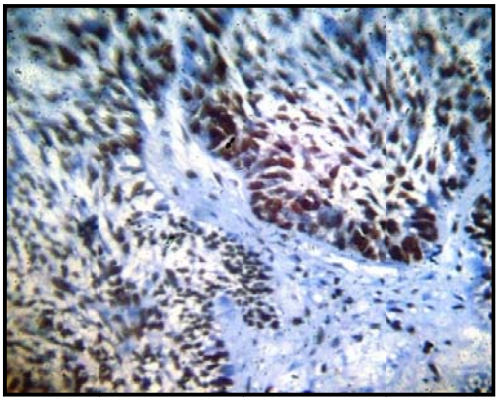


Figure 22: PCNA stain; 40 x



Odontogenic tumors are lesions derived from epithelium and mesenchymal elements of tooth forming apparatus and therefore exclusively found in jaw bones.[1] Odontogenic cysts are classified into developmental and inflammatory depending on their origin. The three kinds of odontogenic epithelium residues are the remnants of dental lamina, reduced enamel epithelium and epithelial rests of Malassez respectively. They give rise to dentigerous cyst, radicular cyst and odontogenic keratocyst. The potential for further differentiation and proliferation of the epithelial cells during formation of cyst may differ and lead to differentiation in epithelial expression of various proliferation which subsequently alters the biological behavior among different types of cyst.[12]

Odontogenic Keratocyst, appears to express an intrinsic higher growth potential. Active mural growth of OKC lining epithelium could be one of the main factors contributing to the development and enlargement of this cyst type.[6]

Ameloblastoma is the most common epithelial odontogenic tumour known to originate from residual odontogenic epithelial cells, cystic epithelial cells, basal cells of mucosal epithelium and enamel organ of developing tooth germ, histologically characterized by structures stimulating enamel organ. In spite of a benign cytological feature, the infiltrative growth still contributes to the high recurrency.[21]

Proliferating cell nuclear antigen (PCNA) is a cell cycle related antigen frequently used in the study of cell kinetics. PCNA is a 36KDa acidic nuclear non-histone nuclear protein associated with the cell cycle. It is an auxillary protein of DNA polymerase necessary for synthesis of DNA. The distribution of PCNA in the cell cycle increases through G1, peaks at the G1/S interphase and decreases through G2 phase. PCNA expression is used as a marker of cell proliferation because the cells remain for a longer time in late G1/S phase when proliferating. [7,8] An increase in PCNA levels may be induced by growth factors or as a result of DNA damage in the absence of cell cycle.[10] PCNA is also expressed in process of DNA replication and repair mechanisms as well and so it may be expressed in cells not synthesizing DNA. [9] PCNA expression is increased in cell cycle dysregulation. Immunostaining with monoclonal antibody PC10 against this antigen has been shown to demonstrate the proliferative compartment of normal tissue. An increased expression of these markers are expressed actively in proliferating cells, particularly in neoplasia.[9,10]

The present study was done to evaluate and compare the expression of PCNA in dentigerous cyst (n=15), radicular cyst (n=15), odontogenic keratocyst (n=15) and ameloblastoma (n=15), using immunohistochemistry in formalin fixed paraffin embedded archival tissues.

PATIENT CHARACTERISTICS

In the present study the mean age in radicular cyst cases was 25 years, varying between 12-43 years with 66.6% males and 33.3% females. ***Gulten usala et al*** [31] studied 20 cases of of radicular cyst and reported that the mean age in their study group was 34.4 ± 8.16 years of which 16 cases were males and 4 were females. ***Shear et al***[32] studied 948 cases of radicular cyst and reported peak age in third decade**, a**mong which 58.5% were males and 41.5% in were females. In our study posterior mandibular region was the involved in 40% of the cases, 13.3% in posterior maxilla, 33.3% in anterior mandible and 20% in the anterior maxilla respectively. ***Gulten usala et al*** [31] in his study reported 60% of cases in the maxilla and 40% in the mandible and ***Shear et al***[32] reported 12 cases in maxilla and 8 cases in the mandible.

In the present study, the age distribution in dentigerous cyst cases ranged from 12 to 47 years with a mean of age of 39.2±7 years 73.3% of the cases were seen in males and 26.6% in females. ***Banu et al*** [33] studied 13 cases of dentigerous cyst ranging from 15-65 with mean age of 32.4 ± 10.4 years. In a study done by ***Shear et al*** [32]in 343 cases of dentigerous cyst, most of the cases were in the second and third decades of life, with 60% under 20 years of age, among them 64% were males and and 36% were females.

In the present study, the mean age in odontogenic keratocyst cases ranged from 25 to 51 years with a mean of 25 years and male: female ratio of 4:1. ***Ataollah et al*** [34] **studied** 74 patients with age group of 5-82 years with mean age of 27.08±3.68, among which 59.5% were males and 40.5 % were females. ***Twafik.A et al*** [35] in a study of 16 patients reported the mean age as 32.56±11.79 years and male: female ratio of 1.7:1. In our study the site involved was posterior mandible region in 66.6% of the cases, 20% in posterior maxilla and 13.3% in anterior mandible respectively. ***Ataollah et al*** [34] reported 67.5% cases in the mandible and 32.5% cases in the maxilla. ***Twafik. A et al*** [35] reported that all 16 cases occurred in the mandible.

In the present study, the mean age was 24.97 years in 15 cases of ameloblastoma, and among them 86.6% were males and 13.3% were females. In a study conducted by ***Ponniah et al*** [36] in 31 cases of ameloblastoma, the mean age was 32.64 years, among them and 51.94% cases were seen in males and 48.06% were seen in females. ***Reichert et al*** [38] in a study reviewed 3,667 cases of ameloblastoma the mean age among the cases was 37.4 years.In their study 53.5% were males and 46.7% were females. In our present study the most common site for ameloblastoma was the posterior mandible (100%). In a study conducted by ***Ponniah et al*** [36] posterior mandible was the most common anatomic location

comprising 67.69%. In a study done by ***Reichert et al*** [38] mandible is to maxilla ratio was 2.2:1.

STAINING CHARACTERISTICS OF ODONTOGENIC CYSTS

In the present study, epithelial expression of PCNA was confined to the nucleus. Expression of PCNA in dentigerous cyst were positive in 66.6 % cases and negative in 33.3% of cases, with mean labeling index of 2.31±1.63 with a median of 1.75 for positive cases. ***Kolar et al*** [20] studied 10 cases of dentigerous cyst and found that there was lower level of basal cell proliferative activity in these cysts as measured by expression of PCNA with a mean labeling index of 23.2±23.85 when compared to other than odontogenic cysts, in which the mean labeling indices were 47.5±31.56. ***Browne*** *et al*[4] also studied and quantified PCNA expression in 10 cases of dentigerous cyst and found that the total PCNA positive counts were 5.1±3.0/mm. In another study by ***Jin Kim et al*** [22] study in 20 cases of dentigerous cyst, only 11 cases demonstrated PCNA positivity. The PCNA positive cells were counted, which were found to be 8.6 to 21.3 cells per 2000 positively stained epithelial cells.

Marcia Gaiger et al [21] explained that lower PCNA expression or negative expression is because of the inflammatory stimuli as a result of eruptive process. This may make cells to proliferate, but may be inconstant and present only for a short time.

In the present study in radicular cyst, the expression of PCNA positive cells in the epithelium were positive in 80% of cases with a mean labeling index of 9.58±4.42 and median of 3.80. ***A.Patelli et al***[7] in his study of 12 cases of radicular cyst, the positivity of PCNA was found to be 6.10±1.12 with median of 6. Similarly in another study done by ***Browne et al*** [4] in 10 cases of radicular cyst, the positive PCNA count per millimeter of basement membrane was 11±4.

Marcia Gaiger et al [21]in their study suggested that in radicular cyst, the inflammatory stimulus increases cell proliferation from the continous aggression caused by the bacterial contamination of the root canal, which may inhibit degradation of p53 protein and increase the level of PCNA in the absence of cell cycling. In contrast to which, the stimuli is only for short periods in dentigerous cyst. He also suggested that the p53 protein is expressed by proliferating cells, but its accumulation in the cell may be caused by several factors. Cell stress, is one of these factors since p53 is a primary mediator of cell response to stress.

In the present study, among odontogenic keratocyst, the expression of PCNA positive cells in the epithelium was 100% with a mean labeling index of 9.58 ±4.42 with a median of 9.30 per 1000 cells, and all the cases showed basal and suprabasal staining in the epithelium. ***A.Patelli et al*** [7]in his study of 12 cases of OKC, the positivity was 23.48±2.04 with median of 23.50. ***Main et al***[32] in

their study done by autoradiography and mitosis, observed that the mitotic figures per centimeter length of basement membrane in OKC lining ranged from 0-19 cms with mean of 8.0. In a study done by ***Li et al***[12] the total PCNA count in the OKC epithelium had a mean value of 94.4±22.7 cells/mm.

Li et al [12]suggested that in comparison to other cyst types, a higher level of coexpression of TGF-α and EGF receptor was detected in OKC epithelium (via autocrine and /or paracrine pathways) which could contribute to higher cell proliferation and differentiation in this cyst type. Higher levels of G6PDH and a lower level of LDH than radicular cyst also suggest that the OKC are metabolically more active, which is consistent with their higher level of mitotic and synthetic activity of the cyst and also suggested that the epithelial linings of OKC expresses higher levels of p53 protein than other cyst types. ***Kichi et al***[19] studied expression of apoptotic protein bcl2. They reported that 96% of bcl2 was in the basal cell layers of OKC and 3% in dentigerous cyst and suggested from their findings that apoptosis do not occur in basal layers to facilitate cellular proliferation of lining epithelium of OKC.

BASAL AND SUPRABASAL DISTRIBUTION AMONG THE EPITHELIUM OF ODONTOGENIC CYST

In our study the localization of PCNA stained cells were only in the basal layers in the radicular and dentigerous cyst among the positive cases of the epithelium .In odontogenic keratocyst both the

basal and suprabasal cells were positive for PCNA in all the cases . ***A.Patelli et al***[7] in their study also showed predominant suprabasal staining in comparison with the dentigerous and radicular cyst in which the distribution of positive cells were in the basal layer. In another study by ***Li et al*** [12] the epithelial linings of OKC contained the highest number of PCNA positive cells most of which were in suprabasal layers with less than 5% in the basal layers. ***Odgen et al***[32] suggested that predominant suprabasal distribution in face of low basal positivity is due to increased expression of EGFr and p53 in OKC epithelium, suggesting a unique proliferative and differentiation process which occurs in this cyst lining. In a study by ***Browne et al*** [4] in his studies suggested that predominant suprabasal distribution of PCNA positive cells in OKC epithelial lining could represent an intermediate stage between normality and dysplasia, where both qualitative and quantitative changes in proliferative activity have occurred in the absence of histological evidence of epithelial disruption. This ability is further supported by recent immunohistochemical studies, showing increased expression of EGFr by supra basal cells in OKC linings.

STAINING CHARECTERSTIC IN AMELOBLASTOMA

In our study all ameloblastomas showed 100% positivity. The mean labeling index were 12.25±12.64 with a median of 4.30. Among the 15 cases, 5 cases were follicular ameloblastoma, 2 cases of plexiform, 1case of acanthomatous, 2 cases of basaloid variant and 5 cases of unicystic ameloblastoma . All the solid multicystic ameloblastomas showed higher labeling index compared to unicystic variant of ameloblastomas.There was a significant difference between these two clinical variants(p=0.003).

Sandra *et al* [25]studied 32 cases of ameloblastoma, among which 16 cases were solid type of ameloblastoma, 9 cases were unicystic and 8 cases were mixed type of ameloblastomas.Solid type of ameloblastoma had significantly higher PCNA labeling index than cystic type (p=0.005). Similar results were seen in a study done **by *A Piatelli et al.,*** [7]·He studied 17 cases of solid ameloblastoma and 5 cases of unicystic ameloblastoma. The mean labeling index was 28.18±3.84 for unicystic ameloblastoma which was lower than solid variants.

In another study by ***Jin Kim et al*** [20] they found no differences between the histological variants of solid ameloblastomas, but among the unicystic variants the area of plexiform intraluminal growth showed the highest proliferative activity.Although both proliferative epithelium with or without ameloblastic change showed higher immunoreactivity than the area of flat epithelium

lining, there was still no statistical significance found.The ameloblastic epithelium, which fit into histological criteria suggested by Vickers and Gorlin showed no significant difference compared to epithelium without ameloblastic change. This result showed that PCNA immunoreactivity might be independent of the cytological differentiation, ***Funaoka et al*** [23] study showed that the mean positive PCNA index in the follicular type was higher than plexiform type ($p<0.10$) and the cystic type showed a low positive PCNA index.

In contradiction to all of the studies above, ***Shabnum et al*** [28] study suggested that the unicystic ameloblastoma showed statistically significantly higher PCNA labeling indices than the solid and multicystic variant ($p< 0.002$).The reason for inconsistent results might be the differences in the morphology of the tumors, with solid lesions providing large follicles or plexiform sheets for analysis,whereas only a thin lining is available in the unicystic cases. This may have resulted in the inclusion of greater numbers of basal and parabasal cells in the unicystic group, thus resulting in higher mean labeling indices.

A total of 60 cases were included in the study, comprising of 15 cases of dentigerous cyst, (group I), 15 cases of odontogenic keratocyst (group II), 15 cases of radicular cyst (group III) and 15 cases of ameloblastoma(GroupIV)

- The mean ages of patients in group I, group II , group III and group IV were 35.53 ± 7.83, 39.07 ± 7.05, 31.40 ± 8.68 and 40.2±8.55 years respectively
- In group I (Dentigerous cyst) 73.3% were males and 226.6% were females.
- In group II (Odontogenic keratocyst) 80% were males and 20% were females.
- In group III (Radicular cyst) 70% were males and 30% were females.
- In group IV (Ameloblastoma) 86.6% were males and 13 were females.
- Among dentigerous cyst, 66.6% of cases exhibited positivity for PCNA and 33.3% of cases were negative. In all positive cases stain was present in basal layer.
- In odontogenic keratocyst; all cases showed PCNA positivity in the epithelium. The stain was present in the basal and suprabasal layers of epithelium.
- In radicular cyst, there was 80% of positivity of PCNA expression in the epithelium. All of the positive cases showed staining in the basal layers of the epithelium.

- All cases of ameloblastoma were positive for PCNA.
- There was statistical differences in the labeling indices among all the four groups (p=0.004).
- There was no statistically significant difference in the epithelial staining intensity of PCNA between localization of stain in the basal and suprabasal layers of odontogenic cysts. (p= 0.131)
- There was no statistical significant difference in the labeling indices of odontogenic keratocyst and ameloblastoma (p=0.520)

In the present study, we could not ascertain the PCNA expression as a marker to differentiate between odontogenic keratocysts and ameloblastomas. However, differential markers such as calretenin can be used to differentiate between ameloblastomas and odontogenic keratocysts.

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Anti-Proliferating cell nuclear antigen (PCNA) [PC10]

AM252-5M
AM252-10M
MU252-UC

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Doc. No. 932-252M-4 Rev. E
Release Date: June 11, 2007

CE

ENGLISH

Specifications:	Anti-Human Proliferating cell nuclear antigen (PCNA)	Catalog No.	Description
Immunogen:	Rat PCNA synthesized with the protein A expression vector pR1T2T	AM252-5M	6 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems.
Clone:	PC10	AM252-10M	10 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems and BioGenex Automated Staining Systems.
Species:	Mouse	MU252-UC	1 ml of Concentrated Antibody for Use with BioGenex Super Sensitive Detection Systems or Other Equivalent Detection Systems.
Immunoglobulin Class	IgG2a	**Recommended Detection System:** LINK-LABEL *Lot specific Ig concentration available upon request.	
Protein Concentration	10 – 15 mg/ml*		

Intended Use
This antibody is currently available for in vitro diagnostic use. This monoclonal antibody is designed for the specific localization of PCNA in formalin-fixed, paraffin-embedded tissue sections.

Summary and Explanation
PCNA, also known as cyclin, is a 36 kD nonhistone nuclear protein. It has been demonstrated that PCNA is an auxiliary protein of DNA polymerase-delta and plays a fundamental role in the initiation of cell proliferation. PCNA is a cell cycle-regulated protein that preferentially occurs in dividing cells and is undetectable or present in small amounts in resting cells. Its cell cycle distribution has been well described. Elevated expression of PCNA appears in the nucleus during late G 1 phase immediately before the onset of DNA synthesis, becomes maximal during S-phase and declines again during G 2 and M phases. Therefore, its level correlates directly with rates of cellular proliferation and DNA synthesis. Immunoperoxidase staining for PCNA in benign tissues has revealed positive nuclear staining in normal colonic crypt epithelium, gastric glandular cells, germinal center cells of lymph node, basal cells of skin, and renal tubular epithelial cells. Robbins et al detected PCNA in 42 of 64 human malignancies of different histologic types and related benign tissues. In a panel of 35 tumors and 11 hyperplastic normal tissues, Dervan et al found that immunostaining with PC10 correlated strongly with proliferative rate data obtained by Ki-67 labeling and concluded that this monoclonal antibody to PCNA might be an acceptable alternative to Ki-67 labeling in routinely processed tissues. PCNA expression in leukocytes isolated in chronic myeloid leukemia (CML) has been found to be elevated during blast crisis. In addition, other cell types that are associated with blast crisis have been identified that express PCNA. Findings indicate that PCNA-positive activated peripheral blood mononuclear cells are present in systemic lupus erythematosus.

Principles of the Procedure
The demonstration of antigens by immunohistochemistry 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. The primary antibody may be used in immunohistochemistry using manual techniques or using BioGenex Automated Staining System. BioGenex offers a variety of Super Sensitive detection systems including link-label and polymer based technologies to detect the chromogenic signal from the stained tissues and cells.

Reagents Provided
Mouse monoclonal antibody diluted in phosphate buffered saline, pH 7.6, containing 1% BSA and 0.09% sodium azide.

Dilution of Primary Antibody
This Ready-to-Use antibody has been optimized for use with detection system as indicated above and should not require further dilution. Further dilution may result in loss of sensitivity. The user must validate any such change. BioGenex Concentrated antibodies must be diluted in accordance with the staining procedure when used with BioGenex Super Sensitive Detection Systems. Use of non-BioGenex systems other than recommended systems and protocols require validation by the user. Antibody dilutions should be appropriately adjusted and verified according to the detection system used.

Materials Required But Not Provided
All the reagents and materials required for immunohistochemistry are not provided. Pre-treatment reagents, Super Sensitive detection systems, control slides, control reagents and other ancillary reagents are available from BioGenex. Please refer to the product insert(s) of the BioGenex Super Sensitive Immunohistochemistry detection systems for detailed protocols and instructions. The immunohistochemistry procedure may need other lab equipment that are not provided including oven or incubator (capable of maintaining 56-60°C), BioGenex Automated Staining System, Humidity Chamber, Microwave oven, Staining Jars or baths, Timer (capable of 3-20 minute intervals), Wash Bottles, Absorbent Wipes, Microscope slides (pre-treated with poly-L-Lysine), Coverslips, Lens paper and Light microscope with magnification of 200X.

Storage and Handling
Antibodies should be stored at 2-8°C without further dilution. Fresh dilutions, if required, should be made prior to use and are stable for up to one day at room temperature (20-26°C). Unused portions of antibody preparations should be discarded after one day.
This antibody is suitable for use until expiry date when stored at 2-8°C. Do not use product after the expiration date printed on vial. If reagents are stored under a condition other than those specified in the package insert, they must be verified by the user (U.S. Congress, 1992).
The presence of precipitate or an unusual odor indicates that the antibody is deteriorating and should not be used. Positive and negative controls should be run simultaneously with all patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact BioGenex Technical Support at 925-275-0550 or your local distributor.

Specimen Collection and Preparation
Tissues fixed in 10% (v/v) formalin are suitable for use prior to paraffin embedding. Consult references (Kiernan, 1981: Sheehan & Hrapchak, 1980) for further details on specimen preparation.

Treatment of Tissues Prior to Staining
Pretreatment of tissues if any, should be done as suggested in the staining procedure section.

Precautions
This antibody contains no hazardous material at a reportable concentration according to U.S. 29 CFR 1910.1200, OSHA Hazard Communication Standard and EC Directive 91/155/EC. However, this product contains sodium azide, at concentrations of less than 0.1%. Sodium azide is not classified as a hazardous chemical at the product concentrations. However, toxicity information regarding sodium azide at product concentrations has not been thoroughly investigated. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing (Center for Disease Control, 1976, National Institute for Occupational Safety and Health, 1976). For more information, a Material Safety Data Sheet for sodium azide in pure form is available upon request. Do not pipette reagents by mouth, and avoid contact of reagents and specimens with skin and mucous membranes. If reagents or specimens come in contact with sensitive area, wash with copious amounts of water. Minimize microbial contamination of reagents or increase in nonspecific staining may occur. Refer to appropriate product inserts for instructions of use and safety information on detection reagents and other materials, which may be used with the antibody.

Staining Procedure
Refer to the following table for conditions specifically recommended for this antibody. Refer to the detection system package insert for guidance on specific staining protocols or other requirements.

Parameter	BioGenex Recommendations
Control Tissue	LYMPHOMA
Tissue Type	Formalin-fixed, Paraffin-embedded
Concentrated Dilution	100-200
Pretreatment	AR Citra
Incubation Time and Temperature	30 min. @ RT

Quality Control
The recommended positive control tissue for this antibody is LYMPHOMA. FB-252M/ FG-252M tissues are available from BioGenex for QC. Refer to the appropriate detection system package inserts for guidance on general quality control procedures.

Troubleshooting
Refer to the troubleshooting section in the package inserts of BioGenex Super Sensitive Detection Systems (or other equivalent detection systems) for remedial actions on detection system related issues, or contact BioGenex Technical Support Department at 925-275-0550 to report unusual staining.

Expected Results
This antibody stains PCNA in the nucleus of proliferating cells in formalin fixed paraffin embedded tissue sections. Interpretation of the staining result is solely the responsibility of the user. Any experimental result should be confirmed by a medically established diagnostic product or procedure.

Limitations of the Procedure
Immunohistochemistry (IHC) is a complex technique involving both histological and immunological detection methods. Tissue processing and handling prior to immunostaining can also cause inconsistent results. Variations in fixation and embedding or the inherent nature of the tissue may cause variations in results (Nadji and Morales, 1983). Endogenous peroxidase activity or pseudoperoxidase activity in erythrocytes and endogenous biotin may cause non-specific staining depending on detection system used. Tissues containing Hepatitis B Surface Antigen (HBsAg) may give false positive with horseradish peroxidase systems (Omata et al, 1980). Improper counterstaining and mounting may compromise the interpretation of results.

Performance Characteristics
BioGenex has conducted studies to evaluate the performance of the antibody with BioGenex detection systems and accessories. The antibodies have been found to be sensitive and show specific binding to the antigen of interest with minimal to no binding to non-specific tissues or cells. BioGenex antibodies have shown reproducible and consistent results when used within a single run, between runs, between lots and wherever applicable between manual and automated runs. The products have been determined to be stable for the periods specified on the labels either by standard real time or accelerated methods. BioGenex ensures product quality through 100% quality control for all products released and through surveillance programs.

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Specifiche tecniche:	Proliferating cell nuclear antigen (PCNA) anti-umano	N. di catalogo	Descrizione
Immunogeno:	PCNA del topo sintetizzata con l'espressione della proteina A vettore pR1T2T	AM252-5M	6 ml di anticorpo pronto per l'uso con i diversi BioGenex Super Sensitive Detection Systems.
Clone:	PC10	AM252-10M	10 ml di anticorpo pronto per l'uso con i diversi BioGenex Super Sensitive Detection Systems e BioGenex Automated Staining Systems.