IMMUNOHISTOCHEMICAL EVALUATION OF CYTOKERATIN 19 AND CALRETININ IN ODONTOGENIC CYSTS AND TUMORS

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BRANCH VI

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CERTIFICATE

This is to certify that this dissertation titled "**IMMUNOHISTOCHEMICAL EVALUATION OF CYTOKERATIN 19 AND CALRETININ IN ODONTOGENIC CYSTS AND TUMORS**" is a bonafide dissertation performed by **Aesha** 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.

Dr. K. Ranganathan, MDS, MS (Ohio),PhD
Professor & HOD
Dept. of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai

Dr. Elizabeth Joshua, MDS
Professor
Dept. of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai

Dr. S. Ramachandran, MDS
Principal
Ragas Dental College & Hospital
Chennai

Odontogenic cysts and tumors are a diverse group of lesions arising from the odontogenic apparatus, which is responsible for tooth development under physiologic conditions[1]. Odontogenic tumors, which arise from odontogenic epithelium, odontogenic ectomesenchyme or both constitute a group of heterogeneous lesions that range from hamartomatous or non-neoplastic tissue proliferations to malignant neoplasms with metastatic capabilities.[2] Ameloblastoma is the most frequently encountered tumor arising from odonotgenic epithelium and is characterized by a benign but locally invasive behavior with high tendency to recur.[1]

Odontogenic cysts include three main groups of cysts, whose epithelial linings are thought to have differing origins. The epithelial lining of radicular cyst is presumed to arise from epithelial rests of Malassez, that of dentigerous cyst from reduced enamel epithelium and that of odontogenic keratocysts from dental lamina or its remnants.[3]The odontogenic keratocyst is a destructive cystic lesion that has a propensity for recurrence. The term Keratocystic odontogenic tumor is given because of its aggressive nature, rapid growth rate and high recurrence rate.[4]

Ameloblastomas are usually lined by a variable epithelium ranging from one that has typical ameloblastic characteristics to one that is metaplastic and which appears completely nondescript consisting of several layers of non keratinizing squamous cells.

Overlapping clinical and radiographic features further add to this diagnostic difficulty. Many techniques have been used in an attempt to distinguish odontogenic cysts from ameloblastoma.[2]

Although the cyst linings may have lost their typical ameloblastic features, the cells retain their immunophenotypic characteristics like continued expression of cytokeratin 19 and calretinin.[5]

Cytokeratins (CKs) are specific intermediate filaments of epithelial cells. In humans, they comprise a complex family of atleast 20 different polypeptides. Based on their charges, immunoreactivity and amino acid sequence, they fall into two subfamilies: acidic proteins with low molecular weight and basic proteins with high molecular weight. Cytoplasmic intermediate filaments are involved in differentiation of mammalian cells. The expression pattern of intermediate filaments has been investigated in normal and neoplastic human cells including oral epithelial cells, odontogenic epithelia, tumors and cysts. These investigators hypothesize that intermediate filaments expression patterns are characteristic for each kind of cells.[6]

Cytokeratin 19 is the smallest known acidic type of cytokeratin, having molecular mass of 40kD. It is not paired in epithelial cells .It is expressed in all kinds of odontogenic epithelial

cells in developing tooth germs and in neoplastic epithelial cells in some odontogenic tumors. It is also detected in cell rests of Malassez, Serre's and odontogenic cyst lining.[1]

Calretinin is a calcium binding protein of 29 kilodalton (29kDa) and is a member of the large family of EF-hand proteins. EF- hand proteins are characterized by a peculiar amino acid sequence that folds up into a helix-loop-helix which acts as the calcium binding site; calretinin contains six such EF-hands stretches.[7]Calretinin is widely expressed in neural tissue and is also a specific marker of mesothelial cells, mast cells, cutaneous mastocytomas, neural elements of the tooth pulp and periodontal ligament. The viscerosensory nerve fibers of oral and pharyngeal tissues have also demonstrated the expression of calretinin in rats[8].This calcium-binding protein may be a specific marker of ameloblastic tissues and may be an important diagnostic aid in differential diagnosis of cystic odontogenic lesions and ameloblastic tumors.[9]

This study was done to evaluate the expression of cytokeratin 19 and calretinin in odontogenic cysts and ameloblastoma.

Aims and Objectives:

To assess cytokeratin 19 and calretinin expression from archival paraffin embedded sections of radicular cyst, dentigerous cyst, odontogenic keratocyst and ameloblastoma by immunohistochemistry.

Hypothesis:

1. Cytokeratin 19 is expressed in all odontogenic cysts and ameloblastoma.
2. Calretinin is expressed in ameloblastoma and not in odontogenic cysts.

Study setting

The study was conducted in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai.

A retrospective study was done to evaluate the expression of calretinin and cytokeratin19 using immunohistochemistry in formalin fixed, paraffin embedded tissue specimens of odontogenic cysts (radicular cysts, dentigerous cysts, odontogenic keratocysts) and ameloblastoma.

Sample size

The study material comprised of 60 formalin fixed, paraffin embedded tissue specimens (archival blocks).

Group I: Fifteen clinically, radiographically and histologically confirmed dentigerous cysts.

Histological criteria: The epithelial lining consists of two to four layers of flattened nonkeratinising squamous cells and a fibrous connective tissue capsule.

Group II: Fifteen clinically, radiographically and histologically confirmed odontogenic keratocysts.

Histological criteria: Odontogenic keratocyst is characterized by a thin fibrous connective tissue capsule and a lining of corrugated parakeratinized/orthokeratinized stratified squamous epithelium

usually about five to eight cell layers in thickness. Basal layer exhibits a palisaded pattern.

Group III: Fifteen clinically, radiographically and histologically confirmed radicular cysts.

Histological criteria: Radicular cyst is lined by stratified squamous epithelium and consists of a dense fibrous connective tissue capsule with a mixed inflammatory infiltrate.

Group IV: Fifteen clinically, radiographically and histologically confirmed ameloblastoma specimens.

Histological criteria: Odontogenic epithelium proliferating in the form of islands or follicles or strands, embedded in a mature connective tissue stroma.

Immunohistochemistry (IHC) procedure:

Armamentarium:

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jars
- Weighing machine
- APES coated slide

- Slide carrier
- Aluminium foil
- Micro-pipettes
- Tooth forceps
- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover –slips
- Light microscope

Reagents used:

- Conc.Hcl
- Laxbro solution
- APES (3 amino propyl tri epoxy silane)
- Acetone
- Citrate buffer(for Calretinin) and Pepsin 1: 3000 (for CK19)
- Phosphate buffer saline(PBS)
- 3% Hydrogen peroxide
- Deionized distilled water
- Harris Haematoxylin
- Absolute alcohol
- Xylene
- DPX (distrene , dibutyl phthalate, xylene)

Antibodies used:

1. Primary antibody - Mouse monoclonal Anti Human Cytokeratin 19 (Biogenex)
   Rabbit polyclonal Anti Human Calretinin (Biogenex)
2. Secondary antibody – Super Sensitive Polymer/ HRP/ DAB

Procedure:

Pretreatment of the slides:

- The slides were first washed in tap water for few minutes.
- They 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.
- Slides were washed in two changes of distilled water.
- The slides were then 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 aluminum foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES coating:

Slides were 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 were left to dry

Preparation of sections:

After the slides were dry, tissue sections of 0.5 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 positive (P) and the other negative (N).

Immunohistochemistry procedure:

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 15 minutes. The slides were allowed to cool and then washed in cold phosphate buffer (PBS)

solution for 5 minutes. Slides were treated with 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non-specific staining. After blotting the excess, the slides were treated with protein block reagent for another 10 minutes. The slides were then wiped carefully without touching the tissue section with gauze to remove excess protein block reagent. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The primary antibody (Calretinin) (Biogenex) was added only to P tissue on the slide and PBS was added to the N tissue to prevent drying. The petridish containing the slides was incubated at room temperature for overnight. For Ck19, primary antibody (Biogenex) was added to P tissue and incubated for 2 hours at room temperature. The sections taken out were washed in three changes of cold PBS for 5 minutes each to remove the excess antibody. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of enhancer from the secondary antibody kit (Biogenex) was added on both the sections and the slides were incubated for 20 minutes. Later slides were washed in three changes of cold PBS for 5 minutes each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of Streptavidin from the secondary antibody kit (Biogenex) 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 each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of freshly prepared DAB (3' Diaminobenzidine Tetrahydrochloride - a substrate chromogen) was added on both sections. Slides were washed in PBS to remove excess DAB and then counter stained with Hematoxylin. The slides were placed in a tray of tap water for 5 minutes for the process of blueing. Then the slides were transferred to 70% alcohol, 100% alcohol and xylene. The tissue sections were mounted with DPX. Slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

IHC procedure for Calretinin:

APES coated slides with 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

↓

Washed in cold PBS (Phosphate buffer solution) (5 min)

↓

Placed in 3% hydrogen peroxide

↓

Washed in PBS thrice (5 min each)

↓

Protein block

↓

Washed in PBS thrice (5 min each)

↓

Primary antibody added and incubated overnight

↓

Washed in PBS thrice (5 min each)

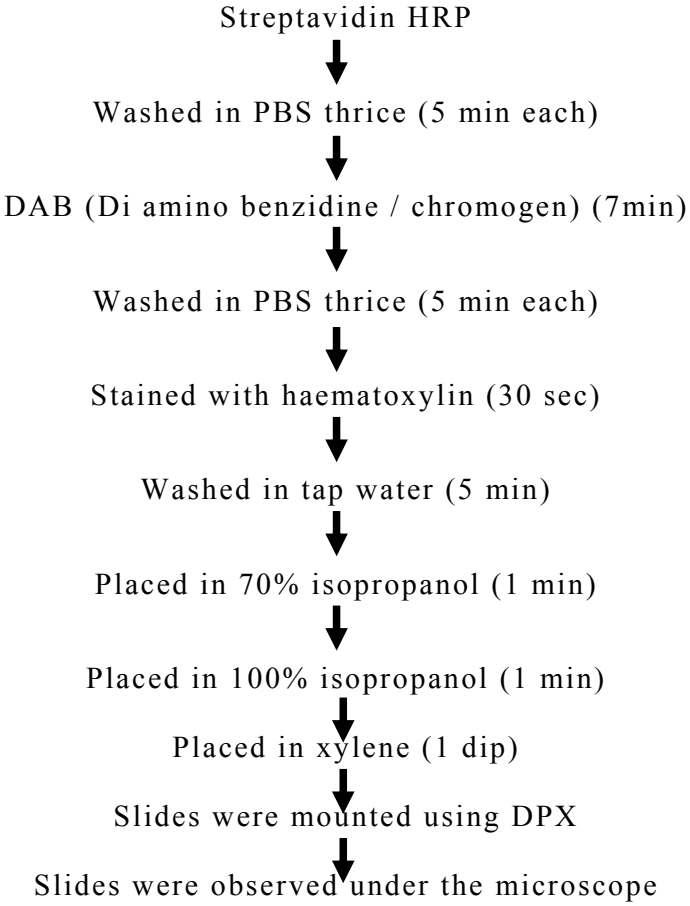
↓

Secondary antibody (biotinylated link)

↓

Washed in PBS thrice (5 min each)

↓


Streptavidin HRP
Washed in PBS thrice (5 min each)
DAB (Di amino benzidine / chromogen) (7min)
Washed in PBS thrice (5 min each)
Stained with haematoxylin (30 sec)
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

IHC procedure for Cytokeratin 19:

APES coated slides with paraffin embedded tissue

↓

Placed in xylene I (5min)

↓

Placed in xylene II (5 min)

↓

Placed in 100% isopropyl alcohol (1min)

↓

Placed in 80% isopropyl alcohol (5min)

↓

Placed in 70% isopropyl alcohol (5min)

↓

Washed in distilled water twice (5min each)

↓

Kept in citrate buffer, autoclaved and allowed to cool

↓

Washed in PBS thrice (5 min each)

↓

Antigen retrieval by pepsin digestion solution (2.0 pH) at room temp (20min)

↓

Washed in PBS buffer thrice (5min each)

↓

Peroxidase block (15 min)

↓

Washed in PBS thrice (5 min each)

↓

Primary antibody added and incubated at room temperature for 2hours

↓

Washed in PBS thrice (5 min each)

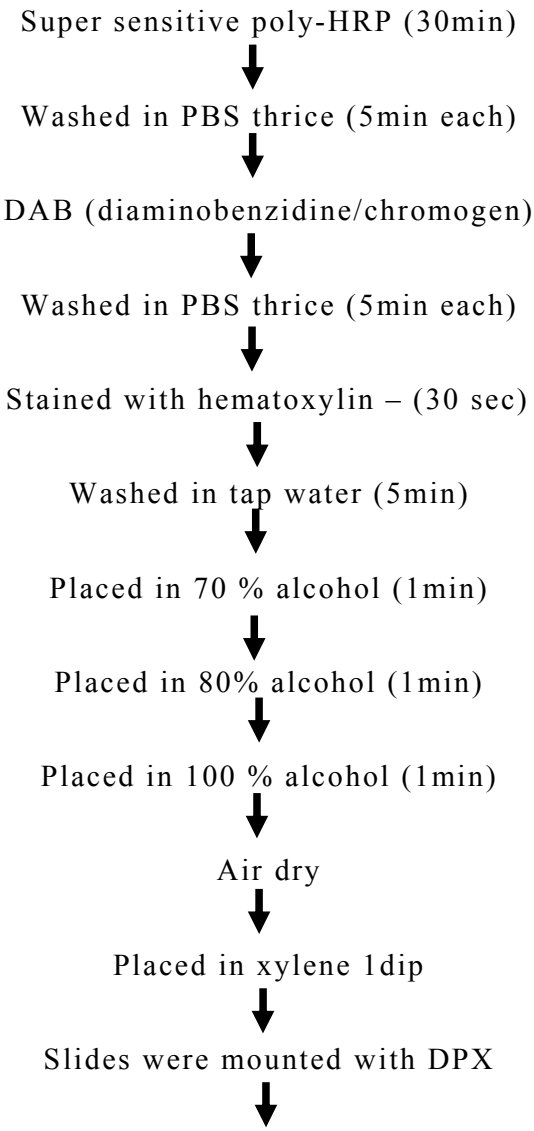
↓

Power block (15min)

↓

Washed in PBS thrice (5min each)

↓


Super sensitive poly-HRP (30min)
Washed in PBS thrice (5min each)
DAB (diaminobenzidine/chromogen)
Washed in PBS thrice (5min each)
Stained with hematoxylin – (30 sec)
Washed in tap water (5min)
Placed in 70 % alcohol (1min)
Placed in 80% alcohol (1min)
Placed in 100 % alcohol (1min)
Air dry
Placed in xylene 1dip
Slides were mounted with DPX
Observe slides under microscope

Positive controls:

Colon cancer tissue was used as a positive control for CK19 and ameloblastoma for calretinin. These were used as a standard benchmark to evaluate the intensity of staining among the study groups.

Criteria for evaluation of Calretinin and CK19 staining:

- Labelling index and staining intensity was performed for calretinin, whereas only staining intensity was done for CK19
- Labelling index (LI) was calculated by dividing the number of positive cells by the total number of cells counted in the slide and expressed as percentage. A minimum of thousand cells was counted for each slide.

$$LI = \frac{\text{Number of positive cells}}{\text{Total number of cells counted}} \times 100$$

Staining Intensity was performed, for the cells stained were both nuclear and cytoplasmic.

Intensity of cytoplasmic staining (mild, moderate, intense staining) of the cells was noted and graded as '+', '++' and '+++' respectively. Mild staining is denoted by light brown colour, moderate by brown colour and intense by dark brown colour. The cells which did not take up any brown stain were considered negative and were given a negative (-) score.

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, intensity of stain and the percentage of cells stained among the study groups

Jaws are a host to a wide variety of cysts and neoplasms, due to the tissues involved in tooth formation. Many benign jaw tumors and several cysts, of both odontogenic and nonodontogenic origin, can exhibit a biologically aggressive course and are diagnostically difficult. The odontogenic lesions are uncommon, accounting for < 2-3 % of all Oral and Maxillofacial specimens sent for diagnosis to oral pathology services. If viewed as a percentage of all tumors in the human body, this figure is reduced to a conservative estimate of approximately 0.002 -0.003%.[10]

Odontogenic tumors are a group of lesions arising from the tooth-producing apparatus or its remnants. They may originate from odontogenic epithelium and/or ectomesenchyme with varying degrees of inductive tissue interaction. These tumors are found exclusively within the jawbones or in soft mucosal tissue overlying tooth bearing areas and may be generated at any stage of individual's life.[2]

Ameloblastomas are usually benign but locally invasive odontogenic tumors with a strong tendency to recur. Distant metastasis of ameloblastoma is a rare occurrence. They are the most common clinically significant odontogenic tumors. The tumor constitutes approximately 1% of all cysts and tumors of the jaws. The recurrence rate after conservative treatment ranges from 30 - 93% for solid/multicystic tumors, which is significantly higher than

the recurrence rate after more aggressive radical treatment , which ranges from 8.3- 21%.[12]

Pathogenesis:

Ameloblastoma although it is a benign neoplasm, it is a locally invasive and destructive tumor of the jaw bone. Clinically they are described into three types: solid, unicystic, and peripheral. The solid type can be subdivided histologically into follicular, plexiform, acanthomatous, desmoplastic, granular, and basal cell forms. The unicystic type can be classified into luminal, intra-luminal, and intramural depending on the direction of invasion of the tumor. Malignant counterparts of ameloblastoma are classified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features. Essentially the tumor consists of epithelial neoplastic islands or strands made up of peripheral columnar or cuboidal cells surrounding a central core of loosely arranged, angular cells. The peripheral cells resemble ameloblasts or pre-ameloblasts while central cells are similar to the stellate reticulum cells of the tooth germ.

Jaw cysts have a variety of origins and the multiple origins represent the multiple sources of the lining epithelium e.g. Malassez's epithelial rests, reduced enamel epithelium and dental

lamina are regarded as the source of the lining epithelium in odontogenic cysts.

Odontogenic cysts are classified into three main groups whose epithelial linings are thought to have different origins. The epithelium lining the radicular cysts is presumed to arise from proliferating root sheath residues (epithelial rests of Malassez), dentigerous / follicular cysts from reduced enamel epithelium and keratocysts from the dental lamina or its remnants.

A radicular cyst is usually lined by stratified squamous epithelium and arises in focus of inflammation in the periodontal ligament caused by pulpal necrosis of an associated tooth. This most commonly occur periapically, but if the tooth has a lateral root canal, it may occur laterally to the tooth. Pathogenesis of radicular cysts takes place in three stages: the phase of initiation, the phase of cyst formation and the phase of enlargement.

In the phase of initiation the epithelial cell rests initiates and proliferates by inflammation as a result of necrotic debris and bacterial antigens derived from the dead pulp. A key factor, which may initiate the inflammation and immune response and may directly cause epithelial proliferation, is now thought to be bacterial endotoxins released from the necrotic pulp. Infiltrates of T lymphocytes, indicating that cellular immune reactions are involved

in their pathogenesis. Studies have also shown an important role for inflammatory cytokines in the proliferation of epithelial cell rests. The epithelial lining of radicular cysts may synthesize cytokines that are known to be important in bone resorption. As well as a direct effect on epithelial proliferation, endotoxins initiate an inflammatory response resulting in production of cytokines with pro-inflammatory and bone-resorbing activities. The major cytokines identified, IL-1 and IL-6, also have a direct effect on epithelial proliferation.

Phase of cyst formation is the next phase in the pathogenesis of a radicular cyst which is the process by which a cavity comes to be lined by the proliferating odontogenic epithelium. Two possibilities have been recognized where one concept proposes that the epithelium proliferates and covers the bare connective tissue surface of an abscess cavity or a cavity which may occur as a result of connective tissue breakdown by proteolytic enzyme activity. The other, and perhaps more widely supported theory, postulates that a cyst cavity forms within a proliferating epithelial mass in an apical granuloma by degeneration and death of cells in the centre. The proliferating epithelial masses show considerable intercellular oedema. These intercellular accumulations of fluid coalesce to form microcysts containing epithelial and inflammatory cells. Microcysts

may increase in size by coalescence with adjacent microcysts and once established, the cyst increases in size.

Phase of growth and enlargement of the cyst, as explained by Toller's studies with evidence to hypothesis for osmolality of fluid to increase the size of the cyst. Electrophoretic studies demonstrated higher protein molecules than patients own sera. Epithelial proliferation continues as long as there is an inflammatory stimulus, contributed to enlargement of the cyst. When the stimulus to epithelial proliferation ceased, the epithelium was able to differentiate to a certain extent, although keratinization was very rare. Growth of the cyst must also be accompanied by degradation of adjacent connective tissues and bone resorption.

Pathogenesis of odontogenic keratocyst is an abnormality arising from the odontogenic keratocyst. Two main sources from where the cyst is derived is dental lamina or its remnants; extensions of basal cells from the overlying oral epithelium. A keratocyst is always lined by a layer of stratified epithelium which is keratinized and has a characteristic structure. Occasionally odontogenic keratocysts arise in place of teeth. Such cysts were originally described as primordial cysts. The epithelium of the keratocyst showed a higher rate of proliferation than the radicular cyst. Connective tissue showed both slowly and rapidly proliferating areas. Toller considered osmolality of the cyst fluid in

the enlargement of the keratocysts. Smith et al concluded from series of studies that release of molecules into luminal fluid contributes to osmotic and hydrostatic pressure and hence is expansile growth.[12] Odontogenic keratocyst is an aggressive lesion that has the propensity fro recurrence if not adequately removed. It suggested a multicentric pattern of cyst growth about by the proliferation of local groups of epithelial cells against the semi-solid cyst contents. Odontogenic keratocyst as a benign neoplasm drew attention to the infolding of the epithelial lining into the capsule suggesting that it was the result of active epithelial proliferation.[5] The discovery of increased mitotic activity in the cyst epithelium , the potential for epithelial budding from the basal layer or daughter cysts in the cell wall , the presence of chromosomal abnormalities and role of the mutation of the PTCH gene in etiology of keratinizing odontogenic tumors resulted in reclassification of this lesion as a neoplasm in the WHO reclassification of head and neck tumors in 2005.[4]

A dentigerous cyst is usually lined a layer of stratified squamous epithelium which encloses the crown of an associated unerupted tooth. It arises as a result of impeded eruption of the associated tooth. This usually occurs within the bone but occasionally takes place in the overlying soft tissue, where it is called as eruption cyst, [7]pathogenesis is unknown. These cysts are

caused by expansion of dental follicles resulting from accumulation of fluid between the tooth crown and epithelial components.[15]

Identification of the proliferating activity in tumors is useful to predict the biological behavior of different lesions.[16] Assessment of cell proliferation in many types of tumors is an important adjunct to histologically based tumor classification and has potential relevance as an indicator of tumor behavior and treatment response and relapse.[40]

Proliferating cell nuclear antigen (PCNA), AgNOR and Ki-67 are the commonly used proliferating markers. [18]

Proliferating cell nuclear antigen is a 36kD nuclear protein associated with the cell cycle. Nuclear PCNA is found in the proliferative compartment of normal tissues. PCNA has also been known as cyclin or as auxillary protein for DNA polymerase δ .Immunofluorescent studies show the existence of two populations of PCNA during S phase of the cell cycle, one that is nucleoplasmic as in quiescent cells and another that is associated to specific nuclear structures. Immunostaining with a monoclonal antibody (PC10) against this antigen and has shown to demonstrate the proliferative compartment of normal tissue and to correlate with prognosis in some tumors.[18]

A more specific marker of proliferating cells, maximally expressed during S phase, is Ki67 antigen, which is rapidly degraded after mitosis. The original antibody raised against this 395 kD Ki67 protein (pKi67) is referred to as Ki67 antibody, a prototype for other antibodies that also identify epitopes of pKi67, eg: MIB-1.17 MIB-1 is an antibody that detects Ki67 antigen in formalin-fixed, paraffin-embedded tissue after antigen retrieval.Ki67 is a nonhistone protein initially expressed in mid G1, increasing in level through S and G2 and peak in M. It has been suggested that Ki67 staining is more accurate than the counting of mitoses or PCNA staining. Its estimated half-life is 60 to 90 minutes.[17]

Nucleolar organizer regions (NORs) are loops of DNA that encode ribosomal RNA and are considered important in the synthesis of proteins. Silver staining methods demonstrate NOR-associated proteins called argyrophilic NORs (AgNORs). The number of Ag-NORs rises with the increasing proliferative activity of cells. Quantitative and qualitative changes of NORs can imply the degree of cell nucleolar activity in hyperplastic and neoplastic conditions. Actively proliferating cells have impaired nucleolar association and, therefore, exhibit a higher AgNOR count, regardless of the ploidy state of the cell. Recent histopathologic studies of NORs have resulted in successful diagnosis,

categorization, and prognostication of various benign and malignant lesions. [19]

The differentiating markers include Calretinin and Cytokeratins.

Calretinin is a calcium-binding protein of 29 kDa and a member of the large family of EF-hand proteins to which S100 also belongs. EF-hand proteins are characterized by a peculiar amino-acid sequence that folds up into a helix-loop-helix, which acts as the calcium-binding site. Calretinin contains six such EF-hand stretches. The exact biological function of Calretinin remains unknown but possible roles as a calcium buffer and/or calcium sensor and regulator of apoptosis have been postulated. Calretinin is expressed in neural tissue and is now established as a marker of neuronal differentiation in central nervous system tumors. It is more specific marker of both benign and malignant mesothelial cells and is expressed in mast cells and cutaneous mastocytomas. Calretinin has been found to be expressed in a high proportion of unicystic, solid, and multicystic ameloblastomas, whereas no positive staining has been found in radicular cysts, dentigerous cysts, and odontogenic keratocyst.[9]

M. Altinin, H Coleman *et al* 2000, conducted study to determine Calretinin expression in the epithelium of ameloblastomas and its

possible specificity as an immunohistochemical marker of ameloblastic differentiation. 27 unicystic ameloblastomas and 31 solid and multicystic ameloblastoma were included. The 27 cases of unicystic ameloblastoma included examples of three clinicopathological variants: cyst only, cyst with intraluminal epithelial proliferation, cyst with transmural tumor growth. Staining was positive in 22 cases (81.5%). Focal positivity of epithelial cells was present in epithelial linings in four cases. Luminal layer did not stain in some cases. Single basal and parabasal cells stained positively in only two cases. Only one case which did not stain was lined by typical ameloblastic epithelium. In type 2 lesions (plexiform unicystic ameloblastoma), the intraluminal nodules of proliferating epithelium showed focal staining of cells while in type 3 lesions, mural follicles were variable. In all cases, numerous individual cells in connective tissue wall of the cyst were strongly positive.

Solid and Multicystic ameloblastomas: the sample consisted of 31 cases comprising equal numbers of follicular, plexiform and mixed histological variants. Of these 29 (93.5%) showed intense positive staining of ameloblastic epithelium while two cases were negative. In all cases staining were restricted to stellate reticulum – like epithelium while in only one case basal cells showed focal positivity.

In this study they demonstrated the expression of Calretinin in epithelium of Ameloblastomas. The expression of Calretinin was almost always very intense and diffuse being band-like in areas but was not always equally distributed throughout the section with some areas completely devoid of staining. Calretinin expression was particularly intense in the epithelium lining the micro cysts and macro cysts and also some areas of squamous metaplasia.[8]

Adriano Piattelli, Massimiliano Fioroni *et al* 2003, evaluated Calretinin expression in odontogenic cysts. The material used for study included a total of 70 odontogenic cysts, 24 radicular cysts, 24 follicular cysts and 22 odontogenic keratocyst. Of the 22 odontogenic keratocyst, 10 were orthokeratotic odontogenic keratocyst and 12 were parakeratotic odontogenic keratocyst.

Parakeratotic odontogenic keratocysts seem to have a neoplastic potential and are found in patients with Marfan's syndrome and nevoid basal cell carcinoma syndrome. Parakeratotic odontogenic keratocyst constitutes 3.3% to 11.2% of all cysts of the jaws and from 83.2% to 86.2% of odontogenic keratocyst. Histologically it is characterized by a cavity with different amounts of keratin and surrounded by a thin, uniform lining of stratified squamous epithelium presenting a corrugated layer of parakeratin. The basal layer is well-defined and consists of palisaded cuboidal or columnar cells, with hyperchromatic nuclei that are polarized away

from the basement membrane. Parakeratotic odontogenic keratocyst have very destructive potential and may invade the adjacent bony structures and soft tissue, whereas orthokeratotic odontogenic keratocyst are less aggressive and respond well to conservative treatment. The positivity to Calretinin in parabasal layer in parakeratotic odontogenic keratocyst is similar to that found for other markers like p53. Proliferating cell nuclear antigen explains the differences in the clinical and pathological behavior of odontogenic keratocyst. All 24 radicular cyst, 24 follicular cysts and 10 orthokeratotic odontogenic keratocyst were negative in all the cyst wall components.[20]

M Alaeddini , S Etemad-Moghadam *et al* 2008, concluded a study to determine the expression of Calretinin in selected odontogenic neoplasms. 55 odontogenic tumors consisting of 20 solid ameloblastomas , 5 calcifying epithelial odontogenic tumors, 10 adenomatoid odontogenic tumors, 10 ameloblastic fibromas and 10 odontogenic myxomas. In the present study Calretinin expression was observed in all ameloblastomas. Immunopositivity was seen almost exclusively in the stellate reticulum of the studied cases.

Mistry *et al* studied Calretinin in developing rat molars and demonstrated that it is weakly expressed in some tooth germs at the cap stage. As the tooth development progresses the intensity of reactivity increases from weak to intense in the late bell stages.

Calretinin immunoreactivity is present in the inner enamel epithelium and presecretory ameloblasts from the late cap stage onwards. Calretinin immunoreactivity was not observed in the peripheral layers of the ameloblastic islands.

Gotzos *et al* demonstrated that Calretinin may act as an anti-apoptotic factor. Various investigations regarding the expression of apoptotic proteins in ameloblastoma exist in literature, which indicate two relatively distinct patterns for ameloblastoma: an anti-apoptotic proliferating area in the outer layer (periphery) and a pro-apoptotic differentiating region in the inner layer (centre).

Tumor cells of Calcifying epithelial odontogenic tumors bear a close morphological resemblance to the cells of stratum intemedium of enamel organ. The stratum intermedium is weakly immunoreactive in early bell stage of normal rat tooth germs, comparable to the lack of reactivity observed in the specimens of Calcifying epithelial odontogenic tumors.

The origin of Adenomatoid odontogenic tumor remains uncertain. It is suggested that this lesion arises from postsecretory ameloblasts subsequent to amelogenisis. In the present study they showed no immunoreactivity for Calretinin. In contrast, normal rat molars, expression was observed mainly in the inner enamel

epithelium, presecretory and secretory ameloblasts, which assumed the cells of origin of this tumor.

Neither the epithelial nor the cellular mesenchymal components of Ameloblastic fibroma showed immunoexpression of Calretinin.

Odontogenic myxoma which originated from odontogenic ectomesenchyme, showed no immunoreactivity for Calretinin. When compared to cardiac myxoma with jaw myxomas, Calretinin was negative for jaw myxomas.

Ameloblastoma is characterized by locally invasive behavior with a high risk of recurrence. Most odontogenic tumors originate from successional and accessional dental laminae, but differentiate into various entities. The mechanisms that trigger the proliferation of odontogenic epithelial rests or produce ameloblastomas, adenomatoid odontogenic tumors, calcifying epithelial odontogenic tumors or other tumors are unknown. Various subcellular, cellular and developmentally related factors may be responsible for differentiation. Among neoplasms studied in this investigation, Calretinin expression was observed only in ameloblastomas. This protein may have a role in the transition of the dental lamina remenants to ameloblastoma. Evaluation of Calretinin expression in these tumors and their comparison with ameloblastoma may provide

additional information on behavior and tumorigenesis of odontogenic neoplasms. Considering that ameloblastomas were consistently reactive for Calretinin, whereas other tumors were invariably non-reactive, it can be hypothesized that this protein is one of the factors responsible for the differences between this aggressive neoplasm and other tumors.[9]

H.Coleman, M Altini *et al* 2001, conducted a study to determine whether Calretinin was expressed in the lining epithelium of odontogenic keratocyst, residual and dentigerous cysts and to determine whether this calcium binding protein could be used to distinguish these cysts from unicystic ameloblastoma. Unicystic ameloblastomas are usually lined by a variable epithelium ranging from one that has typical ameloblastic characteristics to one that is metaplastic and which appears completely nondescript consisting of several layers of nonkeratinizing squamous cells. Such squamous metaplasia is a relatively frequent phenomenon in unicystic ameloblastomas and many of these lesions are lined predominantly by such nondescript epithelium. In such cases the differentiation of odontogenic cysts, such as residual cyst and dentigerous cyst from the unicystic ameloblastomas can be a problematic. Overlapping clinical and radiographic presenting features adds to diagnostic difficulty. Many techniques have been used in an attempt to distinguish odontogenic cysts from the unicystic ameloblastoma.

These include : demonstration of cell surface carbohydrates with blood group specificity ; determination of alkaline phosphotase activity in the stroma ; distribution of lectins and involucrin in the epithelium ; characterization of cytokeratin profiles ; counting of AgNORs and quantification of PCNA and Ki 67.

Although the metaplastic cyst linings may have lost their typical ameloblastic features, the cells have retained their immunophenotypic characteristics resulting in the continued expression of Calretinin. Variable numbers of individual darkly staining non epithelial cells were observed in all cases. These cells were found to be both within tumor or cyst epithelium and in fibrous connective tissue walls. The biochemical properties of Calretinin are well characterized, its biological role remains unknown. In conclusion, it is suggested that Calretinin may be a specific immunohistochemical marker for neoplastic ameloblastic epithelium and may be an important diagnostic aid in the differential diagnosis of cystic odontogenic lesions and ameloblastic tumors.

Intermediate filaments: All mammalian cells contain a complex intracytoplasmic cytoskeleton composed of three prinicipal structural units and associated protein: actin-containing microfilaments, tubulin-containing microtubules, and intermediate

filaments. These are six distinct types of intermediate filaments; keratin filaments constitute type I and type II intermediates filaments with atleast 20 subtypes. A number of two gel electrophoresis experiments on keratin subunits extracted from various epithelial tissues have shown a total of 20 different subunits in any mammalian species, with molecular weight varying within the range 40-70 kDa. [18]Moll *et al* categorized a total of 19 human epithelial keratins. The keratins can be divided into low and high molecular forms based on molecular weight, and divided into acidic and basic forms based on isoelectric point. Low molecular weight keratins will pair with a specific high molecular weight keratin and most basic keratins will pair with an acidic keratin, as defined by coexpression. All epithelia (simple and complex) can be classified based upon cytokeratin (CK) protein expression. The genes coding for keratins represent one of such large multigene families comprising atleast 30 genes. The smaller and acidic type I keratins (K9- K20) are encoded on chromosome 17q, while the larger and more basic type II keratins (K1-K8) are encoded on chromosome 12q. [5]

Cytokeratin 19 is the lowest molecular weight and is widespread in various simple epithelia, including mesothelium and their neoplasms. CK19 is absent in a few simple epithelia, such as hepatocytes, most kidney tubules, and thyroid follicles. Renal cell

carcinomas are positive. It is of 40 kDa cytokeratin. CK 19 is expressed in all kinds of odontogenic epithelial cells in developing tooth germs and in neoplastic epithelial cells in some odontogenic tumors.[1]

Morgan PR,Shirlaw PJ *et al* 1987, odontogenic epithelium and its derivatives, both normal and pathological, prove to exhibit a basic keratin profile which can be detected immuncytochemically, and may be of value in helping to distinguish odontogenic from non-odontogenic cysts and tumors. Odontogenic lesions prove to be a demanding context to evaluate the potential of keratins as histogenic markers. Odontogenic epithelium may present as a classically stratified, squamous epithelium or simple, as in the early tooth germ and various neoplastic and non-neoplastic lesions in which odontogenic epithelial strands and rests are present. Keratin19, a keratin present in some basal cells of stratified mucosal cells as well as most simple epithelia, cell rests of Malassez and serre, odontogenic cysts and junctional epithelium are positive for CK19. Keratin is of diagnostic value in discriminating between Odontogenic cysts and non-odontogenic cysts and tumors.[23]

A W Mac Donald, A Fletcher 1989, Dentigerous and odontogenic keratocysts both arise from odontogenic epithelium and may have similar histological appearance their behavior in terms of recurrence is different to warrant different treatment and follow up. The

expression of cytokeratin to a given epithelium or epithelial cell is characterized by a specific pattern. The focal inflammation within the stroma of odontogenic keratocysts affects the local expression of cytokeratins.[24]

MM Crivelini, VC de Araujjo *et al* 2003, aimed to describe the immunohistochemical expression of cytokeratin 7, 8, 10, 13, 14, 18, 19 and vimentin in epithelial components of the dental germ and of five types of odontogenic tumors. These neoplasms included 10 ameloblastomas, 4 calcifying epithelial odontogenic cysts, 5 ameloblastic fibromas and 3 odontomas. Different epithelial structures in the tooth germ included dental lamina, outer epithelium, stellate reticulum, stratum intermedium, internal enamel epithelium and Hertwig root sheath. Positive results were obtained with CK 7, 13, 14 and 19, but not with CK 8, 18 and vimentin. Odontomas were the compound type, with immature enamel, dentin and dental papillae. CK14 was present in all antibodies against other polypeptides and vimentin had negative reactions. Ameloblastomas were classified into follicular, acanthomatous, plexiform, solid and granular cell variants. They exhibited CK 13, 14, and 19positivity. CK 14 was detected in most tumoral cells, except in part of the central stellate cells and peripheral columnar cells with vacuolated cytoplasm, or metaplastic squamus cells and keratinizing cells. CK19 in some tumors exhibited remarkable

positivity in bundles of metaplastic squamous cells, or weak reaction in part of the central stellate cells and flattened cells of the cystic structures. Adenomatoid odontogenic tumor composed of sheets, cords, and nests of epithelial cells had proliferated to form solid masses and duct-like structures. The antibody against CK 14 was the only one to label cytokeratin polypeptides, embracing all tumor epithelial cells. Calcifying epithelial odontogenic tumors contained sheets or strands of eosinophilic polyhedral cells connected by distinct intercellular bridges. CK 14 was detected in all cells. CK 7, 13 and 19 were weakly positive. Ameloblastic fibroma consisted of mesenchyme and odontogenic epithelium forming islands and cords. CK 14 was detected in all epithelium, unlike CK 8, 18, 19 which were negative.

CK 14 was the main intermediate filament of odontogenic epithelium. CK 7 was a filament of stellate reticulum and Hertwig root sheath proliferating cells. Ck 19 had positivity in preameloblasts and sercretory ameloblasts, showing association with the secretory differentiation. It was also present in the dental lamina. It is hypothesized that CK19 characterizes only ameloblasts and preameloblasts with complete differentiation.[25]

T Lombardi, C Lock *et al* 1995, compared the expression of S 100 protein, α-smooth muscle actin and keratin 19 in odontogenic myxomas and non-odontogenic myxoid lesions. Odontogenic

myxoma is a characteristic gelatinous, slow-growing, expansile benign jaw tumor. 2 normal tooth germs, 7 normal dental follicles, 2 benign intramuscular myxomas,12 cardiac myxomas, 3 soft tissue myxomas from the head and neck, 1 lesion from a case of oral focal mucinosis and 7 odontogenic myxomas were taken. All myxomas were histologically typical. Odontogenic myxomas comprised stellate and spindle cells lying in copious myxoid matrix. The myxoid tissues of the dental papilla and dental follicle in embryonic tooth germs, adult dental follicles and follicles showing myxoid enlargement and were negative for S100, except one myxoid follicle which showed a weak but generalized positivity. α-SMA labeled cells were present in all but one in three of seven of the normal follicles. Cytokeratin 19 was found in the odontogenic epithelium of tooth germs, in odontogenic epithelial rests of all normal dental follicles and in all the myxoid follicles which contained epithelium. No glandular differentiation or epithelium was present in the cardiac myxomas. CK 19 was expressed basally in non-cornified, stratified and many simple epithelia, and is not specific to odontogenic epithelium. However, all odontogenic epithelium and tumors derived from it are thought to express this cytokeratin and this finding adds to the circumstantial evidence which suggests that the epithelium is odontogenic.

Christian Stoll, Carolin Stollenwerk *et al* 2005, evaluated cytokeratin expression patterns as an additional tool for characterization of different cysts as the histomorphologic appearance often is not decisive. Thirty cases of dentigerous and radicular cysts and 15 cases of odontogenic keratocysts were considered. Expression of CK 5/6, 7, 10, 13, 17, 19 and 20 was determined in addition to Ki-67 immunohistochemically. Differences in expression of cytokeratins may allow a given epithelium to be characterized by a specific pattern of its cytokeratin components. CK19 is the smallest known acidic type CK and differentially expressed in various human tissues without association with a basic CK. It is a useful tool in discriminating carcinomas from tumors of different origin and for carcinoma subtyping using immunoblotting or immunocytochemical techniques. The antibody is also a marker of pre – malignant lesions of the oral epithelium. In this study staining was completely absent in odontogenic keratocysts and 68% positive for radicular cysts and 71% positive for dentigerous cysts. CK 19 is a valuable additional parameter in making a decision between odontogenic keratocytsts and other odontogenic cysts which clinically is likely the most significant differential diagnosis in this context.[25]

Yoshifumi Tajima, Mina Kuroda-Kawasaki *et al* 2001, described a case report of 40 year old man with atypical features of peripheral

ameloblastoma along with an immunohistochemical profile of its cytokeratin. Peripheral ameloblastoma presents as a small gingival mass. The tumor consisted of densely packed round or spindle cells with a number of abnormal mitotic figures and foci of squamous metaplasia. Final diagnosis of peripheral ameloblastoma with potentially malignant transformation was made, because of its atypical features clinically and histopathologically. The tissues were examined immunohistochemically for several cytokeratins. CK 14 and CK 19 were positive in basal cells, suprabasal cells, and central stellate reticulum like cells of the tumors. CK expressions in this case suggested strongly an odontogenic origin of the tumor cells. The high expression of CK 19 in this case reflect the tumor cells retaining an immature phenotype of their differentiation.[27]

H Kumamoto, M Yoshida *et al* 2001, evaluated the expression of amelogenin and cytokeratin 19, that are potentially useful polypeptides for identification of odontogenic epithelial components, in various types of epithelial odontogenic tumors. 33 ameloblastomas, 33 CEOTs, 2 CCOTs and 5 malignant ameloblastomas. Ameloblastomas were divided into 20 follicular and 13 plexiform types, including 9 acanthomatous, 5 granular cell, 2 basal cell and 4 desmoplastic subtypes. Malignant ameloblastoma were classified into one metastasizing ameloblastoma and four ameloblastic carcinomas. Specimens of five tooth germs and 10 non-

odontogenic epithelial tumors were compared with the epithelial odontogenic tumors. Immunohistochemical reactivity for amelogenin was detected in the cytoplasm of the odontogenic epithelial cells and their associated extracellular components in the tooth germs and epithelial odontogenic tumors. CK 19 was expressed in cytoplasm of odontogenic epithelial cells. Immunoreactivity for CK 19 was detected in all epithelial cells of enamel organ and dental lamina. CK expression patterns differ according to cell type, developmental stage, differentiation status and anatomical site. The diverse types of epithelial odontogenic tumors, including benign and malignant lesions, express amelogenin and CK 19. The non-odontogenic tumors were negative for amelogenin and CK19. Based on these, it was considered that coexpression of amelogenin and CK 19 was one of the characteristics of these epithelial odontogenic tumors.[1]

Gao Z, Mackenzie IC *et al* 1988, evaluated the patterns of cytokeratin expression in the epithelium of 5 dental follicles, 7 dentigerous cyst, 5 odontogenic keratocyst, 3 nasopalatine cysts, and an epidermoid cyst. The large family of cytokeratins forming the intermediate filaments of epithelia shows a high degree of tissue specificity in their patterns of expression. Developmental cysts that arise from odontogenic epithelium, share the expression of certain keratin polypeptides, but differ from cysts originating from the

epithelium of non-odontogenic origin. The changing of pattern of cytokeratin expression as the odontogenic epithelium of rests of Malassez proliferates to give rise to a radicular cyst. The simplest pattern of cytokeratin expression in odontogenic epithelium, of the quiescent epithelium forming the rests of Malassez, shows strong expression of K5 and K 19 and lack of expression of other cytokeratins except occasional and very weak expression of those typical of simple epithelia K7, K8, K18 and of noncornifying stratified epithelia K4, K13. In the epithelial lining of cysts derived from odontogenic epithelium share the basic pattern of expression of odontogenic epithelium but there is increased, but usually only moderate, staining for K4 and K13, K8 and K18. In the epithelial lining of these cysts, staining for K8, K18, K19 tends to become localized to the superficial cells. Keratin 19, a frequent component of simple epithelia, has been demonstrated in the basal cells of some stratified mucosal epithelia. The results reported here indicate that coexpression of K5 and K19 together with variable and weaker expression of keratins typical of non-cornifying stratified epithelium and simple epithelium may be characteristic for odontogenic epithelia. The expression of keratins other than K5 and K19 increased as the epithelial lining became thicker, suggesting that their expression in odontogenic epithelium is associated with differentiation and maturation of the epithelium. Demonstration of cytokeratin patterns may be of value in differentiating proliferated

odontogenic epithelium from epithelium which is not of odontogenic origin and small differences present between the different types of cysts of odontogenic origin.[37]

The expression of cytokeratin 19 and calretinin was evaluated by immunohistochemistry in 60 cases of formalin fixed, paraffin embedded archival tissues, which were histologically confirmed as odontogenic cysts and tumor. The tissue specimens constituted 4 groups. Group I Dentigerous cysts (n=15), Group II Odontogenic keratocysts (n=15), Group III Radicular cysts (n=15) and Group IV Ameloblastoma (n=15).

Patient characteristics:

Group I:

The age distribution of the patients presenting with dentigerous cysts was 12-47years (Mean age-35.53±7.83 years). (**Table 1, Graph 1)**There were 11 males (73.3%) and 4 were females (26.6%) **(Table 2, Graph 2).** Sites involved were posterior mandibular region in 13 cases and posterior maxilla in 2 cases**. (Table 3, Graph 3).**

Group II:

The age distribution of the patients presenting with odontogenic keratocysts was 25-51years (Mean age-39.07±7.05years). (**Table 1, Graph 1)**There were 12 males (80%) and 3 females (20%). **(Table 2, Graph 2).** Sites involved were posterior mandibular region in 10 cases, anterior mandibular region in 2 cases and posterior maxilla in 3 cases. **(Table 3, Graph 3).**

None of the odontogenic keratocysts were associated with nevoid basal cell carcinoma syndrome (NBCCS).

Group III:

The age distribution of the patients presenting with radicular cysts was 12-43years (Mean age-31.40±8.68years). (**Table1, Graph1)**There were 10 males (66.6%) and 5 females (33.3%). **(Table 2, Graph 2).** Sites involved were posterior mandibular region in 6 cases, anterior mandibular region in 5 cases, anterior maxilla in 2 cases and posterior maxilla in 2 cases. **(Table 3, Graph 3).**

Odontogenic cysts:

Staining Intensity of Calretinin in Odontogenic cysts:

Group I and III showed no staining characteristics and Group II showed two cases having mild to intense staining pattern **(Table 4, Graph 4**).

Staining Intensity of Cytokeratin 19 in Odontogenic cysts:

Group II and III showed no staining characteristics and in Group I three cases showed mild to moderate staining pattern **(Table 5, Graph 5).**

Staining Intensity of Calretinin in Ameloblastomas:

66.6% (n=10) showed mild staining, 26.6% (n= 4) moderate staining and 6.6% (n=1) was intense staining.**(Table 6, Graph 6)**

Labeling index of Calretinin in Odontogenic cysts and Ameloblastoma:

Mean labeling index of the study group which had stained positive was calculated as 1.28 ± 3.39 for Group II (Odontogenic keratocyst) and 19.21 ± 13.22 for Group IV (Ameloblastoma)

Staining Intensity of Cytokeratin19 in Ameloblastomas:

In Group IV, 93.3% cases did not show any staining characteristics, 6.6% cases showed moderate staining pattern**. (Table 7, Graph 7)**

Comparison of Calretinin staining in Odontogenic keratocysts (Group II) and Ameloblastoma (Group IV):

Group IV showed 100% positivity of 66.6% (n=10) cases were mild staining, 26.6% (n=4) moderate staining, 6.6% (n=1) were intense when compared with Group II where 86.6% (n=13) cases did not exhibit any staining pattern while 6.6% (n=1) cases were mild staining and 6.6% (n=1) moderate staining**.(Table 8,Graph 8)**

Comparison of Calretinin in Odontogenic cysts and Ameloblastomas:

Group I and III did not exhibit staining characteristics where as all the case of ameloblastoma (Group IV) exhibited calretinin staining and 66.6% (n=10) of cases were mild, 26.6% (n=4) moderate and 6.6% (n=1) intense. In Odontogenic keratocyst (Group

II) 6.6% (n=1) of cases showed mild staining and 6.6% (n=1) showed moderate staining. However in Group II 86.6% (n=13) cases did not take up any staining characteristics. **(Table 9, Graph 9)**

Figure 1: Armamentarium

Figure 2: Antibody Kit

CK 19 STAINING IN COLON CANCER

Figure 3: 10 x

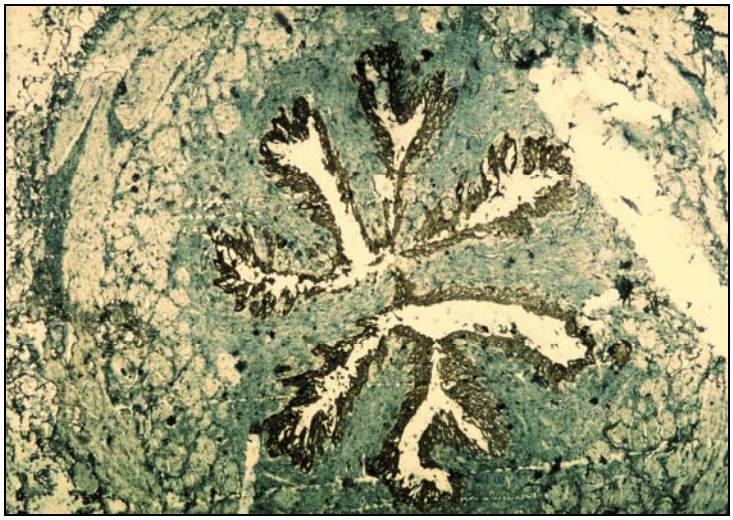
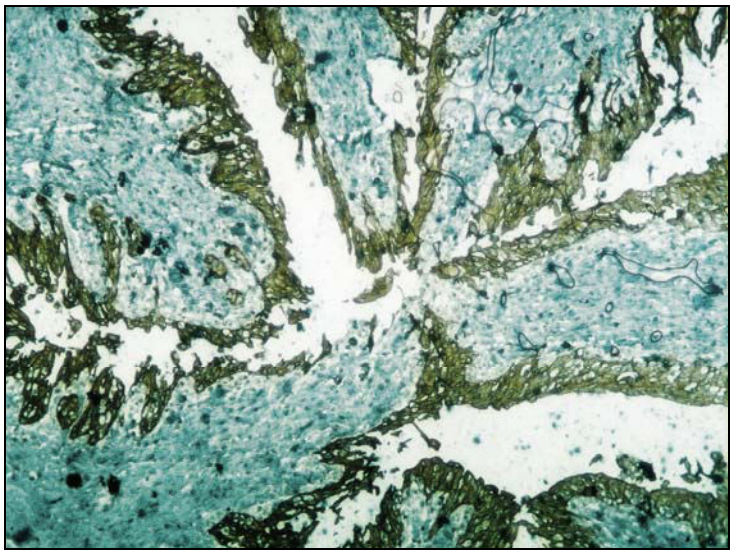


Figure 4 : 40 x



CK 19 STAINING IN DENTIGEROUS CYST

Figure 5: H & E; 10 x

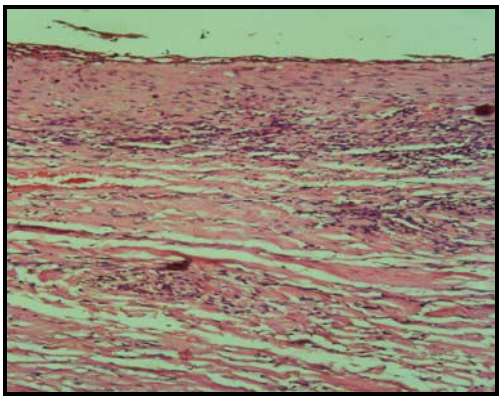


Figure 6: Negative control; 10x

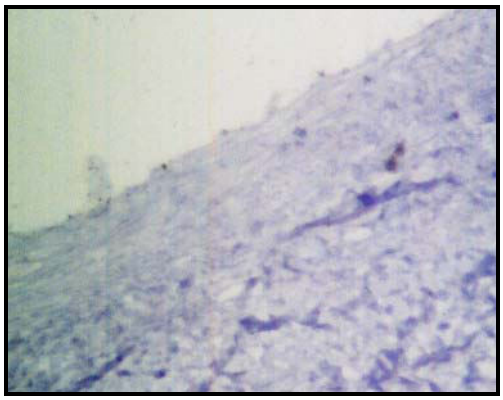


Figure 7: Positive stain10x

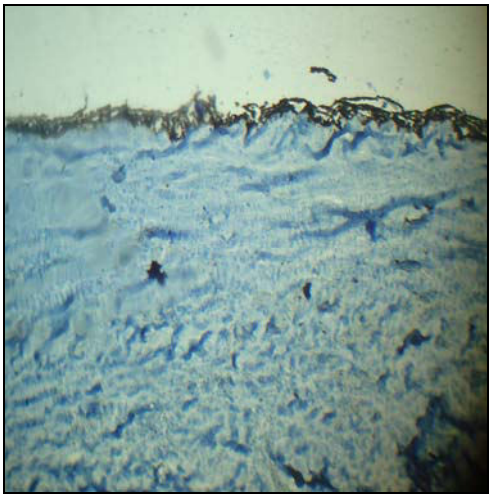
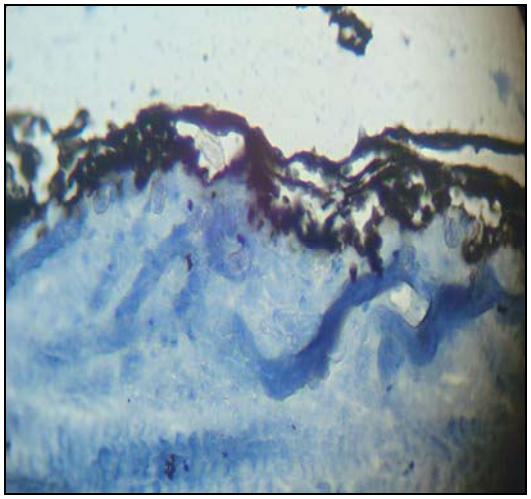


Figure 8: Positive stain 40x



CK 19 STAINING IN ODONTOGENIC KERATOCYST

Figure 9: H & E; 10 x

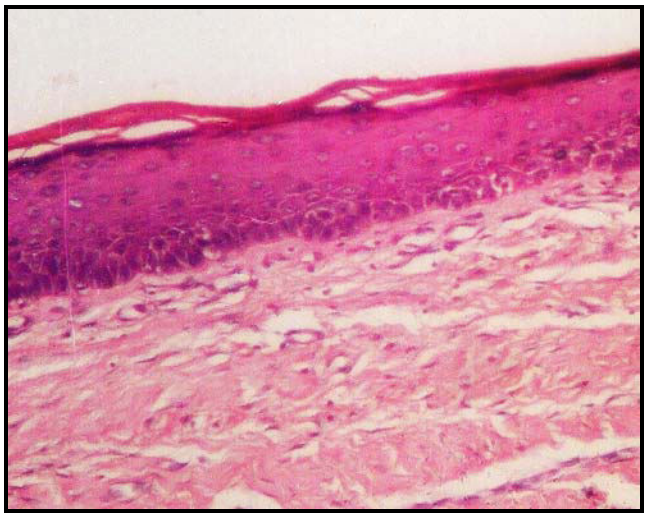
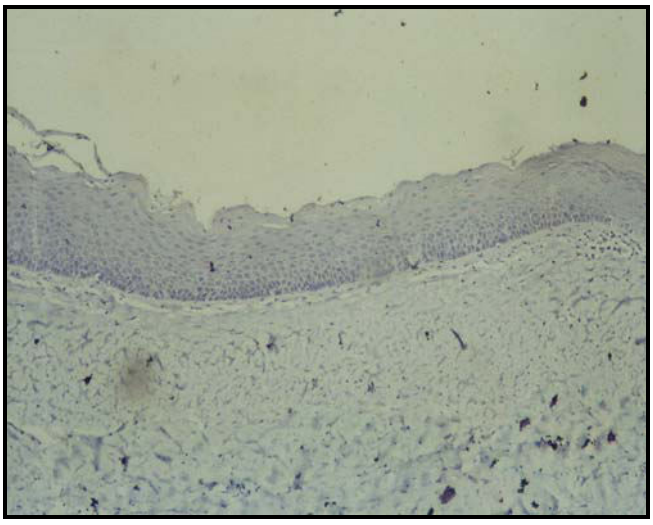


Figure 10: Negative control; 10 x



CK 19 STAINING IN RADICULAR CYST

Figure 11: H & E; 10 x

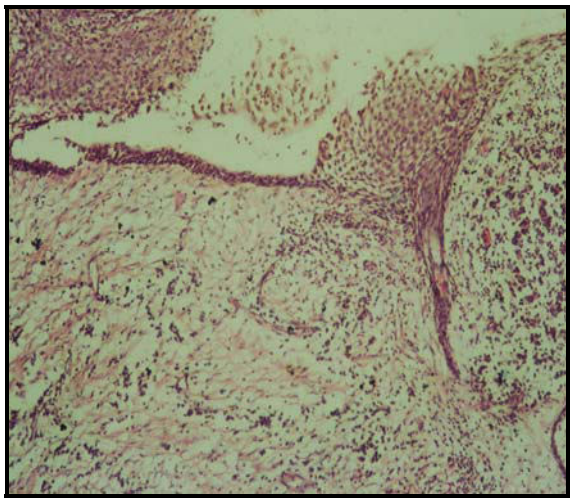
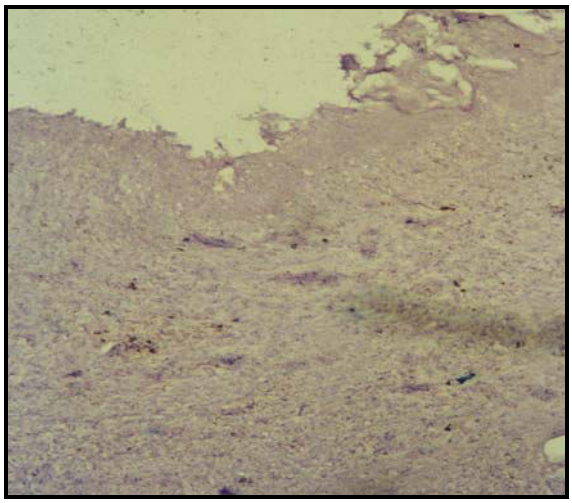


Figure 12: Negative control; 10 x



CK 19 STAINING IN AMELOBLASTSOMA

Figure 13: H & E; 10 x

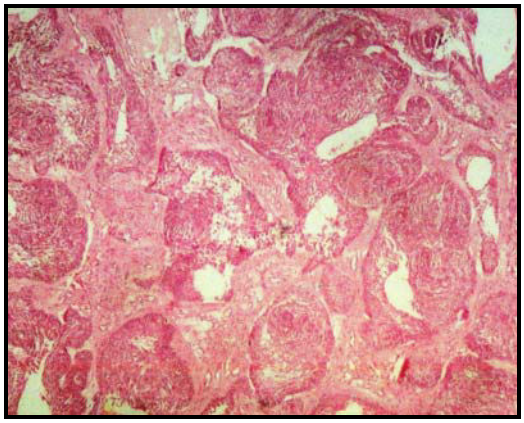


Figure 14: Negative control; 10x

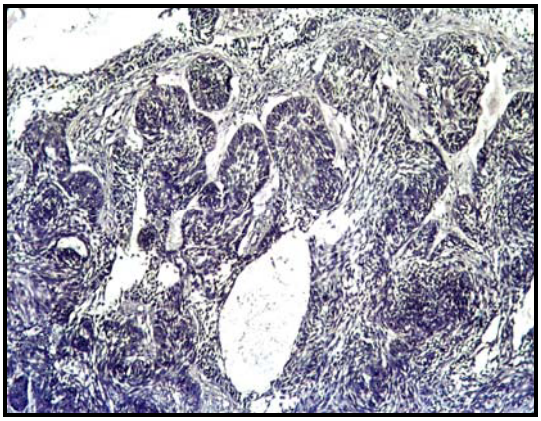


Figure 15: Positive stain 10 x

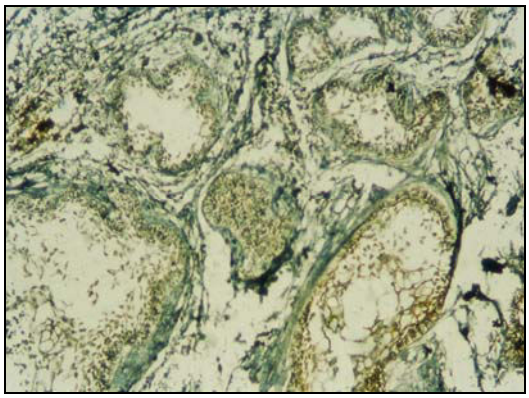
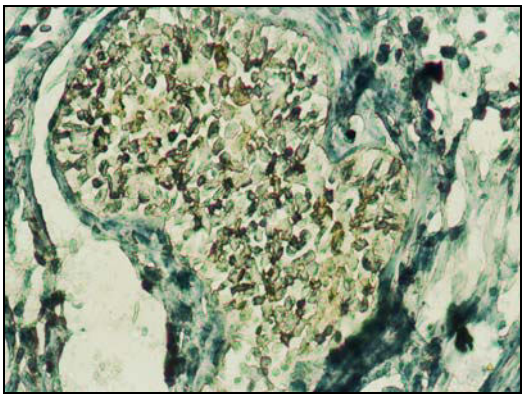


Figure 16: Positive stain 40 x



CALRETININ STAINING IN DENTIGEROUS CYST

Figure 17: H & E; 10 x

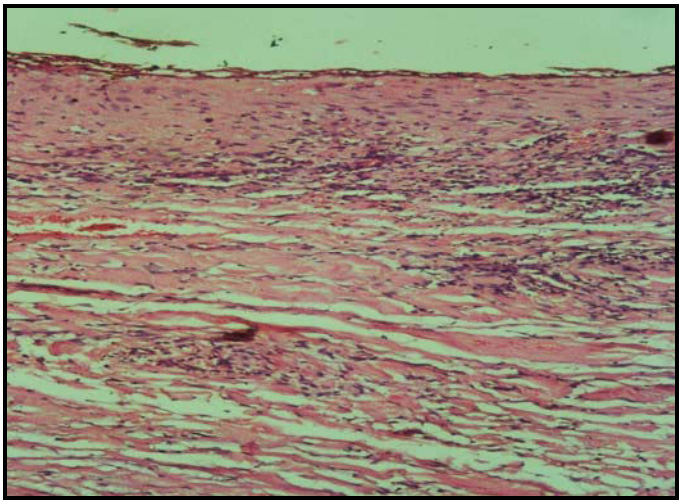
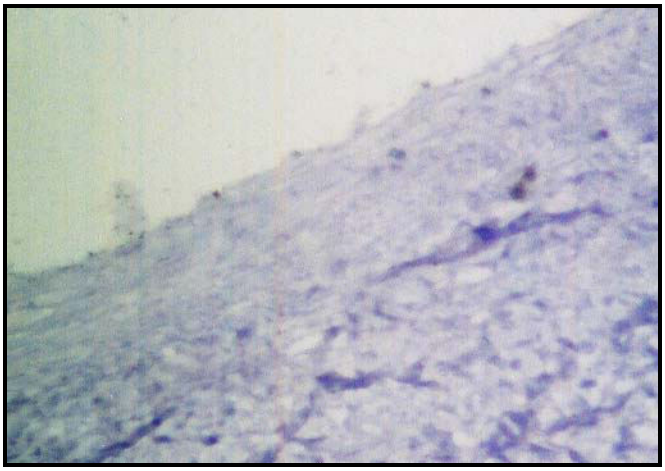


Figure 18: Negative control; 10 x



CALRETININ STAINING IN ODONTOGENIC KERATOCYST

Figure 19: H & E; 10 x

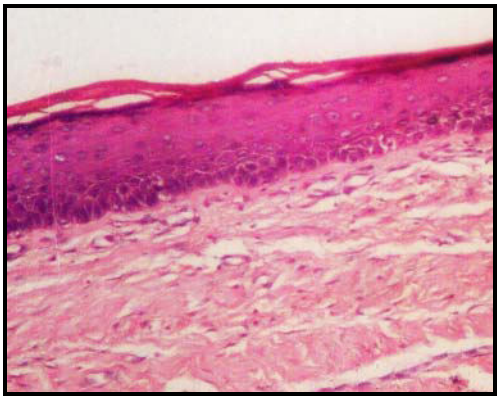


Figure 20: Negative control; 10x

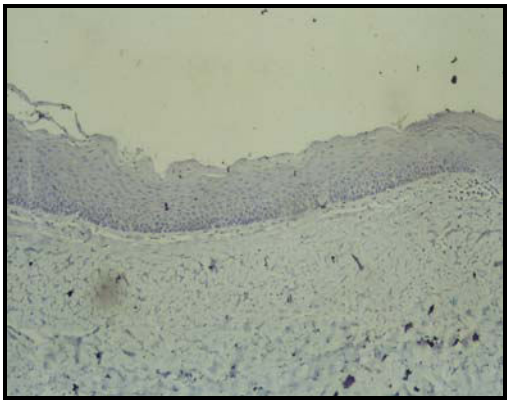


Figure 21 : Positive stain 10x

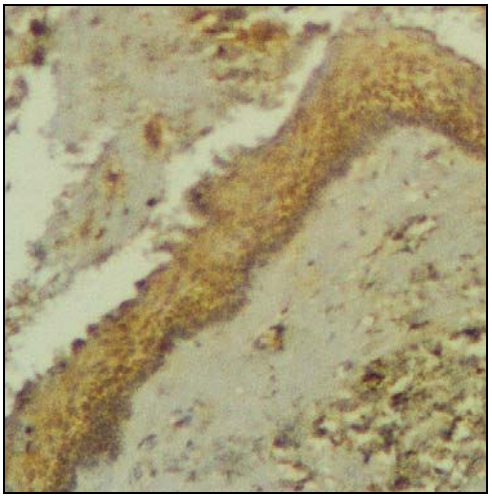
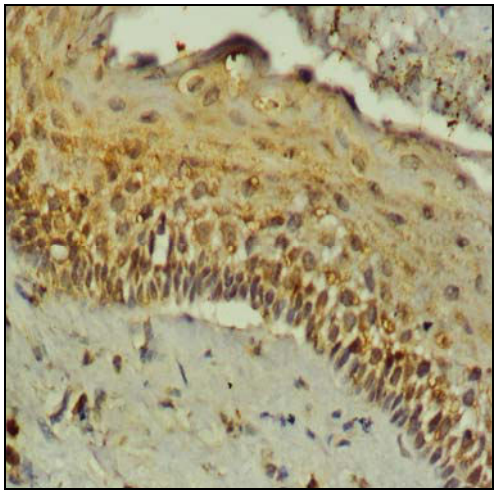


Figure 22 : Positive stain 40x



CALRETININ STAINING IN AMELOBLASTOMA

Figure 23: H & E; 10 x

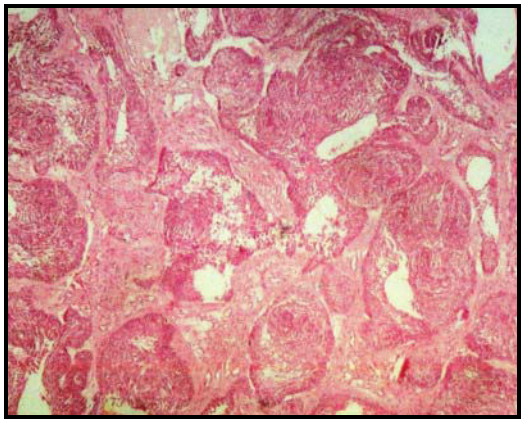


Figure 24: Negative control; 10 x

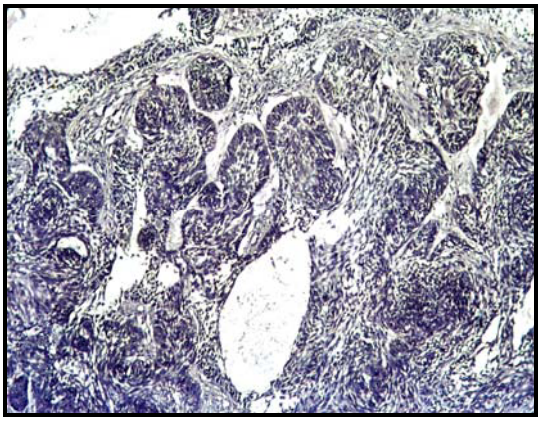


Figure 25: Positive stain10 x

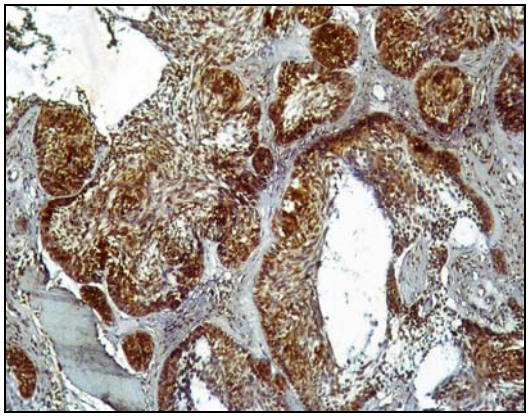
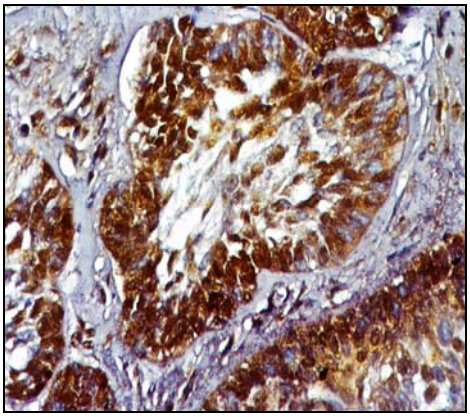


Figure 26: Positive stain 40 x



Cysts are classified based on their origin into developmental and inflammatory origin. Although, dentigerous cysts, radicular cysts and odontogenic keratocysts have characteristic histopathology which helps in identifying them, in many cases, identification is made difficult by secondary changes in cyst, making appropriate management difficult.[28]

Tumors arising from the epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histological variation and are classified into several benign and malignant entities. Ameloblastoma is frequently a tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with high tendency for recurrence.[1]

Dentigerous cyst is the second most common type of cyst of developmental origin, after radicular cyst, which develops by pericoronal or intraepithelial accumulation of fluid spawned by the reduced enamel epithelium and encloses the crown of an unerupted tooth and is attached to its neck.[28]. Although dentigerous cysts can involve any unerupted tooth, they usually involve unerupted deciduous teeth, supernumerary teeth or odontomas.Odontogenic tumors, such as ameloblastomas or epidermoid carcinomas, occasionally arise from the lining of the dentigerous cysts. About 70% of dentigerous cysts occur in the mandible and 30% in the maxilla.[15].**Koseoglu *et al* (2004)** also reported cases which were

involved in mandibular third molar region. In our study, posterior mandible was involved in 86% of the cases and posterior maxilla was involved in the 13% of the cases which was similar to the above mentioned study.

Odontogenic keratocysts(OKCs), classified under developmental odontogenic keratocyst, develop from odontogenic epithelium or its remnants.[3] The discovery of increased mitotic activity in the cyst epithelium, the potential budding from the basal layer or daughter cysts in the cyst wall, the presence of the PTCH gene in the etiology resulted in reclassification of this lesion as a neoplasm in the WHO classification of head and neck tumors in 2005, and was renamed as Keratocystic Odontogenic Tumor. **Habibi *et al* (2007)** conducted a study where odontogenic keratocysts occurred in any part of the jaw with a considerable predilection of 40% in posterior body of the mandible and ascending ramus[13]. **Koseoglu *et al* (2004)** and **Nakamura T *et al* (1995)** also conducted a study where 62.5 % cases were most common in posterior mandibular region. In our study, the site involved was in posterior mandibular region 66.6%, posterior maxilla 20% and anterior mandible 13.3% which was similar to the above mentioned study.[38,39]

Radicular cysts classified under inflammatory cysts, arise from the epithelial residues (rests of Malassez) in the periodontal

ligament as a consequence of inflammation which usually follows the death of the dental pulp and represent a part of more than half of all odontogenic cysts.[28]. In study conducted by **Usalan *et al* (2009),** 45% were in maxilla which was affected three times as frequently as the mandible.[30] Most cysts were located in the maxilla, especially 45 % anterior region followed by 15% maxillary posterior region, 15% mandibular posterior region and 25% in mandibular anterior region**. Nakamura *et al* (1995)** reported 41% of their cases in the maxilla whereas in our study, 40% of cases were seen in posterior mandible, 33% in anterior mandible, 20% in anterior maxilla and 13% in posterior maxilla.[39]

Ameloblastomas are benign odontogenic tumors of epithelial origin. However, as ameloblastomas sometimes arise as direct proliferation of oral mucosa or dentigerous cyst epithelium, these tissues are also considered as potential sources of the tumor epithelium**.**[31]

Rosenstein *et al* (2001), Ponniah *et al* (2010),Reichart reported cases in which all were located in posterior mandible, similar to our study group. [32,33,2]

Dentigerous cysts usually present in the second or third decade of life .The age range of the cases reported by **Buyukkurt *et al* (2010),** ranged from 4years to 57 years of age. The mean age of the previously reported cases were 24.70 years[15]. The age of the

patients reported by **Koseoglu *et al* (2004)** ranged from 15 to 65 years[38.] In our study, nearly 50% patients presenting with dentigerous cysts were in third and fourth decades of life and ranged from 12 years to 47 years of age which was similar to their study.

There was a wide range of age distribution of patients who presented with odontogenic keratocyst in our study with a peak incidence in the third and fourth decade when compared to **Habibi *et al* (2007)** study, who reported in a wide age range[13], but with a peak incidence in the second and third decades and **Browne *et al* (1975)** reported peak incidence in the second and third decades.[14]

Radicular cysts are rarely seen in individuals younger than 10 years of age, and were the most common between the ages of 20 to 45years in our study which was similar to reports of **Usalan et al** and **Koseoglu *et al***.[30,38]

Rosenstein *et al* (2001) and Ponniah *et al* (2010) conducted study on ameloblastomas where the age group was from 12 to 72 years with a mean age of 35 years[32,33]. **Ladeinde *et al* (2005)** conducted a study where the age group was from 4 to 82 years with mean age of 31.7 years.[45] In our study, 40% of patients were in the age group of 35-44 years and 33% in ≥ 45 years with mean age of 24.97 years which was similar to the studies conducted.

Dentigerous cysts are frequently seen in 64% of males and 36% of females as mentioned by **Shear *et al*** and this finding is similar to our study where 73% were males and 26% were females.[12] **Koseoglu *et al* (2004)** in their study on odontogenic keratocysts reported a male to female ratio of 1.1:1.[38] In the study done by **Shear et al,** males were 65%. But in our study 80% of our patients were males and 20% were females.[12]

According to **Shear *et al*** in radicular cysts males were 58.5% and females were 41.5% .In our study 66.6% were males and 33.3% were females which concurred with the above mentioned study.[12] **Ponniah *et al* (2010)** conducted study on ameloblastomas; of the patients they studied were 52% males and 48% were females where as in our study males were 86% and females were 13% .[33]

Immunohistochemical methods have revolutionized the approach to tumors of uncertain origin. Many antibodies recognize antigens that are expressed by cells of specific histogenesis. The antibodies with widest use are those that indicate the embryologic origin of cells such as epithelial, mesenchymal and neural.[41]

In this study, we evaluated the expression of cytokeratin 19 and calretinin in odontogenic cysts and Ameloblastomas.

Calretinin Expression:

All cases of ameloblastoma (100%) and 13.3% in odontogenic keratocyst while radicular and dentigerous cyst did not show any immunoexpression.

Altini *et al* (2000), in their study on 27 cases of unicystic ameloblastomas and 31 cases of solid multicystic ameloblastomas reported 93.5% positivity of calretinin.[8]

H Coleman and Altini *et al* (2001), conducted a study on 22cases of odontogenic keratocyst, 26 cases of residual cyst and 20 cases of dentigerous cyst. In their study none of the cases were positive except three cases of odontogenic keratocyst which showed staining of occasional intraepithelial cells and similar staining were seen in single darkly stained cells which was interpreted as mast cells.[21]

Adriano Piattelli *et al* (2003), conducted a study on radicular cysts, follicular cysts, odontogenic keratocysts which were negative except for two cases of odontogenic keratocysts which showed positivity. This study for calretinin explains the differences in clinical and pathologic behavior, in particular the differences found between orthokeratinised and parakeratinised keratocysts. Parakeratinised odontogenic keratocyst have a very destructive potential and invade the adjacent bony structures and soft tissue, whereas orthokeratinised are less aggressive and respond well to conservative treatment. [20]

De Villiers *et al* (2008), in their study on ameloblastomas and keratocystic odontogenic tumor reported 19 cases of ameloblastomas which showed positive staining and of the 18 cases of keratocystic odontogenic tumor reported one case ,had a recurred twice within two years and the staining of calretinin was seen in the recurrent sample .[42]

In our study, all cases showed positive staining in ameloblastomas as compared with **De Villiers *et al* (2008)** whose study suggested the possible biologic role of calretinin as a calcium buffer and regulator, playing a role in the process of enamel formation; behavior and tumorigenesis of odontogenic neoplasms. It has been hypothesized that calretinin plays a role in the aggressiveness of ameloblastoma. The 13.3% of positive staining in the odontogenic keratocysts as compared with the authors suggest their destructive potential. The keratocystic odontogenic tumor deserves a special consideration because of its destructive behavior, in a lesser degree when compared to ameloblastoma. There are clinical and radiographic similarities which may also be reflected at the histologic level if the tissue sample is small and if neoplastic epithelium displays reactive changes induced by inflammation.

Cytokeratin 19 expression:

The technique of antigen retrieval was performed by enzyme digestion, to evaluate the expression of cytokeratin 19 in odontogenic cysts and tumors as recommended by Biogenex and was also performed by **Kumamoto *et al* (2001)**[.1] Formalin fixed , paraffin embedded tissue section of normal skin were used but because of the presence of melanin pigments the positive stain was not appreciated and so colon cancer tissue provided by Biogenex was used which showed positive stain in the ductal region. The staining intensity for cytokeratin 19 in the ductal epithelium of the colon cancer tissue was intense (+++).

In our study, 80% of cases of Group I did not show any staining characteristics, 3.3% cases showed mild staining and 6.6% cases showed moderate staining intensity. **Stoll *et al,* (2005)** reported that, 48.3% of dentigerous cyst showed positive staining of the superficial layers for cytokeratin 19. 70.6% of dentigerous cysts showed positive immunoreactivity.[28] Moderate staining for cytokeratin 19, was also reported in majority of epithelial cells irrespective of their level within the epithelium and/or differentiation by **Mathews *et al* (1998)**[.3]

In our study, no staining was detected for cytokeratin 19, in any layer in Group II. In contrast to the study conducted by **Mathews *et al* (1998)** where cytokeratin 19 expression was detected

in the epithelial linings of the odontogenic keratocysts irrespective of their level within the epithelium and/or differentiation. Recent studies done by **Stoll *et al* (2005)** on odontogenic keratocysts showed a complete negative staining.[28]

Cytokeratin19 is a marker of simple epithelia and is an obligatory constituent of all normal epithelium, odontogenic epithelium, junctional epithelium and odontogenic cysts, odontogenic keratocysts is an exception. **Morgan *et al* (1987)** suggested a reason for negative staining as it could be due to the superimposition of the cornification of markers, cytokeratin1 and 10.[23]

Shear *et al* (2007) suggested that there could be change in the pattern of cytokeratin profiles in odontogenic epithelial cells during odontogenesis and also when quiescent cells proliferate in certain pathological situations like odontogenic cysts and tumors[12]. Hence negative staining could be due to the epithelial characteristic, modification or absence of epitope. The results of our study, was consistent with the studies of **Stoll *et al* (2005)**.[28]

In our study, none of the cases of Group III showed any staining characteristics. However, 48.3% of the radicular cysts showed positive immunoreactivity, in suprabasal cell layers in a study conducted by **Stoll *et al* (2005). Mathews *et al* (1998),** also

reported moderate staining in all specimens, in majority of epithelial cells, irrespective of their level within epithelium or differentiation[3].

In our study, 6.6% of ameloblastomas, expressed cytokeratin19 staining characteristics were seen, 93.3% of cases did not show immunoexpression. **Fukumashi *et al* (2002),** conducted study on all variants of ameloblastoma where follicular type and desmoplastic type showed immunoexpression whereas plexiform showed 95% positivity and granular 67%. The probable explanation for negative staining could be due to direction of the differentiation to the odontogenic epithelium may be diminished, because the cells of tumor cell nest negatively stain with cytokeratin19 and would be same reason for negativity in our study.

Matsuo *et al* (1991), reported in their study negative staining in 12 cases of follicular ameloblastoma, 2 cases of acanthomatous and one case of basal cell type which showed partial staining. Whereas the 15 cases of plexiform type conducted were positive. The tendency of squamous metaplasia in follicular type is thought to correspond to the fact that the enamel organ finally undergoes dedifferentiation into squamous epithelium and plexiform is in a primitive stage of differentiation.[46]

Kumamoto *et al* (2001), conducted a study on all histological types of ameloblastoma and found decreased expression in keratinizing cells of acanthomatous type suggesting the dedifferentiation from odontogenic epithelial characteristics. The reasons for the absence of staining in our study could be due to the dedifferentiation of cells in ameloblastoma which is similar to the results of above mentioned authors.[1]

Morgan *et al* (1987), stated that a diagnostic interpretation based on the use of a single antibody or even evaluation of a single keratin with several antibodies may not be conclusive. Keratin profile of an epithelium is linked to several factors such as differentiation, proliferation and histogenesis. The biological rules of keratin expression are yet to be defined fully and often there may be subtle differences between keratin at the molecular levels that may be reflected as absence of immunoreactivity with antibodies that have generated to known epitopes. [23]

- A total of 60 cases were included in our 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 (Group IV). The mean ages of study groups in group I,II,III,IV were 35.53± 7.83,39.07± 7.05,31.40± 8.68 and 40.2± 8.55 years respectively.
- In Group I, 73% were males and 27% were females. In Group II, 80% were males and 20% were females. In Group III, 67% were males and 33% were females. In Group IV, 87% were males and 13% were females.
- In Group I, none of the dentigerous cyst cases showed Calretinin stain.

  Cytokeratin 19 showed 13.3% of mild staining and 6.6% showed moderate staining pattern.
- In Group II, 6.7% cases of odontogenic keratocyst showed mild staining and 6.7% showed moderate staining for Calretinin.

  Cytokeratin 19 did not exhibit any staining characteristic.
- In Group III, there was no immunoexpression for calretinin and cytokeratin 19.
- In Group IV, all the cases showed immunoexpression for calretinin. 6.7% of the cases showed moderate staining for cytokeratin 19.
- In comparison of staining intensity of Group II and IV the p value 0.004 was considered to be statistically significant.

In conclusion, the results of the current study show that calretinin could be a immunohistochemical marker for neoplastic ameloblastic epithelium and it may be an important diagnostic aid in the differential diagnosis of cystic odontogenic lesions and ameloblastic tumors.

Cytokeratin 19 is a marker of simple epithelia. Absence of cytokeratin 19 in odontogenic keratocyst could be due to the nature of epithelium or give to different differentiating pattern of epithelium.

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Anti-Cytokeratin 19 [RCK108]

AM246-5M
MU246-UC

BioGenex

4600 Norris Canyon Road
San Ramon, CA 94583 USA
Tech Support: 925-275-0550
Fax: 925-275-1999
support@biogenex.com

EC REP
Emergo Europe
Molenstraat 15
NL-2513 BH The Hague
The Netherlands
Tel: (+31) 70-345-8570
Fax: (+31) 70-346-7299

Doc. No. 932-246M-4 Rev. G
Release Date: June 11, 2007

CE

ENGLISH

Specifications:	Anti-Human Cytokeratin 19	Catalog No.	Description
Immunogen:	Total cell extract from human bladder cancer cell line	AM246-5M	6 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems.
Clone:	RCK108	MU246-UC	1 ml of Concentrated Antibody for Use with BioGenex Super Sensitive Detection Systems or Other Equivalent Detection Systems.
Species:	Mouse	Recommended Detection System: LINK-LABEL	
Immunoglobulin Class:	IgG1, Kappa		
Protein Conc.:	10 - 15 mg/ml*	*Lot specific Ig concentration available upon request.	

Intended Use
This antibody is currently available for in vitro diagnostic use. This monoclonal antibody is designed for the specific localization of cytokeratin 19 in formalin-fixed, paraffin-embedded tissue sections.

Summary and Explanation
Cytokeratin 19 (molecular mass 40 kD) is a marker of simple epithelia. Cytokeratin 19 has been found in mesothelial and mesothelioma cells, and in ovarian cysts, cystadenomas, and ovarian carcinomas. It has been shown to be present in the basal layer of non-keratinizing stratified squamous epithelia such as the oral cavity and the ectocervix. Cytokeratin 19 has also been found in adenocarcinomas of the lung and in tumor cells of pulmonary metastases. Schüssler et al.reported that cytokeratin 19 can be found in the ductal cells of normal pancreas and in pancreatic cancers. The RCK108 antibody to cytokeratin 19 reacts with many types of epithelial cells including many ductal and glandular epithelia. It has been shown that RCK108 stains mammary gland luminal cells and prostate epithelia. RCK108 does not stain stratified squamous epithelium of the epidermis, sebaceous glands, liver hepatocytes, the cells of some endocrine glands, or some testicular cell types. Nor does it stain non-epithelial tumors, basaliomas, or seminomas.

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 from ascites 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	COLON CARCINOMA
Tissue Type	Formalin-fixed, Paraffin-embedded
Concentrated Dilution	100-200
Pretreatment	Pepsin, 37 C, 5 min.
Incubation Time and Temperature	2 hrs. @ RT

Quality Control
The recommended positive control tissue for this antibody is COLON CARCINOMA. / FG-246M 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 cytokeratin 19 in cytoplasm of many types of epithelial 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.

ITALIANO, ITALIAN

Specifiche tecniche:	Cytokeratin 19 anti-umano	N. di catalogo	Descrizione
Immunogeno:	Estratto cellulare totale da una linea cellulare di tumore della colecisti	AM246-5M	6 ml di anticorpo pronto per l'uso con i diversi BioGenex Super Sensitive Detection Systems.
Clone:	RCK108	MU246-UC	1 ml di anticorpo concentrato per l'uso con i diversi BioGenex Super Sensitive Detection Systems o altri sistemi di rilevamento equivalenti.
Specie:	Topo	Raccomandato Sistema di Rivelazione: LINK-LABEL	
Classe immuno-globulina:	IgG1, Kappa		
Conc. proteine:	10 - 15 mg/ml*	*Concentrazione delle Ig specifica per il lotto disponibile su richiesta.	

Anti-Calretinin [POLYCLONAL]

AR413-5R
AR413-10R
PU413-UP

BioGenex

4600 Norris Canyon Road
San Ramon, CA 94583 USA
Tech Support: 925-275-0550
Fax: 925-275-1999
support@biogenex.com

EC REP
Emergo Europe
Molenstraat 15
NL-2513 BH The Hague
The Netherlands
Tel: (+31) 70-345-8570
Fax: (+31) 70-346-7299

Doc. No. 932-413P-4 Rev. F
Release Date: September 27, 2004

CE

ENGLISH

Specifications:	Anti-Human Calretinin	Catalog No.	Description
Immunogen:	Recombinant human calretinin.	AR413-5R	6 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems.
Clone:	POLYCLONAL	AR413-10R	10 ml of Ready-to-Use Antibody for Use with BioGenex Super Sensitive Detection Systems and BioGenex Automated Staining Systems.
Species:	Rabbit	PU413-UP	1 ml of Concentrated Antibody for Use with BioGenex Super Sensitive Detection Systems or Other Equivalent Detection Systems.
Immunoglobulin Class:	N/A	Recommended Detection System: POLYMER-HRP and LINK-LABEL	
Protein Conc.:	~50 mg/ml*	*Lot specific Ig concentration available upon request.	

Intended Use
This antibody is currently available for in vitro diagnostic use. This antibody is designed for the specific localization of Calretinin antigen in formalin-fixed, paraffin-embedded tissues.

Summary and Explanation
Calretinin, encoded by gene calb2, also known as Calbindin 2, is a calcium-binding protein belonging to the troponin C superfamily and calbindin subfamily. It consists of 271 amino acids and has a molecular weight of 31.5 kD. Among the many calcium-binding proteins in the nervous system, calretinin, together with parvalbumin and calbindin-D28K, are particularly striking in their abundance and in the specificity of their distribution. They can be found in different subsets of neurons in many brain regions and are considered valuable markers of neuronalsubpopulations for anatomical and developmental studies. Calretinin is approved as a highly sensitive and specific marker for mesothelial cells and one of the best positive markers for differentiating epithelial malignant mesotheliomas. This polyclonal antibody specifically recognizes calretinin in tissue originating from human, monkey, rat and mouse. It does not cross-react with other known calcium-binding proteins as determined by Western Blot analysis and by its distribution in the brain with immunohistochemistry.

Principles of the Procedure
The demonstration of antigens by immunohistochemistry is a two-step process involving first, 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
Rabbit immune serum diluted in PBS, pH 7.6, containing 5% 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	BRAIN
Tissue Type	Formalin-fixed, Paraffin-embedded
Concentrated Dilution	10-30 (Use HK941-YAK as Ab. Diluent)
Pretreatment	AR Citra Plus, Power Block, 5 min
Incubation Time and Temperature	30 min. @ RT

Quality Control
The recommended positive control tissue for this antibody is BRAIN. / FG-413P tissues are available from BioGenex for QC. Refer to the appropriate detection system package inserts for guidance on general quality control procedures.

Troubleshootng
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 calretinin antigen in cytoplasm of various neurons in normal brain and mesothelial 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.

ITALIANO, ITALIAN

Specifiche tecniche:	Calretinin anti-umano	N. di catalogo	Descrizione
Immunogeno:	Calretinina ricombinante umana	AR413-5R	6 ml di anticorpo pronto per l'uso con i diversi BioGenex Super Sensitive Detection Systems.
Clone:	POLYCLONAL	AR413-10R	10 ml di anticorpo pronto per l'uso con i diversi BioGenex Super Sensitive Detection Systems e BioGenex Automated Staining Systems.
Specie:	Coniglio	PU413-UP	1 ml di anticorpo concentrato per l'uso con i diversi BioGenex Super Sensitive Detection Systems o altri sistemi di rilevamento equivalenti.
Classe immuno-globulinaa:	N/A	Raccomandate Sistema di Rivelazione: POLYMER-HRP and LINK-LABEL	
Conc. proteine:	~50 mg/ml*	*Concentrazione delle Ig specifica per il lotto disponibile su richiesta.	