EVALUATION OF EXPRESSION OF P63 AND AMELOGENIN IN HUMAN TOOTH GERM AND AMELOBLASTOMA

A Dissertation submitted in
partial fulfillment of the requirements
for the degree of

MASTER OF DENTAL SURGERY

BRANCH – VI
ORAL PATHOLOGY AND MICROBIOLOGY

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY
CHENNAI – 600 032

2015 - 2018

CERTIFICATE

This is to certify that **Dr. P. SRI KANTHA LAKSHMI,** Post Graduate Student (2015-2018) in the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003 has done this dissertation titled "**EVALUATION OF EXPRESSION OF P63 AND AMELOGENIN IN HUMAN TOOTH GERM AND AMELOBLASTOMA**" under my direct guidance and supervision in partial fulfillment of the regulations laid down by **The Tamil Nadu Dr. M.G.R. Medical University, Chennai – 600 032** for M.D.S., (Branch – VI)**Oral Pathology and Microbiology** degree examination.

Dr.I. PONNIAH, MDS,
Professor and Head,
Department of Oral Pathology
and Microbiology,
Tamil Nadu Government Dental College and
Hospital, Chennai – 600 003.

Dr. B. SARAVANAN MDS, Ph.D.
Principal,
Tamil Nadu Government Dental College
and Hospital,
Chennai – 600 003.

DEPAMENT OF ORAL PATHOLOGY AND
MICROBIOLOGYTAMIL NADU GOVERNMENT
DENTAL COLLEGE AND HOSPITALCHENNAI – 600 003

DECLARATION

I **Dr. P. Sri Kantha Lakshmi,** do hereby declare that the dissertation titled **"EVALUATION OF EXPRESSION OF P63 AND AMELOGENIN IN HUMAN TOOTH GERM AND AMELOBLASTOMA"** was done in the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai – 600003. I have utilized the facilities provided in the Tamil Nadu Government Dental College and Hospital, Chennai - 600 003 for the study in partial fulfillment of the requirements for the degree of Master of Dental Surgery in the specialty of Oral Pathology and Microbiology (Branch VI) during the course period 2015-2018 under the conceptualization, design, guidance and supervision of Professor and Head Dr. I. PONNIAH, MDS.

I declare that no part of the dissertation will be utilized for gaining financial assistance, for research or other promotions without obtaining prior permission from the Tamil Nadu Government Dental College and Hospital, Chennai- 600 003.

I also declare that no part of this work will be published either in the print or electronic media except with those who have been actively involved in this dissertation work and I firmly affirm that the right to preserve or publish this work rests solely with the permission of the Principal, Tamil Nadu Government Dental College and Hospital, Chennai – 600003, but with the vested right that I shall be cited as author(s).

Signature of the PG Student

Signature of the Head of the Department

Signature of the Head of the Institution

TRIPARTITE AGREEMENT

This agreement herein after the "Agreement" is entered into on this day, 15th November, 2017 between the Tamil Nadu Government Dental College and Hospital represented by its Principal having address at Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, (hereafter referred to as, 'the College')

and

Dr. I. PONNIAH MDS., aged 49 years working as Professor and Head of the Department of Oral Pathology and Microbiology at the college, having residence address at Plot No. 164E, 7th Cross Street, 2nd Main Road, "Ring Road Housing Sector," Madhavaram in Chennai - 600 060 (herein after referred to as the 'Researcher and Principal investigator')

and

Dr. P. Sri Kantha Lakshmi, aged 28 years currently studying as Post Graduate student in the Department of Oral Pathology and Microbiology (herein after referred to as the 'PG/Research student and Co- investigator').

Whereas the 'PG/Research student as part of his curriculum undertakes to research on the study titled **"EVALUATION OF EXPRESSION OF P63 AND AMELOGENIN IN HUMAN TOOTH GERM AND AMELOBLASTOMA"**for which purpose the Researcher and Principal investigator shall act as Principal investigator and the College shall provide the requisite infrastructure based on availability and also provide facility to the PG/Research student as to the extent possible as a Co-investigator.

Whereas the parties, by this agreement have mutually agreed to the various issues including in particular the copyright and confidentiality issues that arise in this regard.

Now this agreement witnessed as follows:

1. The parties agree that all the research material and ownership therein shall become the vested right of the college, including in particular all the copyright in the literature including the study, research and all other related papers.
2. To the extent that the College has legal right to do go, shall grant to license or assign the copyright do vested with it for medical and/or commercial usage of interested persons/entities subject to a reasonable terms/conditions including royalty as deemed by the college.
3. The royalty so received by the college shall be equally by all the parties.
4. The PG/Research student and PG/Principal Investigator shall under no circumstances deal with the copyright, confidential information and know how generated during the course of research/study in any manner whatsoever, while shall sole vest with the manner whatsoever and for any purpose without the express written consent of the college.
5. All expenses pertaining to the research shall be decided upon by the Principal investigator/Co-investigator or borne sole by the PG/Research student (Co-investigator).
6. The College shall provide all infrastructure and access facilities within and in other institutes to the extent possible. This includes patient interactions, introductory letters, recommendation letters and such other acts required in this regard.
7. The principal investigator shall suitably guide the student research right from selection of the research topic and area till its completion. However the selection and conduct of research, topic and area research by the student researcher under guidance from the principal investigator shall be subject to the prior approval, recommendations and comments of the Ethical Committee of the college constituted for this purpose.
8. It is agreed that as regards other aspects not covered under this agreement, but which pertain to the research undertaken by the Student Researcher, under guidance from the Principal Investigator, the decision of the college shall be binding and final.
9. If any dispute arises as to the matters related or connected to this agreement herein, it shall be referred to arbitration in accordance with the provisions of the Arbitration and Conciliation Act, 1996.

In witness whereof the parties herein above mentioned have on this the day month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

College represented by its Principal

Principal investigator

Student Researcher

Witnesses

1.

2.

ACKNOWLEDGEMENT

The dissertation owes its existence to the help, support and inspiration of several people. I would like to express my sincere gratitude to all of them.

I wish to express my sincere and grateful thanks to **The Director, Institute of Obstetrics and Gynaecology, Chennai-600 008,** who permitted me to avail foetus tissue to use as a subject material in my dissertation from her esteemed institution.

I wish to express my sincere and heartfelt gratitude to **Dr. B. Saravanan M.D.S., Ph.D.,** Principal, Tamil Nadu Government Dental College and Hospital, Chennai, who forwarded a requisition letter to The Director, Global Hospital and Health City, Perumbakkam, Chennai- 600 100 to permit me to observe the immunohistochemistry procedure in the Department of Pathology.

I also extend my sincere thanks to **The Director, Global Hospital and Health City, Perumbakkam, Chennai- 600 100** and **Dr. Mukhul Vij Professor, Department of General Pathology, Global Hospital and Health City, Perumbakkam, Chennai- 600 100** for their permission to allow me as a student observer in the Department of Pathology of the esteemed hospital to learn the immunohistochemistry procedure.

I wish to place on record my deep sense of gratitude to my guide **Dr. I. Ponniah M.D.S**, for the keen interest, inspiration, immense help and expert guidance throughout the course of this study as Professor and HOD of the Dept. of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003.

I would like to thank my teacher, **Dr. R. Bharathi M.D.S.,** Professor of Oral Pathology and Microbiology for her support and encouragement.

I would like to thank my teachers, **Dr. S. Gnanadeepam, M.D.S. and Dr. M.P. Sumathy M.D.S.**, Associate professors, who were there throughout my educational career have supported and encouraged me to believe in my abilities.

I would also like to thank all my teachers, **Dr. S. Kuzhali M.D.S., Dr. Selva Jothi M.D.S., Dr.V.Shanthi M.D.S., Dr. Dhanalakshmi M.D.S.,** Assistant Professors, for all the encouragement and generous help rendered by them.

I would like to thank my batch mate and dear friend **Dr. P. Suganya**, who was always with me throughout the dissertation work and for her full support.

I would like to thank my dear junior P.G. colleagues Dr. Juliet Deepa, Dr. Devaki, Dr. N. Hema Malini and Dr. K. Kalpana for believeing in me and many precious memories along the way.

I thank our lab technicians **Mrs. Meenakshi, Mrs. Hilda Margaret and Mrs. Sargunavathy** for their timely help in providing the archival samples from the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai -600 003. I also wish to thank **Mr. Kavivanan**, lab technician, Global hospital, Chennai and **Mr. Senthil Kumar**, lab technician, Government Veterinary College, Chennai for their help.

Finally I would like to express my heartfelt gratitude to my family. I would like to thank my father **R. Piramanayagam Kannan**, mother **P. Gangeswari,** brothers **P. Selva Nivas and P. Sri Sheshathri**, who all supported and helped me along the course of this dissertation by providing the moral, emotional and financial support that I needed to complete my thesis.

Above all, I am always grateful to God Almighty, who is always beside me.

TAMIL NADU GOVERNMENT DENTAL COLLEGE & HOSPITAL, CHENNAI – 3.
TELEPHONE : 044-253403343
FAX: 044- 25300681
date : 25-11-2016

Ref No: R. C. NO: 0420/DE/2016
Sub: IEC review of the research proposals,

Title of the work: Evaluation of expression of p63 and Amelogenin in human tooth germ and Ameloblastoma

Investigator: Dr.P.Sri Kantha Lakshmi
II year , MDS

Department : Department of Oral Pathology and Microbiology
Tamil Nadu Govt. Dental College & Hospital, Chennai-3

Thank you for submitting your research proposal , which was considered at the Institutional Ethics Committee meeting held on 30-09-2016, at TN Govt. Dental College. The documents related to the study referred above were discussed and the modifications done as suggested and reported to us through your letter On 15-11-2016 have been reviewed. The decision of the members of the committee , the secretary and the Chairperson IEC of TN Govt. Dental College is here under:

Approved	Approved and advised to proceed with the study
Approved with suggestions	----------

The principal investigators and their team are advised to adhere to the guide lines given below:

1. You should get detailed informed consent from the patients / participants and maintain confidentiality.
2. You should carry out the work without affecting regular work and without extra expenditure to the Institution or the Government.
3. You should inform the IEC, in case of any change of study procedure, site, and investigating guide.
4. You should not deviate from the area of work for which you have applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions. You should abide to the rules and regulations of the institution(s) .
6. You should complete the work within specific period and if any extension of time is required, you should apply for permission again to do the work.
7. You should submit the summary of the work to the ethical committee every 3 months and on completion of the work.
8. You should not claim any kind of funds from the institution for doing the work or on completion/ or for any kind of compensations.
9. The members of the IEC have the right to monitor the work without prior intimation.
10. Your work should be carried out under the direct supervision of the guide/ Professor.
11. The investigator and Guide should each declare that no plagiarism is involved, in this whole study and enclose the undertaking in dissertation/ thesis.

MEMBER SECRETARY,
INSTITUTIONAL ETHICS COMMITTEE
Tamil Nadu Govt. Dental College & Hospital
Chennai

CHAIRPERSON
INSTITUTIONAL ETHICS COMMITTEE
Tamil Nadu Govt. Dental College & Hospital
Chennai

To
Dr. Srikantha Lakshmi

Dr. I. PONNIAH, MDS.,
(Reg No: 1667)
Professor & Head
Department of Oral Pathology
Tamil Nadu Government
Dental College & Hospital
Chennai - 600 003.

Permitted
5/12/16

To DRMO / DRMO

Chennai,
28 November, 2016

From	To
Dr. I. Ponniah M.D.S., Professor and Head, Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital, Chennai 600 003.	The Director, Institute of Obstetrics and Gynaecology, IOG Campus, Chennai 600 008.

Madam,

Subject: Permission to avail foetus tissue - reg.

I kindly request you to provide unclaimed foetal tissue to use as a subject material for the purpose of doing MDS dissertation.

I also bring to your kind notice and consideration that the foetus tissue for research purpose (DISSERTATION) was previously obtained from the Institute of Obstetrics and Gyneacology and the remaining foetus tissue was normally stored and preserved in the museum of the Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital. However if for certain reasons the remaining tissues have to be disposed, it will be carefully handled and placed in leak proof yellow bins for disposal in accordance with biomedical waste management protocol followed by the Tamil Nadu Medical Service. Therefore, I kindly request you to permit my student, Dr. P. Sri Kantha Lakshmi, (MDS Postgraduate Student) to avail foetus tissue from your Institute of Obstetrics and Gynaecology, for the purpose of doing dissertation. I humbly submit that your permission in this regard will be appropriately mentioned in the Materials and Methods and in the acknowledgement section of the proposed disser ation.

Thanking you,

Sincerely,

Ponniah 28/11/16.

Dr. I. PONNIAH, MDS.,
(Reg No: 1667)
Professor & Head
Department of Oral Pathology,
Tamil Nadu Government
Dental College & Hospital,
Chennai - 600 003.

5/12/16.

RESIDENT MEDICAL OFFICER
GOVERNMENT WOMEN & CHILDREN HOSPITAL
EGMORE, CHENNAI-600 008.

From,
Dr.B.Saravanan,MDS, Ph.D.,
PRINCIPAL
Tamilnadu Government Dental College and Hospital, Chennai-3.

To,
The Director, (Academics & Research).
Department of Pathology,
Global Hospital and Health city,
Perumbakkam, Chennai-600 010.

Ref no: 1714 / DE / 2017

Date:- 11-04-2017.

Respected Sir/ Madam

Sub:- Requisition for Student observer in Department of General Pathology – part of the MDS curriculum - Permission to observe the immuno - histochemistry procedure - post graduate of the Department of Oral & maxillofacial pathology Tamilnadu Government Dental College and Hospital, Chennai - at Global Hospital and Health city, Chennai - Regarding;

Ref: Letter from Dr.Sri Kantha Lakshmi .P, postgraduate, Department of Oral & Maxillofacial pathology dated 11.04.2017, forwarded by the Professor & HOD, TNGDCH.

With reference to the letter cited above, the second year post graduate of the Department of Oral & maxillofacial pathology Dr.Sri Kantha Lakshmi, has requested to observe immunohistochemistry procedures for four days, as part of the MDS curriculum, & dissertation purpose in the Department of General Pathology, at Global Hospital and Health city, Chennai. Hence I request you to consider her request and permit Dr.Sri Kantha Lakshmi, to be an observer in the Department of General Pathology at your esteemed Hospital. We will be very thankful to you if you could help our student to fulfill the norms.

Thanking you,

Yours faithfully,

11/4/17

PRINCIPAL

To,

1. Dr.Sri Kantha Lakshmi P. - through the HOD,

Copy to,

The Prof.& HOD, Dep. Of oral pathology & Microbiology

Forwarded to Dr. Murali raj for Permission

12/04/2017

Allowed to learn in Dept of Pathology

12/4/17

PLAGIARISM CERTIFICATE

Urkund Analysis Result

Analysed Document:	Thesis - IMRAD 2.docx (D34298509)
Submitted:	1/3/2018 8:16:00 AM
Submitted By:	kantha889@gmail.com
Significance:	5 %

Sources included in the report:

http://europepmc.org/articles/PMC4587831
https://www.ncbi.nlm.nih.gov/pubmed/1734713
http://onlinelibrary.wiley.com/doi/10.1002/ar.20520/full
https://link.springer.com/article/10.1007/s004180050360
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4959624/
http://www.intjmorphol.com/wp-content/uploads/2017/06/art_08_352.pdf
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4261713/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3247901/
https://www.ncbi.nlm.nih.gov/pubmed/26421144

Instances where selected sources appear:

29

CERTIFICATE II

This is to certify that this dissertation work titled **" EVALUATION OF EXPRESSION OF P63 AND AMELOGENIN IN HUMAN TOOTH GERM AND AMELOBLASTOMA"** of the candidate **DR. SRI KANTHA LAKSHMI P**. with registration number **241521001** for the award of **M.D.S. ORAL PATHOLOGY AND MICROBIOLOGY** in the branch of **VI**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and the results show **5%** of plagiarism in the dissertation.

Guide and Supervisor Sign with Seal

ABSTRACT

Background:

The study was undertaken to evaluate the expression of p63 and amelogenin in human tooth germs and ameloblastomas by immunohistochemistry to find out whether the pattern of expression was related to differentiation of ameloblasts.

Materials and Methods:

In this study fifteen human tooth germs of late bell stage, six human tooth germs of early bell stage and fifteen ameloblastoma samples were included and expression pattern of p63 and amelogenin were evaluated individually in all the samples.

Results:

During the early bell stage, p63 expression was intense throughout the enamel organ. But, during the late bell stage, the number and intensity of p63 expression decreases in the cells of enamel organ. The peripheral cells in the ameloblastoma shows variable pattern of p63 expression. Expression of amelogenin was first evident in the presecretory ameloblasts at the cusp tip, followed by secretory ameloblasts and progresses cervically. But in ten late bell stage tooth germs, amelogenin expression was negative in the secretory ameloblasts and positive only at the secreting end i.e. tomes process. Intense staining was evident at the enamel matrix and in some dentinal tubules nearer to enamel matrix secretion. No amelogenin expression was evident in the dental papilla cells or odontoblasts throughout odontogenesis with the exception of dental follicle. Amelogenin expression was absent in eleven cases of

ameloblastoma, but in four cases positive expression was evident at the peripheral cells.

Conclusion :

In the present study, the expression pattern of p63 and amelogenin in human tooth germ and ameloblastoma correlates with cytodifferentiation of ameloblasts.

Keywords : human tooth germ, ameloblastoma, immunohistochemistry, p63, amelogenin,

ABBREVIATIONS

IEE – Inner enamel epithelium

OEE – Outer enamel epithelium

PA – Preameloblasts

tPSA – Transitional presecretory ameloblasts

PSA – Presecretory ameloblasts

SA – Secretory ameloblasts

LBS – Late bell stage

EBS – Early bell stage

DPX- di butyl pthalate xylene

H&E- Hematoxylin and Eosin

IHC - Immunohistochemistry

PBS- Phosphate buffered saline

AMELX- Amelogenin

APES- 3-aminopropyltriethoxysilane

H_2O_2 – Hydrogen peroxide

HCl- Hydrochloric acid

CONTENTS

INTRODUCTION

Human teeth develop after a series of tightly regulated, sequential steps. At about 7th week thickening of the dental epithelium starts to form the dental lamina or dental placode, the first stage in tooth development. Subsequently, the lamina invaginates into the underlining neural crest derived mesenchyme and assumes a ‘‘bud’’ conformation, surrounded by condensed mesenchyme. Then the developing tooth matures into the cap stage, with the dental epithelium folding to embrace the mesenchyme. In this structure, the epithelium above the mesenchyme assumes a button-like morphology known as the enamel knot. The further shaping of the tooth germ results from asymmetric proliferation i.e, cells outside the enamel knot actively proliferate, while inner cells cease to proliferate and thus act like an anchor to enforce moulding of the cap stage. This asymmetric proliferation also splits the enamel knot into the inner and outer enamel knots; the mesenchyme adjacent to the inner knot will form the dental papilla, whereas that on the outside will develop into the dental follicle. Incisor teeth assume a conical shape at this stage and lose their enamel knot at the end of the cap stage. Molars have a more complex destiny, after loss of the first enamel knot, they develop secondary enamel knots, which shape the tooth into a multicuspid. ‘‘bell’’ stage. Finally, deposition of enamel by the epithelial derived ameloblasts and dentin by the mesenchyme-derived odontoblasts lead to the final mature tooth **(1)**.

Ameloblastoma is a benign but locally aggressive odontogenic tumor believed to arise from the odontogenic epithelium of tooth germ and presents with variable clinico-pathologic behavior and tendency for recurrence. Histologically, ameloblastoma shows considerable variation, including follicular,

plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types which needs different management.

The p63 gene has a high degree of amino acid sequence homology to the p53 and p73 genes and produces at least six different proteins with distinct properties that affect the function of other p63, p53 and p73 proteins expressed in the same cell. It has both amino- and carboxy terminals. At the C-terminal of p63, isofoms such as α, β, and γ exists. The N-terminal of p63 is characterized by two different isoforms viz; TA form (or transactivated form) and ΔN form (or truncated form). TAp63 contains acidic N-terminal transactivation domain and ΔNp63 lacks this domain. Various isoforms of p63 has different roles. ΔNp63 contributes to cell proliferation while TAp63 isoforms induce cell differentiation **(2).** Expression of p63 has been previously reported in human tooth germ as well as in ameloblastoma **(3,4,5)**. Although several studies indicate the expression of p63 in the respective tissues, the pattern of expression in different types of cells in enamel organ is lacking.

Amelogenin is the predominant enamel matrix proteins involved in biomineralization and organization of developing enamel. It functions by regulating crystallite formation during the secretory stage of enamel development. Although several studies indicate the expression of amelogenin in different animal species such as rat, bovine, porcine and dog by using different techniques including immunohistochemistry, only three studies regarding the immunohistochemical pattern of expression of amelogenin in human tooth germs have been reported so far (**6,7,8**) to my knowledge. There are so many studies regarding the expression of amelogenin in ameloblastoma have been reported, but still the results are inconclusive **(9,10,11,12).**

Therefore the present study is under taken to evaluate the expression of p63 and amelogenin in the inner enamel epithelial cell lineage and compare it with ameloblastoma.

AIM AND OBJECTIVES

Aim :

To evaluate the expression of immunohistochemical markers p63 and amelogenin in human tooth germ and ameloblastoma.

Objectives:

The objective of the study is to

1. To compare the expression pattern of immunohistochemical markers p63 and amelogenin in human tooth germ and ameloblastoma.
2. To determine whether the pattern of expression is related to differentiation.

REVIEW OF LITERATURE

I. EXPRESSION OF P63 IN HUMAN TOOTH GERM

In 1999, **Parsa et al.** studied the expression of p63 in normal human epidermis, hair follicles, stratified squamous epidermis and squamous cell carcinoma by immunohistochemistry and other techniques. Mouse monoclonal antibody to p63 clone 4A4 was used in the study. The author's found out that p63 expression was localized to the basal cells of keratinocytes and hair follicles. But the intensity of p63 expression varied in different cells of the basal layer. The basal cells which strongly stained for p63 were clustered in patches, separated by basal cells weakly stained or unstained. Moreover, p63 expression was absent from the spinous layer where terminal differentiation begins. In well differentiated squamous cell carcinoma p63 expression was confined to the nuclei of basal cells arranged in a ring, at a distance from the centres of terminal differentiation. But, in poorly differentiated tumors the number of cells containing p63 increased and the distribution of p63 was disorganized with respect to the centres of terminal differentiation. The author's concluded that p63 protein helped in maintaining the proliferative potential of cells and prevents it from terminal differentiation **(13).**

In 2000, **Pellegrini et al.** studied the expression of p63 and PCNA in the proliferative compartment of stratified squamous epithelia which consists of stem and transit amplifying (TA) keratinocytes by immunohistochemistry, clonal analysis and other techniques. A p63 specific mAb and PCNA specific mAb were used in the study. The author's found out that by immuunohistochemistry, the basal cells with higher content of nuclear p63 were interspersed by cells with little or no p63. The authors also found out that among the basal cells, some cells express both p63 and PCNA, some express PCNA but not p63 and some express

p63 but not PCNA. By clonal analysis, the author's found out that both p63 and PCNA expression were high in holoclones (consist primarily of stem cells), high levels of PCNA and low levels of p63 in meroclones (contain slightly more differentiated yet highly proliferative cells called transit- amplifying (TA) cells) and PCNA expression alone in paraclones (consists of committed, terminally differentiating cells). From these findings the author's concluded that p63 protein was principally restricted to keratinocyte stem cells (**14)**.

In 2004, **Cernochova et al.** studied the expression of p53, p63 and p73 in the orofacial region such as dental lamina, dental organ, dental papilla, dental follicle, vestibular lamina, epithelium of the palate and palatal suture, differentiating mucosa of the dorsum of the tongue and respiratory and olfactory regions of the developing nasal cavity of five human embryos aged 7 -18 weeks of intrauterine development using a three step immunohistochemical method using monoclonal antibodies. The author's found out that the expression of p63 in all the above said areas increased from seventh to eleventh week, where a maximum of positive nuclei was found and p63 expression dropped down from thirteenth week. A decrease in p53 and p73 proteins also occured in the 13-week-old material with the exception of the tooth germ where a drop in p73 appeared in the ninth week **(15)**.

In 2005, **Kumamoto et al**. studied the expression of p63 and p73 in human tooth germs and ameloblastoma by immunohistochemistry and reverse transcriptase polymerase chain reaction. Mouse anti p63 monoclonal antibody in 1:100 dilution and rabbit anti p73 polyclonal antibody in 1:500 dilution were used in the study. The author's found out that the p63 expression was evident in most cells of outer and inner enamel epithelium, dental lamina and fewer cells of

stratum intermedium and stellate reticulum. In ameloblastoma p63 expression was evident in most peripheral columnar or cuboidal cells and in some central polyhedral cells. The authors also found out that in both tooth germ and ameloblastoma p63 positive cells were more numerous than p73 positive cells in stratum intermedium and stellate reticulum. The authors concluded that p53 homologs i.e. p63 and p73 played a role in the proliferation and differentiation of odontogenic epithelial cell **(3).**

In 2005, **Kock et al**. studied the expression of the p63 in normal human tooth germs at different gestational stages by immunohistochemistry. Monoclonal mouse anti human p63 protein, clone 4A4 in 1: 50 dilution was used in the study. Incisors representing the cap stage were identified at GA 11 and 15 weeks and incisor representing the bell stage with hard tissue formation was identified at GA 21 weeks. The author's found out that expression of p63 was evident in the outer and the inner enamel epithelium, dental lamina as well as in the proliferating cells of the stratified epithelium in the overlying mucosa. at GA 11 and 15 weeks. In addition there was a strong expression of p63 in the enamel knot and at the stellate reticulum cells at GA 11 weeks, which was absent at GA 15 weeks. The author's indicated that p63 might play a role in signaling in the morphogenesis of the human tooth. At GA 21 weeks, strong p63 immunostaining was evident at the outer and inner enamel epithelium, secondary dental lamina, proliferating cells of oral mucosa and weak staining was evident at the nucleus of stellate reticulum cells. The spatio-temporal expression of p63 indicated that p63 might play a role in the tooth formation **(4).**

In 2006, **Laurikkala et al**. studied the expression of p63 during tooth development in wild type embryos from E10 to postnatal day 3 using insitu

hybridization, immunohistochemistry and other techniques. Immunohistochemical analysis was done with antibodies that recognize all p63 isoforms (pan p63) and ΔNp63 isoforms.The primary antibodies used were anti-p63 (4A4,1:500) and anti-ΔNp63 (sc-8609, 1:100). During the initiation (E11), bud (E12) and cap (E15) stages of mandibular molar development, both pan-p63 and ΔNp63 expression were seen throughout the dental epithelium, and it extended into the oral epithelium. At the bell stage (E17), pan-p63 and ΔNp63 expression were intense in the outer enamel epithelium, whereas the intensity of expression reduced in the stellate reticulum and inner enamel epithelium when it differentiated into ameloblasts. But, no expression of TAp63 was seen in the developing teeth. The author's concluded that ΔNp63 was the major p63 isoform expressed during embryonic tooth development and only one percent of all p63 isoforms represented the TA isoform. The author's also concluded that in the absence of p63, tooth development arrests at the dental lamina stage, suggesting that p63 is required in the ectoderm for the formation of tooth **(16)**.

In 2012, **Matsuura et al.** determined the role of p51/63 isoforms in ameloblastic differentiation of mouse tooth germ by RT-PCR and western blotting using epithelium of molar tooth germ cell lines established from a mandibular tooth germ of p53 deficient mice. The authors' described that the expression of ΔNp51β/ΔNp63α (an isoform without transactivation domain), was detected at high level in immature cells, while the expression of TAp51/TAp63 isoforms, (isoform with the transactivation domain), was detected at high level in mature cells. Moreover, induction of TAp51α/TAp63γ expression led to down-regulation of ΔNp51β/ ΔNp63α expression and up-regulation of ameloblastin expression, a differentiation marker of amelogenesis. The authors' concluded that

p63 has important role in regulation of the proliferation and differentiation of tooth germ epithelial cells and its contribution towards tooth development **(17)**

II. EXPRESSION OF P63 IN AMELOBLASTOMA

In 2006, **Lo Muzio et al.** evaluated p63 expression in the epithelial lining of different odontogenic tumors such as unicystic ameloblastoma, solid ameloblastoma, peripheral ameloblastoma, ameloblastic carcinoma, adenomatoid odontogenic tumor, calcifying odontogenic cyst, primary intraosseous carcinoma and clear cell odontogenic carcinoma by immunohistochemistry for a better understanding of its biological role in oncogenesis. Monoclonal mouse anti-human p63 protein, clone 4A4 raised against aminoacids 1-205 of the N- terminal portion of human ΔNp63 protein (does not recognize TAp63 isoforms) was used in the study. The authors' described that p63 expression was found in the cells of basal layer and in most cases both in the cells of basal as well as superficial layer of unicystic ameloblastoma and in the peripheral as well as central epithelial cells of solid ameloblastoma, recurrent ameloblastoma and ameloblastic carcinoma. The authors' concluded that benign odontogenic, locally aggressive tumours with a high risk of recurrence exhibited statistically higher p63 expression than benign odontogenic, non-aggressive tumours with a low risk of recurrence. However, p63 expression did not significantly differ between benign, locally aggressive odontogenic tumour with a high risk of recurrence and malignant odontogenic tumour **(18)**.

In 2007, **Gratzinger et al.** evaluated the expression of p63,calponin, CK 7, CK 5/6, GFAP, SMA, S100 in odontogenic tumors such as ameloblastoma, calcifying epithelial odontogenic tumor, glandular odontogenic cyst, and

keratocystic odontogenic tumor. Mouse monoclonal, clone 4A4, p63 antibody in 1 : 200 dilution was used in the study. The authors' described that both CK 5/6 and p63 were strongly expressed in the basal half of the oral epithelium and generally not expressed in the mature superficial layers. This polarized loss of expression of p63 in superficial layers of oral epithelium is absent in solid odontogenic neoplasms such as ameloblastoma and CEOT, but is maintained in the cystic lesions such as KOT and GOC **(5)**.

In 2008, **Gurgel et al.** investigated the immunohistochemical expression of Ki-67, p53 and p63 in keratocystic odontogenic tumors (Nevoid basal cell carcinoma syndrome, sporadic KOTs, primary KOTs and recurrent KOTs) in order to contribute to the biological profile of the tumor. Monoclonal antibody against p63, clone 4A4 (recognizes both TAp63 and ΔNp63 isoforms) was used in the study. The authors' described that p63 was expressed more intensely in all epithelial layers of KOT with no difference in immunostaining observed between primary and recurrent KOTs or between NBCCS and sporadic KOTs.. The author's concluded that the p63 plays a role in the regulation of epithelial cell differentiation, and may favor tumorigenesis **(19)**.

In 2011, **Seyedmajidi et al.** evaluated the expression of p63 in odontogenic cysts such as dentigerous cyst, radicular cyst and keratocystic odontogenic tumor based on the differences in their clinical behavior by immunohistochemistry. The monoclonal mouse anti-human p63 protein, clone 4A4 was used in the study. The authors' described that the intensity of staining by p63 was greater in the nucleus of epithelial cells lining KCOT in comparison with radicular cyst and dentigerous cyst. The authors' concluded that the higher

expression of p63 in KCOTs, could be useful to explain the difference in the clinical and pathological behavior of KCOTs **(20).**

In 2013, **Moghadam et al.** investigated the immunohistochemical expression of p63 in odontogenic cysts and tumors such as odontogenic keratocyst, ameloblastoma, radicular cyst, dentigerous cyst and calcifying odontogenic cyst. P63 monoclonal antibody, clone 4A4 (Code N 1604 that recognizes Np63 isoforms) in 1:25 dilution was used in the study and p63 positive cells was calculated in the lining of odontogenic cysts and islands of ameloblastoma. The authors' described that all lesions showed intense reactivity in the odontogenic epithelium, as it was expressed through out the lining epithelium of odontogenic keratocyst except the surface parakeratinised layer, all layers of epithelial cells in calcified odontogenic cysts, basal and parabasal layers of radicular and dentigerous cysts (upper layers show weaker expression) and in peripheral cells of ameloblastoma except stellate reticulum which shows weaker immunostaining. They concluded that p63 protein is important in the proliferation and differentiation of odontogenic epithelial cells but it could not be a useful marker to differentiate between aggressive and non aggressive lesions. They also concluded that p63 can be a basal cell marker as it was not expressed in mature differentiated cells **(21)**.

In 2014, **Varsha et al.** investigated the expression of p63 in odontogenic lesions such as odontogenic keraocyst, solid ameloblastoma, unicystic ameloblastoma Type I and Type III and follicular tissue by immunohistochemistry. The authors' described that p63 was expressed in the peripheral columnar and central stellate reticulum – like polyhedral cells in ameloblastic islands of solid ameloblastoma. P63 expression was also seen in the

cystic lining of unicystic ameloblastoma Type I and Type III and intramural nodules in Type III ameloblastoma The authors' concluded that expression of p63 in OKC was comparable with that of solid ameloblastoma and unicystic ameloblastoma type III and significantly higher than that of unicystic ameloblastoma type I. They concluded that the higher expression of p63 in odontogenic lesions correlates well with their aggressive behavior and thereby suggesting alterations in treatment modalities **(22).**

In 2015, **Jaafari - Ashkavandi et al.** investigated the expression of p63 and Ki-67 in dentigerous cyst, unicystic and conventional ameloblastoma using immunohistochemistry. Mouse monoclonal anti-p63 antibody (clone 7JUL) was used in the study. The authors' described that the p63 was expressed in the basal as well as suprabasal layers of luminal and mural types of unicystic ameloblastoma and in the peripheral and **central** cells of follicular and plexiform subtypes of solid ameloblastoma. They found out that intensity of p63 expression was significantly higher in solid ameloblastoma and mural type of unicystic ameloblastoma than luminal type of unicystic ameloblastoma and dentigerous cysts. They concluded that the expression of p63 is directly related to the aggressive and invasive behavior of odontogenic lesions **(23).**

III. EXPRESSION OF AMELOGENIN IN HUMAN TOOTH GERM

In 1988, **Kircham et al.** did a study on maturation in developing permanent porcine enamel by assessing the mineral content per tissue volume and found out that the levels of mineralization correlated directly with the histological appearance of enamel organ. The author's found out that the tall ameloblasts associated with the secretary stage of enamel development was characterized by

relatively low levels of mineral (30% by volume) and high levels of organic matrix. Relatively shortened ameloblasts associated with the transition stage was characterized by massive loss of organic matrix but mineral content did not rise significantly on a volume basis. Ameloblasts with 50% reduction in height, associated with the maturation stage of enamel development was characterized by total loss of organic matrix but the mineral content was relatively low on a volume basis. The author's concluded that permanent porcine teeth erupt in a partially immature porous condition and thereafter the mineralization occurs by ingress of mineral ions from saliva **(24).**

In 1991, **Inai et al**. examined the synthesis and secretion of enamel protein by ameloblasts in the early stages of their development by immunohistochemistry in rat molar tooth germs. Immunohistochemical localization of amelogenin in the early stages of ameloblast differentiation was carried out at light and electron microscopic levels using amelogenin monoclonal antibody. The authors found out that the first appearance of amelogenin was detected in the presecretory ameloblasts prior to the breakdown of the basement membrane, whereas the expression of amelogenin was detected both prior to and after the breakdown of the basement membrane in the odontoblasts. However, the intensity of the staining increased in the predentin, intercellular spaces of odontoblasts and dental pulp cells after the breakdown of basement membrane, which indicated penetration of amelogenin from the presecretory ameloblast layer to the dental pulp. On the other hand, the staining in the odontoblast layer decreased markedly and spot-like staining was detected in the predentin once the enamel matrix was secreted. From these observations, the author's concluded that the penetration of enamel protein

toward the dental pulp and odontoblasts plays a role in the interaction between ameloblasts and odontoblasts **(25).**

In 1992, **Salido et al**. investigated the characterization of the AMGX and AMGY genes on the short arms of the human X and Y chromosomes which encode the amelogenins in the incisor tooth buds from six human fetuses ranging from 19 to 24 wk of gestation by fluorescence in situ hybridization. The author's found out that the AMGY locus on the Y chromosome also encodes a functional protein, although its level of expression was only 10% of that of the AMGX locus on the X chromosome **(26).**

In 1996**, Inage et al.** studied the gene expression and localization of amelogenin in the developing rat incisor by insitu hybridization and immunohisotchemistry. Polyclonal antibody against bovine amelogenin and antibody against C telopeptide of amelogenin were used in immunohistochemistry. The author's found out that both by insitu hybridization and immunohistochemistry, a weak reaction for amelogenin was evident in the inner enamel epithelium of the cervical loop and the expression became more intense in the cytoplasm of secretory ameloblasts, ameloblasts in the terminal secretion and transitional zones. The expression became weak in the shortening ameloblasts and at the ruffled border of the ruffle ended ameloblasts in the maturing zone and it became negative in the smooth ended ameloblasts in the maturing zone. No expression was found in the cells of the stratum intermedium and stellate reticulum throughout amelogenesis **(27).**

In 1997, **Karg et al**. studied the expression of amelogenin in embryonic and neonatal hamster teeth by in situ hybridisation and immunohistochemistry. During the bell stage, expression of amelogenin both by in situ hybridisation and

immunohistochemistry was detected in the presecretory ameloblasts, predentine and between odontoblasts. However, the expression was limited to the secretory ameloblasts and as well as transitional/ maturation ameloblasts once enamel is formed. The authors conclude that expression of amelogenin marks the differentiation of PSA, but is not expressed in the mesenchymal component of the tooth germ **(28).**

In 1997, **Zeichner David et al.** studied the timing of expression of enamel protein tuftelin in relation to amelogenin in developing mouse molars by immunohistochemistry, PCR and other techniques. The author's found out that by immunohistochemistry, tuftelin expression was first detected at E17 in the preameloblasts where it is synthesized and secreted into the developing extracellular matrix and dental papilla mesenchymal cells. In contrast, amelogenin expression was first detected at E19 in the polarized, non dividing, fully differentiated ameloblast cells. But mRNA transcripts of tuftelin was first detected at E13 (equivalent to bud stage of tooth development) and amelogenin transcripts was first detected at E15 (equivalent to early cap stage of tooth development). The author's concluded that the discrepancy between transcript detection and translation product detection might represent a difference in method sensitivity i.e., PCR is extremely sensitive, whereas immunodetection requires a larger number of molecules for detection **(29).**

In 1999, **Papagerakis et al**. studied the expression on amelogenin mRNA in rat incisors by insitu hybridization and other techniques and compared the expression pattern with vitamin-D deficient rats. The author's found that amelogenin mRNA was highly expressed in differentiated ameloblasts during the secretion stage as well as the maturation stage. In addition, amelogenin mRNA

was also detected in apparently smaller amounts in pre-ameloblasts located near the apical loop. No amelogenin mRNA expression was detected in the odontoblasts. But the steady state levels of amelogenin mRNA was decreased in vitamin -D deficient rats and an upregulation of mRNA expression was noted in the animal tissue section injected with 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$). From these findings the author's concluded that amelogenin gene expression is regulated by vitamin D **(30).**

In 1999, **Wakida et al.** studied the expression of amelogenin in maturation stage ameloblasts of porcine tooth germs by insitu hybridization and immunohistochemistry and compared the expression pattern with rat tooth germs. Amelogenin antiserum in 1:1000 dilution was used in the study. The author's found out that in the pigs, the secretory ameloblasts showed immunoreactivity in the infranuclear and supranuclear cytoplasm and also intense immunoreactivity was found in the enamel matrix from the surface upto a depth of about 40 μm and deeper to this only a faint reaction was observed. In the transition stage ameloblasts, immunoreactivity was detected only in the infranuclear cytoplasm. Immunoreactivity of immature enamel under the transition stage ameloblasts was found to decrease gradually and disappeared at the end of this stage. At the early maturation stage, immunoreactivity was absent in the maturation ameloblasts as well as in the surface layer of immature enamel. Other cells of the tooth germ such as outer enamel epithelium, stratum intermedium and odontoblasts showed no immunoreactivity throughout the developmental stages. But in the rat tooth germ, immunoreactivity of amelogenin was detected in ameloblasts till the early maturation stage by using the same antiserum. The author's concluded that the reason for persistence of enamel protein expression till the early maturation stage

in rodents was due to the rapid rate of enamel formation and continuously growing rat incisors compared with pigs. These results also indicated that the secretory activity of the maturation ameloblast is varied depending upon animal species **(31).**

In 2000, **Ravindranath et al.** demonstrated that a conserved GMp mimicking peptide(N-acetyl-D-glucosamine mimicking peptide) motif of cytokeratins specifically binds to the amelogenin trityrosyl motif peptide (ATMP) and suggested that amelogenins are "cytokeratin14 (intermediate filament)-binding proteins," similar to plectin, nestin, and desmocalmin. Phosphorylation of serine 16 of amelogenins resulted in their dissociation from intermediate filament. From these findings the author's postulated that intracellular amelogenin-keratin binding may be functionally involved in inhibiting amelogenin assembly and that the reason for such interaction could be to facilitate amelogenin secretion and also the polypeptide motifs, comparable with the cytokeratin GMp1, occur within currently unidentified matrix proteins and have a functional role in normal enamel biomineralization **(32).**

In 2001, **Ravindranath et al.** demonstrated the co-assembly of amelogenin-CK14 in the secretory ameloblasts, its migration from the perinuclear region to the apical region of ameloblasts and it's subsequent dissociation at Tomes' process using confocal laser microscopy,. The author's suggested that CK14 functions as a chaperon for nascent amelogenin polypeptides during amelogenesis **(33).**

In 2001, **Hu et al.** studied the expression of amelogenin and enamelin in developing molars of postnatal mouse by insitu hybridization. The author's found out that the amelogenin mRNA was first detected on preameloblasts in the cusp

slopes on day 2 and continued till day 14 throughout the secretory, transition and early maturation stages and ceased at the CEJ at day 21. Amelogenin mRNA hybridization signals were not detected in odontoblasts or dental pulp, bone, stratum intermedium, stellate reticulum and outer enamel epithelial cells of the enamel organ and also along the developing root **(34).**

In 2002, **Oida et al.** studied the expression of amelogenin mRNA in porcine tooth germs by RT-PCR. The author's found out that the amelogenin gene expression was detected in the secretory ameloblasts only after 20 cycles of PCR. However, when the PCR was carried out for 30 cycles, amelogenin gene products were found in the secretory and maturation stage ameloblasts, as well as in the odontoblast layer and a little expression was also detected in dental pulp cells. But, no amelogenin amplification products were detected in oral epithelium cells, connective tissue cells and muscle cells after 30 cycles. However, a trace of amelogenin gene expression was detected in the oral epithelium sample only after 40 cycles. The author's concluded that the secretory ameloblasts expressed amelogenin at a rate over 1000 times than that of odontoblasts. The positive reaction in the odontoblast layer was attributed to background staining **(35).**

In 2003, **Hatakeyama et al**. studied the expression of RANKL in wild-type controls and amelogenin- null mice by RT-PCR and immunohistochemistry. They reported that two mRNA splice forms of amelogenin, M180 and LRAP (Leucine Rich Amelogenin Peptide) were expressed in cementoblast/PDL cells of wild-type mice (control). However in amelogenin-null mice the expression of these two mRNA splice forms of amelogenin were absent. In addition, the amelogenin-null PDL cells showed more intense staining for RANKL near the bone and cementum surface, suggesting that the amelogenin played a key role in

the maintenance of cementum through the RANKL/RANK mediated osteoclastogenic pathway **(36).**

In 2003, **Nagano et al**. examined, semi-quantitatively the mRNA levels of various enamel proteins such as amelogenin, enamelin, sheathlin (ameloblastin/ amelin), enamelysin (MMP-20), and KLK4 (EMSP-1) in enamel organ epithelia and odontoblasts at different developmental stages of porcine permanent tooth germs by in situ hybridisation. In this study, the ratio of amelogenin mRNA for secretory EOE (SA), maturation EOE (MA), young odontoblast (YO), and mature odontoblast (MO) samples was found to be 4096:2:64:1. The author's concluded that, in the pig, the secretory ameloblasts express amelogenin mRNAs at levels thousands of times higher than do maturation ameloblasts and odontoblasts lining dentin beneath maturing enamel, and hundreds of times higher than do odontoblasts lining dentin beneath secretory-stage enamel. From these findings the author's demonstrated that amelogenin mRNAs were expressed also by odontoblasts and suggested that they might play a role in the formation of dentin at the EDJ **(37).**

In 2003, **Papagerakis et al.** studied the developmental expression pattern of amelogenin in the odontoblasts of rat tooth germs through various in vitro and in vivo approaches at both the mRNA and the protein level. The author's found out that amelogenin expression was detected not only in the ameloblasts, but also in the young odontoblasts and stratum intermedium, concomitant with the deposition of mantle dentin. Once the formation of mantle dentin was complete, expression of Amel had diminished progressively within the maturing odontoblast and stratum intermedium cell layers. In contrast, amelogenin expression was not found in the preodontoblasts, mature odontoblasts, dental pulp

cells and odontoblasts associated with root formation. The author's concluded that the odontoblasts express Amel in a highly restricted development-dependent pattern associated with early mantle dentin formation and it had been characterized as a useful marker to distinguish different stages of odontoblast cell lineage **(38).**

In 2004, **Denbesten et al.** characterized a culture system to identify the human primary enamel organ epithelial cells and evaluated the expression of cytokeratin 14, amelogenin, ameloblastin, enamelin, MMP- 20, KLK-4 by RT-PCR. Tooth organs dissected from approximately 21 week old human fetal cadaver tissues was used in the study. The pooled tooth organs were washed in phosphate buffered saline, digested with 2mg/ml of collagenase/ dispase and cultured in supplemented keratinocyte media KGM-2 with or without serum and with different calcium concentrations. The authors' described that the cells isolated from this media were mixture of cobblestone ameloblast like cells and spindle shaped cells. The cobblestone ameloblast like cells were found to be immunopositive for cytokeratin 14, amelogenin, ameloblastin, enamelin, MMP-20 and KLK-4 by RT-PCR. The spindle cells are likely to be derived from mesenchymal cells and they are not immunopositive for cytokeratin 14. They also described that the increasing calcium concentration in the culture increases the presence of spindle shaped cells and formation of nodules. They suggested that analysis of mRNA with cDNA array for osteoblast/ odontoblast related mRNA confirmed that these cells did not synthesize type I collagen or other noncollagenous matrix proteins characteristic of osteogenesis (odontogenesis) related cells **(39).**

In 2005**, Quintana et al.** studied the expression of amelogenin and ameloblastin in developing molars of mice from postnatal days 1-15 by insitu hybridization. The author's found out that the amelogenin mRNA was detected in the secretory ameloblasts on the cusptip at day 3 and continues till day 7, after which the level of amelogenin mRNA gradually decreased, and at day 10 the expression was completely absent. The author's concluded that the amelogenin expression disappeared by the time the ameloblast morphology indicates the beginning of the maturation stage and ceases at the cementoenamel junction **(40).**

In 2006, **Yan et al.** characterized a culture system to identify differentiated ameloblast-lineage cells and evaluated the expression of cytokeratin 14, amelogenin and ameloblastin in human ameloblast- lineage cells for their synthesis of matrix proteins by immunoflourescence technique. Tooth organs from 19–24-wk-old human fetal cadavers were either frozen and cryosectioned for immunostaining, or digested in collagenase/ dispase for cell culture. Protein A affinity - purified antibody developed in rabbit exposed to recombinant human amelogenin in 1 : 2000 dilution was used in the study. Epithelial clones with two distinct morphologies, including smaller cobblestone-shaped cells and larger (5–15 times in size) rounded cells were formed. Both cell types stained for cytokeratin 14 indicating the epithelial phenotype of these culture cells and the larger cells appeared more differentiated, showing stronger staining for amelogenin and ameloblastin. The authors' also described that the expression of cytokeratin 14 begins to decrease as the expression of amelogenin increases and suggested that cobblestone- shaped cells are derived from undifferentiated enamel epithelium (inner and outer), and the larger cells are characteristic of preameloblast and ameloblast like cells **(41).**

In 2006**, Ye et al.** studied the expression of amelogenin in human developing and mature dental pulp tissue by in situ hybridization, immunohistochemistry and other techniques. The author's found out that amelogenin expression was evident in the developing dentin of a developing tooth organ where enamel matrix had not formed yet and also in the odontoblast layer of the adult human dentin. From these results the author's suggested that amelogenins might not directly interact with pulp cells during development, but they had a role in epithelial/mesenchymal interactions related to enamel and dentin formation **(42).**

In 2007, **Haze et al**. studied the expression of amelogenin in long bone cells, cartilage cells and in bone marrow progenitor cells by insitu hybridization and indirect immunohistochemistry in rats and dogs. LF-108, a polyclonal rabbit amelogenin antibody, raised against a synthetic peptide corresponding to 10 amino acids at the C-terminus of human amelogenin (cross-reacts with dog and rat, diluted to 1/1000 for rat tissues and 1/2000 for dog tissues) and 110BQ, a monoclonal mouse antibody raised against human amelogenin (diluted to 1/1000) were used in the study. Amelogenin mRNA and protein were found to be expressed in long bone periosteum, which is composed of progenitor cells, bone marrow cells, osteoblasts and osteoclasts lining the bone trabeculae and by some osteocytes. Amelogenin mRNA and protein were also found to be expressed in articular cartilage chondrocytes, epiphyseal bone cells, and differentially in specific cell layers of the epiphyseal growth plate. From these findings the author's suggested that amelogenin has a crucial role in the processes of bone development and remodeling **(43).**

In 2009, **Cohen et al.** focused on the spatio-temporal expression of amelogenin in different tissues of the developing embryonic mouse craniofacial complex, such as the tooth germ, brain, eye, ganglia, peripheral nerve trunks, cartilage and bone using immunohistochemistry. The antibodies used were: (a) 270- Polyclonal rabbit antibody, raised against amelogenin N-terminus (identical in mouse, human etc.) diluted in Phosphate buffered saline to 1:500 - 1:1000. (b) 859 polyclonal rabbit antibody raised against rat amelogenin. The authors' described that amelogenin is expressed in various tissues of the developing mouse embryonic craniofacial complex such as brain, eye, ganglia, peripheral nerve trunks, cartilage and bone, and is first expressed at E10.5 in the brain and eye, long before the initiation of tooth formation. Application of amelogenin (recombinant human amelogenin protein) beads together with DiI, on E13.5 and E14.5 embryonic mandibular mesenchyme and on embryonic tooth germ, revealed recruitment of mesenchymal cells. The authors' concluded that amelogenin is expressed in many tissues of the cranio-facial complex during mouse embryonic development and differentiation, pointing to the possibility that it might be a multifunctional protein **(44).**

In 2011**, Nel et al**. studied the immunohistochemical expression of amelogenin in odontogenic epithelium of developing dog teeth. Amelogenin (FL-191, Santa Cruz Biotechnology) in 1:50 dilution was used in the study. The author's found out that amelogenin staining was not found at the bud and cap stage of tooth development, but during the late bell stage small amounts of amelogenin staining was observed in the presecretory ameloblasts in close approximation to the cell nucleus. All secretory ameloblasts show diffuse granular staining for the amelogenin protein. In contrast amelogenin staining was not

detected in the IEE, OEE, stellate reticulum, stratum intermedium, dental laminae, Serres rests, or alveolar epithelium. Also no staining was observed in and around odontoblasts at advanced stages of odontogenesis, but the odontoblasts that were associated with the mantle dentin stained with amelogenin. The author's concluded that amelogenin was a promising marker to distinguish between odontogenic and nonodontogenic tissues in dogs **(45).**

In 2011, **Gutierrez-Cantu et al**. studied the extracellular and intracellular spatial distribution of amelogenin and enamelysin in the enamel organ of the human tooth germ during its development by immunoflourescent labeling using confocal microscope. During the early bell stage amelogenin labeling was seen more than enamelysin, as free immunereactive granular patches towards basal membrane between ameloblast and odontoblast. Increased amelogenin expression and secretion was found towards extracellular matrix formation region whereas enamelysin distribution was only perinuclear in early bell stage. But, during the late bell stage decreasing amelogenin labeling was found in contrast with enamelysin, suggesting specific temporal amelogenin degradation. Enamelysin expression was evident in the ameloblast and stellate reticulum cytoplasm whereas, amelogenin expression was observed inside ameloblasts, stellate reticulum, and stratum intermedium cells in the enamel organ as well as in the newly formed dentin extracellular matrix **(7).**

In 2012, **Landin et al.** studied the expression of genes such as amelogenin, ameloblastin and enamelin during early (presecretory stage) stages of murine tooth development (i.e.) starting at the 11^{th} embryonic day and ending at 7^{th} day after birth by microarray analysis, insitu hybridization, real time PCR and western blotting. The author's found out that in all the above mentioned methods Ambn,

Amelx, and Enam had similar expression pattern throughout the developmental stages, showing low pre-natal levels of expression compared to post-natal levels. Although expression levels of Ambn, Amelx, and Enam were lower during early tooth development compared to secretory stages, enamel proteins were detectable by Western-blotting **(46).**

In 2013, **Zheng et al.** conducted a study and found out that clock genes direct ameloblast function by controlling several ameloblast-specific genes including amelogenin (marker of secretory ameloblasts) and klk4 (marker of maturation ameloblasts) at RNA and protein level. The author's found out that amelogenin and klk4 undergo a regular 24 hr oscillations. This circadian rhythm was evident in the human enamel as cross-striations traversing enamel prisms. The authors also suggested that higher level of amelx protein expression occurs in phase with the awake status of rodents i.e) 10p.m. to 10a.m. The author's concluded that genetic modification in clock genes could account for variation in enamel characteristics such as shape, thickness and hardness among individuals **(47).**

In 2014, **Mitsiadis et al.** studied the expression of amelogenin protein in human developing, injured and carious teeth by immunohistochemistry. Human fetus tissues in 18-30 gestational week and rabbit polyclonal antibody against mouse amelogenin in 1:1000 dilution were used in the study. During the early bell stage (18^{th} – 21^{st} gestational week) amelogenin staining was evident at the differentiating IEE cells at the cusp tip, newly deposited predentin, proliferating inner enamel epithelial cells of the cervical areas and few stratum intermedium and stellate reticulum cells. During the late bell stage (30^{th} gestational week) strong amelogenin staining was evident at the functional ameloblasts (secreting

enamel matrix), differentiating odontoblasts and to a lesser extent in the cells of outer enamel epithelium. In the enamel matrix a zebra like pattern of amelogenin protein distribution was observed i.e.) two amelogenin positive layers at the ends splitted by an amelogenin negative zone. The author's concluded that the zebra like pattern of amelogenin protein distribution in the enamel could be due to differential amelogenin processing during the secretory stage **(6)**.

In 2015, **Gasse et al**. studied the expression of four enamel matrix protein genes amelogenin, ameloblastin, enamelin and amelotin during amelogenesis in the lizard by in situ hybridization. The author's found out that the *AMEL* expression was first evident in the secretory ameloblasts located at the cusp tip during early enamel matrix deposition. But, during the maturation stage AMEL expression was absent in the ameloblasts at the tooth tip but was still present in those towards the tooth base, on which enamel matrix is still deposited. In addition, AMEL transcripts were also detected in the odontoblasts facing the recently deposited predentin matrix at this stage. Also, when the tooth was nearly functional *AMEL* transcripts were no longer detected in the reduced ameloblasts, but were still faintly present in odontoblasts in the upper region of the pulp cavity **(48).**

In 2016, **Kim et al**. studied the differential expression of amelogenin, enamelin and ameloblastin proteins in rat tooth germ development by western blotting and immunoflourescence. Rabbit polyclonal antibody against amelogenin was used in the study. In western blotting the author's found out that the expression of both amelogenin and enamelin began at the crown stage and increased in an time dependent manner. In contrast, the expression of ameloblastin began at the early bell stage and maintained it's expression till the root stage. In

immunofluorescence, expression of amelogenin was evident in the tall columnar preameloblasts/ ameloblasts and in the secretory ameloblasts at the crown stage. But, the expression of amelogenin in the secretory ameloblasts stopped, when the enamel began to mature. In addition, amelogenin expression was also evident occasionally in the preodontoblasts or odontoblasts but not in the dentin matrix **(49).**

In 2016, **Frasheri et al**. did a research with the title as "full length amelogenin influences the differentiation of human dental pulp stem cells". The author's treated human DPSCs with amelogenin at a concentration of 10ng/ml, 100ng/ml and 1000 ng/ml and compared it with the unstimulated control in terms of cell morphology, proliferation, mineralization and gene expression for ALP(alkaline phosphatase), DMP-1 (dentin matrix protein -1) and DSPP (dentin sialophospho protein). The author's found out that the proliferation of human DPSCs at 2 weeks was significantly reduced in the presence of highest concentration of amelogenin when compared to the unstimulated control. From these findings the author's suggested that full length amelogenin influences differentiation of human DPSCs in relation to various concentrations **(50).**

In 2016, **Mazumder et al**. studied the spatial interaction of amelogenin and ameloblastin around maturing enamel rods by immunochemical analysis. Chicken anti-amelogenin (Amel-FL [amelogenin full-length antibody], goat anti-amelogenin (Amel-C19 [amelogenin C-terminal antibody],goat antiamelogenin-Nt (Amel-Nt [amelogenin N-terminal antibody], rabbit anti-ameloblastin (Ambn-M300 [ameloblastin C-terminal antibody] and goat anti-ameloblastin (Ambn-N18 [ameloblastin N-terminal antibody] were used in the study. The results revealed that the Amel-FL and Amel-Nt immunostained the entire thickness of enamel

matrix while Amel-C19 stained only the matrix adjacent to the secretory face of ameloblasts. Immunostained with Ambn-M300 revealed that the cleaved fragments generated from major C-terminal processing leave the enamel layer, while immunostaining with Ambn-N18 showed that the N-terminal portions of ameloblastin persist for longer periods in the enamel layer. These results suggested that N-terminal proteolytic products of ameloblastin and amelogenin colocalized around enamel rods, forming a "fish net" pattern of the matrix throughout the enamel thickness **(51).**

In 2017, **Gutierrez-Cantu et al**. studied the expression of enamel proteins amelogenin and enamelysin in rough endoplasmic reticulum and golgi complex in human dental germs by histochemistry. Anti amelogenin polyclonal antibody prepared from amelogenin of 11 day old rats in 1:1000 dilution was used in the study. The author's found that in early stages a colocalization of both proteins was found inside the Golgi apparatus with amelogenin being more evident in relation with golgi complex and in late stages, a colocalization of both proteins was found in the rough endoplasmic reticulum with enamelysin being more evident in relation with rough endoplasmic reticulum. The author's also demonstrated the presence of amelogenin and enamelysin in odontoblast and ameloblast and in dental papilla cells. However, the presence of these two proteins in odontoblast and dental papilla cells remain unknown.The author's suggested that the amelogenin expression in papilla could be due to degradation of amelogenin by enamelysin, and these are nothing but the rests of amelogenin in dental papilla cells **(8).**

IV. EXPRESSION OF AMELOGENIN IN AMELOBLASTOMA

In 1991, **Mori et al.** evaluated the immunohistochemical expression of amelogenin in odontogenic cysts and tumors such as ameloblastomas, granular cell ameloblastomas, adenomatoid odontogenic tumours, ameloblastic fibromas, squamous odontogenic tumours and odontomas. A monoclonal antibody mAb to bovine amelogenins (IgM) in 1:1000 dilution was used. The authors' described that amelogenin was expressed more intensely in the peripheral cells of follicular ameloblastomas, granular cells in granular ameloblastoma, pseudoglandular and ductal structures of adenomatoid odontogenic tumor, non-keratinized tumour cells of squamous odontogenic tumor, epithelial cells of ameloblastic fibroma, eosinophilic amorphous material and tumour cells of CEOT, ghost cells of COC and reduced ameloblasts of odontoma. The results of the study indicate that amelogenin is not only expressed in ameloblastoma but also in other odontogenic tumours and as well as in the eosinophilic amorphous matrix and ghost cells **(52).**

In 1992, **Snead et al.** investigated the gene expression parameters for human ameloblastomas with the techniques of mRNA phenotying such as RT-PCR, insitu hybridization and northern blot analysis. The author's observed that both strands of the human amelogenin genes are transcribed by epithelia of the ameloblastoma, which results in the detection of RNAs that correspond to either the sense or antisense orientation. But in developing mouse molars, the sense orientation of the amelogenin probe fails to produce hybridization signals. The author's concluded that in ameloblastoma the antisense RNA had the ability to bind to the sense strand of mRNA thus preventing it from being translated to proteins and this might account for the inability of ameloblastoma to produce matrix **(53).**

In 1992, **Saku et al.** analysed the expression of amelogenin and enamelin in odontogenic tumours such as ameloblastoma AOT, CEOT and COC and compared with that of expression of keratin. Twenty human tooth germs of various stages, from bud stage to the early stage of apposition and mineralization of enamel and dentin and twenty bovine tooth germs with mineralization were used as control. The antibodies to amelogenin and enamelin were prepared from immature enamel of bovine tooth germs and were raised in rabbits and purified by passing through columns of antigen coupled Sepharose 4B. The authors' described that the amelogenin and enamelin were expressed intensely in the cytoplasm of secretory ameloblasts and in the extracellular enamel matrix of tooth germs. Keratin was expressed in the ameloblasts irrespective of the stages of maturation but, enamel matrix was not stained with antikeratin antibodies in tooth germ. In ameloblastoma, neither amelogenin nor enamelin was expressed in either the extracellular matrix or the tumor cells of basal- columnar and stellate types. Keratin was expressed intensely in most of the tumor cells especially those located in the centre of tumor cell nests. The author's concluded that negative immunostaining in ameloblastoma was due to the absence of participation of ameloblasts in the histogenesis of ameloblastoma and the ameloblastoma cells are too immature to express a detectable amount of enamel proteins **(54).**

In 2001, **Kumamoto et al.** detected the immunohistochemical expression of amelogenin and cytokeratin 19 in epithelial odontogenic tumors such as ameloblastomas, calcifying epithelial odontogenic tumors, clear cell odontogenic tumors and malignant ameloblastomas and compared with that of five human tooth germs (crown mineralization stage) and ten non-odontogenic epithelial tumours. Rabbit antibovine amelogenin polyclonal antibody in 1:200 dilution was

used in the study. The authors described that the amelogenin was expressed intensely in the peripheral columnar or cuboidal cells and in some central polyhedral cells of follicular and plexiform ameloblastoma, granular cells of granular cell ameloblastoma and less intensely in the basal cell ameloblastoma and desmoplastic ameloblastoma. Amelogenin was expressed diffusely in neoplastic cells and faintly in intercellular amyloid like material of CEOT and also it is faintly expressed in the neoplastic cells of CCOT. In tooth germ amelogenin was expressed more intensely in the enamel matrix and inner enamel epithelium and less intensely in the stratum intermedium and stellate reticulum. Outer enamel epithelium, dental lamina of tooth germ and non-odontogenic epithelial tumours were negative for anti-amelogenin antibody **(55).**

In 2001, **Abiko et al**. investigated the immunolocalization of amelogenin in odontogenic tumours such as ameloblastoma, adenomatoid odontogenic tumour, calcifying odontogenic cysts, odontomas and primary intraosseous carcinoma using an anti-amelogenin polyclonal antibody raised against bovine amelogenin in 1:200 dilution. The authors' revealed that positive immunoreactivity for amelogenin was observed in adenomatoid odontogenic tumour, calcifying odontogenic cysts, primary intraosseous carcinoma and odontomas, but no such positive reaction was detected in ameloblastoma. The authors' concluded that polyclonal anti-amelogenin antibody against bovine amelogenin used in this study is useful for the determination of odontogenic tumours in which small mineralized foci are present in the epithelial nests **(9).**

In 2005, **Chen et al**. studied the expression of amelogenin and calcium transport proteins such as plasma membrane calcium pump and calbindin in ten conventional ameloblastoma (solid) and tooth germ of rat mandible by

immunohistochemistry and the presence of calcium in tissue sections by von kossa. Polyclonal anti-amelogenin antibody prepared against the full-length recombinant M179 mouse amelogenin, which cross reacts with all known amelogenin isoforms was used in the study at a dilution of 1:750. The authors' described that the expression of amelogenin was minimal during the presecretory ameloblasts, but gradually increased during the secretory stage ameloblast suggesting that enamel matrix formation begins only after the presecretory stage in rat incisor tooth. In ameloblastoma amelogenin is expressed in both stellate reticulum-like cells and ameloblast-like cells, which suggests differentiation beyond the presecretory stage. Based on the similarity of expression of amelogenin in tooth germ and ameloblastoma, the authors' conclude that ameloblastomas recapitulate a stage of differentiation beyond the presecretory stage. However, the mechanisms of extracellular matrix formation and mineralization associated with normal amelogenesis may be uncoupled in ameloblastomas **(10).**

In 2005, **Tsujigiwa et al**. analysed the expression of amelogenin in chromosome X and Y in both male and female patients with ameloblastoma by RT-PCR, in situ hybridization and immunohistochemistry. However, expression of amelogenin was detected with both RT-PCR and in situ hybridization techniques but not with immunohistochemistry. The authors' also showed that all male patients with ameloblastoma displayed predominance of Y chromosome which is unlike the expression pattern in tooth germ. The authors' postulate that epigenetic alteration in chromosome may correlate with tumorigenesis in ameloblastoma **(56).**

In 2011, **Ren et al.** investigated the gene expression profile of enamel matrix proteins and osteogenic associated proteins in ameloblastoma, keratocystic odontogenic tumor and calcifying epithelial odontogenic tumor by immunohistochemistry, cell culture, RT-PCR, Insitu alkaline phosphatase activity. Antibodies for cytokeratin 14, amelogenin, ameloblastin, enamelin, and odontogenic ameloblast associated protein in 1 : 50 dilution were used in the study. The authors' described that all cell populations were positive for cytokeratin 14, confirming their epithelial phenotype. Among the enamel matrix proteins, more intense staining was seen for AMEL and ODAM A and less intense staining was seen for AMBN in all the tumors studied. In ameloblastoma and keratocystic odontogenic tumor amelogenin staining was intracellular whereas in calcifying epithelial odontogenic tumor, amelogenin stains the nuclei **(57).**

In 2012, **Crivelini et al.** evaluated the expression of odontogenic ameloblast-associated protein (ODAM), amelotin (AMTN), ameloblastin (AMBN) and amelogenin (AMEL) by immunohistochemistry in samples of adenomatoid odontogenic tumor (AOT), calcifying epithelial odontogenic tumor (CEOT), developing odontoma, ameloblastoma, calcifying cystic odontogenic tumor (CCOT), ameloblastic fibroma (AF), myxoma, odontogenic fibroma (OF) and reduced enamel epithelia (REE). Mouse anti-human amelogenin (AMEL) in 1:500 dilution was the primary antibody used in the study. Ameloblastoma and CCOT were negative to amelogenin, both of which show ameloblast-like cells, while it was positive in the ameloblast-like cells of ameloblastic fibroma. In addition, amelogenin was negative in the ghost cells in CCOT and in mineralized tissue in AOT. The authors' regard the lack of amelogenin expression in

ameloblastoma was due to undifferentiated state of the peripheral columnar cells, but were silent about the expression in ameloblastic fibroma **(11).**

In 2016, **Anigol et al.** evaluated the expression of amelogenin immunohistochemically in rat tooth germ, odontogenic epithelium of follicular tissue, odontogenic cysts and tumours such as dentigerous cysts, radicular cysts, solid/ multicystic ameloblastoma, unicystic ameloblastoma, desmoplastic ameloblastoma, squamous odontogenic tumor, adenomatoid odontogenic tumor, keratocystic odontogenic tumor, odontoma, calcifying cystic odontogenic tumor and ameloblastic carcinoma. Rabbit polyclonal antibody raised against AMELX/ AMELY in 1 : 200 dilution was used in the study. The authors' described that the amelogenin was expressed in secretory ameloblasts and odontoblasts with moderate intensity, stellate reticulum with less intensity of rat tooth germ. They also described that amelogenin is moderately expressed in the peripheral ameloblast like cells compared with stellate reticulum like cells in all ameloblastomas except unicystic ameloblastoma where amelogenin is more intensely expressed in basal layer (ameloblast like cells) and moderately expressed in stellate reticulum like cells. This explains the mature state of ameloblastoma like cells with high differentiation in unicystic ameloblastoma which correlates with its less aggressive clinical behavior. The authors' concluded that the amelogenin expression in hard tissue formative lesions indicate the advancing differentiation of the tissue. The diffuse and constant expression in the epithelium predict the less likelihood of recurrence of the lesion **(12).**

MATERIALS AND METHODS

- The research project was reviewed and approved by the Institutional Review Board/ Institutional Ethical Committee before the study.
- Six spontaneously aborted and unclaimed dead fetal tissues from 12th week to 24th week was obtained from Institute of Obstetrics and Gynaecology, Chennai with prior permission. From these dead foetal tissues, jaws were dissected and separated into upper and lower and segmented into left and right sections thus obtaining 24 samples. These samples, were decalcified in 8% HCl for one hour, fixed in 10% formalin, dehydrated, embedded in wax block and sectioned sagitally in 3.5 microns thick serially cut sections. Among the 24 wax blocks which were obtained from the dead fetus tissues, twelve late bell stage (LBS) tooth germ sections and six early bell stage (EBS) tooth germ sections were made and confirmed with histological observation. Along with this, three wax blocks of late bell stage (LBS) tooth germ were retrieved from the archival samples of Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai.
- Fifteen archival samples of different histological variants of ameloblastoma were retrieved from Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai and confirmed with histological observation.
- Immunohistochemical markers purified anti-p63 antibody 4A4 clone (BioCare) and amelogenin (Biorbyt) were purchased and stored at 2-8˚ C in the refrigerator.

Materials Required:

- Soft tissue rotary microtome – manual
- Tissue floatation bath
- Glass slides (both normal and APES coated)
- Coplin jars
- Cover slip
- pH meter
- Tissue paper
- Induction stove
- Pressure cooker
- Weighing machine
- Micropipette (1-10µl and 5-50µl)
- Research microscope with photographic attachment

Reagents used:

- Hydrochloric acid
- Formalin
- Xylene
- Alcohol
- Distilled water
- Hydrogen peroxide
- PBS (Sodium Chloride (8gm), Potassium Dihydrogen Orthophosphate (1.5 gm), Disodium Hydrogen Orthophosphate Anhydrous (8.5 gm) Distilled water 1000 ml at pH – 7.4)
- Tween 20

- Citrate buffer (Tri- sodium citrate dihydrate (2.94 gm), 1N HCl (5ml), Distilled water (1000 ml) at pH - 6)

METHODOLOGY:

- Immunohistochemical staining was performed with the following markers, anti-p63 antibody (Mouse Monoclonal, clone 4A4, 1:25 dilution, CM163 – BioCare, USA) and AMELX antibody (Rabbit Polyclonal, 1:200 dilution, orb140077 – Biorbyt, UK) diluted in PBS, for 3.5 microns thickness foetus tissue sections and ameloblastoma sections mounted on APES coated glass slides as per manufacturer's instructions.
- Sections were dewaxed and deparaffinized by three changes of xylene and hydrated through descending grades of alcohol. Endogenous peroxidase was blocked in 3% H_2O_2 for 20 minutes and washed properly in distilled water 5 mins twice. Antigen retreival was done in pressure cooker at 120˚ C for 15 mins at pH-6 (citrate buffer) and washed in distilled water 2 changes - 5 mins each followed by PBS 3 changes – 5 mins each. Primary antibody in the above said dilutions was incubated for one hour in a moist chamber at room temperature and washed in PBS 2 times, 2 mins each, followed by incubation with secondary antibody (Mouse/Rabbit Polydetector Plus DAB HRP Brown Detection System – BSB 0257- Bio SB, USA) as per manufacturer's instructions. The sections were rinsed properly in distilled water and counterstained with Ehrlich hematoxylin for 10 seconds, dehydrated in ascending grades of alcohol and mounted using DPX mountant.
- Evaluation was done based on staining as mild, moderate and intense in cells of interest by visual identification of the immunoreactivity in the

tissue sections using the Olympus Research Microscope (Model BX43F). In tooth germ the cells of the enamel organ such as inner enamel epithelium (IEE), preameloblasts (PA), presecretory ameloblasts (PSA) and secretory ameloblasts (SA) were selected and observed for immunoreactivity. In ameloblastoma, the peripheral cells were selected because they resemble the normal development of enamel organ such as inner enamel epithelium (IEE), preameloblasts (PA), presecretory ameloblasts (PSA) and secretory ameloblasts (SA).

Immunohistochemical controls:

Oral squamous cell carcinoma (well- differentiated) was used as a positive control for p63. Human late bell stage tooth germ with formation of enamel matrix was used as a positive control for amelogenin. For negative control the primary antibody was replaced by antibody diluent and confirmed to be unstained.

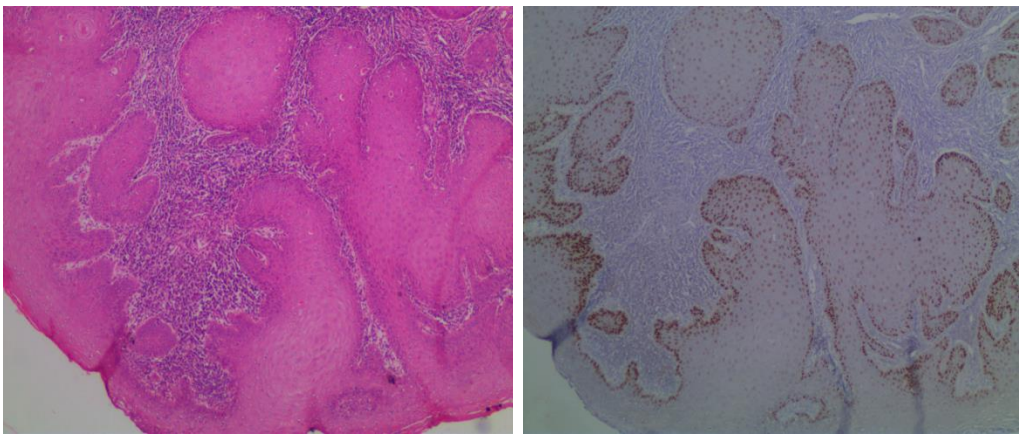
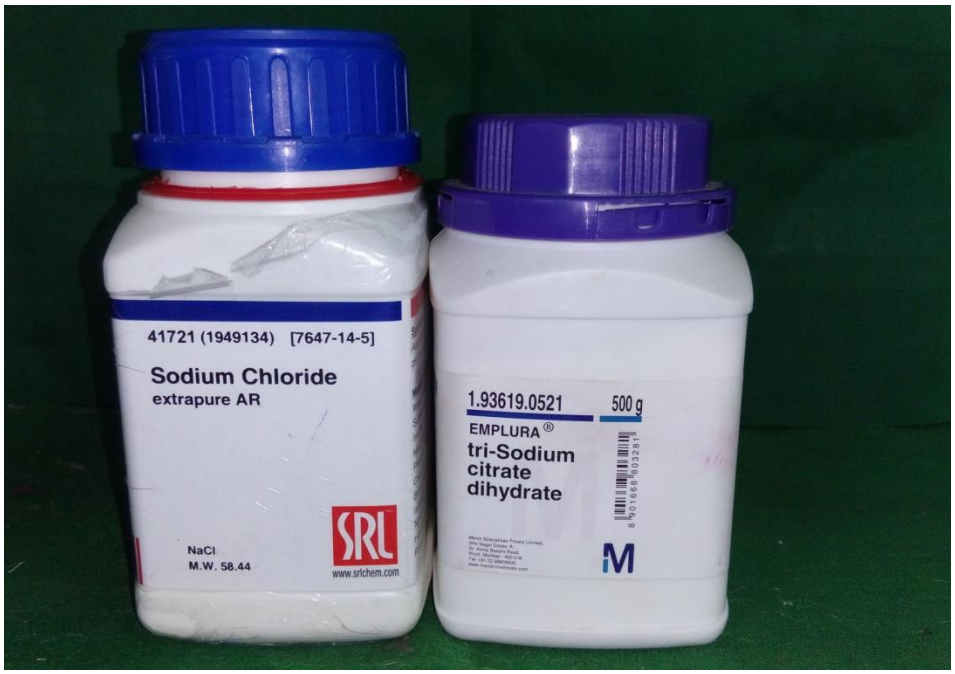


Figure : Oral squamous cell carcinoma (well - differentiated) showing expression of p63 (control).

ARMAMENTARIUM


41721 (1949134) [7647-14-5]
Sodium Chloride
extrapure AR
NaCl
M.W. 58.44
SRL
www.srlchem.com
1.93619.0521
500 g
EMPLURA®
tri-Sodium
citrate
dihydrate
M


sartorius
sartorius
Proline 5 - 50 µl


BioSB
BIOSCIENCE
HRP LABEL
REF BSB 0257
Volume 15 ml
DAB BUFFER-SUBS
biosb.com


NICE
LABORATORY REAGENT
HYDROGEN PEROXIDE 30% W/V
H_2O_2
100 VOLUMES
Assay 30 - 32% W/V
N.V.M. 0.1% max
Chloride (Cl) 0.005% max
Heavy Metals (Pb) 0.0005% max
PINHOLE IS PUT IN THE INNER LID TO AVOID PRESSURE DEVELOPMENT.
KEEP THE BOTTLE UPRIGHT.
No liability accepted for accidents in use or handling.
NICE CHEMICALS Pvt.Ltd., P.B.No.2217, Manimala Road, Ettupatta, Kochi - 682024, Kerala, India, E-mail : mail@nicechemicals.com
Customer Care Manager : Ph: 0484 - 2800212
E-mail: marketing@nicechemicals.com
NICE
D.P.X. MOUNTANT
NICE CHEMICALS (P) LTD.

RESULTS

Expression of p63 in human tooth germ and ameloblastoma

Immunohistochemical expression of p63 was intensely positive in the proliferating cells of the tooth bud, dental lamina, basal and supra basal cells of oral epithelium and skin epithelium and also in the basal cells of hair follicle and salivary duct.

Early bell stage:

During the early bell stage, expression of p63 was intensely positive at the cervical loop, stratum intermedium, stellate reticulum and outer enamel epithelium. But, in the inner enamel epithelial lineage, as the cell progresses from the cervical loop to the cusp tip, both the intensity and number of p63 positive cells decreases **(Figure 1).**

Late bell stage:

During the late bell stage, expression of p63 was intensely positive only at the cervical loop region. In stellate reticulum, stratum intermedium and in outer enamel epithelium the number of p63 positive cells decreases and only a sporadic expression was evident **(Figure 2).**

Ameloblastoma:

Among the peripheral cells of ameloblastoma, the intensity of p63 expression varies, indicating that the peripheral cells of ameloblastoma are at different levels of differentiation **(Figure 3).**

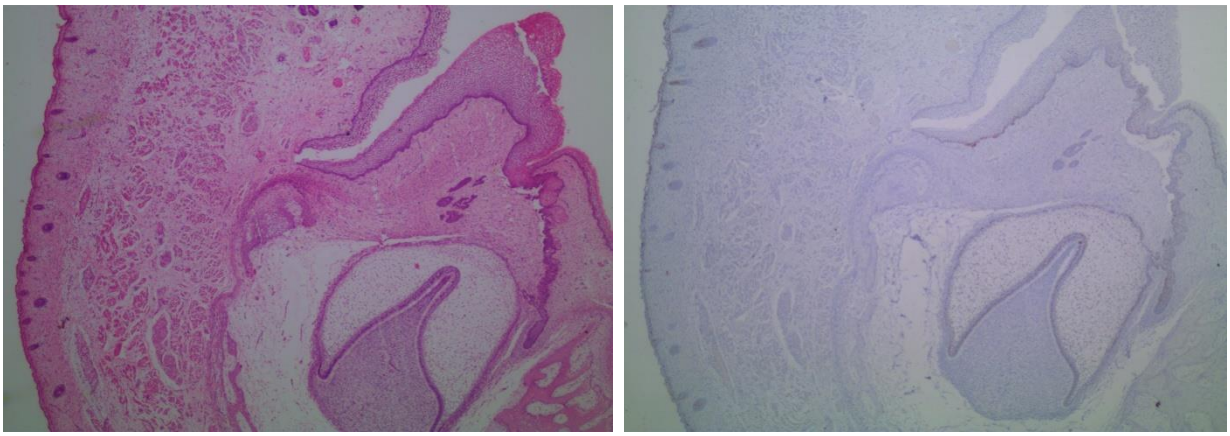


Figure 1A

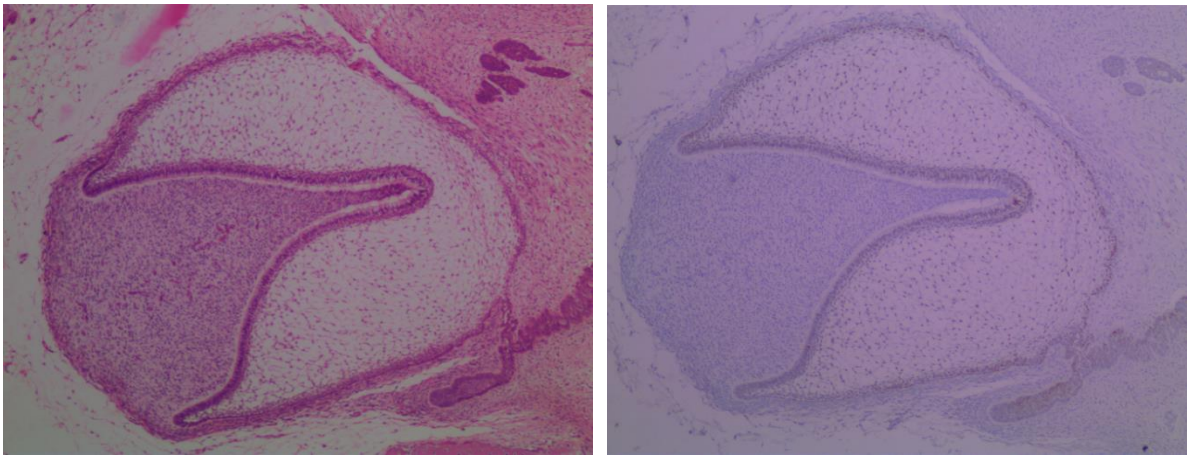


Figure 1B

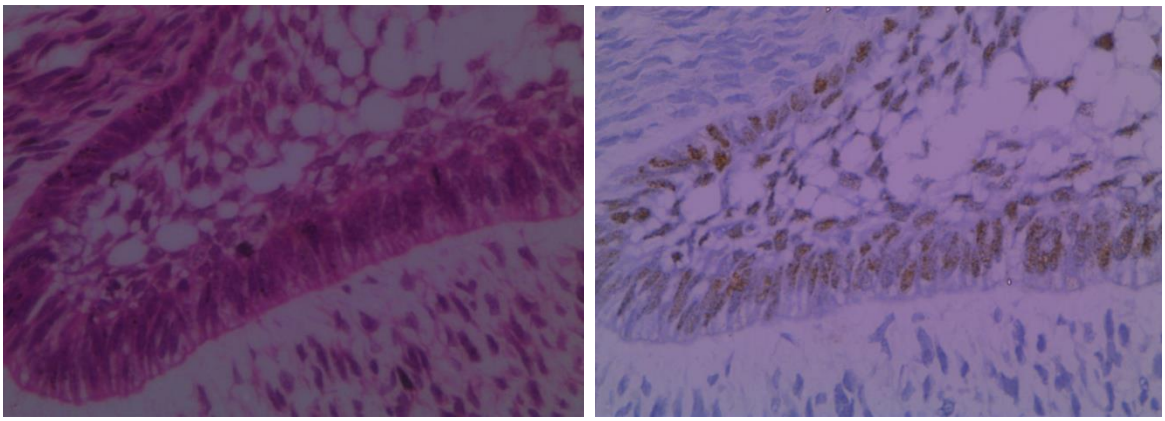


Figure 1C

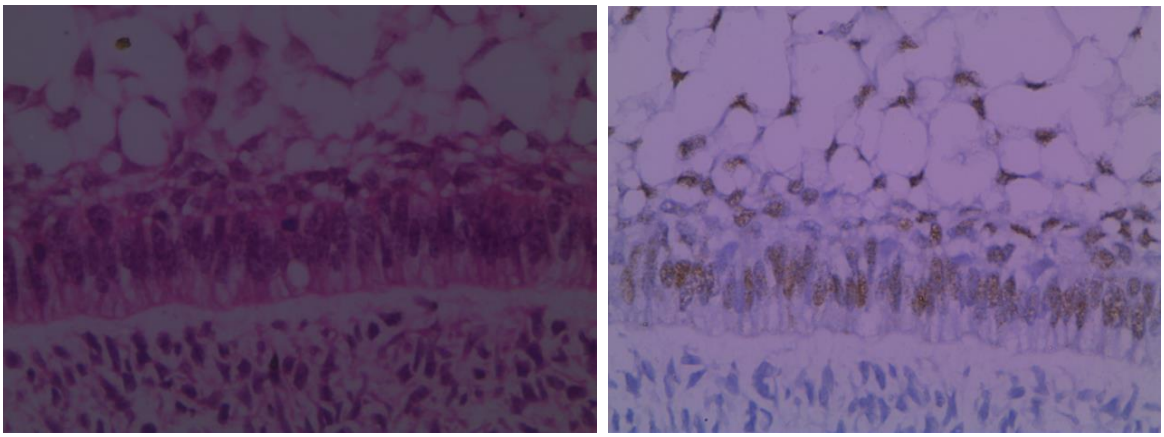


Figure 1D

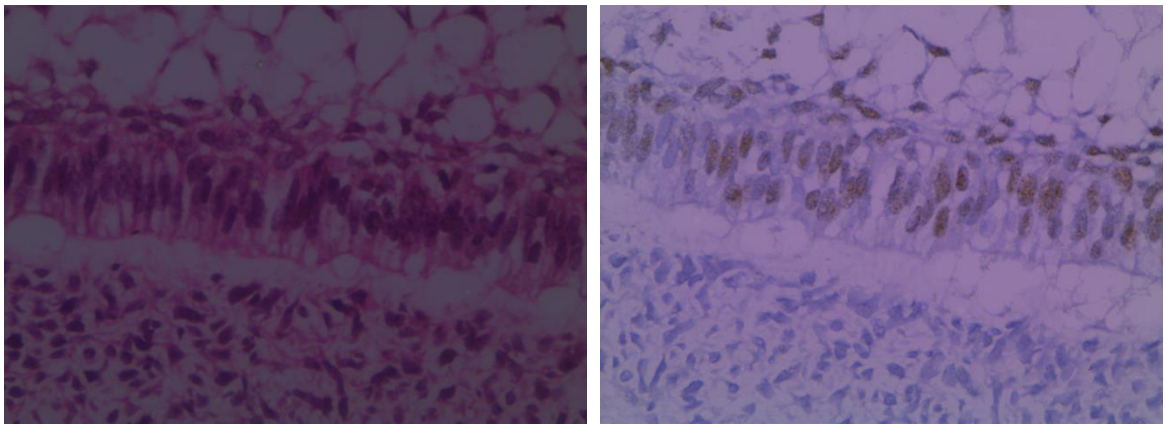


Figure 1E

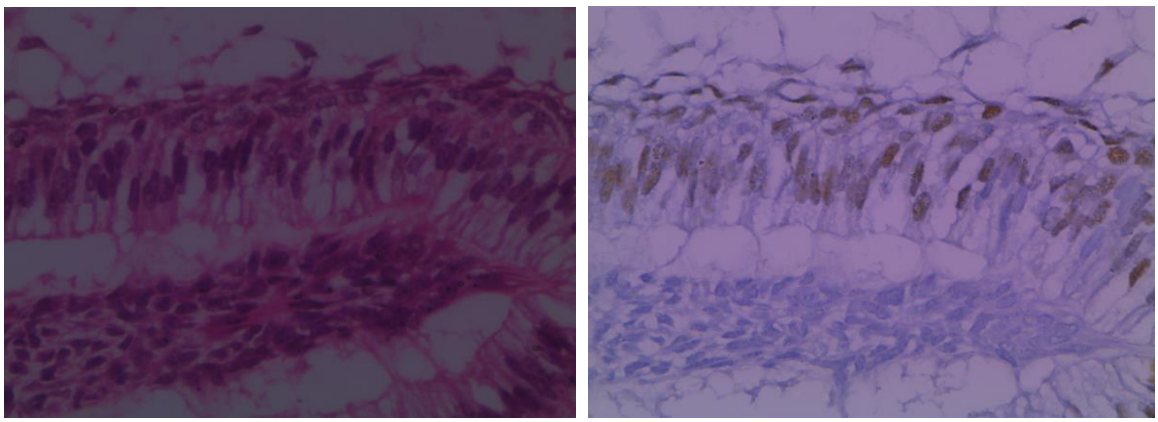


Figure 1F

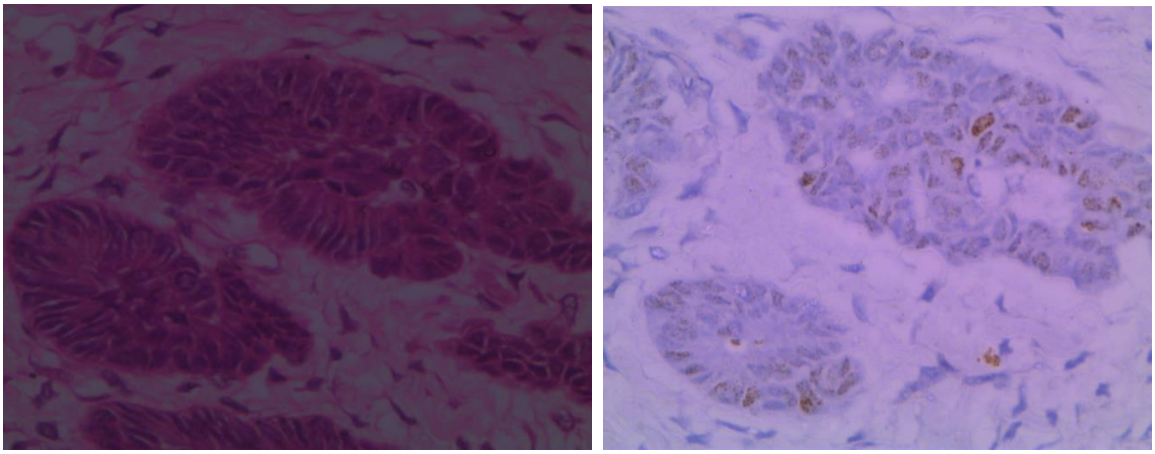


Figure 1G

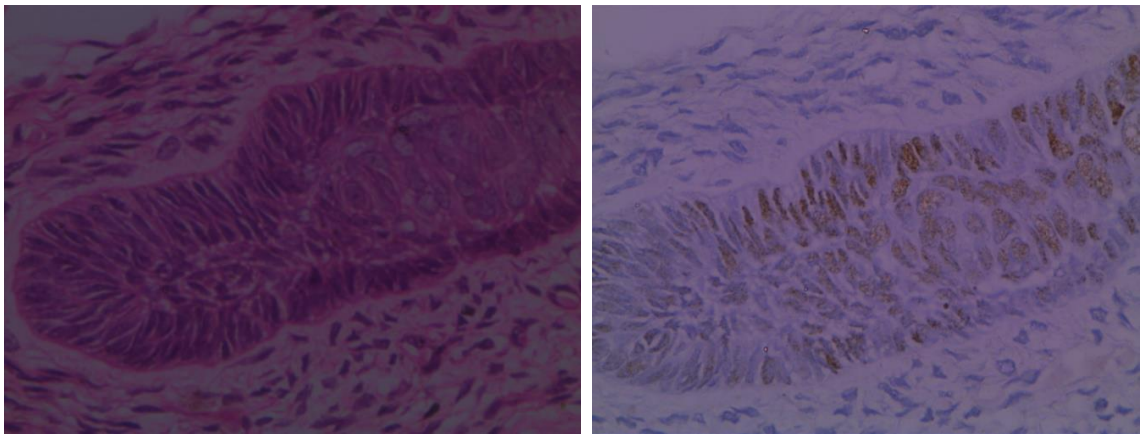


Figure 1H

Figure 1: A shows the p63 expression in early bell stage tooth germ (x2 magnification). B. shows the p63 expression in the inner enamel epithelium, stratum intermedium, stellate reticulum and outer enamel epithelium of early bell stage tooth germ (x4 magnification). C. shows the p63 expression in cervical loop region (x40 magnification). D. shows the p63 expression in preameloblasts (x40 magnification).E. shows the p63 expression in transitional presecretory ameloblasts (x40 magnification). C,D,E,F. showing the p63 positivity decreasing in number and intensity as the cell progresses from the cervical loop to the cusp tip (x40 magnification) G. shows the p63 expression in dental lamina rests(x40 magnification).H. shows the p63 expression in tooth bud(x40 magnification)

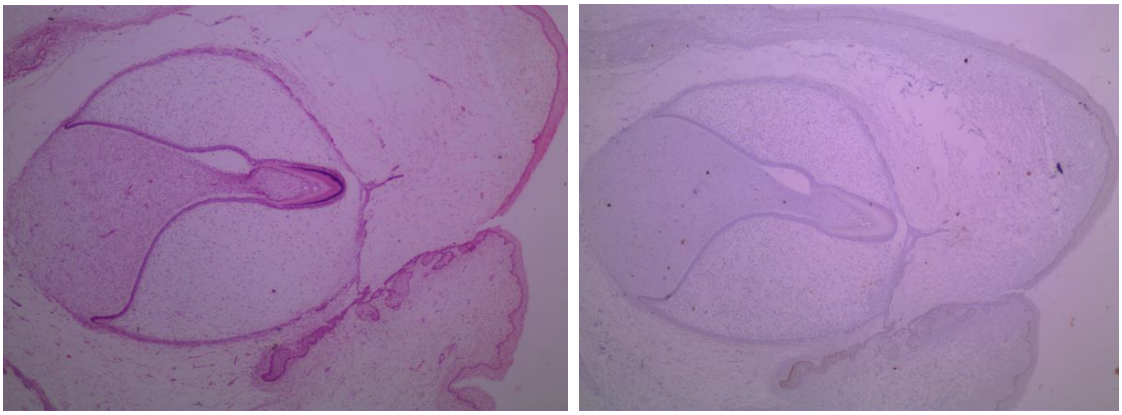


Figure 2A

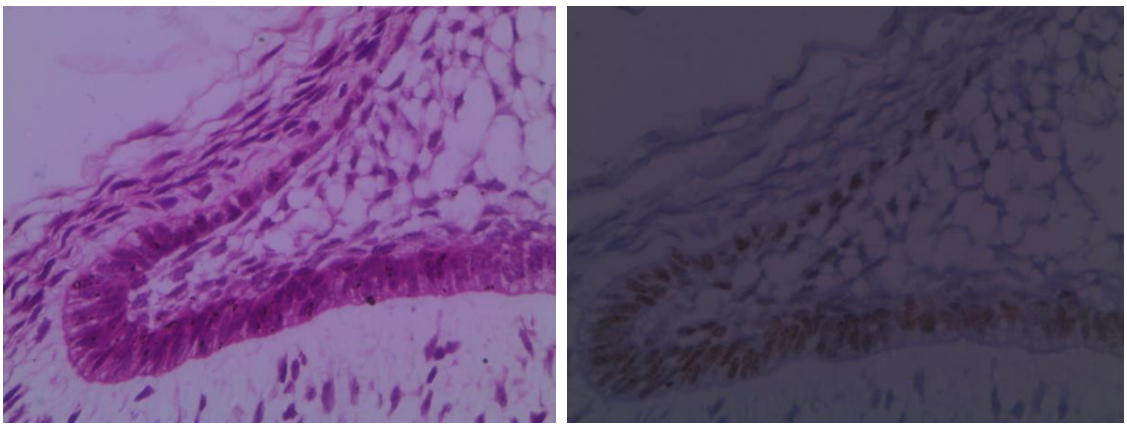


Figure 2B

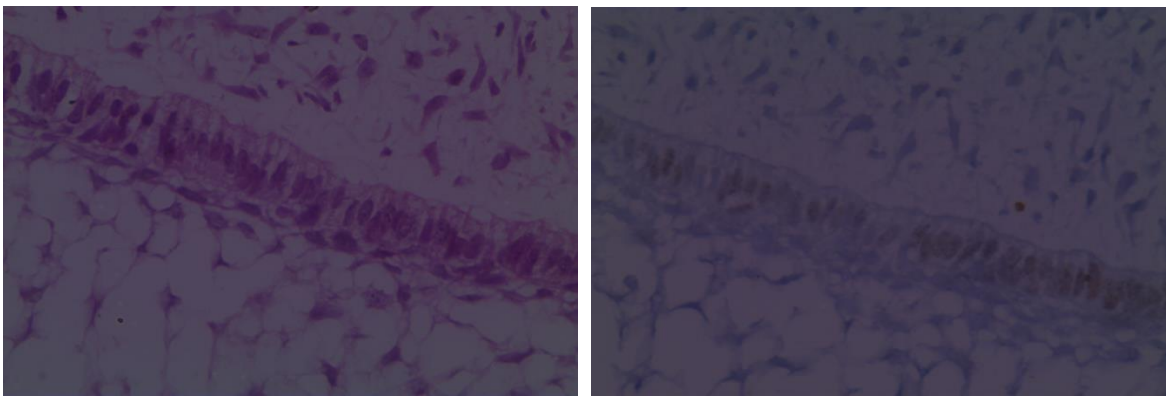


Figure 2C

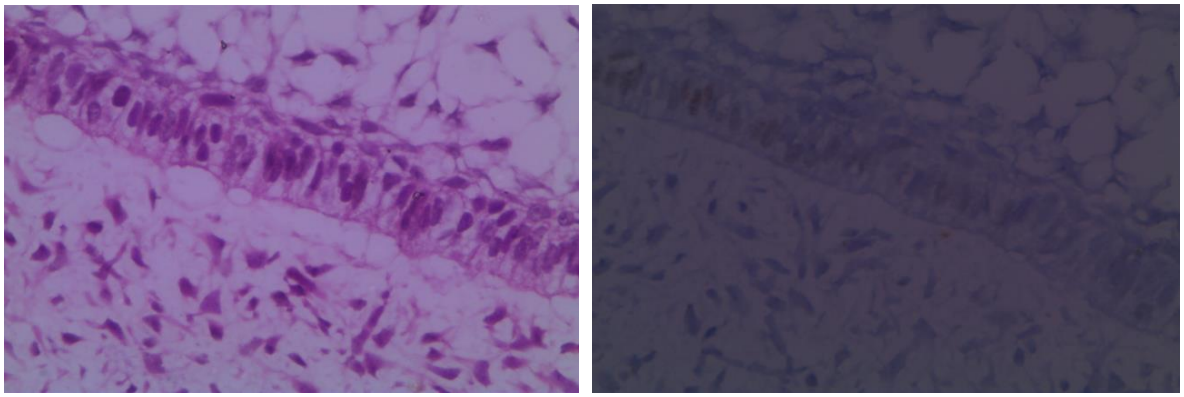


Figure 2D

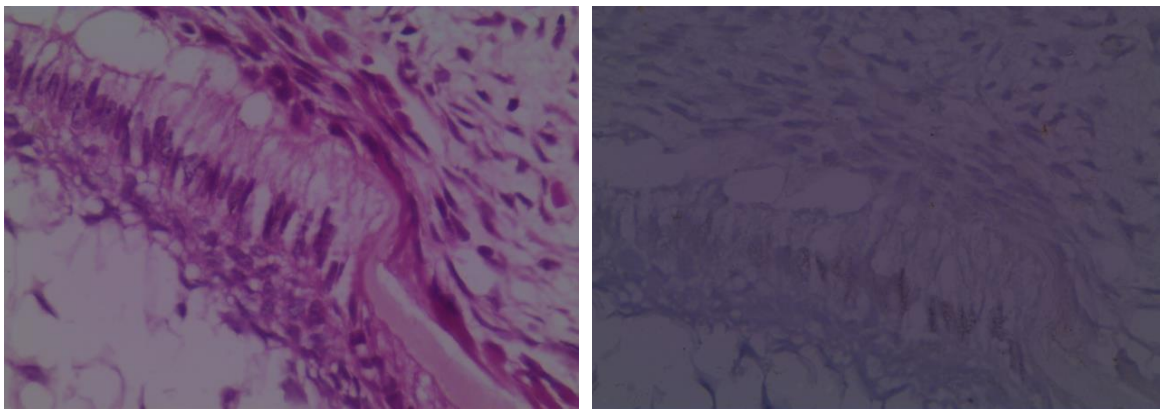


Figure 2E

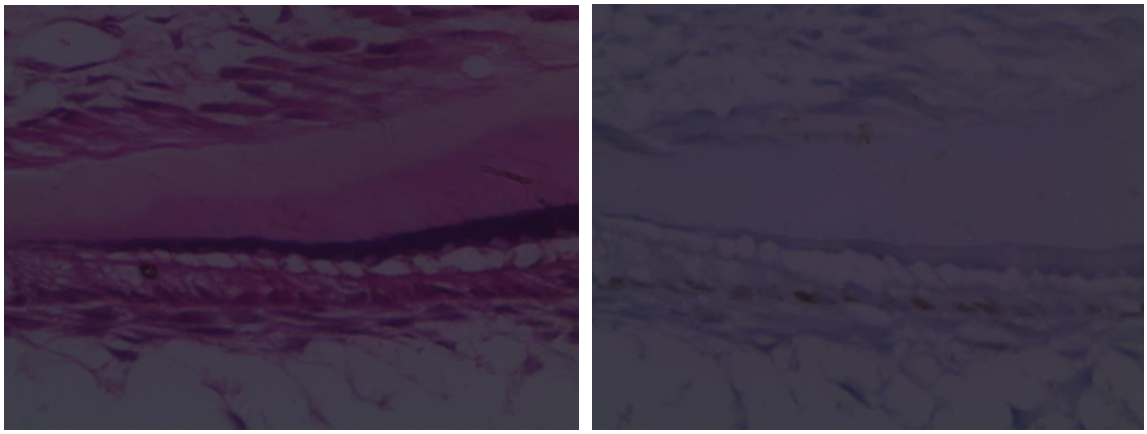


Figure 2F

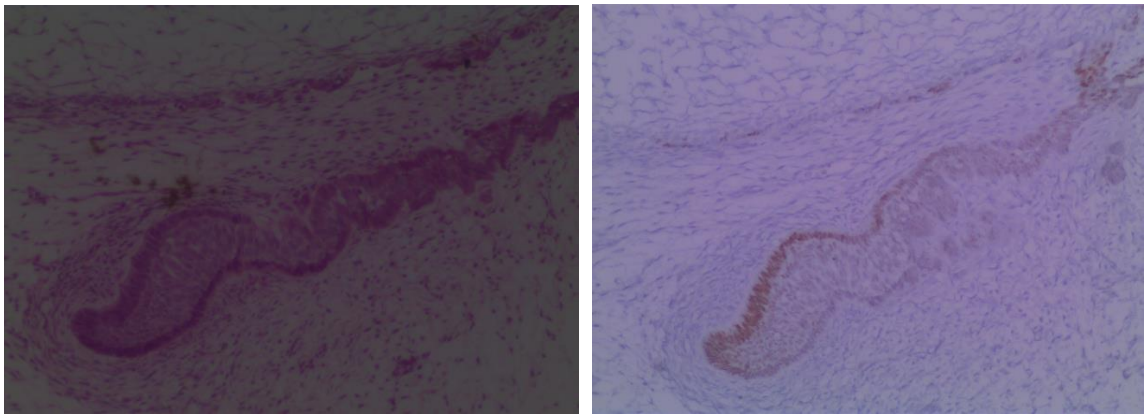


Figure 2G

Figure 2. A. shows the p63 expression in late bell stage tooth germ (x2 magnification). B. shows the p63 expression in cervical loop region of late bell stage tooth germ (x40 magnification). C. shows the p63 expression in preameloblasts (x40 magnification). D. shows the p63 expression in transitional presecretory ameloblasts (x40magnification).E.shows the p63 expression in presecretory ameloblasts(x40 magnification). F. shows the p63 expression in secretory ameloblasts. B,C,D,E,F shows p63 positivity decreasing in intensity as the cell progresses from the cervical loop to the cusp tip (x40 magnification) G. shows the sporadic p63 expression in outer enamel epithelium and intense p63 expression in proliferating cells of the tooth bud.(x40 magnification).

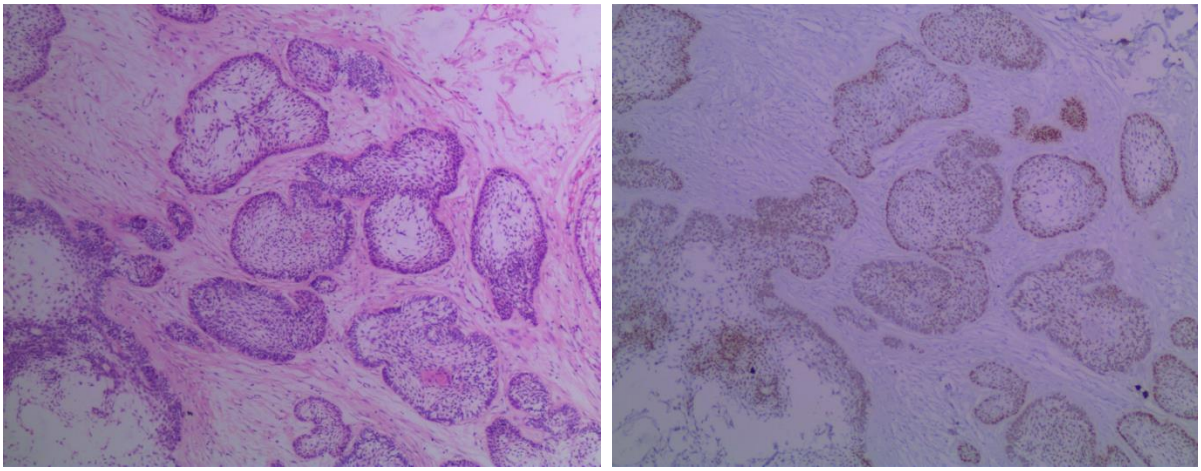


Figure 3A

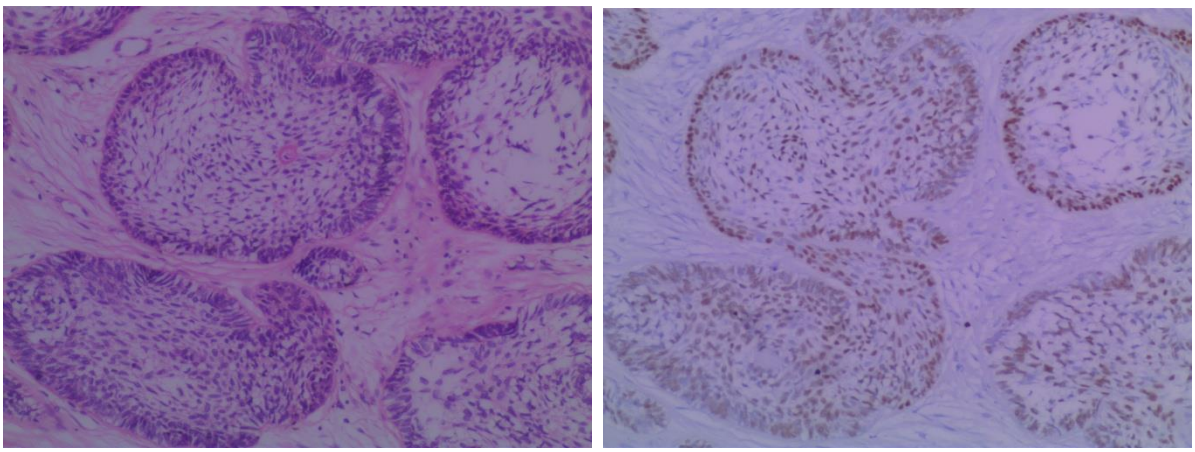


Figure 3B

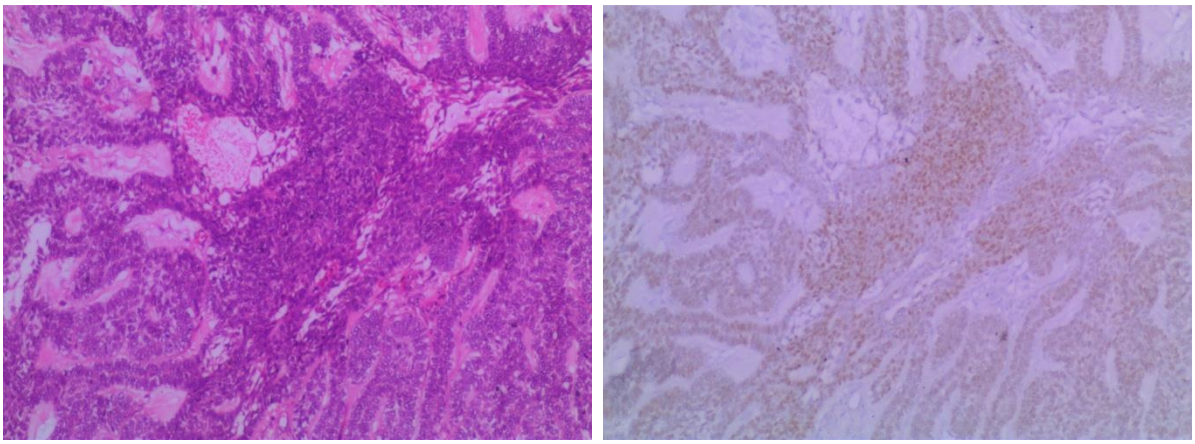


Figure 3C

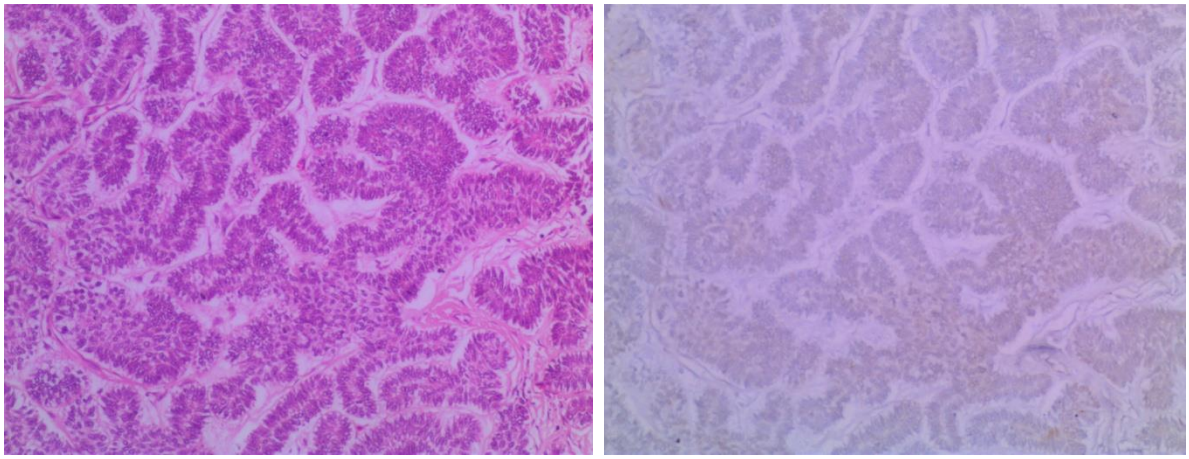


Figure 3D

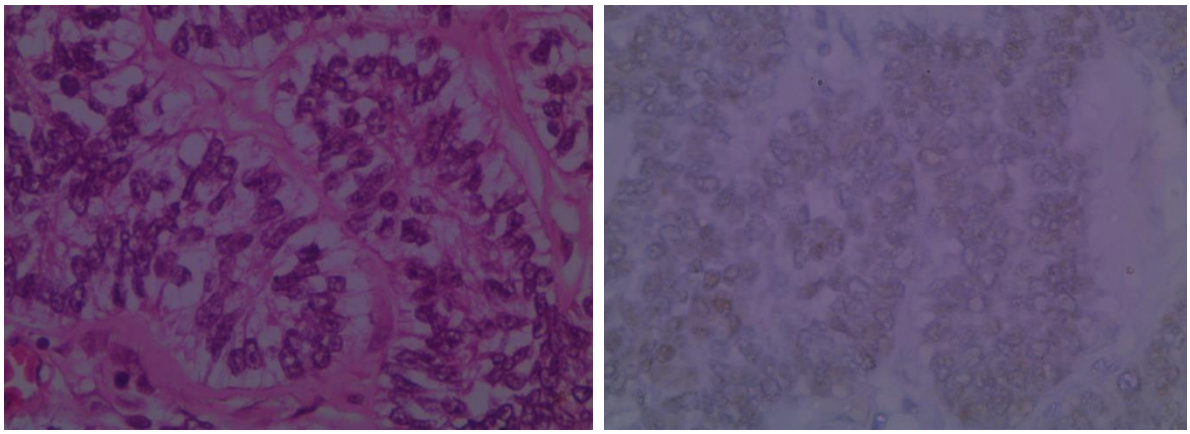


Figure 3E

Figure 3: A shows p63 expression in peripheral cells of ameloblastoma some stained intense and some stained mild (x4 magnification) B. shows mild p63 expression in peripheral cells showing morphology of presecretory ameloblasts (x10 magnification).C shows intense p63 expression in the undifferentiated cells and moderate p63 expression in the peripheral cells (x10 magnification).D. shows mild p63 expression in peripheral cells showing morphology of secretory ameloblasts (x10 magnification). E. D in (x40 magnification).

Expression of amelogenin in human tooth germ and ameloblastoma

Early bell stage:

No expression of amelogenin was detected in the enamel organ and dental papilla cells except for a mild reaction in the dental follicle cells in all the six EBS's evaluated in the current study **(Figure 4).**

Late bell stage

Immunohistochemical expression of amelogenin was first evident at the presecretory ameloblasts at the cusp tip before enamel matrix secretion and the expression in these cells became negative once the enamel matrix is secreted, but by this time the secretory ameloblasts begin to react intensively with amelogenin **(Figure 5).**

The expression of amelogenin in the secretory ameloblast starts at the cusp tip and progresses cervically and was evident only in five of the fifteen late bell stages studied. In ten LBS's the expression of amelogenin in the secretory ameloblasts was negative and in some cases granular positivity of amelogenin was evident at the tomes process **(Figure 6,7).**

Enamel matrix stains intensively with amelogenin with more intense staining at the DEJ in all the late bell stages studied. Mild expression of amelogenin was evident at the dentinal tubules near the forming enamel matrix **(Figure 7).**

No expression of amelogenin was evident at the dental papilla cells or odontoblasts, stratum intermedium and stellate reticulum cells throughout odontogenesis. Mild expression of amelogenin was evident at the dental follicle and condensing mesenchyme around bud. Expression of amelogenin was also evident at the differentiated cells of oral epithelium and in the central squamous cells of the tooth bud **(Figure 6).**

In addition, expression of amelogenin was also evident in the condensing mesenchyme around bone forming region, osteoid matrix, osteoblasts, osteocytes, differentiated cells of skin epithelium and hair follicles. In general cartilage was negative, but for a mild reaction in the perichondrial cells **(Figure 8).**

Ameloblastoma:

Out of fifteen cases studied, immunohistochemical expression of amelogenin was absent in eleven cases of ameloblastoma, but in four cases, expression of amelogenin was evident in the cytoplasm of peripheral cells of ameloblastoma **(Figure 9).**

In addition to this, in three cases amelogenin expression was evident in the collagen fibres located around the follicles in ameloblastoma, which was further confirmed by massons trichrome special stain (stains collagen fibres blue). Amelogenin expression was also evident at the follicles of ameloblastoma showing central squamous differentiation **(Figure 10).**

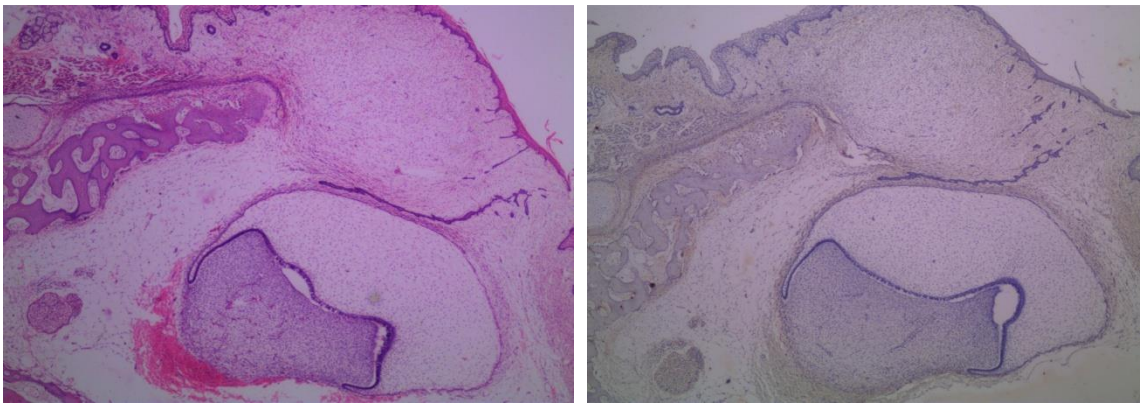


Figure 4A

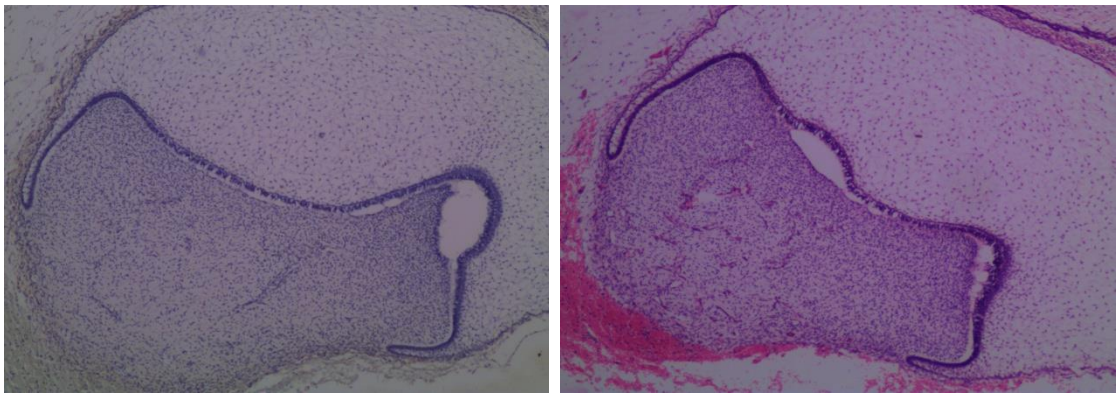


Figure 4B

Figure 4: A shows amelogenin expression in early bell stage tooth germ (x2 magnification). B. Shows mild expression of amelogenin in the dental follicle cells (x4 magnification).

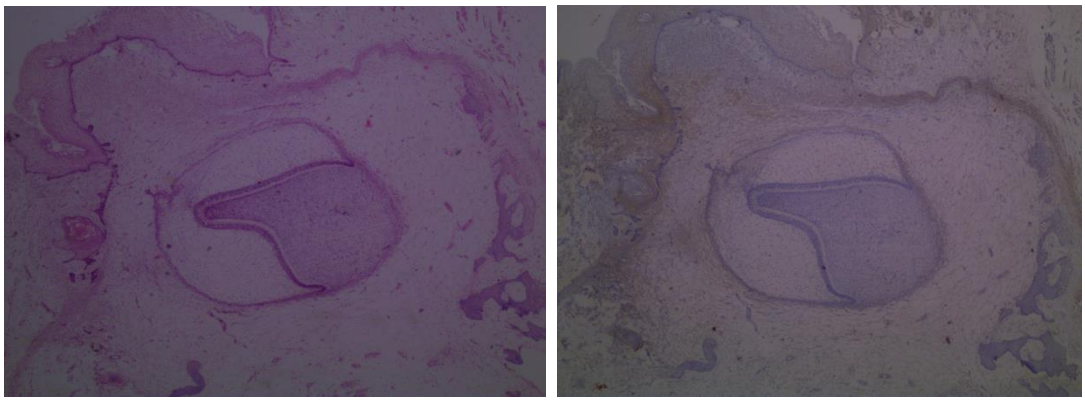


Figure 5 A

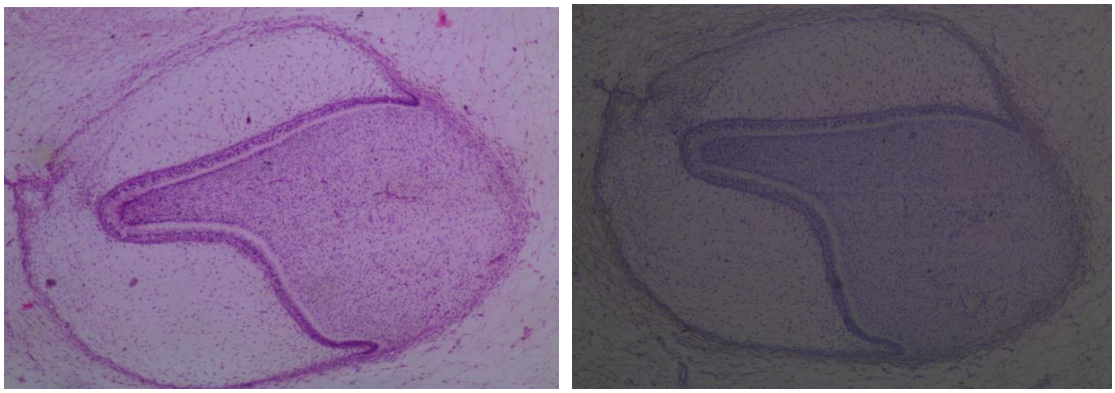


Figure 5 B

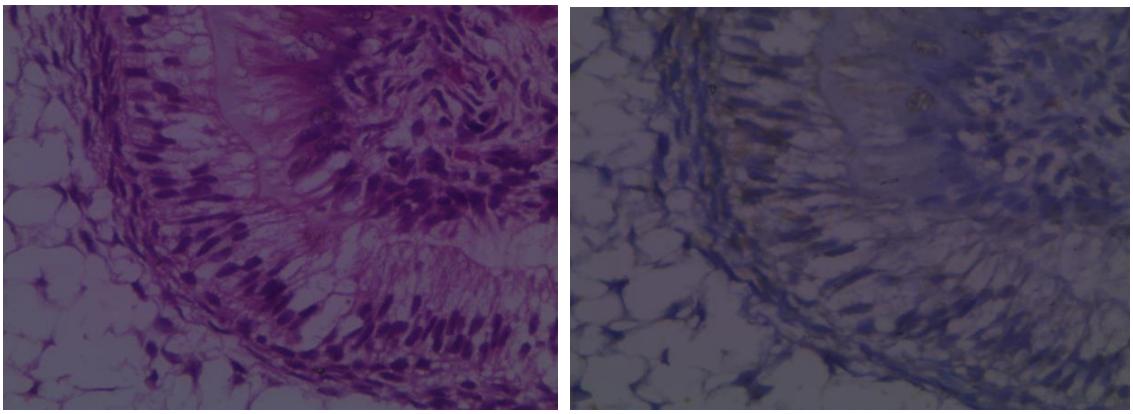


Figure 5 C

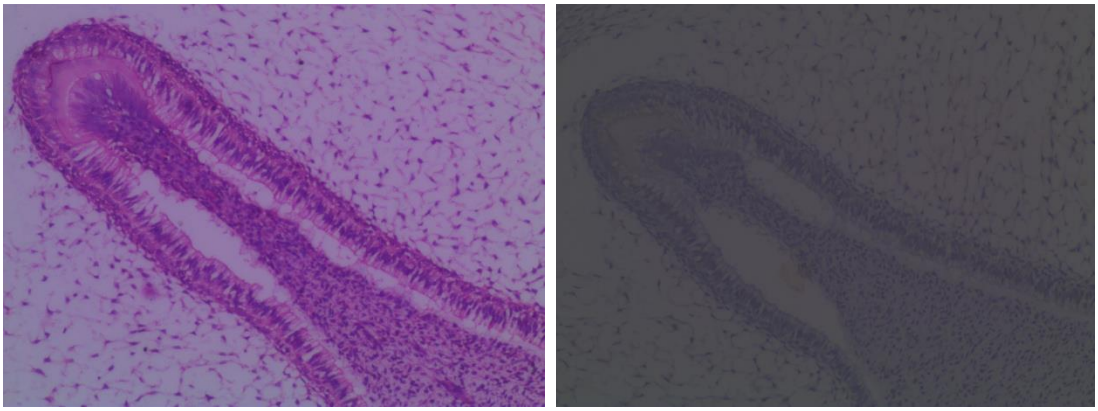


Figure 5D

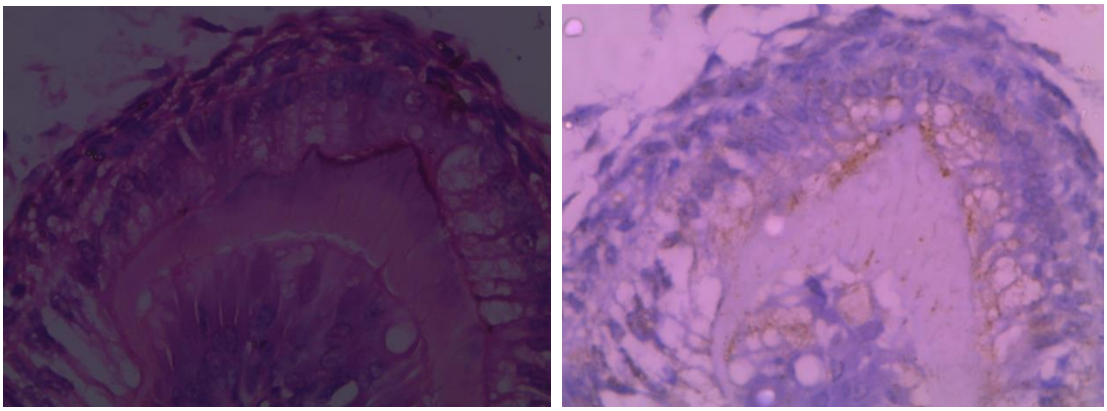


Figure 5 E

Figure 5 : A Shows the late bell stage tooth germ with only dentin formation at the cusp tip.(x2 magnification) B. A in (x4 magnification). C. shows mild expression of amelogenin in presecretory ameloblasts at the cusp tip (x40 magnification). D.Shows intense expression of amelogenin in the secretory ameloblasts and also shows secretion of enamel matrix at the cusp tip (x10 magnification). E. D (in x40 magnidication)

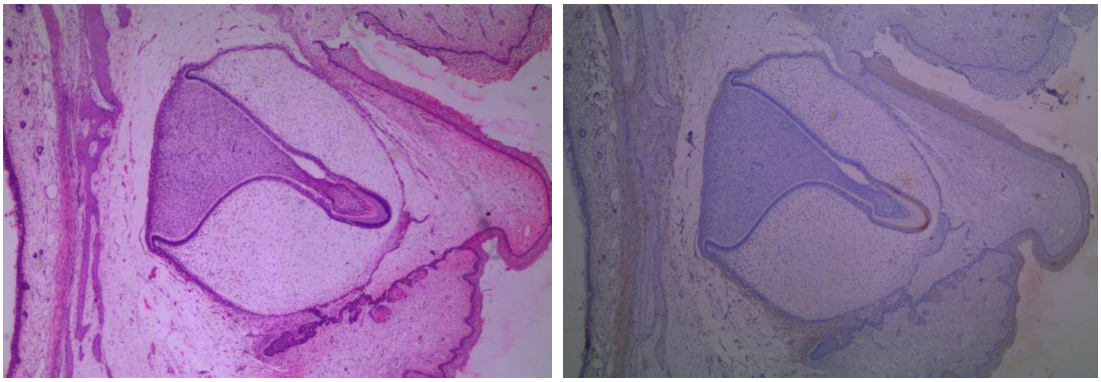


Figure 6A

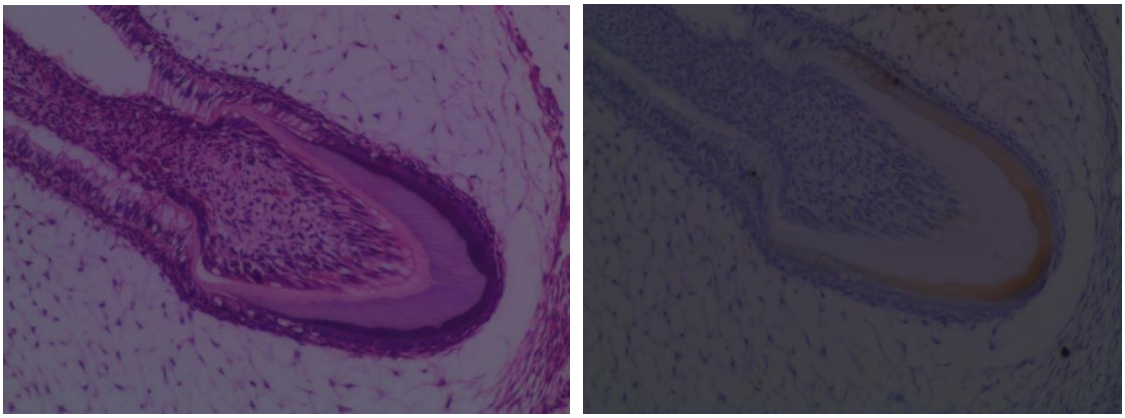


Figure 6B

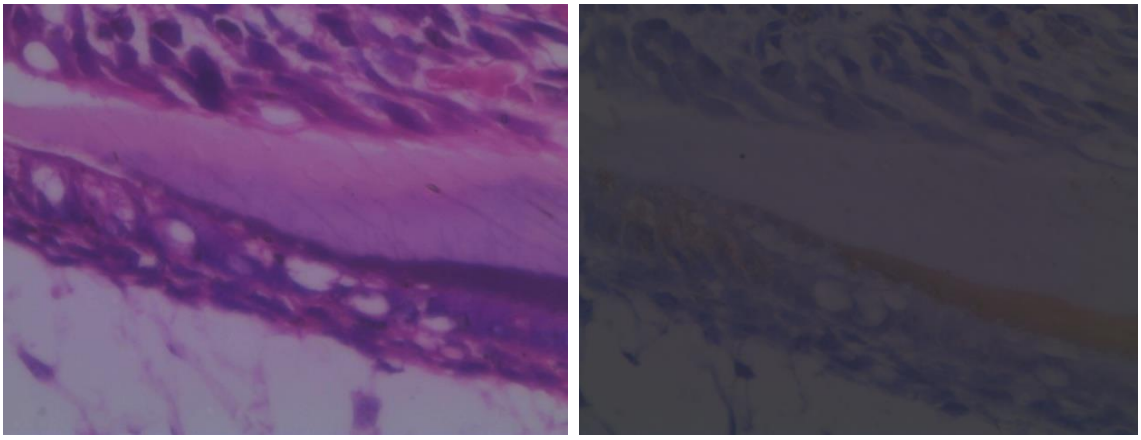


Figure 6C

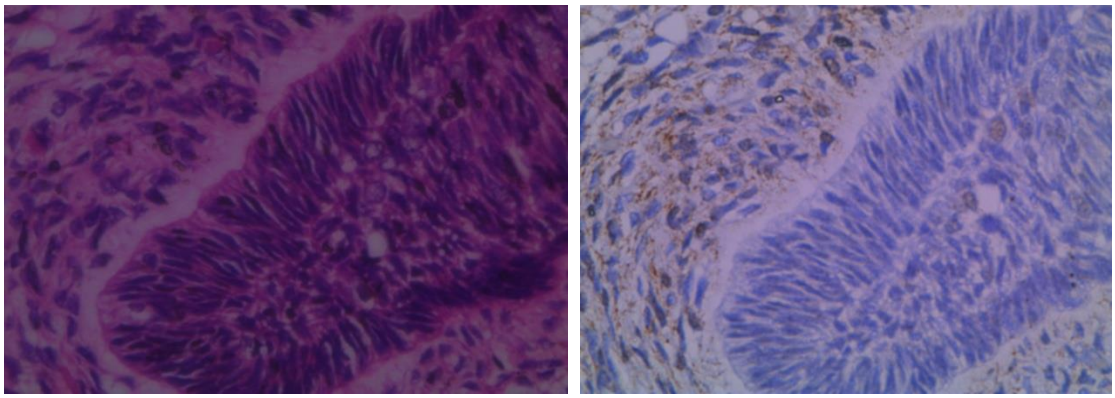


Figure 6D

Figure 6: A shows amelogenin expression in late bell stage tooth germ and it's associated structures(x2 magnification). B shows intense expression of amelogenin in the enamel matrix (x10 magnification). C shows moderate expression of amelogenin in the secretory ameloblasts progressing cervically(x40 magnification). D shows mild expression of amelogenin in the central squamous cells of the bud and condensing mesenchyme around bud(x40 magnification).

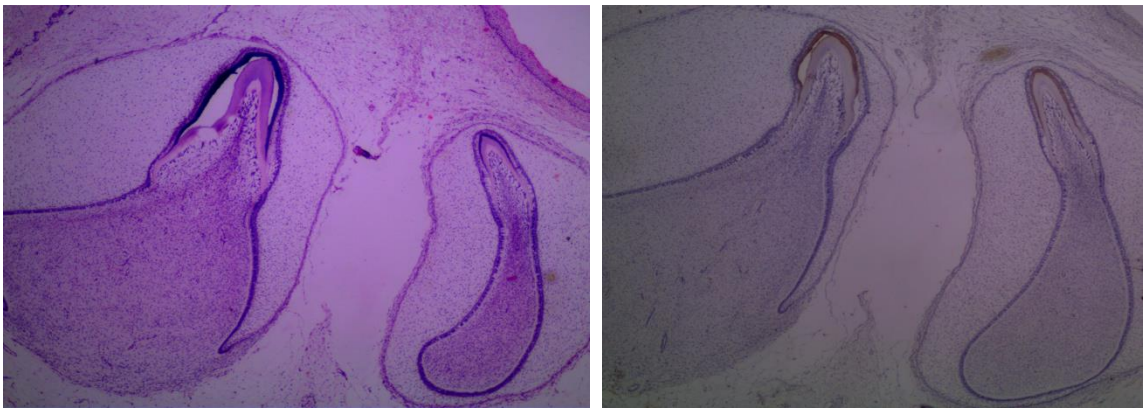


Figure 7A

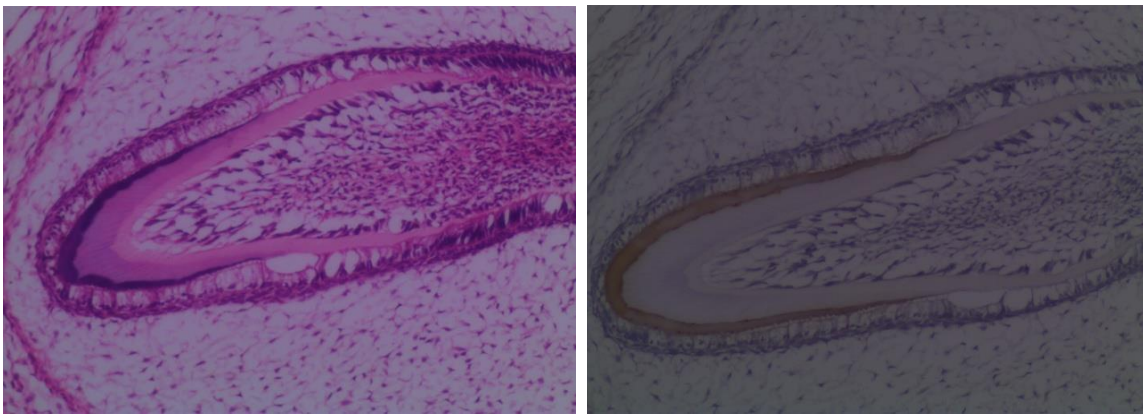


Figure 7B

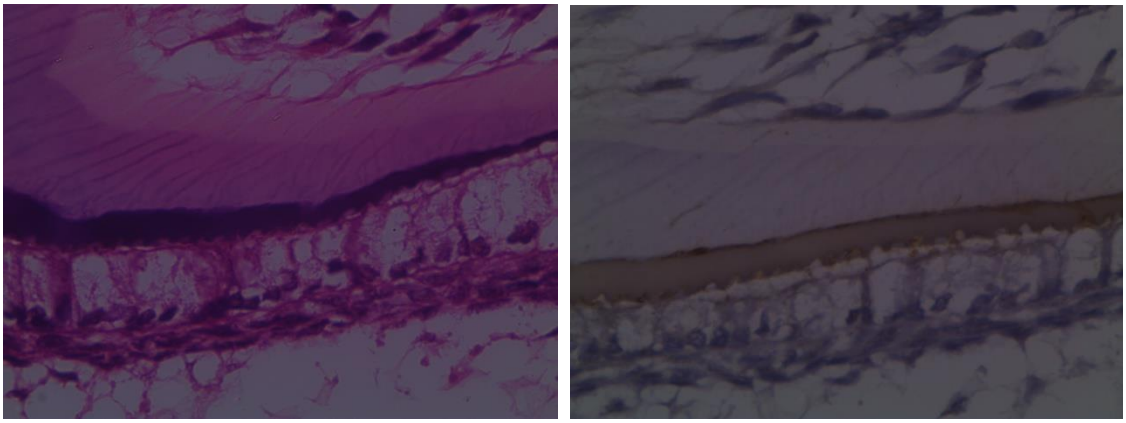


Figure 7C

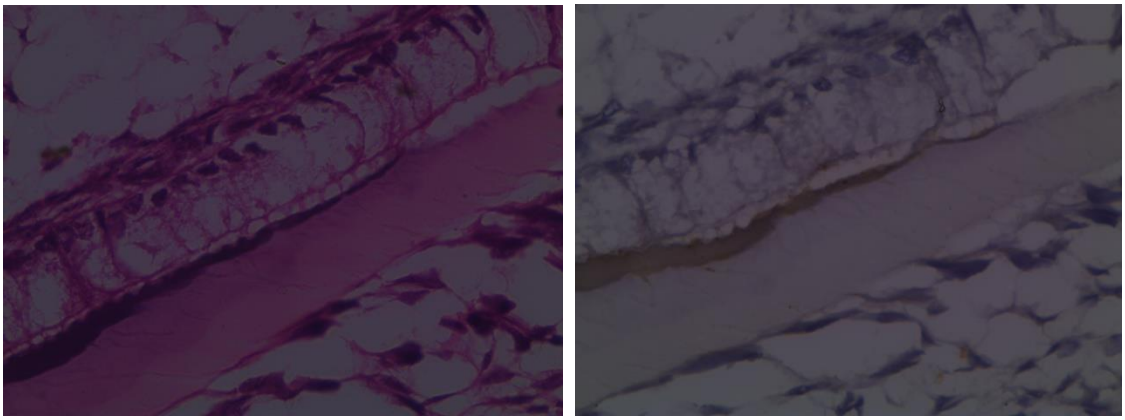


Figure 7D

Figure 7: A Shows expression of amelogenin in two late bell stage tooth germs (x2 magnification).B. Shows intense staining of amelogenin in the enamel matrix (x40 magnification). C. Shows moderate amelogenin expression at the tomes process and absence of amelogenin expression in the secretory ameloblasts and mild expression of amelogenin at the dentinal tubules (x40 magnification). D. Shows absence of expression of amelogenin in the secretory ameloblasts at the matrix forming end (x40 magnification).

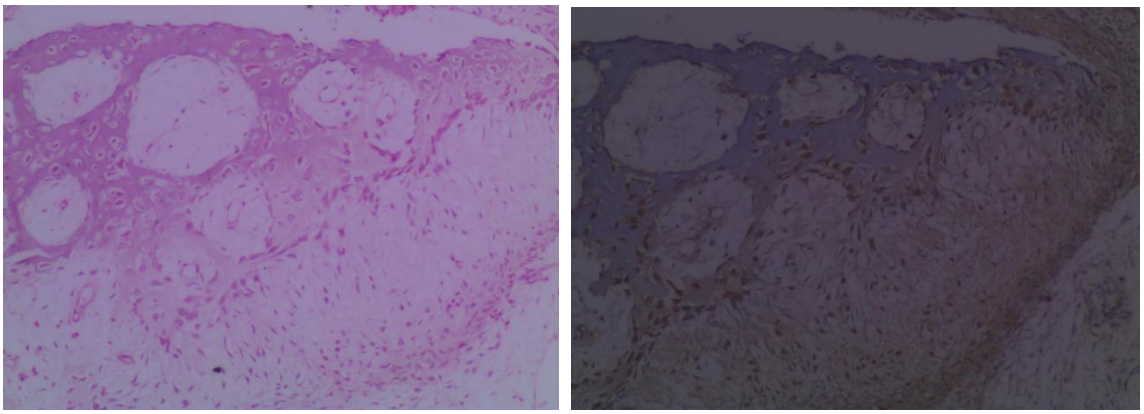


Figure 8A

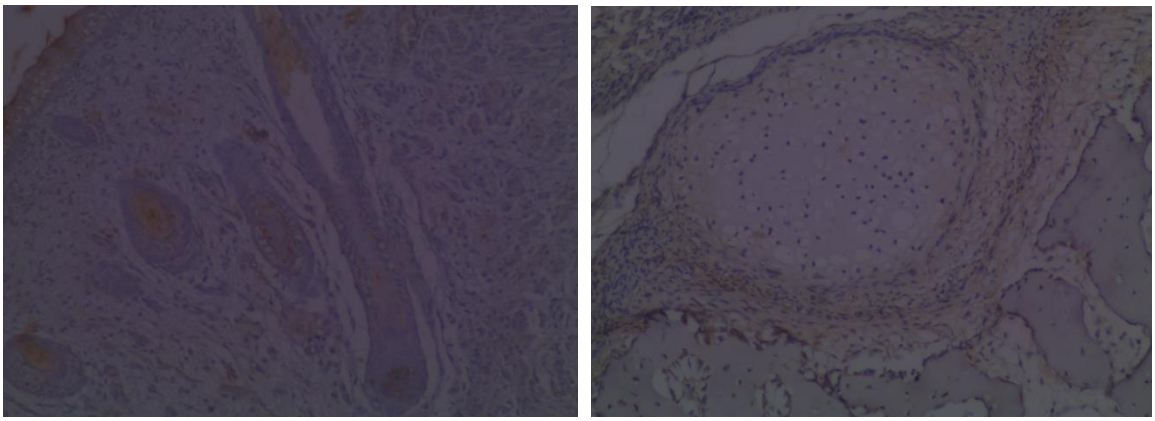


Figure 8B and 8C

Figure 8: A shows expression of amelogenin in the condensing mesenchyme around bone, osteoid matrix, osteoblasts and some osteocytes(x40 magnification). B Shows expression of amelogenin in the differentiated cells of skin epithelium and hair follicles (x40 magnification). C Shows expression of amelogenin in the condensing mesenchyme around cartilage(x40 magnification).

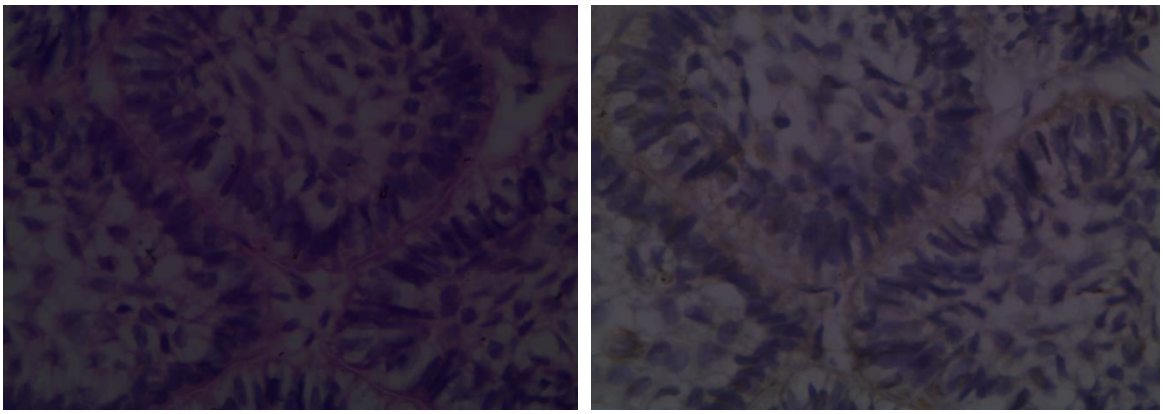


Figure 9 A

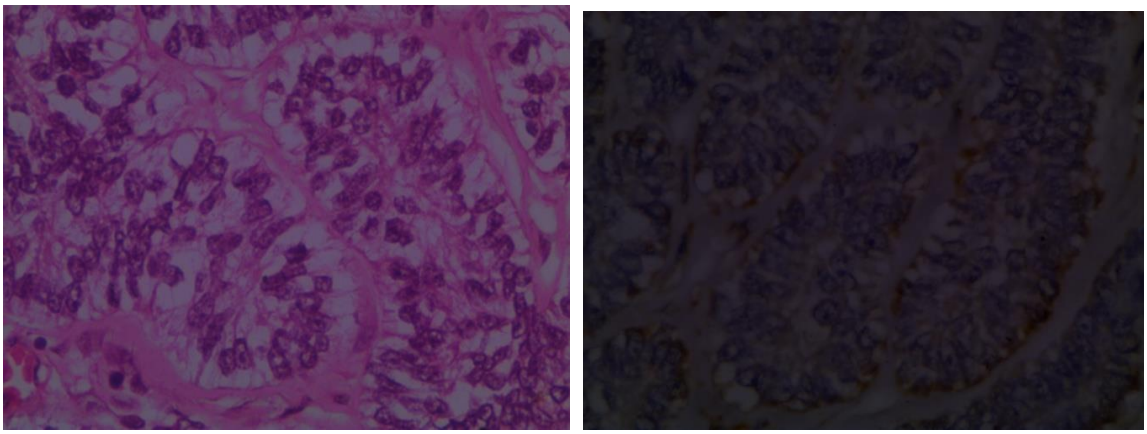


Figure 9 B

Figure 9: A shows mild expression of amelogenin in the peripheral cells of ameloblastoma showing morphology of presecretory ameloblasts (x40 magnification). B. shows intense expression of amelogenin in the peripheral cells of ameloblastoma showing morphology of secretory ameloblasts (x40 magnification).

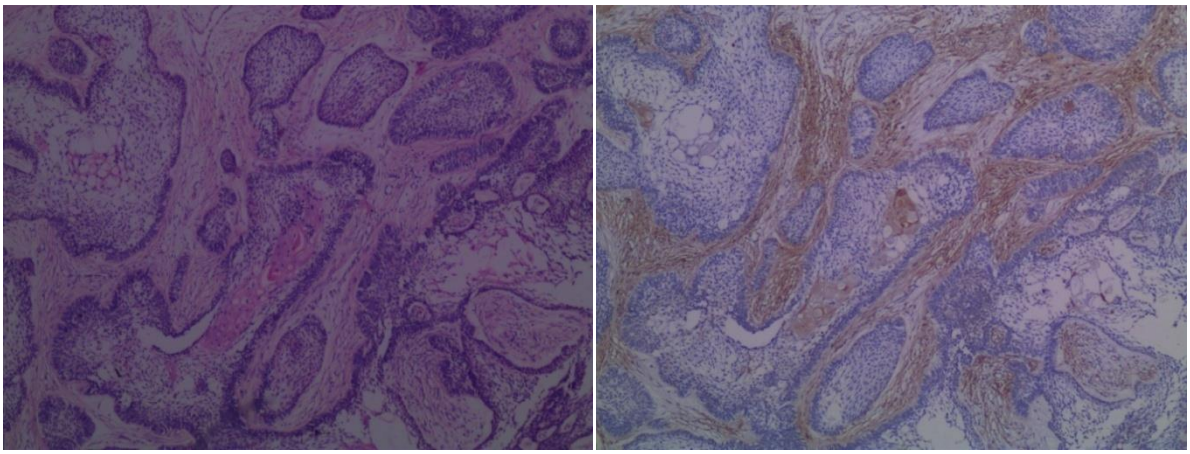


Figure 10 A

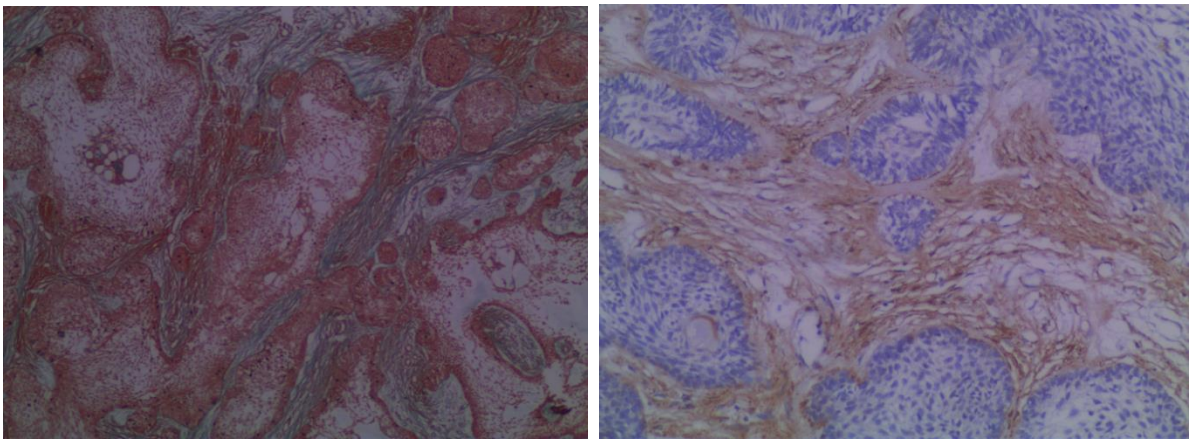


Figure 10 B and 10 C

Figure 10: A shows amelogenin expression in the follicles of ameloblastoma showing central squamous differentiation and in the connective tissue around the follicles(x4 magnification).B shows A stained with masson's trichrome (stains collagen fibres blue) (x4 magnification). C shows expression of amelogenin in connective tissue around the follicles of ameloblastoma (x10 magnification).

DISCUSSION

Previous studies have mentioned that basal cells are composed of heterogenous cell population and p63 is expressed only by the cells that possesss the ability to proliferate. Strong p63 expression is restricted to keratinocyte stem cells and the transit- amplifying cells after their withdrawal from the stem cell compartment show reduced p63 expression even though they possess appreciable proliferative capacity **(13,14).** Similar to this study we also found strong p63 expression in some of the basal cells of oral epithelium and as the cells differentiate and move upward we noticed reduced p63 expression.

Pindborg 1959 **(58)** divided the enamel organ of rat into five main sectors histologically, extending from the epithelial loop till the fusion with the gingival epithelium. Among the five main sectors, the first sector comprises the proliferative zone of odontogenic epithelium which extends from the cervical loop (junction of outer enamel epithelium and inner enamel epithelium) to the point where dentin formation begins and is characterized by rapid mitotic activity of undifferentiated ameloblasts. During the early bell stage we noticed intense p63 expression in the cervical loop region and as the cell progresses from the cervical loop to the cusp tip, both the intensity and number of p63 positive cells decreases. After the formation of enamel and dentin i.e, during the late bell stage, intense p63 expression was evident only in the cervical loop region, which revealed that cervical loop region might be the stem cell compartment of tooth germ.

Our results in late bell stage tooth germ were consistent with the results of Laurikkala et al. **(16)** who found out that the intensity of p63 expression decreases when the inner enamel epithelium differentiated into ameloblasts. p63 expression was evident throughout

odontogenesis, although the intensity of expression varies suggesting that p63 might play a role in tooth formation.

There are so many studies which showed that p63 is expressed in the basal and suprabasal layers of cystic ameloblastoma, peripheral and central stellate reticulum-like cells of conventional ameloblastoma **(3,5,18,21,22,23).** In addition to this, we noticed that the intensity of p63 expression varies in the peripheral cells of ameloblastoma suggesting that peripheral cells are at different levels of differentiation yet maintaining their proliferative capacity.

Nel et al. **(45)** suggested that amelogenin expression was not found during the bud, cap and early bell stages of tooth development in dog by immunohistochemistry. But, during the late bell stage small amounts of amelogenin expression was observed in the presecretory ameloblasts in close approximation to the cell nucleus. Similar to this study we didn't observe any amelogenin expression during the bud, cap and early bell stages of tooth development and during the late bell stage, amelogenin expression was evident in the presecretory ameloblasts at the cusp tip.

The expression of amelogenin was first detected at the presecretory ameloblasts before enamel matrix secretion **(25).** Amelogenin expression was first detected at the presecretory ameloblasts and the expression was limited to secretory ameloblasts once the enamel matrix is secreted **(28).** As the first evidence of amelogenin protein expression was also detected in the presecretory ameloblasts at the cusp tip in the current study, it is suggested that amelogenin mRNA expression might also be evident in presecretory ameloblasts at the cusp tip before the onset of biomineralization**.**

Amelogenin mRNA expression was first detected at the cusp tip and ceases at the cementoenamel junction **(40).** Amelogenin protein expression was absent during the early bell stage and noted only during the late bell stage **(45).** By in situ hybridization AMEL expression was first detected in the secretory ameloblasts located at the cusp tip during early enamel matrix deposition. But, during the maturation stage AMEL expression was absent in the ameloblasts at the tooth tip but was present in the ameloblasts towards the tooth base, in which enamel matrix was still deposited **(48).** Immunohistochemiclly amelogenin protein expression was first detected at the secretory ameloblasts and ceases when the enamel begins to mature **(49).** Amelogenin protein expression in the current study was first detected at the cusp tip and the secretion progresses cervically towards the cementoenamel junction.

We observed the formation of enamel matrix progressing cervically but, we observed immunostaining in the secretory ameloblasts only in five LBS tooth germs, out of fifteen LBS's evaluated in the current study.

The immunonegativity of secretory ameloblasts might be due to small amount of amelogenin secretion and the synthesized amelogenins are below the sensitivity of immunolocalisation techniques or inability of the antibody to detect the amelogenin which is secreted by secretory ameloblast. During amelogenesis, clock genes (Bmal 1, Clock, Per 1, Per 2) direct ameloblast function by controlling several ameloblast-specific genes including amelogenin (marker of secretory ameloblasts) and klk4 (marker of maturation ameloblasts) at RNA and protein level. It is suggested that amelogenin and klk4 undergo a regular 24 hr oscillations and result in significant alterations in enamel apposition and mineralization and this circadian rhythm was evident in the human enamel as cross-striations traversing enamel prisms

(47). The change in the expression pattern of clock genes might also be the reason for the immunonegativity in the secretory ameloblasts.

Ameloblasts synthesize cytokeratins 5 and 14 along with several matrix proteins. Studies showed that tri tyrosyl motif of N- terminal region of amelogenin binds to N acetyl glucosamine (GlcNAc). N acetyl glucosamine (GlcNAc) mimicking peptide was localized in the highly conserved N-terminal domain of cytokeratins 14, 16, and 17. Amelogenin is translocated from the site of synthesis in the perinuclear region to the apical region of the cell in association with CK14, and at the apical region (Tomes process) the dissociation of the co-assembly complex of CK14-amelogenin occur, prior to amelogenin secretion **(32,33).** Only free amelogenin proteins could be detected by immunohistochemistry. Since amelogenin is bonded to CK14 intracellularly and the dissociation of the CK14-amelogenin complex occurs at the tomes process, this might be the reason that it is not detected in the secretory ameloblasts.

The MMP-20 (matrix metalloproteinase-20) or enamelysin is secreted along with amelogenins and other structural proteins such as ameloblastin and enamelin during the secretory stage and it begins to cleave the enamel proteins **(59).** In human tooth germ, during it's development amelogenin labeling was more in the enamel organ than enamelysin during the early bell stage and enamelysin labeling was more in the enamel organ than amelogenin during the late bell stage **(7).** During early bell stage, a colocalization of amelogenin and enamelysin proteins were found inside the Golgi apparatus with amelogenin being more evident in relation with golgi complex and during late bell stage, a colocalization of both proteins was found in the rough endoplasmic reticulum with enamelysin being more noticeable in relation with rough endoplasmic reticulum **(8).** This study demonstrates that both secretion and maturation of

amelogenin go hand in hand during amelogenesis. Because enamelysin is secreted more than amelogenin during late bell stage, this might be the reason that amelogenin is not detected in the secretory ameloblasts by immunolocalisation techniques. Further studies in this regard in human tooth germ are encouraged**.**

Expression of amelogenin in dentinal tubules depends upon the secretory capacity of SA. If the SA secretes more, it will spread out through channels in dentin to coat the lateral and distal surfaces of odontoblasts. The concentration decreases gradually as the amelogenin spread out further from the point of secretion **(60).** The distribution of first amelogenins secreted onto the dentinal tubules suggest that these proteins could perform a signaling function. Migration of enamel protein toward the dental pulp and odontoblasts plays a role in the interaction between ameloblasts and odontoblasts **(25).**

Oida et al. **(35)** suggested that secretory ameloblasts express amelogenin 1000 times more than odontoblasts. The author also suggested that the positive reaction in the odontoblasts was because of the 1000 times stronger reaction in the nearby secretory stage ameloblasts. Ye et al. **(42)** suggested that amelogenin expression was evident in the forming dentin of a developing tooth germ where enamel matrix formation had not been initiated yet and also in the odontoblast layer of the adult human dentin by in situ hybridization, immunohistochemistry and other techniques. Throughout the present study we didn't observe expression of amelogenin in the odontoblasts. But we observed mild staining of amelogenin in the dentinal tubules. It was suggested that staining of amelogenin in the dentinal tubules could be due to background staining

Mitsiadis et al. suggested that zebra like pattern of amelogenin protein distribution was observed in human tooth germ (two amelogenin positive layers at the ends splitted by an amelogenin negative zone). This pattern of amelogenin protein distribution in the enamel could be due to differential processing of amelogenin during the secretory stage **(6).** We observed intense staining of amelogenin in the enamel matrix of all late bell stage tooth germs with more intense staining at the DEJ. Intense staining at the DEJ in all LBS's studied might be due to intimate contact between enamel and dentin.

We also noticed expression of amelogenin in the condensing mesenchyme around bud, dental follicle cells and condensing mesenchyme around bone and cartilage. The reason for this is unknown but, this suggested that amelogenin was not an enamel specific protein rather a multifunctional protein **(44).** We also noticed ameligenin expression in the differentiated cells of keratinized oral epithelium, central squamous cells of the tooth bud. Amelogenin cross reaction with keratin polypeptides was confirmed by immunoblotting technique. This might be due to the similarities in the antigenic sites of keratin and amelogenin **(54).**

Amelogenin mRNA and protein has a very important role in the processes of bone development and remodeling. Amelogenin play a role in the induction of osteogenesis and inhibition of osteoclastogenesis. Amelogenin was found to be expressed in long bone periosteum, which is composed of progenitor cells, bone marrow cells, osteoblasts and osteoclasts lining the bone trabeculae and by some osteocytes. Amelogenin protein was also found to be expressed in articular cartilage chondrocytes, epiphyseal bone cells and differentially in specific cell layers of the epiphyseal growth plate **(43)**. Similar to this study, we also noticed expression of amelogenin in the condensing mesenchyme around bone forming region, osteoid matrix, osteoblasts, some osteocytes and in the perichondrial cells.

Although the peripheral cells of ameloblastoma nests mimics the normal inner enamel epithelium from the bud to bell stages of human tooth germ, they do not attain differentiation as secretory ameloblasts in amelogenesis **(54)**. This might account for the absence of amelogenin expression in the peripheral cells of ameloblastoma in eleven of the fifteen case studied.

Previous studies also indicated that only one strand of amelogenin gene was transcribed during the normal development of tooth whereas both strands of human amelogenin gene were transcribed in ameloblastoma. Ameloblastoma cells contain antisense mRNA transcribed from the opposite strand of an amelogenin gene. Antisense mRNA bind with the sense strand of mRNA, thereby prevents it from being translated to protein and this might account for the inability of the tumor cells to produce matrix **(53).**

Expression of amelogenin was evident in the peripheral cells of ameloblastoma (four out of fifteen cases studied) demonstrating presecretory and secretory morphology suggesting that ameloblastoma attained functional maturation beyond the presecretory stage **(10).** This also suggested that some matrix proteins were formed intracellularly but it could not be processed or released appropriately. And also enamel matrix secretion by ameloblasts has been found to be dependent on precedent dentinogenesis which was absent in ameloblastoma **(54).**

Amelogenin immunoreactivity was evident in the collagen fibres located around the follicles of ameloblastoma in three cases, which was further confirmed by massons trichrome special stain (stains collagen fibres blue) and at the follicles of ameloblastoma showing central squamous differentiation. The reason for this is unknown. This might be due to the similarities in the antigenic sites of keratin and amelogenin **(54)** or cross reaction between the antigenic sites of any other molecules and amelogenin.

We also observed that different detection kits and different concentrations of primary antibody resulted in slightly different expression pattern in the enamel organ. So it was important to keep the methodology as consistent as possible. Previous studies indicated that antigen preservation was depended on the type of fixation and decalcification. Throughout the present study all these parameters were kept constant.

Limitations of the study:

As amelogenin is a research marker, a number of dilution threshold is required to determine ideal dilutions. This will invariably result in shortage of the marker for the study. In the present study, the reaction at various dilutions are noted as follows

- At dilution 1:25, 1:50 and 1:80 in PBS, the expression was evident throughout the enamel organ from the inner enamel epithelium, preameloblast, presecretory and secretory ameloblasts as well as in the stratum intermedium, stellate reticulum and outer enamel epithelium with the nuclear and cytoplasmic staining pattern although the intensity of staining decreases when the dilution increases. Enamel matrix was not stained as intense as the cells of the enamel organ. Expression was also noted in the odontoblasts, dental papilla cells and dental follicle cells. In addition, we also noted expression in other connective tissue components which was considered to be background staining.
- At dilution 1:100, 1:120 and 1:150 in PBS, the expression was evident in the enamel organ from the preameloblast, presecretory and secretory ameloblasts as well as in the stratum intermedium and stellate reticulum cells with predominantly cytoplasmic and focal nuclear staining pattern. Enamel matrix stained intensely in all the dilutions.

Expression was also noted in the odontoblasts and the peripheral condensed dental papilla cells in the region of the cusp tip. In the dental follicle the expression was restricted to the region of cervical loop and diaphragm. In addition, we also noted expression in other connective tissue components which we believe as background staining.

- At dilution 1:180 in PBS, the expression was evident in the enamel organ from presecretory and secretory ameloblasts with only the cytoplasmic staining pattern. Enamel matrix stained intensely similar to 1:100, 1;120, 1;150 dilutions. A focal expression was seen in the stratum intermedium cells and a very mild expression was detected in the odontoblasts and the peripheral condensed dental papilla cells in the region of the cusp tip. In the dental follicle the expression was restricted to the region of cervical loop and diaphragm. In addition, we also noted mild expression in other connective tissue components which we believe as background staining.
- At dilution 1:200 in PBS, expression was evident only in the presecretory, secretory ameloblasts and in the enamel matrix. No expression was detected in the other cells of the enamel organ. The dental papilla cells and odontoblasts are negative except for a focal reaction in the dental follicle. No background staining was evident in this dilution in the connective tissue components.

Polyclonal antibody has the affinity to bind to different epitopes of the same antigen. Since amelogenin used in this study is polyclonal in nature, it cross reacts with other molecules when it recognizes similar epitopic sites, which could be the reason for its expression in the oral epithelium in the current study.

FIGURE LEGENDS

Figure 1: A shows the p63 expression in early bell stage tooth germ (x2 magnification). B. shows the p63 expression in the inner enamel epithelium, stratum intermedium, stellate reticulum and outer enamel epithelium of early bell stage tooth germ (x4 magnification). C. shows the p63 expression in cervical loop region (x40 magnification). D. shows the p63 expression in preameloblasts (x40 magnification). E. shows the p63 expression in transitional presecretory ameloblasts (x40 magnification). C,D,E,F. showing the p63 positivity decreasing in number and intensity as the cell progresses from the cervical loop to the cusp tip (x40 magnification) G. shows the p63 expression in dental lamina rests (x40 magnification).H. shows the p63 expression in tooth bud(x40 magnification).

Figure 2. A. shows the p63 expression in late bell stage tooth germ (x2 magnification). B. shows the p63 expression in cervical loop region of late bell stage tooth germ (x40 magnification). C. shows the p63 expression in preameloblasts (x40 magnification). D. shows the p63 expression in transitional presecretory ameloblasts (x40magnification).E.shows the p63 expression in presecretory ameloblasts(x40 magnification). F. shows the p63 expression in secretory ameloblasts. B,C,D,E,F shows p63 positivity decreasing in intensity as the cell progresses from the cervical loop to the cusp tip (x40 magnification) G. shows the sporadic p63 expression in outer enamel epithelium and intense p63 expression in proliferating cells of the tooth bud.(x40 magnification).

Figure 3: A shows p63 expression in peripheral cells of ameloblastoma some stained intense and some stained mild (x4 magnification) B. shows mild p63 expression in peripheral cells showing

morphology of presecretory ameloblasts (x10 magnification).C shows intense p63 expression in the undifferentiated cells and moderate p63 expression in the peripheral cells (x10 magnification).D. shows mild p63 expression in peripheral cells showing morphology of secretory ameloblasts (x10 magnification). E. D in (x40 magnification).

Figure 4: A shows amelogenin expression in early bell stage tooth germ (x2 magnification). B. Shows mild expression of amelogenin in the dental follicle cells (x4 magnification).

Figure 5 : A Shows the late bell stage tooth germ with only dentin formation at the cusp tip.(x2 magnification) B. A in (x4 magnification). C. shows mild expression of amelogenin in presecretory ameloblasts at the cusp tip (x40 magnification). D.Shows intense expression of amelogenin in the secretory ameloblasts and also shows secretion of enamel matrix at the cusp tip (x10 magnification). E. D (in x40 magnification).

Figure 6: A shows amelogenin expression in late bell stage tooth germ and it's associated structures(x2 magnification). B shows intense expression of amelogenin in the enamel matrix (x10 magnification). C shows moderate expression of amelogenin in the secretory ameloblasts progressing cervically(x40 magnification). D shows mild expression of amelogenin in the central squamous cells of the bud and condensing mesenchyme around bud(x40 magnification).

Figure 7: A Shows expression of amelogenin in two late bell stage tooth germs (x2 magnification).B. Shows intense staining of amelogenin in the enamel matrix (x40 magnification). C. Shows moderate amelogenin expression at the tomes process and absence of amelogenin expression in the secretory ameloblasts and mild expression of amelogenin at the dentinal tubules (x40 magnification). D. Shows absence of expression of amelogenin in the secretory ameloblasts at the matrix forming end (x40 magnification).

Figure 8: A shows expression of amelogenin in the condensing mesenchyme around bone, osteoid matrix, osteoblasts and some osteocytes(x40 magnification). B Shows expression of amelogenin in the differentiated cells of skin epithelium and hair follicles (x40 magnification). C Shows expression of amelogenin in the condensing mesenchyme around cartilage(x40 magnification).

Figure 9: A shows mild expression of amelogenin in the peripheral cells of ameloblastoma showing morphology of presecretory ameloblasts (x40 magnification). B. shows intense expression of amelogenin in the peripheral cells of ameloblastoma showing morphology of secretory ameloblasts (x40 magnification).

Figure 10: A shows amelogenin expression in the follicles of ameloblastoma showing central squamous differentiation and in the connective tissue around the follicles(x4 magnification).B shows A stained with masson's trichrome (stains collagen fibres blue) (x4 magnification). C shows expression of amelogenin in connective tissue around the follicles of ameloblastoma (x10 magnification).

SUMMARY AND CONCLUSION

The present study was undertaken to evaluate the expression of p63 and amelogenin in human tooth germs and ameloblastomas by immunohistochemistry to find out whether the pattern of expression is related to differentiation of ameloblasts.

Six unclaimed dead fetal tissues from 13th to 24th week were collected from Institute of Obstetrics and Gynaecology, Chennai with prior permission. Jaws were dissected from these dead fetal tissues and were decalcified in 8% HCl for one hour, fixed in 10% formalin, dehydrated, embedded in wax block, sectioned sagitally in the thickness of 3.5 microns and stained with H&E for histological observation. Archival samples of different histological variants of ameloblastoma were retrieved from Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai and sectioned in 3.5 microns thickness and stained with H&E for histological observation.

Accordingly, histologically confirmed 15 human tooth germs of late bell stage, 6 human tooth germs of early bell stage and 15 ameloblastoma samples were included in the study.

Immunohistochemical staining was performed with the following markers, p63 antibody (Mouse Monoclonal, clone 4A4, 1:25 dilution, CM163 – BioCare, USA) and AMELX antibody (Rabbit Polyclonal, 1:200 dilution, orb140077 – Biorbyt, UK) diluted in PBS, foetus tissue sections and ameloblastoma sections mounted on APES coated glass slides as per manufacturer's instructions and assessed individually.

During the early bell stage, p63 expression was intense throughout the enamel organ. But, during the late bell stage, the number and intensity of p63 expression decreases in the cells of enamel organ. The peripheral cells in the ameloblastoma shows variable pattern of p63

expression i.e., some nuclei stained intense and some stained mild. Expression of amelogenin was first evident in the presecretory ameloblasts at the cusp tip, followed by secretory ameloblasts and progresses cervically. But in ten late bell stage tooth germs, amelogenin expression was negative in the secretory ameloblasts and positive only at the secreting end i.e. tomes process. Intense staining was evident at the enamel matrix and in some dentinal tubules nearer to secretion of enamel matrix. No amelogenin expression was evident in the dental papilla cells or odontoblasts throughout odontogenesis with the exception of dental follicle. Amelogenin expression was absent in eleven cases of ameloblastoma, but in four cases positive expression was evident at the peripheral cells showing presecretory and secretory morphology suggesting that ameloblasts attained differentiation beyond the presecretory stage.

The present study concluded that expression pattern of p63 and amelogenin in human tooth germ was found to be consistent with ameloblastoma. p63 was considered to be a proliferation marker whereas amelogenin was considered to be a differentiation marker.

Amelogenin expression was evident only in ameloblasts which show negative or mild p63 expression. Hence downregulation of p63 expression was associated with ameloblast differentiation.

BIBLIOGRAPHY

1. Rufini A et al. p63 in tooth development. Biochemical Pharmacology 2011;82:1256-1261.

2. Nylander K et al. Differential expression of p63 isoforms in normal tissues and neoplastic cells. J Pathol 2002;198:417-427.

3. Kumamoto H et al. p63 and p73 in ameloblastomas. J Oral Pathol Med 2005;34:220-226.

4. Kock M et al. Immunohistochemical expression of p63 in human prenatal tooth primordia. Acta Odontologica Scandinavica 2005;63:253-257.

5. Gratzinger D et al. Ameloblastoma, calcifying epithelial odontogenic tumor, and glandular odontogenic cyst show a distinctive immunophenotype with some myoepithelial antigen expression. J Oral Pathol Med 2008;37:177-184.

6. Mitsiadis T et al. Distribution of the amelogenin protein in developing, injured and carious human teeth. Frontiers in Physiology 2014;5:1-8.

7. Gutierrez-Cantu FJ et al. Amelogenin and enamelysin localisation in human dental germs. In Vitro Cell Dev Biol -Animal 2011;47:355-360.

8. Gutierrez-Cantu FJ et al. Expression of enamel proteins in rough endoplasmic reticulum and golgi complex in human dental germs. Int J Morphol 2017;35:435-441.

9. Abiko Y et al. Immunohistochemical localization of amelogenin in human odontogenic tumors, using a polyclonal antibody against bovine amelogenin. Med Electron Microsc 2001; 34:185-189.

10. Chen et al. Calcium transport protein and amelogenin expression in ameloblastoma: relationship to normal amelogenesis. Taiwan J Oral Med Health Sci 2005;21:85-94.

11. Crivelini MM et al. Expression of odontogenic ameloblast-associated protein, amelotin, ameloblastin and amelogenin in odontogenic tumors: immunohistochemical analysis and pathogenetic considerations. J Oral Pathol Med 2012;41:272-280.

12. Anigol P et al. Amelogenin in odontogenic cysts and tumors: an immunohistochemical study. National Journal of Maxillofacial Surgery 2014;5:172-179.

13. Parsa R et al. Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. J Invest Dermatol 1999;113:1099-1105.

14. Pellegrini G et al. p63 identifies keratinocyte stem cells. PNAS 2001;98:3156-3161.

15. Cernochovo D et al. Expression of p53, p63 and p73 in the orofacial region of human embryos. Biomed Papers 2004;148:203-204.

16. Laurikkala J et al. p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. Development 2006;133:1553-1563.

17. Matsuura T et al. Regulation of proliferation and differentiation of mouse tooth germ epithelial cells by distinct isoforms of p51/p63. Archives of Oral Biology 2012;57:1108-1115.

18. Lo Muzio L et al. p63 expression correlates with pathological features and biological behaviour of odontogenic tumours. Histopathology 2006;49:198-216.

19. Gurgel CAS et al. Expression of Ki-67, p53 and p63 proteins in keratocyst odontogenic tumours: an immunohistochemical study. J Mol Hist 2008;39:311-316.

20. Seyedmajidi M et al. p63 expression in randomized odontogenic cysts. Saudi Med J 2011; 32:463-466.

21. Moghadam SA et al. Immunohistochemical analysis of p63 expression in odontogenic lesions. BioMed Research International 2013;1-4.

22. Varsha BK et al. Evaluation and comparison of expression of p63 in odontogenic keratocyst, solid ameloblastoma and unicystic ameloblastoma. J Oral Maxillofac Pathol 2014;18:223-228.

23. Jaafari-Ashkavandi Z et al. p63 and Ki-67 expression in dentigerous cyst and ameloblastomas. Dent Shiraz Univ Med Sci. 2015;16:323-328.

24. Kircham J et al. Maturation in developing permanent porcine enamel. J Dent Res 1988; 69:1156-1160.

25. Inai T et al. Immunohistochemical demonstration of amelogenin penetration toward the dental pulp in the early stages of ameloblast development in rat molar tooth germs. The Anatomical Record 1991;229:259-270.

26. Salido EC et al. The human enamel protein gene amelogenin is expressed from both the X and Y chromosomes. Am J Hum Genet 1992;50:303-316.

27. Inage et al. Gene expression and localization of amelogenin in the rat incisor. Adv Dent Res 1996;10:2002-2007.

28. Karg HA et al. Gene expression and immunolocalisation of amelogenins in developing embryonic and neonatal hamster teeth. Cell Tissue Res 1997;288:545-555.

29. Zeichner-David M et al. Timing of the expression of enamel gene products during mouse tooth development. Int J Dev Biol 1997;41:27-38.

30. Papagerakis P et al. Evidence for regulation of amelogenin gene expression by 1,25-dihydroxyvitamin D3 in vivo. Journal of Cellular Biochemistry 1999;76:194-205.

31. Wakida K et al. Maturation ameloblasts of the porcine tooth germ do not express amelogenin. Histochem Cell Biol 1999;111:297-303.

32. Ravindranath RMH et al. Tyrosyl motif in amelogenins binds to N-acetyl-D-glucosamine. J Biol Chem 1999;274:2464-2471.

33. Ravindranath RMH et al. Amelogenin - cytokeratin 14 interaction in ameloblasts during enamel formation. J Biol Chem 2001;276:36586-36597.

34. Hu et al. A comparison of enamelin and amelogenin expression in developing mouse molars. Eur J Oral Sci 2001;109:125-132.

35. Oida S et al. Amelogenin gene expression in porcine odontoblasts. J Dent Res 2002;81:103-108.

36. Hatakeyama J et al. The receptor activator of nuclear factor -κB ligand-mediated osteoclastogenic pathway is elevated in amelogenin-null mice. J Biol Chem 2003;278:35743-35748.

37. Nagano T et al. Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. J Dent Res 2003;82:982-986.

38. Papagerakis P et al. Expression of amelogenin in odontoblasts. Bone 2003;32:228-240.

39. Denbesten PK et al. Characterization of human primary enamel organ epithelial cells in vitro. Archives of Oral Biology 2005;50:689-694.

40. Torres-Quintana MA et al. Ameloblastin and amelogenin expression in posnatal developing mouse molars. Journal of Oral Science 2005;47:27-34.

41. Yan Q et al. Differentiation of human ameloblast-lineage cells in vitro. Eur J Oral Sci 2006; 114:154-158.

42. Ye L et al. Amelogenins in human developing and mature dental pulp. J Dent Res 2006; 85:814-818.

43. Haze A et al. Amelogenin expression in long bone and cartilage cells and in bone marrow progenitor cells. The Anatomical Record 2007;290:455-460.

44. Cohen YG et al. Amelogenin in craniofacial development: The tooth as a model to study the role of amelogenin during embryogenesis. 2007;1-21.

45. Nel S et al. Immunohistochemical profile of odontogenic epithelium in developing dog teeth (Canis familiaris). Veterinary Pathology 2011;48:276-282.

46. Landin et al. Gene expression profiling during murine tooth development. Frontiers in Genetics 2001;3:1-12.

47. Zheng L et al. Circadian rhythms regulate amelogenesis. Bone 2013;55:158-165.

48. Gasse B et al. Comparative expression of the four enamel matrix protein genes, amelogenin, ameloblastin, enamelin and amelotin during amelogenesis in the lizard anolis carolinensis. EvoDevo 2015;6:1-9.

49. Kim et al. Differential expression of amelogenin, enamelin and ameloblastin in rat tooth germ development. International Journal of Oral Biology 2016;41:89-96.

50. Frasheri I et al. Full-length amelogenin influences the differentiation of human dental pulp stem cells. Stem Cell Research and Therapy 2016;7:1-12.

51. Mazumder P et al. Amelogenin - ameloblastin spatial interaction around maturing enamel rods. Journal of Dental Research 2016;95:1042-1048.

52. Mori M et al. Immunohistochemical expression of amelogenins in odontogenic epithelial tumours and cysts. Virchows Archiv A Pathol Anat 1991;418:319-325.

53. Snead LM et al. Human ameloblastoma tumors express the amelogenin gene. Oral Surg Oral Med Oral Pathol 1992;74:64-72.

54. Saku T et al. Immunohistochemical demonstration of enamel proteins in odontogenic tumors. J Oral Pathol Med 1992;21:113-119.

55. Kumamoto H et al. Immunohistochemical detection of amelogenin and cytokeratin 19 in epithelial odontogenic tumors. Oral Diseases 2001;7:171-176.

56. Tsujigiwa H et al. Analysis of amelogenin gene (AMGX, AMGY) expression in ameloblastoma. Oral Oncology 2005;41:843-850.

57. Ren C et al. Differential enamel and osteogenic gene expression profiles in odontogenic tumors. Cells Tissues Organs 2011;194:296-301.

58. Pindborg JJ et al. Morphologic and functional correlations in the enamel organ of the rat incisor during amelogenesis. Acta anat 1959;36:367-381.

59. Gibson CW et al. The amelogenin proteins and enamel development in humans and mice. J Oral Biosci. 2011;53:248-256.

60. Simmer JP et al. Isolation and characterization of mouse amelogenin expresssed in escherichia coli. Calcif Tissue Int. 1994;54:312-319.