EVALUATION OF EXPRESSION OF AMELOBLASTIN AND NOTCH IN HUMAN TOOTH GERM AND ASSOCIATED STRUCTURES AND IN 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

EVALUATION OF EXPRESSION OF AMELOBLASTIN AND NOTCH IN HUMAN TOOTH GERM AND ASSOCIATED STRUCTURES AND IN 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. SUGANYA**, **BDS,** 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 AMELOBLASTIN AND NOTCH IN HUMAN TOOTH GERM AND ASSOCIATED STRUCTURES AND IN 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 **MDS in 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.

TAMIL NADU GOVERNMENT
DENTAL COLLEGE AND HOSPITAL CHENNAI – 600 003

DECLARATION

I, Dr. P. Suganya, BDS, do hereby declare that the dissertation titled "**Evaluation of expression of ameloblastin and Notch in human tooth germ and associated structures and in ameloblastoma"** was done in the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003. I have utilized the facilities provided in the Government Dental College and Hospital and Institute of Obstetrics and Gynaecology, Madras Medical College, Egmore, Chennai - 600 008 for the study in partial fulfilment 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 and guidance of the Principal investigator, Dr. I. Ponniah, MDS, Prof. and Head of Oral Pathology and Microbiology.

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 – 600 003, 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 the 22nd January, 2017 between the Tamil Nadu Government Dental College and Hospital represented by its Principal having address at the Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, (hereafter referred to as, 'the college')

and

Dr. I. PONNIAH, MDS, aged 50 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, 3rd Main Road, "Ring Road Housing Sector", Madhavaram in Chennai - 600 060 (herein after referred to as the 'Researcher and Principal investigator')

and

Dr. P. SUGANYA, BDS, aged 26 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 ameloblastin and Notch in human tooth germ and associated structures and in ameoblastoma"** 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 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. The PG student and Principal investigator undertake not to divulge (or) cause to be divulged any of the confidential information or, know – How to anyone in any manner whatsoever and for any purpose without the express written consent of the college.
6. 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).
7. The college shall provide all infrastructure and access facilities within and in other institutes to the extent possible. This includes patient interactions, introductory letters and such other acts required in this regard.
8. 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.

9. 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.
10. 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

My sincere thanks to Prof. Dr. Saravanan, MDS, Principal, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, for granting me permission as Chairman of the Institutional Ethical Committee (IEC) to undertake this study.

I would like to thank all the members of the Institutional Ethical Committee (IEC), Tamil Nadu Government Dental College and Hospital, Chennai - 600 003 for their critical scientific comments and granting permission to undertake this study.

I extend my heartfelt gratitude to the Director & Professor and other faculties of the Institute of Obstetrics and Gynaecology, Madras Medical College, Egmore, Chennai – 600 008, for graciously according permission to utilize unclaimed human fetuses for this study.

I owe thanks to Dr. P. Srikantha Lakshmi, BDS, co-postgraduate student, for encouragement and support during immunohistochemistry.

My sincere thanks to Mrs. Meenakshi, Mrs. Hilda Margaret, B.Sc and Mrs. Sargunavathy, the laboratory technicians of Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, for their laboratory assistance during this study.

I also express my sincere gratitude for the kind encouragement showered on me during my post graduate course by Dr. R. Bharathi, MDS, Professor of Oral Pathology, Tamil Nadu Government Dental College and Hospital.

My sincere thanks to the support extended by Dr. S. Gnanadeepam, MDS, Associate Professor of Oral Pathology, and Dr. M. P. Sumathy, MDS, Associate Professor of Oral Pathology, Tamil Nadu Government Dental College and Hospital.

I also thank Dr. S. Kuzhali, Tutor/Assistant professor of Oral Pathology, MDS, Dr. S.P. Selvajothi Ranjitham, MDS, Tutor/Assistant professor of Oral Pathology, Dr. J. Dhanalakshmi, MDS, Tutor/Assistant professor of Oral Pathology, and Dr. V. Shanthi, MDS, Tutor/Assistant professor of Oral Pathology, for their support and encouragement.

I thank Dr. I. Ponniah, MDS, Professor and Head of Oral Pathology, Tamil Nadu Government Dental College and Hospital, Chennai for his help in the conceptualization, design, and as well as for his guidance as Principal investigator during all stages of this study.

I would fail in my duty if I fail to recognize all the qualified teaching faculty who had served in the department in the order as found below; Prof. Dr. R. Vishwanathan, Prof. Dr. T. R. Saraswathi, Dr. Shantha Bharathan, Prof. Dr. V. L. Indirani, Prof. Dr. R. Chandrabai, Prof. Dr. Shaheen Ahmed, Prof. Dr. I. Ponniah, Dr. M. R. C. Rajeshwari, Prof. Dr. R. Bharathi, Dr. S. Gnanadeepam, Dr. M. P. Sumathy, Dr. J. Dhanalakshmi, Dr. V. Shanthi, Dr. J. Jude, Dr. S. Jayalakshmi, Dr. S. Kuzhali and Dr. S.P. Selvajothi Ranjitham for their invaluable endeavor towards contribution to the diagnosis and preservation of vital source of information and materials to accomplish this study with ease.

Lastly, my deep appreciation to my parents for their financial support.

Permitted
TORMO/DRMO
5/12/16

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 fordisposal 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. Suganya, (MDS Postgraduate Student) to avail foetus tissue from your Institute of Obstetrics and Gynaecology, for the purpose of doing dissertation. I humblysubmit that your permission in this regard will be appropriately mentioned in the Materials and Methods and i.1 the acknowledgement section of the proposed dissertation.

Thanking you,

Sincerely,

November 28, 2016.

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.

Permitted
TORMO/DRMO
5/12/16

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 fordisposal 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. Suganya, (MDS Postgraduate Student) to avail foetus tissue from your Institute of Obstetrics and Gynaecology, for the purpose of doing dissertation. I humblysubmit that your permission in this regard will be appropriately mentioned in the Materials and Methods and i.1 the acknowledgement section of the proposed dissertation.

Thanking you,

Sincerely,

November 28, 2016.

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.

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 Ameloblastin and Notch in human tooth germ and associated structures and in odontogenic tumors.

Investigator: Dr. P. Suganya,
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.

To
Dr. P. Suganya 1st yr
Approved
17/11/17

Dr. I. PONNIAH, MDS.,
(Reg No. 16...)
Professor & Head
Department of Oral Pathology
Tamil Nadu Government Dental College & Hospital
Chennai - 600 003.

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

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

URKUND

Urkund Analysis Result

Analysed Document:	upload.docx (D34781813)
Submitted:	1/17/2018 4:21:00 PM
Submitted By:	sughik1629@gmail.com
Significance:	4 %

Sources included in the report:

Thesis - IMRAD.docx (D34330736)
http://onlinelibrary.wiley.com/doi/10.1111/j.1469-7580.2009.01167.x/full
https://www.researchgate.net/publication/264793570_Expression_of_cytokeratin_8_vimentin_syndecan-1_and_Ki-67_during_human_tooth_development

Instances where selected sources appear:

14

CERTIFICATE - II

This is to certify that this dissertation work titled "**EVALUATION OF EXPRESSION OF AMELOBLASTIN AND NOTCH IN HUMAN TOOTH GERM AND ASSOCIATED STRUCTURES AND IN AMELOBLASTOMA"** of the candidate **Dr. P. SUGANYA, BDS,** with registration Number **241521002** for the award of **MDS** in the branch **VI – Oral Pathology and Microbiology.** 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 result shows **4%** percentage of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

ABSTRACT

OBJECTIVES: The present study was to compare the expression pattern of ameloblastin and Notch-1 in human tooth germ and associated structures, and in ameloblastoma to determine cytodifferentiation between tooth germ and ameloblastoma and to determine the stem cell pool in the human tooth germ.

STUDY DESIGN: The study included 11 tooth germs (EBS-4 & LBS-7) from 7 fetuses (20 to 26 weeks old) and 6 tissue samples of ameloblastoma retrieved from the archives. The morphological characteristics of the tooth germ and ameloblastoma in hematoxylin and eosin sections were studied under light microscopy and immunohistochemistry was performed using ameloblastin and Notch-1 antibodies against human tooth germ and ameloblastoma tissue sections.

RESULTS: Information regarding expression of ameloblastin and Notch in human tooth germ is limited or negligible. In the present study, in the human tooth germ, ameloblastin was expressed in the acellular zone of the dental papilla with simultaneous fluctuating reaction within the IEE lineage that parallels with the similar type of IEE lineage cells present within the ameloblastoma. In the human tooth germ, Notch-1 was expressed in all cell layers of the enamel organ in both EBS (cytoplasm and nucleus) and LBS (nucleus) with reduced number of positive cells in the LBS when compared to EBS. In ameloblastoma, Notch-1 was expressed in both cytoplasm and nucleus of peripheral and central cells similar to the reaction in the tooth germ. Notch-1 neither identifies stem cell pool in the human tooth germ nor restricted to SI. Ameloblastin and Notch-1 was expressed in the immature osteoid and chondroid matrix but not in the mature bone.

CONCLUSION: Ameloblastin in the acellular zone of the dental papilla are required for the differentiation of odontoblasts and ameloblasts. The expression of ameoblastin in human tooth germ and ameloblastoma showed similar reaction pattern related to the common cytodifferentiation occurring among them. Notch-1may have a role in the differentiation of IEE lineage based on the reduced intensity and decrease in the number of positively stained cells in LBS compared to EBS. The expression of Notch-1 in ameloblastoma parallels the reaction pattern in the EBS and LBS of human tooth germ but difficult to determine the cytodifferentiation within the tumor. Notch-1 was not specific to identify neither the SI nor the stem cell pool in the human tooth germ rather helped in enamel and bone formation.

ABBREVIATIONS

EBS	**:**	Early bell stage
LBS	**:**	Late bell stage
IEE	**:**	Inner enamel epithelium
OEE	**:**	Outer enamel epithelium
PA	**:**	Preameloblasts
PSA	**:**	Presecretory ameloblasts
SA	**:**	Secretory ameloblasts
SI	**:**	Stratum intermedium
SR	**:**	Stellate reticulum
DP	**:**	Dental papilla
DF	**:**	Dental follicle
PBS	**:**	Phosphate buffer solution
HRP	**:**	Horse Radish Peroxidase

CONTENT

INTRODUCTION

Odontogenesis, in literal sense, means the formation of tooth. However, it is not only the formation of tooth but also its supporting structures. For proper odontogenesis to occur, two distinct tissues are required for induction and reciprocation. At the beginning of inception of odontogenesis, the odontogenic epithelium segregates from oral epithelium and instruct the underlying mesenchymal cells (ectomesenchymal cells) to acquire the ability to form tooth. However, soon thereafter, it is the mesenchyme that adopts the instructive role for further tooth development. Both, odontogenic epithelium and ectomesenchyme interact with each other during every stages of tooth development that begins with initiation of bud through cap and bell stages.

The tooth germ includes enamel organ, dental papilla and dental follicle each contributing enamel, dentin and pulp, cementum, periodontal ligament and alveolar bone, respectively. The tooth germ is enclosed in a bony case which eventually becomes the future alveolar bone. Of the tooth germ, the enamel organ is an epithelial product whereas the dental papilla and the dental follicle are derived from neural crest cells. The mesenchyme in the head and neck region is referred to as ectomesenchymal cells because they are derived from neural crest cells.

The formation of tooth germ starts with the epithelial-mesenchymal interactions that determine the shape of the tooth and differentiation of the formative cells of the tooth and their eventual secretions at the appropriate time. Dental lamina is formed by the proliferation of certain areas of basal ectoderm at about 6 weeks which divides into inner lingual process and outer vestibular process at about 7^{th} week. The ectodermal cells along the dental lamina multiply still more rapidly and forms little knobs that grow into the underlying mesenchyme

represents enamel organ. The enamel organ changes its shape due to its differential growth providing 4 developmental stages namely bud, cap, early and advanced bell stages.

The epithelium of the dental lamina is separated from the underlying ectomesenchyme by a basement membrane, from where round or ovoid swellings develop called tooth buds. They represent the primordia of the enamel organ which consists of peripheral low columnar and central polygonal cells. The tooth bud eventually condenses due to the increased mitotic activity and the migration of neural crest cells into the area of ectomesenchymal cells. Dental papilla represents the condensed area immediately adjacent to the enamel organ whereas dental sac represents the condensed area surrounding tooth bud.

The cap stage is characterized by shallow invagination on the deep surface of the bud which consists of outer enamel epithelium, inner enamel epithelium, stellate reticulum, dental papilla and dental sac. The outer enamel epithelium is cuboidal in shape that covers the convexity and concavity comprises of tall, columnar cells that represent inner enamel epithelium. The stellate reticulum are polygonal shaped cells located in the center of the enamel organ and eventually separate from each other as a result of water being drawn into it giving star-shaped cells. They have cushion like consistency and acts as a shock absorber that may support and protect the delicate enamel forming cells. The dental papilla represents the formative organ of the dentin and the primordium of the pulp. The dental papilla shows changes during the development of the enamel organ. The peripheral cells of the dental papilla enlarge and later differentiate into odontoblasts.

The enamel organ resembles bell as the epithelium invaginates and the continuous growth of the margins. It consists of inner enamel epithelium, stratum intermedium, stellate reticulum, outer enamel epithelium, dental lamina and dental sac. The inner enamel epithelial cells in the future cusp tip or incisal region stop dividing earlier and begin to differentiate

first. Cell differentiation proceeds gradually from the cusp tip to the cervix. The cells of inner enamel epithelium exert an organizing influence on the underlying mesenchymal cells in the dental papilla, which differentiate into odontoblasts. The cells of stratum intermedium along with the inner enamel epithelium combined as a single functional unit and helps in the formation of enamel. The stellate reticulum collapses before the enamel formation, reducing the distance between the ameloblasts and outer enamel epithelium in order to provide nutrients necessary for the formation of enamel. The cells of the outer enamel epithelium flatten and laid into folds during the formation of enamel. The acellular zone in between the inner enamel epithelium and the dental papilla disappear with subsequent formation of the dentin.

The final developmental stage is an advanced bell stage characterized by the initiation of mineralization and root formation. The cervical portion gives rise to Hertwig's epithelial root sheath which is responsible for the shape, length, size and the number of roots. The tooth germs are enclosed in a bony case which is a mineralized connective tissue.

The contact between the enamel organ and the ectomesenchyme acts as the primary source for the differentiation of ameloblasts and odontoblasts. The dental papilla cells controls the shape of the tooth and gained the ability to direct the differentiation of the epithelial cells. Electron microscopic studies showed that cell-to-cell contacts were formed between ameloblasts and odontoblasts which further initiate differentiation. The basement membrane matrix undergoes modifications during the successive steps of odontogenesis. Fibronectin, fibronectin receptors, syndecan and tenascin are participated in matrix-mediated signaling during odontogenesis.

Ameloblastin, also known as amelin or sheathelin is the most abundant non-amelogenin enamel matrix protein. It is expressed at higher levels at the inception of

secretory stage and continues throughout the secretory stage and diminishes during maturation stage of ameloblasts.[1] In addition, it is also expressed transiently by the alveolar bone.[2]

Notch encodes a transmembrane protein important for various cell fate decisions during development. The literature shows that Notch signal maintains stem cell character of cells. In the tooth germ of rat, it was shown that cells that express Notch are situated in the central core of cells in the cervical loop region.[3] This observation was based on the fact that when the inner enamel epithelium in the cusp tip region was removed, cells migrated and reached cusp tip from the cervical loop region, but when the reverse experiment was performed no such event took place.[3] In addition, it has been reported that in rat incisor, the stratum intermedium cells diverge from a subpopulation of inner enamel epithelium.[4] In the rat tooth germ, immunohistochemical observation revealed that Notch-1 was expressed in stratum intermedium and Notch-2 was expressed by stellate reticulum.[4] In the human tooth germ, RT-PCR and in-situ hybridization techniques revealed that Notch-1 was expressed in all cells of the enamel organ except IEE lineage.[5] However, there are no published studies as of yet regarding the immunohistochemical expression of Notch-1 in human tooth germ.

Therefore, the purpose of the present study is to evaluate the expression of ameloblastin and Notch-1 by immunohistochemistry in the human tooth germ and associated structures and in ameloblastoma.

AIMS AND OBJECTIVES

AIM:

To evaluate the expression of ameloblastin and Notch-1 in the human tooth germ and associated structures and in ameloblastoma.

OBJECTIVES:

1. To determine the expression of ameloblastin in the human tooth germ and associated structures, and in ameloblastoma.
2. To determine the expression of Notch-1 in the human tooth germ and associated structures, and in ameloblastoma.
3. To determine the stem cell pool in the enamel organ.
4. To compare the expression pattern of ameloblastin and Notch-1 in the human tooth germ and in ameloblastoma.

REVIEW OF LITERATURE

I. EXPRESSION OF AMELOBLASTIN

In 1996, Lee et al, studied the expression of ameloblastin in rat incisor and human tooth germ using in-situ hybridization, IHC and western blot techniques. The authors' showed that ameloblastin in rat incisor was first expressed in the pre-secretory ameloblasts (in-situ hybridisation) and pre-ameloblasts (IHC), became intense in secretory ameloblasts and gradually reduced in late maturation stage. In the human tooth germ, it was intensely expressed in the cuspal region in early bell stage and secretory ameloblasts and enamel in late bell stage. According to the authors', the expression of ameloblastin is believed to have a role in enamel formation.[6]

In 1996, Krebsbach et al, studied expression of ameloblastin in rat incisor using Northern blot analysis of RNA, in-situ hybridization and IHC techniques. The authors' showed that ameloblastin mRNA was not detected in any other mineralized structures such as bone and cartilage but was detected only in the incisor tissue by Northern blot analysis. The authors' also showed that ameloblastin was expressed in the distal cytoplasm of secretory ameloblasts by in-situ hybridization. The authors' showed that expression of ameloblastin was positive in the secretory ameloblasts, tome's processess and enamel matrix. According to the authors', ameloblastin is believed to play a role in matrix secretion.[7]

In 1997, Uchida et al, studied the fate of ameloblastin in rat incisor by IHC techniques using various antibodies such as Nt (residue 27-47), M1 (residue 98-107), M2 (residues 224-232), M3 (residues 386-399) and Ct (residues 406-419) that recognizes the aminoacid residues in ameloblastin. The authors' showed that these antibodies

immunostained the cytoplasm of secretory ameloblasts and enamel matrix (except M3) but did not stain either dentin or odontoblasts prompting the authors' to suggest that ameloblastin may have a possible role in anchoring the ameloblast to the newly synthesized enamel matrix during secretory stage of amelogenesis.[8]

In 1998, Kirn et al, studied the expression of DSP, DPP, enamelysin and ameloblastin in 4-day old mice molars using in-situ hybridization and immunohistochemistry. The authors' showed that ameloblastin transcripts were expressed in the polarizing and functional ameloblasts, pre-odontoblasts, odontoblasts and weakly in the dental papilla cells. The authors' also showed that the ameloblastin was expressed extracellularly concomitant with the differentiation of ameloblasts. Thus, the authors' concluded that the expression of ameloblastin may play an important role in the differentiation of ameloblasts and mineralization of an enamel matrix.[9]

In 1998, Fong et al, studied the expression of amelin in rats using insitu hybridization and IHC techniques. The authors' showed that the expression of amelin was detected in the differentiating ameloblasts only after the formation of dentin with gradual fading of expression in the odontoblasts and neighbouring cells. In addition, it was also expressed in the secretory ameloblasts and post-secretory ameloblasts and enamel matrix with patchy distribution in the DEJ. According to the authors', the expression of amelin appeared earlier in the undifferentiated mesenchymal cells of dental papilla and pre-odontoblasts before it was expressed in the IEE lineage indicating the epithelial-mesenchymal interaction in the differentiation of IEE lineage.[10]

In 1998, Nanci et al, compared the expression of ameloblastin and amelogenin in rat incisors using insitu hybridization, immunoblotting and immunochemistry techniques. The authors' showed that both amelogenin and ameloblastin were expressed from pre-secretory to the maturation stage but the latter sustains the intensity till late maturation stage. The authors' also showed that both the proteins are expressed in the enamel matrix. According to the authors', both the proteins are involved in the differentiation of IEE lineage and secretion of enamel matrix.[11]

In 2000, Takata et al, studied the expression of sheathelin in human tooth germ and odontogenic tumors using IHC techniques. The authors' showed that the sheathelin was expressed in the enamel matrix with less intense staining in the cytoplasm of secretory ameloblasts. The authors' also showed that sheathelin was expressed in the eosinophilic droplets of AOT, immature enamel in odontomas and ameloblastic fibro-odontoma, ghost cells of COC but not in ameloblastoma. According to the authors', the tumor cells of ameloblastoma donot attain full differentiation into functional ameloblasts.[12]

In 2000, Mac Dougall et al, studied the expressionof ameloblastin in human tooth germ using IHC techniques. The authors' showed that the expression of ameloblastin was first detected in the secretory ameloblasts with less intensity when compared to enamel matrix and DEJ. The authors' also showed faint immunostaining in the pre-dentin and developing odontoblasts. According to the authors', the ameloblastin is believed to have a role in the enamel formation.[13]

In 2003, Nagano et al, studied relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts in porcine tooth germ using techniques such aas

histochemistry, RT-PCR. The authors' divided the samples as EOE containing SA, SI-like cells and a thick layer of SR and the odontoblast samples contains mostly odontoblasts. The authors' showed that mRNAs encoding enamel structural proteins such as amelogenein, enamelin, sheathelin and enamelysin were found more or less both in the EOE and odontoblasts sample. However, the PCR products of enamel structural proteins were not detected in the pre-odontoblast cell layers and dental pulp cells. The DSPP PCR product was found only in the young and mature odontoblasts. The expression of the amelogenin, ameloblastin and sheathelin were found in decreasing order from secretory ameloblasts, maturation ameloblasts, young odontoblast and mature odontoblast. Thus, the authors' showed that structural enamel proteins were expressed in both the EOE and odontoblast cell layer samples. The authors' also demonstrated the same in an erupted young first molar in the root forming stage where the ameloblasts disappeared after enamel formation and the authors' have also found that inspite of the absence of ameloblasts, all the enamel matrix proteins namely amelogenin, enamelin, sheathelin and enamelysin were detected in the odontoblast sample. The authors' concluded that, in the porcine, secretory ameoblasts express mRNA encoding enamel structural proteins at levels thousands of times higher than do maturation ameloblasts and odontoblast lining the dentin beneath the secretory ameloblasts enamel and also younger odontoblasts lining dentin closer to the dentinoenamel junction express mRNAs encoding enamel structural proteins at levels over 30 fold higher than do older odontoblasts forming dentin further from the dentinoenamel junction. Thus, the authors' suggest that enamel structural proteins secreted by odontoblasts may play a role in the formation of dentin, particularly at the dentinoenamel junction.[14]

In 2003, Lee et al, studied the structure of ameloblastin gene and its expression in amelogenesis in rat tooth germ using RNA isolation and Northern blot analysis, RT-PCR,

Motif analysis, in-situ hybridization, immunohistochemistry and western blot analysis techniques. The authors' showed that ameoblastin I (exon 11) was positive from the preseretory to the maturation stages of ameloblast differentiation and the reaction was more intense in the cytoplasm of the secretory ameloblasts, whereas hybridization using other transcript ameloblastin II (exon 3a) gave a relatively weak reaction in the secretory ameloblasts but an intense reaction in the distal cytoplasm of post secretory ameloblasts and maturation ameloblasts. The authors' also showed strong reaction of ameloblastin antibody in presecretory to post secretory ameloblasts with more intense reaction in the distal cytoplasm of secretory ameloblasts in addition to the early and late maturation stage of ameloblasts. The authors' also showed weak staining in presecretory ameloblasts and in the distal cytoplasm of secretory ameloblasts, but a strong positive reaction in post secretory ameloblasts against exon 3a with increased intensity during late maturation. Thus, the authors' observed differences in the pattern of ameloblastin transcripts and translation products from the secretory to the maturation stage of ameloblast differentiation. Ameloblastin I was expressed in the secretory ameloblasts whereas ameloblastin II was restricted to the maturation stage ameloblast. The authors' suggest that differences in the distribution of ameloblasts among the secretory and maturation ameloblasts are due to the difference in phosphorylation properties which helps in mediating biomineralisation. Thus, the authors' concluded that ameloblastin II, containing exon 3a, was more intensely expressed in the late maturation stage of ameloblasts than the secretory stage, thus suggesting its role in biomineralisation during maturation stage.[15]

In 2004, Fukomoto et al, studied the expression of ameloblastin in mice using IHC techniques. The authors' observed that there was no differences between normal and double mutant knock-out mice tooth germ until the presecretory stage. During the secretory stage,

the ameloblasts in the knock-out mice formed enamel matrix, but detached sooner from the enamel matrix and start proliferating to form a multilayer of cells that positively stained for enamel matrix protein. In addition, the authors' found that ameloblastin null mice had defective enamel formation but normal development of craniofacial bones. The authors' also showed that enamel proteins were expressed in the tumors developed in the mutant mice except ameloblastin. Based on their observation, the authors' suggest that ameloblastin is required for the maintenance of a differentiated phenotype during enamel formation and the absence of ameloblastin resulted in unregulated cell proliferation in the tumors.[1]

In 2004, Perdigao et al, compared the ameloblastin gene in epithelial odontogenic tumors and normal mucosa using PCR techniques. The authors' showed that normal cells from the patient's oral mucosa were free from deletions or insertions whereas odontogenic tumors such as ameloblastoma, AOT and SOT revealed mutation in the ameloblastin gene. Thus, the authors' concluded that mutation in the ameloblastin gene may lead to the development of odontogenic tumors.[16]

In 2005, Torres-Quintana et al, studied the expression of ameloblastin and amelogenin in post natal developing mouse molars using in-situ hybridization techniques. The authors' showed that both ameloblastin and amelogenin are expressed in the secretory ameloblasts, but gradually decreased during the maturative stage, when the ameloblasts detach from the newly formed enamel. However, the expression of ameloblastin remained a little longer than amelogenin and ends at CEJ once the decision to form root is made or probably due to lack of signals from stratum intermedium or stellate reticulum.[17]

In 2006, Spahr et al, studied the expression of ameloblastin during craniofacial bone formation in rats using IHC, in-situ hybridization and RT-PCR techniques. The authors' showed that expression of ameloblastin is not only restricted to ameloblasts, odontoblasts and pulpal mesenchymal cells but also expressed in the newly formed bone. However, the expression of ameloblastin show different patterns of expression between intramembranous and endochondral ossifications. In addition, ameloblastin is also expressed in the cartilage and perichondrium during endochondral ossification. On the other hand, it is expressed in the cellular layer surrounding the newly formed bone during intra-membranous ossification, including alveolar bone. The authors' suggest that ameloblastin may regulate craniofacial bone formation. However, the authors' is of the opinion that the expression of ameloblastin is neither sufficient nor necessary for osteogenesis because no bone defect has been noted in ameloblastin-null mice.[2]

In 2009, Wazen et al, studied the expression of ameloblastin in AMBN heterogenous mice, AMBN wild type mice and AMBN mice lacking exons 5 and 6 by using IHC and western blotting techniques. The authors' showed that mice lacking exons 5 and 6 of AMBN gene exhibits problems with amelogenesis due to failure of initial layer of enamel formation and development of Tome's process. Instead, vacoulation appeared between dentin and ameloblasts leading to disorganized enamel organ and loss of columnar morphology of ameloblasts where it is no longer distinguishable as such but eventually forming a dysplastic matrix material and altered tooth associated structures such as porous interdental bone. In addition, ameloblastin was not expressed in the matrix masses present throughout the disorganized enamel organ. Thus, the authors' concluded that ameloblastin may directly or indirectly required for the formation of an organized enamel organ.[18]

In 2009, Smith et al, studied consequences for enamel development and mineralization resulting from loss of function of ameloblastin or enamelin in mice using microweighing of mineral content technique. The authors' revealed that ameloblastin must be present around ameloblasts (extracellular matrix) for induction of mineralization. Further, the authors' showed that failure of enamel formation result from either truncated form of ameloblastin or enamelin-null mice. The authors' also showed that amelogenin-null model mice with the creation of mineralization front by the secretion of normal ameloblastin and enamelin but ameloblasts are unable to generate much appositional growth as there is consequent loss of amelogenin as well. Thus, the authors' conclude that ameloblastin is necessary to initiate mineralization of enamel matrix.[19]

In 2010, Tambursten et al, studied the expression of ameloblastin in mice using RT-PCR, western blot and IHC techniques. The authors' demonstrate that ameloblastin gene and its protein product are expressed in wide variety of cells such as osteoblasts, osteoclast, chondrocytes and CD34+ cells especially in sites close to hard tissue matrix, but did not react with osteocytes in mature bone. The authors' proposed that ameloblastin may have a regulatory role in bone formation during embryogenesis and repair in adult bone.[20]

In 2011, Zhang et al, studied the expression of ameloblastin in the cultured dental follicle cells and periodontal ligament cells from mouse molar using western blot techniques. The authors' showed that ameloblastin enhances adhesion of PDL and DF cells by forming cell processes in the ameloblastin coated dishes and reduces proliferation of the same. According to the authors', ameloblastin maintains the DF and PDL cells in its differentiated state.[21]

In 2012, Crivelini et al compared the expression of enamel proteins such as odontogenic ameloblast associated protein, ameloblastin, amelogenin and amelotin in odontogenic tumors using IHC techniques. The authors' showed that ameloblastin expression was positive in the fusiform cells of AOT, polyhedral tumor cells of CEOT, secretory ameloblasts and enamel surface in odontomas, but negative in ameloblastoma, ameloblastic fibroma, odontogenic fibroma. These findings suggest that tumor cells that possess secretory activity shows positivity for ameloblastin, whereas tumor cells that fails to react did not attain such level of differentiation.[22]

In 2013, Hirose et al, studied the expression of ameloblastin in mice using RT-PCR techniques. The authors' showed that ameloblastin is expressed in the basal cells of HERS and its expression is necessary for proper root formation. The authors' proved their premise by injecting ameloblastin siRNA (small interfering RNA) into the mesial aspect of mandibular molar in a 10 day post-natal mice which lead to the formation of shorter roots and irregular root dentin with multilayer formation in the basal portion of HERS. In addition, ameloblastin depleted cells showed increased proliferative activity as measured by increased bromouridine incorporation. Thus, the authors' conclude that AMBN may regulate not only differentiation state of HERS derived cells, but also function as a trigger for normal root formation.[23]

In 2014, Kim et al, compared peripheral ameloblastoma and oral basal cell carcinoma using IHC techniques with a panel of markers including ameloblastin. The authors' showed differentially expressed proteins in the peripheral ameloblastoma and oral basal cell carcinoma and found that EpCam (epithelial cell adhesion molecule) was exclusively expressed in the oral basal cell carcinoma and matrix related proteins such as KL1 was

expressed in the peripheral amelobastoma. The authors' also showed that immunostaining of ameloblastin, amelogenin, Krox-25, carcino embryonic antigen, E-cadherin, p63, cathepsin K were positive in the peripheral ameloblastomas whereas tumor growth factor β1, surviving, α1-antitrypsin, tumor necrosis factor α, matrix metalloproteinases-1 were positive in the oral basal cell carcinomas. The authors' also showed oral basal cell carcinoma has more tendency for malignant transformation and aggressive behavior due to the over expression of p53, survivin and greater nuclear β-catenin. Thus, the authors' concluded that both the tumors were varied in the process of tumorogenesis.[24]

ACELLULAR ZONE IN THE TOOTH GERM:

In 1969, Slavkin et al, studied the fine structure of extracellular matrix during epithelial-mesenchymal interactions in the incisor tooth germ of rabbit under electron microscope. The authors' showed that the cervical loop consists of rapidly dividing cell population and the undifferentiated mesenchymal cells adjacent to it were progenitors called pre-odontoblasts under light microscope. The electron microscopic study revealed that cytoplasmic extensions from the undifferentiated mesenchymal cells were in close contact with the basal lamina of the enamel epithelium. The basal lamina appeared continuous along the epithelial-mesenchymal interface with small clusters of collagen fibrils in the dental papilla side whereas the lamina near the apex of the cervical loop consists of less number of microfibrils. In addition, the junction between the epithelium and the connective tissue suggested a decrease in the number of filopodia but an increase in the density of the microfibrils adherent to the basal lamina. The authors' also showed that electron-dense basal lamina was separated from the inner enamel epithelium by an electron-transparent space consisting of outer cell coat of the inner enamel epithelium, basal lamina and extracellular collagen fibrils and suggested that the microfibril material may be a product of epithelial cells. Thus, the authors' concluded that selective migration of epithelial and mesenchymal cells towards each other was responsible for the subsequent differentiation to columnar secretory type cells, thus providing evidence that epithelial-mesenchymal interaction was necessary for the odontogenesis.[25]

In 1972, Sisca et al, studied dentin formation in human deciduous teeth using an electron microscope. The authors' showed that the epithelium and connective tissue in the concavity of the cap stage were separated by the basal lamina and an irregular filamentous layer. During the early bell stage, the cell-poor zone consists of few fibrils and cell processes

and the filamentous layer was organized into aperiodic fibers and the density of the latter increased in the intermediate bell stage (matrix maturation). The authors' also showed that fibril-free zone are narrowed down in the late bell stage and finally obscured during matrix formation. The authors' also found that the zone of aperiodic fibers remained dense until the odontoblastic processes were connected to the area and finally organized to form Von Korff's fibers. Thus, the authors' concluded that the basal lamina begins to disappear with the invasion of the collagen fibrils in the aperiodic fibril zone, thus providing epithelial-mesenchymal interaction for the initiation of dentin formation.[26]

In 1995, Sawada et al, studied the expression of basement membrane components in the dental papilla mesenchyme of monkey tooth germ using electron microscope and immunohistochemistry techniques with antibodies such as collagen IV, laminin and heparan sulfate proteoglycans. The authors' showed that the basal lamina consists of lamina fibroreticularis which was poorly developed at the growing-end region, later becomes thick and runs perpendicularly towards the dental papilla. All the three antibodies were expressed in the lamina fibroreticularis but with increasing intensities from the growing-end. The authors' also showed that the dental papilla cells remaining close to the basement membrane were positive for all three antibodies. Thus, the authors' showed that the amount of basement membrane components increases gradually in the lamina fibroreticularis during the tooth development. the authors' also showed that none of the antibodies were positive in the inner enamel epithelial cells and concluded that dental papilla cells in the mesenchyme and inner enamel epithelial cells play a role in the synthesis of basement membrane components.[27]

In 1996, Reith et al, studied amelogenesis in the molar tooth germ of young rats using electron microscope. The authors' showed that a narrow space of 1µ wide appears on the dental papilla side but not in the dental sac side and consists of microvilli of the dental

papilla cells and numerous non-striated fibrils arranged at right angles to the basal lamina. The authors' showed that the basal lamina was penetrated by the epithelial cells present adjacent to it and disrupted just after the odontoblast gets differentiated and before the mineralization of pre-dentin whereas the ameloblasts differentiate irrespective of the disruption of the basal lamina. However, the basal lamina was still contacted by the non-striated fibrils at the central part of pre-dentin. The authors' concluded that the ameloblasts are in close association with the non-striated fibrils as soon as the disruption of the basal lamina with subsequent formation of the vesicles which helps in matrix formation.[28]

In 1999, Sawada et al, studied the basement membrane of the inner enamel epithelium in monkey and rat tooth germ using electron microscope and IHC techniques with antibodies such as fibronectin and amyloid P. The authors' showed that the basement membrane of IEE was composed of lamina fibroreticularis. The authors' showed that the lamina fibroreticularis were composed of basotubules arranged perpendicular to each other and parallel to the cell surface and the dental papilla cells extend their processes towards the basement membrane. The authors' also showed that the narrow space between the basotubules and the cell surface were composed of firm filaments which stained positively for fibronectin along with the lamina fibroreticularis of the basement membrane in the monkey tooth germ. The authors' also showed that the lamina fibroreticularis of the basement membrane of inner enamel epithelial cells showed positivity for amyloid P. Thus, the authors' suggested that close association of dental papilla cells with the basement membrane of inner enamel epithelial cells were necessary for the differentiation of odontoblasts by forming tight grip by means of binding basotubules through fibronectin filaments which leads to the formation of a single layer of cells along the basement membrane that differentiate into odontoblasts. Thus, the authors' concluded that basotubules in lamina fibroreticularis function as an anchoring unit to hold the dental papilla cells during the differentiation of

odontoblasts, thus providing evidence for association of dental papilla cells with the inner enamel epithelial cells.[29]

II. EXPRESSION OF NOTCH

In 1995, Mitsiadis et al, studied the expression of Notch-1, 2 and 3 in the developing mouse tooth germ using in-situ hybridization and IHC techniques. The authors' showed that Notch genes exhibit distinct expression pattern in developing teeth and they are regulated by epithelial-mesenchymal interactions. During the bud stage, the Notch expression was noted in the superficial layers of epithelium and absent in mesenchyme. During the cap stage, Notch-1 and 2 were expressed in the epithelium except in the basal cells which are further differentiated into ameloblasts. Notch-1 was restricted to dental papillae, whereas Notch-3 was not expressed anywhere. During the bell stage, Notch-1 was restricted to cervical loop and stratum intermedium, Notch-2 in dental papillae and SR, Notch-3 in stratum intermedium and dental papilla. However, all three types of Notch are not expressed in the IEE lineage cells. According to the authors', the Notch expression maintains the cells in its undifferentiated state because notch was excluded in the differentiated cells.[30]

In 1997, Luo B et al, studied the expression of Notch-1 and its ligand Jagged-2 in murine embryos using in-situ hybridization, Northern blot analysis, Western blot analysis, C212 myogenesis assay and IHC techniques. The authors' showed that Notch-1 and Jagged-2 was expressed in the dorsal root ganglia, thymus, osteoblasts of developing bone in the skull, hair follicle, epidermis and skeletal muscle during development. According to the authors', Notch-1 and Jagged-2 together leads to differentiation of cells within each organ.[31]

In 1998, Mitsiadis et al, studied the expression of Notch during odonotogenesis using in-situ hybridization and IHC techniques in a mouse model. The authors' detected that the cervical loop of incisor acts as a reservoir of cells as they were capable of differentiation into IEE, OEE, SI and SR. The authors' showed that Notch-1 was restricted to the stratum intermedium, Notch-2 in SI, SR, OEE and mesenchymal cells of dental papillae and Notch-3

was restricted to SI and mesenchymal cells of denta papilla in the early bell stage of mouse molar. The authors' also showed that expression of Notch-1 was noted in SI and sub-odontoblastic layer, Notch-2 was present in SI, OEE and sub-odontoblastic layer and Notch-3 in sub-odontoblastic layer but not in odontoblasts whereas it was expressed in IEE which progressively restricted to SI in the mouse incisor tooth germ. Thus, the authors' detected that Notch was no longer expressed when the cells are differentiated. This was based on the fact that down-regulation of Notch expression occurred in a decreasing gradient from the cervical loop to the cusp tip, which also indicates that Notch signaling inhibits the commitment of cells to differentiate.[32]

In 1999, Harada et al, studied the localization of putative stem cells in dental epithelium using 2 day old mice with the help of in-situ hybridisation and IHC techniques. The authors' showed that the cervical loop epithelium in the apical bud of incisors possibly remains as a stem cell pool. This was proved by their premise that when the differentiating epithelium was removed, new epithelium having the capability to differentiate into ameloblasts, has been synthesized by the cervical loop, whereas there was no evidence of regeneration when the cervical loop is completely removed.[3]

In 2002, Kawano et al, studied the expression of Notch in the dental epithelial cell line (HAT-7) from the cervical loop of incisor germs of rat and its association with cell differentiation using IHC techniques. The authors' showed that Notch-1 was expressed in the SI, whereas in the zone of ameloblast differentiation Jagged-1 was expressed instead of Notch-1. The authors' also showed that cells expressing Notch-1 also expressed ALP, indicating it as a marker of SI. This is because ALP positive cells were actually derived from the population of ALP negative cells which was shown to be dependent on Notch-1 expression. Thus, the choice of cell lineage either towards SI or ameloblasts depends on Notch signaling.[33]

In 2002, Tedzuka et al, studied the role of Notch in osteoblastic cell differentiation using MC3T3-E1 and C3H10T1/2 mouse cell lines with Northern blot, Western blot, transfection and luciferase assay. The authors' showed that Notch-1 was expressed in osteoblast cell line and calcified nodule in the mouse osteoblast cell line. The authors' also showed that Notch-1 induces osteogenic differentiation in multipotent stem cells of mouse under the influence of BMP and also in human bone marrow mesenchymal stem cells. Thus, the authors' concluded that Notch stimulates osteoblastic cell differentiation within the stem cells.[34]

In 2006, Harada et al, studied the ameloblast lineage via Notch signaling in rat incisors using cell-culture, IHC and in-situ hybridization techniques. The authors' showed that Notch-1 was expressed in the SI and in the sub-odontoblast layer of dental papilla. The authors' also showed that Notch-1 expression in SI was associated with ALP as the ALP positive cells are Notch positive. In addition, the authors' stated that IEE cells also produce SI via Notch signaling.[4]

In 2008, Kumamoto et al, studied the expression of Notch-1, 2, & 3 in human tooth germ and ameloblastomas by RT-PCR and in-situ hybridization techniques. The authors' showed that all three types of Notch were expressed in both tooth germ and ameloblastoma by RT-PCR, whereas the expression of Notch 1, 2 and 3 was detected in the cytoplasm of many epithelial cells of the tooth germ such as DL, OEE, SR and SI but not in IEE by in-situ hybridization technique along with weak staining in fibroblastic and endothelial cells of dental papilla and dental sac. The authors' also showed the central polyhedral neoplastic cells were positive to Notch – 1, 2 and 3 but Notch -2 was found in some neoplastic peripheral columnar or cuboidal cells whereas the keratinizing and granular cells of ameloblastoma were negative to Notch. The authors' also showed that Notch ligands such as Delta 1 and Jagged 1 were positive in the central polyhedral cells and peripheral cuboidal or columnar cells but negative to the keratinizing and granular cells of ameloblastoma. The Notch ligands, similar

to the Notch receptors showed mild staining of fibroblast and endothelial cells. The authors' showed that there was no difference in the expression of Notch between tooth germs and ameloblastoma, concluding that Notch signaling doesnot play an oncogenic role in the tumorogenesis of odontogenic epithelium ansd helps in suppression of neoplastic cell proliferation and controls neoplastic cell differentiation in epithelial odontogenic tumors. The authors' conclude that expression of Notch in tooth germ and ameloblastoma are related to cell differentiation.[35]

In 2008, Nakano et al, studied expression of Notch-1 and Ki-67 in ameloblastoma and ameloblastic carcinoma using in-situ hybridization and immunohistochemistry techniques. The authors' showed that immunohistochemically, both tumors expressed Notch-1 which was intensely restricted to the most proliferating cells among the peripheral columnar cells with strong reactions in the budding areas of epithelial nests and moderately to the SR. In addition, some of the tumor cells showed strong positivity of the cytoplasm whereas others show strong positivity in the nucleus. In addition, the authors' also showed that Notch-1 gene expressions were found in the budding nests and peripheral cells of ameloblastoma and most of the neoplastic cells in the ameloblastic carcinoma. However, the intensity of staining reaction in ameloblastic carcinoma was more intense than in ameloblastoma. According to the authors' the expression of Notch-1 is related to cell cycle arrest in ameloblastic carcinoma, but is related to acquisition of morphological characteristics in ameloblastoma.[36]

In 2008, Borkosky et al, studied expression of Notch-1, Jagged 2 and Math 1 in molar tooth germ of mouse using in-situ hybridization. The authors' showed that Notch-1 was intensely expressed in the cervical loop and weakly in the preameloblast and odontoblast layers at the cusp tip. The authors' also showed that the expression of Notch-1 was down-regulated from the cervical loop to the cusp tip with increased intensity of Jagged 2 and Math

1. According to the authors', the absence of Notch-1 during late stages was believed due to the inhibition of differentiation by Notch.[37]

In 2009, He et al, studied the expression of Notch in the human dental pulp stem cells using IHC, western blot, RT-PCR techniques. The human dental pulp stem cells were isolated from impacted third molars and cultured. The authors' found that the culturing of DPSC lead to the formation of multiple layers which gets differentiated into odontoblasts and mineralized nodule. Notch-1, was expressed in most of the non-nodule forming cells, thus suggesting its possible role in maintaining the cells in undifferentiated state.[38]

In 2010, Siar et al, studied the expression of Notch-1, 2, 3 & 4 in solid or multicystic, unicystic and recurrent ameloblastoma by immunohistochemical techniques. The authors' showed that the Notch-1 was variably expressed among the three types with positive reaction in the central cells than the peripheral cells with mild reactivity in the granular cell and acanthomatous types of cells in the tumor, Notch-2 was neagative among the cells in all three types of ameloblastoma with mild staining of stromal elements such as blood vessels and fibroblasts in the tumor, Notch-3 showed positivity in the cytoplasm and cell membrane of the tumor cells irrespective of the type of ameloblastoma with positivity in the granular cell and acanthomatous type, Notch-4 was overexpressed in solid or multicystic ameloblastoma than in unicystic or recurrent ameloblastoma whereas Notch-1 and Notch-3 were variably present in the central cells and Notch-2 was absent in all three types. In addition, among the Notch receptors, Jagged-1 was expressed more in the conventional/solid amelobalstoma than in the unicystic and recurrent types whereas Jagged-2 was absent in all types with Delta-1 expression in the solid and recurrent types and negative in the unicystic ameloblastoma. These findings suggests that the Notch molecules play different roles in the acquisition of different types of ameloblastoma but the action of Notch varying with different types of ameloblastoma has not been clearly explained.[39]

In 2010, Siar et al, studied the differential expression of Notch in desmoplastic ameloblastoma by immunohistochemical techniques using Notch-1 monoclonal antibody and Notch-2, 3 and 4 polyclonal antibody. The authors' showed that Notch-1 stained the cytoplasm or cell membrane of central spindle shaped whorl cells than the peripheral cells with mild staining of the stromal elements such as endothelial lining of blood vessels and fibroblasts, Notch-2 stained only the stromal components of the tumor, Notch-3 showed immunopositivity in the cytoplasm of central spindle shaped whorl forming cells than the peripheral cells with non-reactive intervening basaloid cells in the tumor and Notch-4 were expressed in the central cells of desmoplastic ameloblastoma than in the peripheral cells showing nuclear, cytoplasmic and nuclear staining with the positive reaction in the intervening basaloid cells which stained negative to Notch-3. In addition, the Notch receptors Jagged-1 and Delta-1 were mildly expressed among the tumor cells whereas Jagged-2 was not detected in any of the tumor cells of desmoplastic ameloblastoma. The authors' concluded that Notch plays a role in the acquisition of cellular characteristics in the desmoplastic ameloblastoma.[40]

In 2011, Muraki et al, studied the expression of Notch in follicular and pleiform types of ameloblastomas using IHC techniques. The authors' showed that the peripheral columnar cells were more positive than the peripheral cuboidal cells. The central reticular and squamous (pre-final stage) cells were positive whereas the keratinizing cells in acanthomatous type were negative. The Notch ligands, Jagged showed similar reaction to its receptors. The authors' explained that this difference might result as the keratinizing cells were the terminally differentiated cells suggesting that Notch has a role in the differentiation of neoplastic cells.[41]

In 2011, Chen et al, studied the association between amelogenin and Notch 1 in the process of odontogenesis in wild type mouse, amelogenin null mouse and transgenic mouse

expressing a 180 AA or an amelogenin with a proline to threonine change at codon 70 (TgP70TKO) by using RT-PCR and IHC techniques. The authors' showed that expression of Notch-1 was localized to ameloblasts and stratum intermedium in TgP70TKO. The authors' stated that the mutant amelogenin leads to the proliferation of cells adjacent to ameloblast layer which stains positively for Notch-1 and resembles stratum intermedium. Thus the authors' concluded that mutant amelogenin leads to formation of disrupted odontogenic apparatus and Notch-1 marks the proliferative zone.[42]

In 2013, Bouroncle et al, studied the signaling of Notch-1 in osteogenic differentiation of DF cells using cell culture, RT-PCR and western blotting techniques. The authors' showed that Notch-1, being a specific marker of DF cells induces osteogenic differentiation. The authors also showed that DLX3 pathway induces osteogenic differentiation among the dental follicle cells. On the other hand, Notch signaling impairs ALP and BMP activity which inturn impairs osteogenic differentiation within DF cells and leads to formation of mineralized nodule 4 weeks after the osteogenic differentiation with BMP2 and dexamethasone based osteogenic differentiation media. Thus, the Notch signaling pathway negatively regulates the BMP2/DLX3 pathway with a negative feedback loop. According to the authors', Notch-1 have negative regulation of osteogenic differentiation in the somatic stem cells and the Notch signaling pathway was activated after the induction of DLX3 and the Notch signaling inturn decreases the expression of DLX3 and the osteogenic differentiation of dental follicle cells.[43]

In 2016, Jheon et al, studied the expression of Notch and its ligands in mouse incisor using in-situ hybridization, PCR and electron microscopy. The authors' showed that Notch-1 was expressed in SI and ameloblasts whereas Notch-2 was expressed in SI similar to Notch-1. The authors' also showed the mice injected with blocking antibodies of Notch leads to defects in the interface between ameloblast and SI at the pre-secretory and secretory stage of amelogenesis whereas combination of blocking antibodies and ligands leads to more severe

enamel defects leading to either partial or complete detachment of SI from the ameloblast surface. In addition, the authors' also showed separation of ameloblast from SI with alterations in the microstructure of enamel rods leading to delayed mineralization when the adult mice were injected with blocking antibodies against Notch-1, Notch-2, Jagged-1 and Jagged-2. The electron microscopic study of the same revealed shrinkage and flattening of SI leading to detachment of SI from ameloblast and also increased spacing between the SI cells with loss of desmosomes among the ameoblasts leading to spacing in between the ameloblasts. In addition, the internal rod arrangement in the enamel appeared rounded and enlarged leading to the change in the angle of matrix deposition and mineralization. Thus, the authors' concluded that Notch and its ligands signaling was needed for enamel mineralization.[44]

MATERIALS AND METHODS

After obtaining necessary permission from the Institute of Obstetrics and Gynaecology and Institutional Ethical clearance Committee, the maxilla and mandibular tissues of 20 to 26 months old unclaimed fetuses, 7 in number were dissected and fixed in 10% formalin.

Tissues were decalcified with 8% HCl for 3 days, dehydrated using alcohol in increased gradients (60%, 80% and 100% alcohol), fixed in 10% formalin, cleared with xylene, embedded in the paraffin wax and a total of 11 tooth germs (EBS-4 & LBS-7) were included in the study. Serial sections were made at 3.5 µm on a microtome (RM 2245, Leica) for both histologic (Hematoxylin & Eosin) and immunohistochemical study.

The tumor samples containing six ameloblastomas were retrieved from archival samples from Department of Oral pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai, Tamil Nadu, India.

Routine Hematoxylin and Eosin stains of the tooth germ were made and observed under microscope. The cells of the IEE lineage within the enamel organ were described in relation to the dental papilla as according to our previous study – Table 1[45] and **Figure 1 & 2.**

Table 1. Show the characteristics of IEE lineage cells in human tooth germs.

	EBS	LBS
IEE	The zone of IEE cells extend from the tip of the cervical loop to the point of transition of PA. The IEE cells are cuboidal to short columnar shaped cell with a round to oval centrally placed nucleus. However, not infrequently, the nuclei are overlapped due to cell crowding. A distinct basement membrane zone is visible. The cells are bordered by the SR of the enamel organ and undifferentiated ectomesenchymal cells of the DP with an acellular zone between them**.**	The zones of IEE and PA are similar in extent and morphology as in the EBS. However, the zone of transitional PSA extends from the zone of PA to the zone of PSA.
PA	The zone of PA cells extends from the transition point of IEE to the point of transitional PSA. The zone of PA cells are tall columnar shaped with an oval nucleus occupying almost half of the cell showing reversal of nuclear polarity. It is bordered by the SI and the undifferentiated ectomesenchymal cells of the DP with an acellular zone between them. The visible presence of the SI distinguishes PA from IEE**.**	
tPSA	The zone of transitional PSA is found between	

	the point of termination of PA and the cusp tip, i.e., from the upper 1/3rd of the cusp slopes to the cusp tip. The transitional PSA are tall columnar cells with a distinct pseudostratified appearance and is overlaid by a denser layer of SI compared to the PA. The apical cell membrane of the transitional PSA is irregular and is in contact with the condensed peripheral undifferentiated cells of the DP, but is often detached from it due to vacuole formation**.**	
PSA		The zone of PSA is a short segment of cells that extends from the transition point of transitional PSA to the transition point of SA. The PSA are the tallest cells among the differentiating lineages of IEE. It is a columnar shaped cell with an elongated cytoplasm which contains a long and slender nucleus at the basal 1/3rd characterized by reversed nuclear polarization and palisading, but the latter feature was not a constant finding due to cell crowding. The apical cell membrane is irregular with indistinct cell

		outline. The PSA are overlaid by prominent SI and is in contact with either the differentiated ectomesenchymal cells (preodontoblasts) or dentin matrix and odontoblast.
SA		The zone of SA covers the cusp tip and slopes up to the point of transition from PSA. The SA cells are tall columnar shaped cells containing round or flame shaped nucleus and short conical cytoplasmic projections (Tomes' process) [Note: it was not possible to clearly identify SA with flat apical surface responsible for rodless enamel]. The nucleus occupies basal 1/3rd of the cell with reversed nuclear polarization and prominent palisading, but the latter feature is often masked by nuclear overlapping. The cytoplasm appears slightly basophilic or granular to clear due to the presence of cytoplasmic vacuoles. The cells are

		bordered by the enamel matrix and a thin layer of SI**.**

Early bell stage (EBS); Late bell stage (LBS); Inner enamel epithelium (IEE); preameloblasts (PA); transitional presecretory ameloblasts (tPSA); presecretory ameloblasts (PSA); secretory ameloblasts (SA).

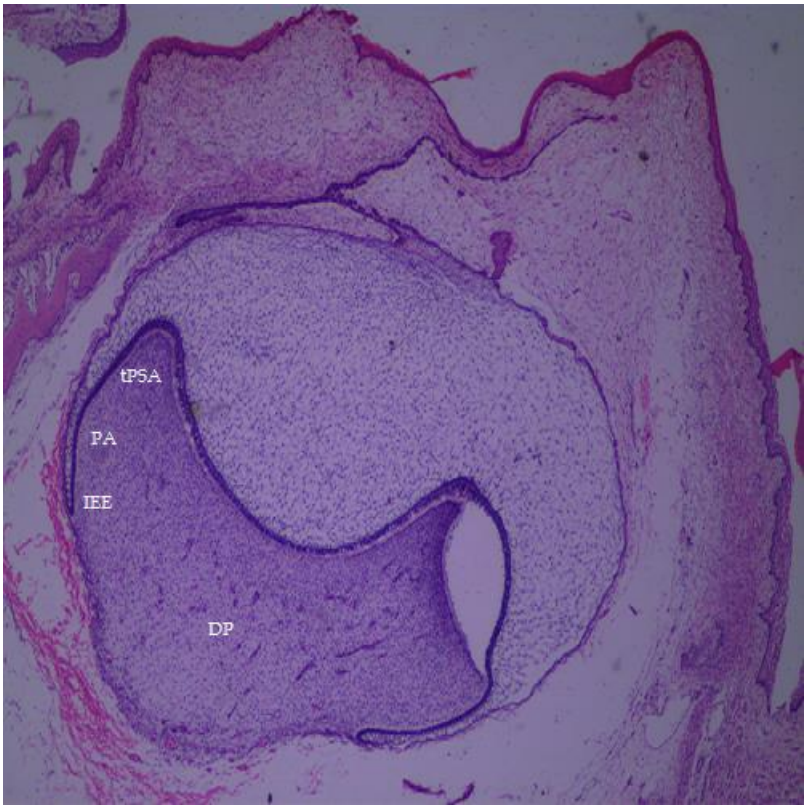

Figure-1. Shows cells of IEE lineage within the enamel organ in relation to the dental papilla in EBS, x20.

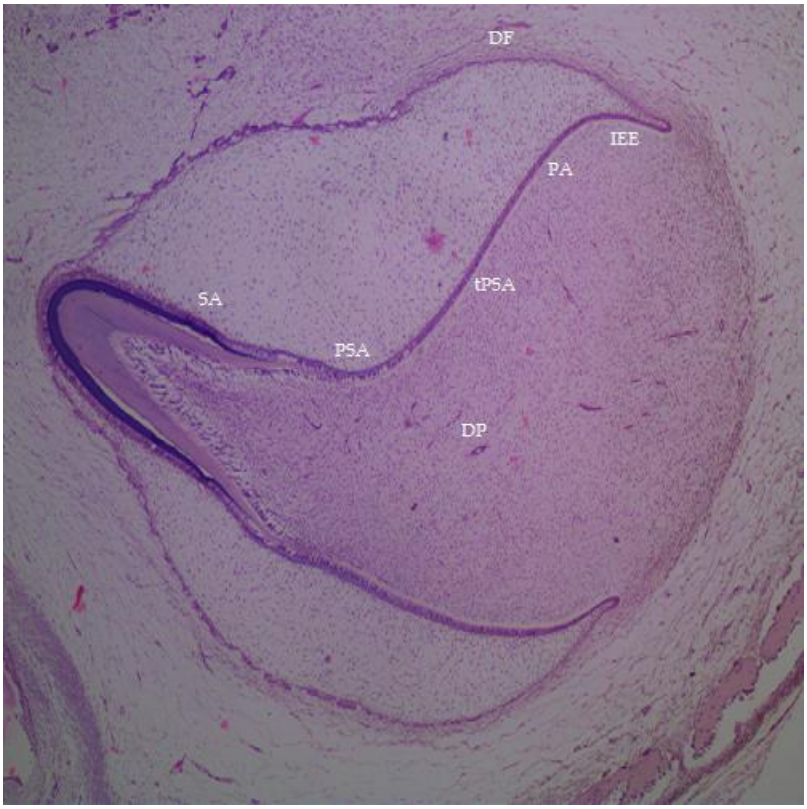

Figure 2. Shows cells of IEE lineage within the enamel organ in relation to the dental papilla in LBS, x20.

IMMUNOHISTOCHEMISTRY PROCEDURE FOR AMELOBLASTIN

Immunohistochemistry was performed on coated slides which were incubated in an oven at 60ºC for one hour. Do not bombard/tap the slide as soon as the section was taken as it may lead to folding of the tissues.

The slides after one hour incubation are rinsed in three changes of xylene with 5 minutes duration between the three changes.

The slides after treatment with the xylene in a coupling jar are rinsed in decreasing gradient of alcohol with 2 changes in 100% alcohol initially, followed by 90% alcohol, 70% alcohol with 2 minutes duration between the changes.

The slides are eventually rehydrated with 2 changes of distilled water in a coupling jar. The slides were washed by shaking the coupling jar gently and in circular motion and vigorous washing has to be avoided which might lead to the tearing of the tissues or the entire tissues might be washed off.

Antigen retrieval was done with citrate buffer solution prepared by using trisodium citrate - 0.735 gm, 1N HCl – 1.25 ml and distilled water of 250 ml.

The slides stacked in a tightly closed coupling jar are placed in a pressure cooker filled with one-third of water was heated at 120ºC for 15 minutes (10 Watts). Tissues are washed off from the coated slides if the temperature or the timings increased.

The coupling jar was cooled down by placing it under running water followed by washing the same with distilled water.

The sections on the coated slides were subjected to **PolyDetector Peroxidase Blocker (BIO-SB, Mouse/Rabbit PolyDetector Plus DAB HRP Brown Detection System)** for 15 minutes.

The slides are then washed with 2 changes of distilled water and incubated for half an hour in PBS prepared by Disodium hydrogen phosphate – 4.25 gm, Potassium dihydrogen phosphate – 0.75 gm, Sodium chloride – 4 gm and distilled water – 500 ml.

The primary antibody used was ameloblastin antibody - 100µl **(HYGENE BIOSCIENCES PVT LTD, BIORBYT, UK)** in 1:200 dilution.

The tissue over the slides were covered with the primary antibody for one hour and washed with PBS twice.

The tissues were covered with **PolyDetector Plus link** for 8 minutes and washed with PBS twice.

The tissues were covered with **PolyDetector HRP Label** for 12 minutes and washed with PBS twice.

Chromogen (DAB) was prepared by adding 1µl of **PolyDetector DAB chromogen** per drop of **PolyDetector DAB buffer**.

The tissues were covered with prepared DAB substrate-chromogen solution for 3 minutes and washed with distilled water gently.

The sections were counterstained using Harri's hematoxylin by dipping the slides into it for one minute.

The slides were dehydrated using increasing gradients of alcohol with a single wash in 70% and 90% and 2 washes in 100% alcohol.

The slides were dipped in xylene, dried and the sections are covered with the coverslip. The slides were examined by using **OLYMPUS (MODEL BX43F)**.

The photographs of the sections were captured by **OLYMPUS U-CMAD3, INFINITY 1** connected to the computer and also by smart phone.

IMMUNOHISTOCHEMISTRY FOR NOTCH-1

Immunohistochemistry was performed on coated slides which were incubated in an oven at 60ºC for one hour. Do not bombard/tap the slide as soon as the section was taken as it may lead to folding of the tissues.

The slides after one hour incubation are rinsed in three changes of xylene with 5 minutes duration between the three changes.

The slides after treatment with the xylene in a coupling jar are rinsed in decreasing gradient of alcohol with 2 changes in 100% alcohol initially, followed by 90% alcohol, 70% alcohol with 2 minutes duration between the changes.

The slides are eventually rehydrated with 2 changes of distilled water in a coupling jar. The slides were washed by shaking the coupling jar gently and in circular motion and vigorous washing has to be avoided which might lead to the tearing of the tissues or the entire tissues might be washed off.

Antigen retrieval was done with citrate buffer solution prepared by using trisodium citrate - 0.735 gm, 1N HCl – 1.25 ml and distilled water of 250 ml.

The slides stacked in a tightly closed coupling jar are placed in a pressure cooker filled with one-third of water was heated at 120ºC for 15 minutes (10 Watts). Tissues are washed off from the coated slides if the temperature or the timings increased.

The coupling jar was cooled down by placing it under running water followed by washing the same with distilled water.

The sections on the coated slides were subjected to **PolyDetector Peroxidase Blocker (BIO-SB, Mouse/Rabbit PolyDetector Plus DAB HRP Brown Detection System)** for 15 minutes.

The slides are then washed with 2 changes of distilled water and incubated for half an hour in PBS prepared by Disodium hydrogen phosphate – 4.25 gm, Potassium dihydrogen phosphate – 0.75 gm, Sodium chloride – 4 gm and distilled water – 500 ml.

The primary antibody used was Notch-1 - 50µl **(HYGENE BIOSCIENCES PVT LTD, BIORBYT, UK)** in 1:50 dilution.

The tissue over the slides were covered with the primary antibody for one hour and washed with PBS twice.

The tissues were covered with **PolyDetector Plus link** for 8 minutes and washed with PBS twice.

The tissues were covered with **PolyDetector HRP Label** for 12 minutes and washed with PBS twice.

Chromogen (DAB) was prepared by adding 1µl of **PolyDetector DAB chromogen** per drop of **PolyDetector DAB buffer**.

The tissues were covered with prepared DAB substrate-chromogen solution for 3 minutes and washed with distilled water gently.

The sections were counterstained using Harri's hematoxylin by dipping the slides into it for one minute.

The slides were dehydrated using increasing gradients of alcohol with a single wash in 70% and 90% and 2 washes in 100% alcohol.

The slides were dipped in xylene, dried and the sections are covered with the coverslip. The slides were examined by using **OLYMPUS (MODEL BX43F)**.

The photographs of the sections were captured by **OLYMPUS U-CMAD3, INFINITY 1** connected to the computer and also by smart phone.

RESULTS

Expression of ameloblastin in human tooth germ

During the early bell stage (EBS), ameloblastin was expressed in the stratum intermedium (SI) and stellate reticulum (SR) but not in the outer enamel epithelium (OEE), inner enamel epithelium (IEE) and pre-ameloblasts (PA). The expression of ameloblastin was first evident only in the transitional pre-secretory ameloblasts (PSA). In contrast, ameloblastin expression was intensely expressed in the acellular zone between the enamel organ and the core of the dental papilla in the region of IEE and gradually decreased in intensity in the region of PA and became negative in the region of transitional PSA (**Figure 3**).

During the late bell stage (LBS), as in EBS, ameloblastin was expressed in the SI and SR but not in the OEE, IEE and PA. However, unlike EBS, ameloblastin was not expressed in the transitional PSA but expressed in the PSA. In contrast, in the acellular zone, ameloblastin was expressed with decreasing intensities from the region of IEE to the transitional PSA. In the region of PSA, the expression of ameloblastin was negative in the dental papilla concomitant with the obliteration of the acellular zone by the elongation of PSA and dentin matrix formation (**Figure 4**). In addition, the expression of ameloblastin was initially less intense and later negative in the SA but with intense staining of enamel matrix and patchy to linear distribution in the DEJ (**Figure 5**). In the surrounding tissues, ameloblastin was expressed in the early osteoid and chondroid matrix as well as in the loose connective tissue in relation to the immature trabeculae of bone but not in the mature bone (**Figure 6**).

Expression of ameloblastin in ameloblastoma

In ameloblastoma, the expression of ameloblastin was negative in the peripheral cells resembling IEE and SA of the tumor component whereas the ameloblastin was expressed in the peripheral cells with transitional PSA morphology. In addition, ameloblastin was expressed in the central SR-like cells and as well as inflammatory cells in the connective tissue but negative in the undifferentiated cells present within the tumor follicles (**Figure 7 & Figure 8**).

Expression of Notch-1 in human tooth germ

During the EBS, the expression of Notch-1 (both cytoplasm and nucleus) was detected intensely in the cervical loop region of the enamel organ comprising OEE, IEE, SI and SR. However, the intensity of staining reaction in these cells decreased as the IEE cells differentiated into PA and transitional PSA. In contrast, the dental papilla cells are moderately and diffusely stained in the region of IEE and PA, whereas the condensing peripheral cells intensely stained in the region of transitional PSA (**Figure 9**).

During the LBS, the expression of Notch-1 (nucleus) was detected with moderate intensity but the number of positively stained cells decreased with increasing differentiation from the zone of IEE to the zone of SA compared to the EBS with positive reaction in the inner part of the enamel matrix (**Figure 10**). In LBS, the dental papilla cells stained moderately and diffusely as in EBS. In addition, the odontoblasts in the zone of PSA and SA stained intensely to Notch-1 including pre-dentin. In the surrounding tissues, Notch-1 was expressed in the osteoblasts, osteoclasts and in the early osteoid and as well as in the loose

connective tissue in relation to the immature trabeculae of bone but negative in the mature bone (**Figure 11**).

Expression of Notch-1 in ameloblastoma

The expression of Notch-1 was detected intensely in both the central and peripheral cells of ameloblastoma with nuclear and/or cytoplasmic staining (**Figure 12**).

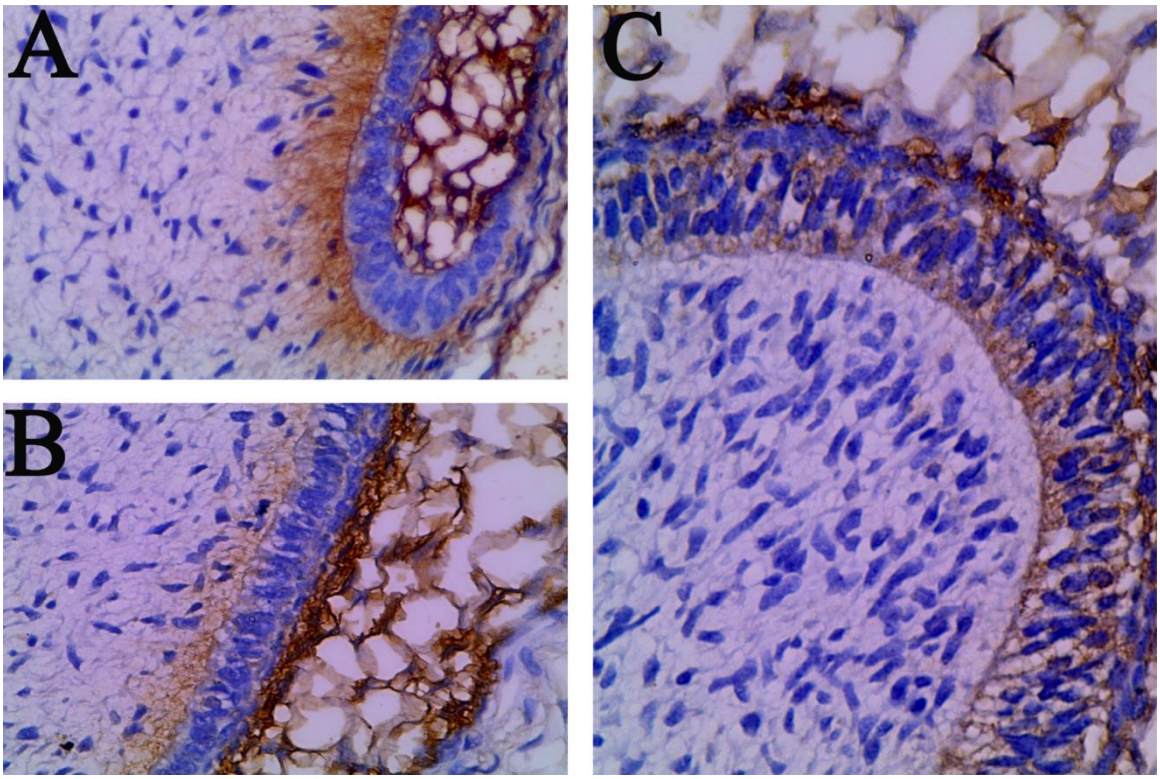

Figure-3. Shows expression of ameloblastin in EBS. A. Shows intense reaction of ameloblastin in the acellular zone of dental papillae in the region of IEE, x400. B. Shows reduced intensity of ameloblastin in the acellular zone of dental papillae in the region of PA, x400. C. Shows intense reaction of ameloblastin in the transitional PSA but negative in the acellular zone of dental papillae, x400.

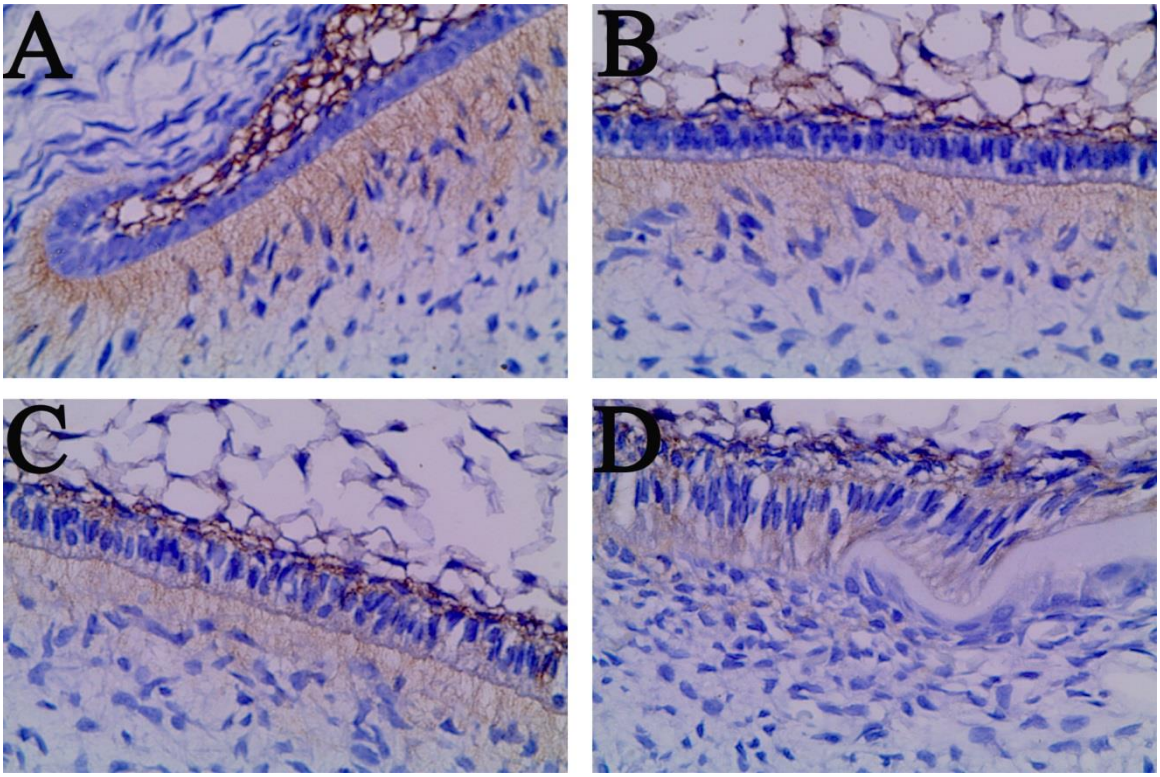

Figure-4. Shows expression of ameloblastin in LBS. A. Shows intense reaction of ameloblastin in the acellular zone of dental papillae in the region of IEE. B. Shows reduced intensity of ameloblastin in the acellular zone of dental papillae in the region of PA, x400. C. Shows reduced intensity of ameloblastin in the acellular zone of dental papillae in the region of transitional PSA, x400. D. Shows positive reaction of ameloblastin in the PSA and obliteration of acellular zone by the cytoplasmic extesions of PSA and dentin formation, x400.

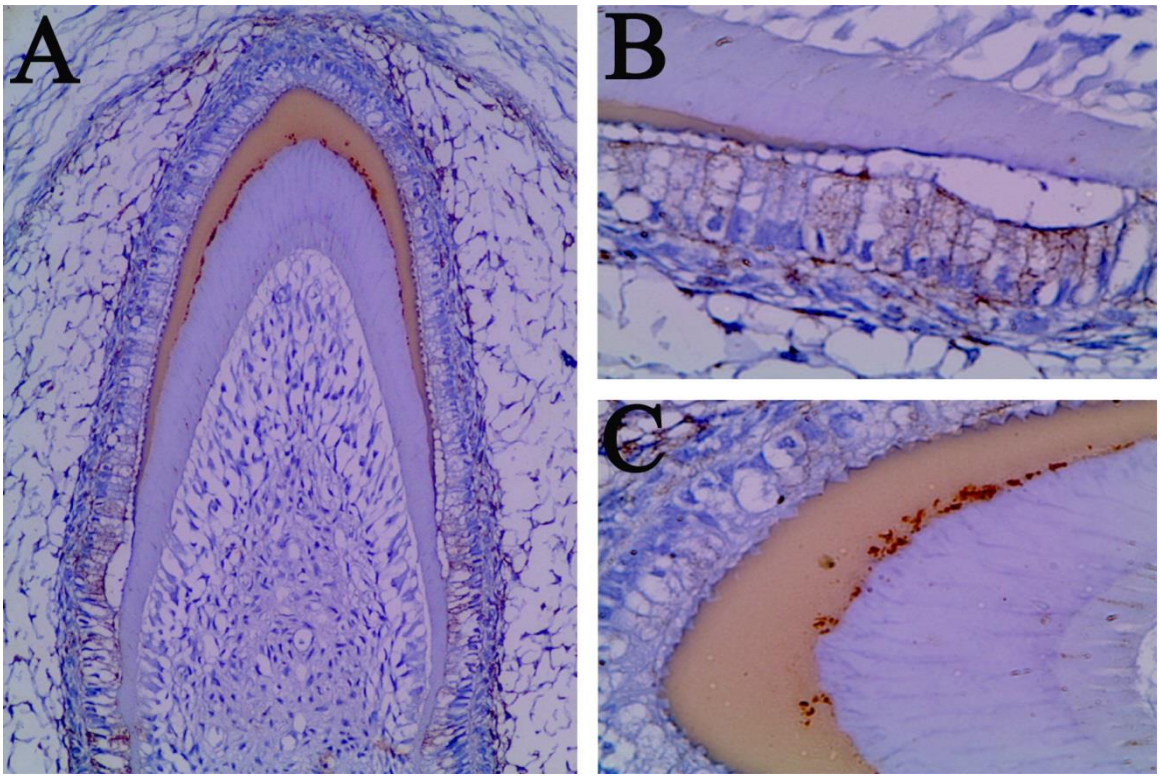

Figure-5. Shows expression of ameloblastin in LBS. A. Shows positive reaction of ameoblastin in the PSA, SA (initial stage) and enamel matrix, x100. B. Shows positive reaction of ameloblastin in the SA at the front of enamel formation, x400. C. Shows negative reaction of ameloblastin in the SA at later stages with intense reaction in the enamel matrix, x400.

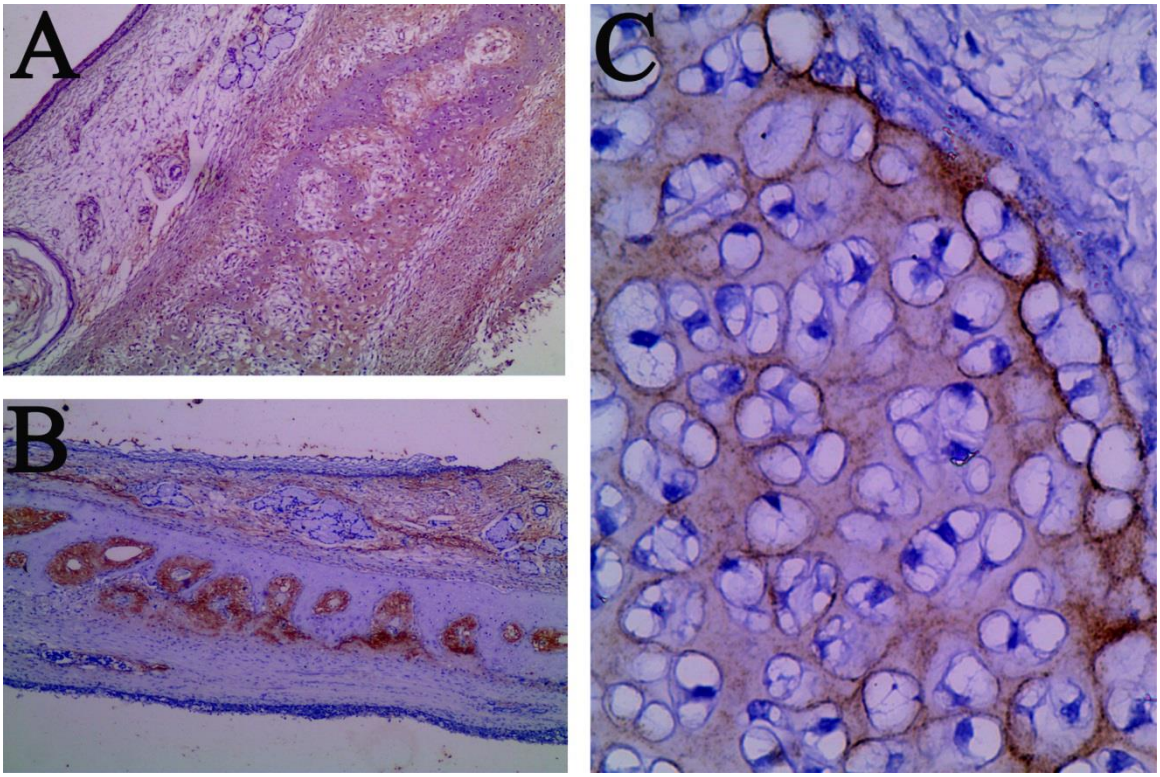

Figure-6. Shows expression of ameloblastin in osteoid and chondroid matrix. A. Shows intense reaction of ameloblastin in the immature bone (osteoid), x100. B. Shows reduced intensity of ameloblastin in mature bone and intense reaction in the loose connective tissue, x100. C. Shows intense reaction of ameloblastin in the chondroid matrix, x400.

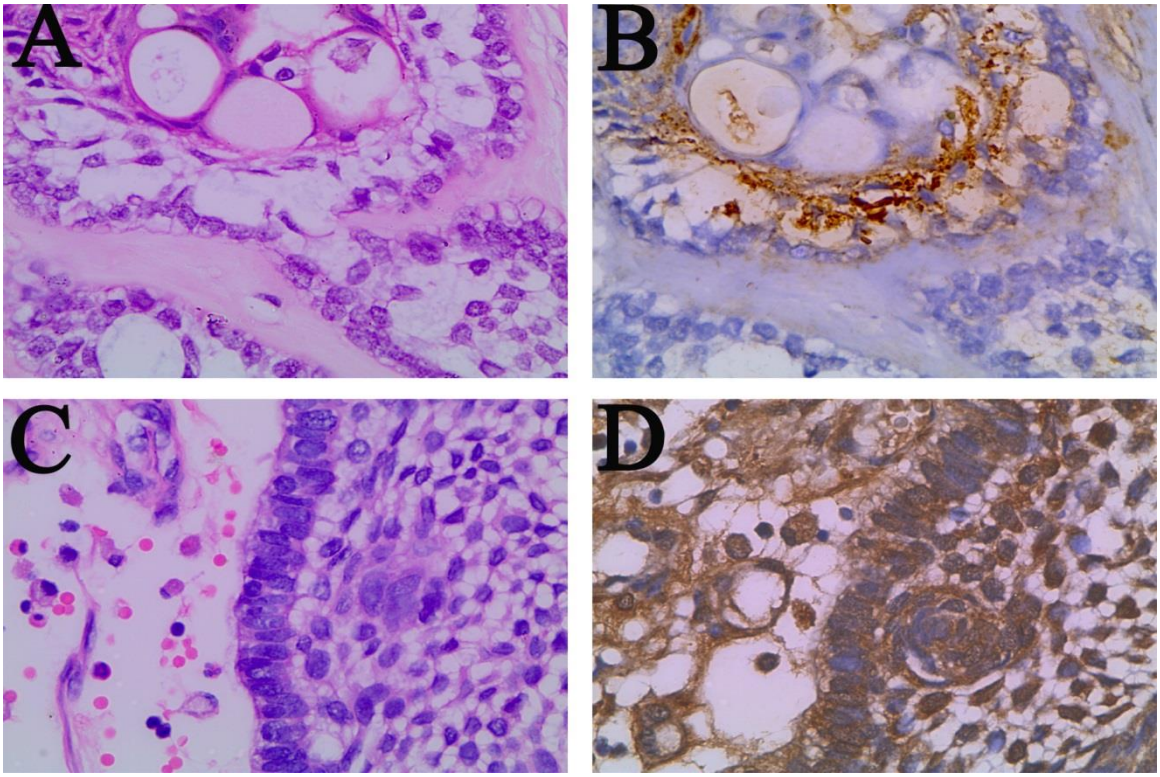

Figure-7. Shows expression of ameloblastin in ameloblastoma. A. Shows tumor islands with IEE morphology of the peripheral cells, which was negative to ameloblastin as shown in B, x400. C. Shows tumor cells with PA morphology which reacted positively to ameloblastin as shown in D, x400.

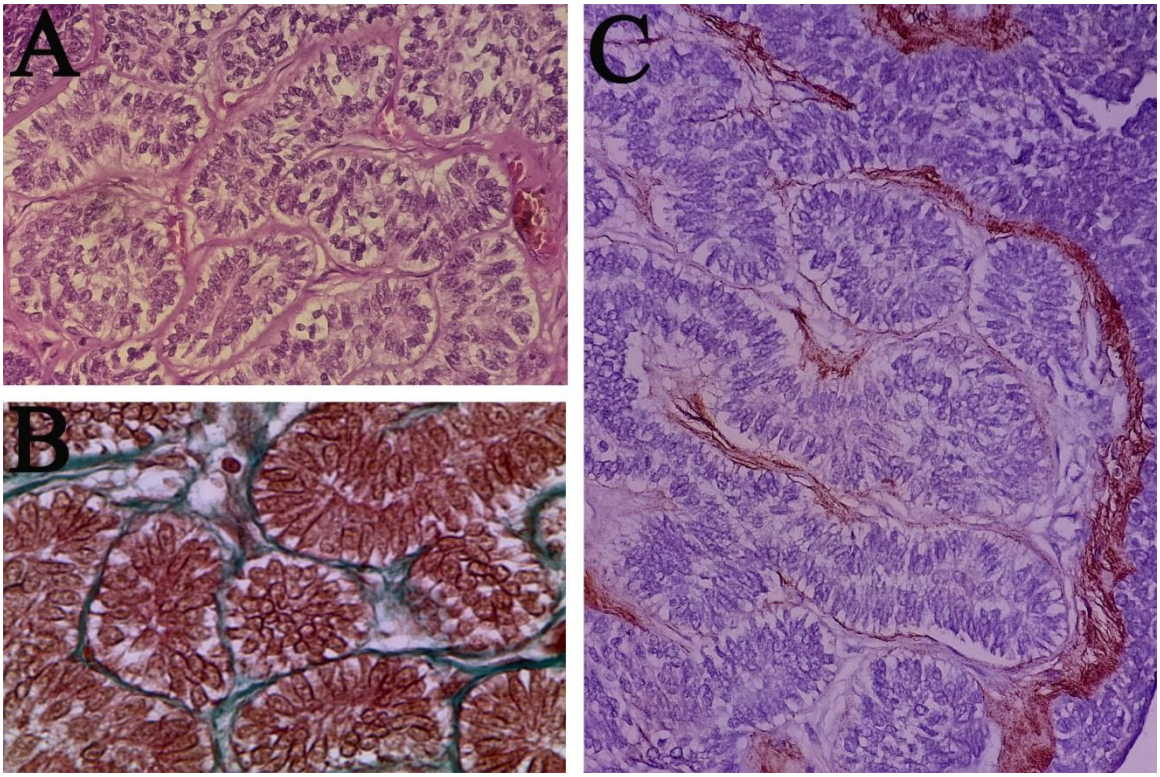

Figure-8. Shows expression of ameloblastin in ameloblastoma. A. Shows tumor islands with SA morphology of the peripheral cells with positively stained collagen fibers for Masson's trichrome in B (x400) which reacted positively to ameloblastin with the cells showing negative reaction in C, x100.

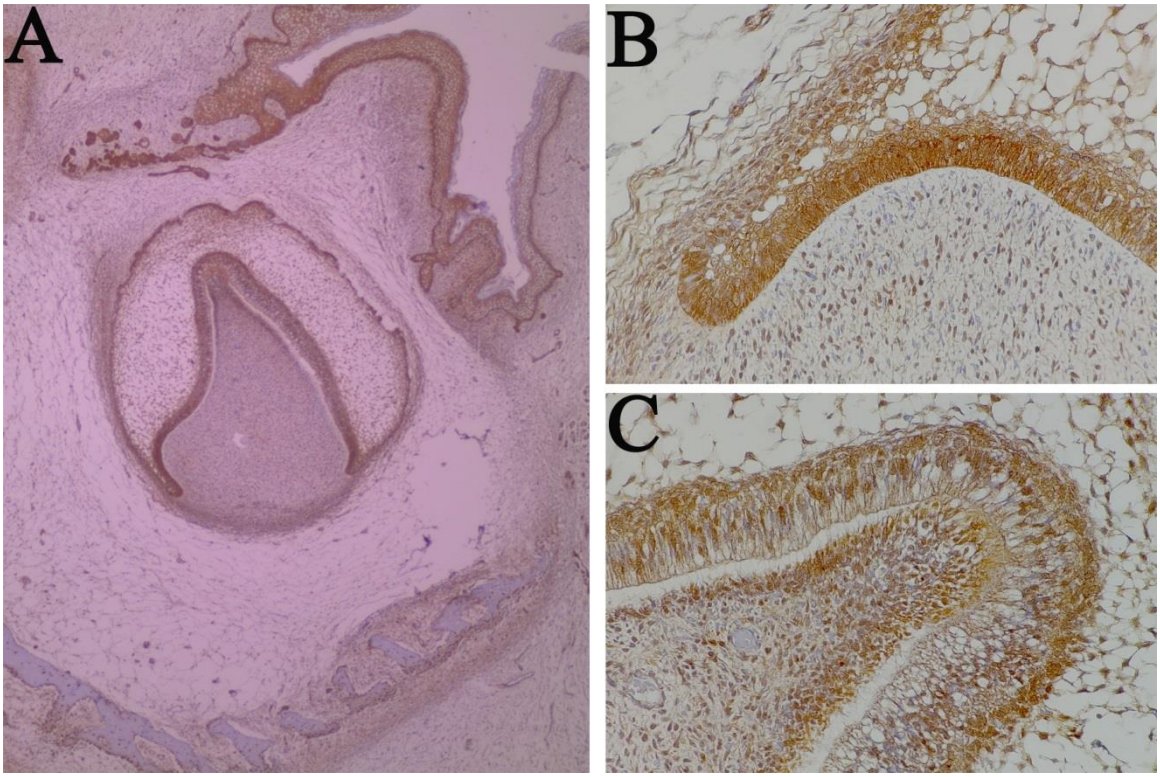

Figure-9. A. Shows expression of Notch-1 in EBS, x20. B. Shows intense reaction of Notch-1 in the cervical loop region of enamel organ, x400. C. Shows reduced intensity of the IEE lineage in the cusp tip with intense reaction of Notch-1 in the condensing peripheral cells of dental papillae, x400.

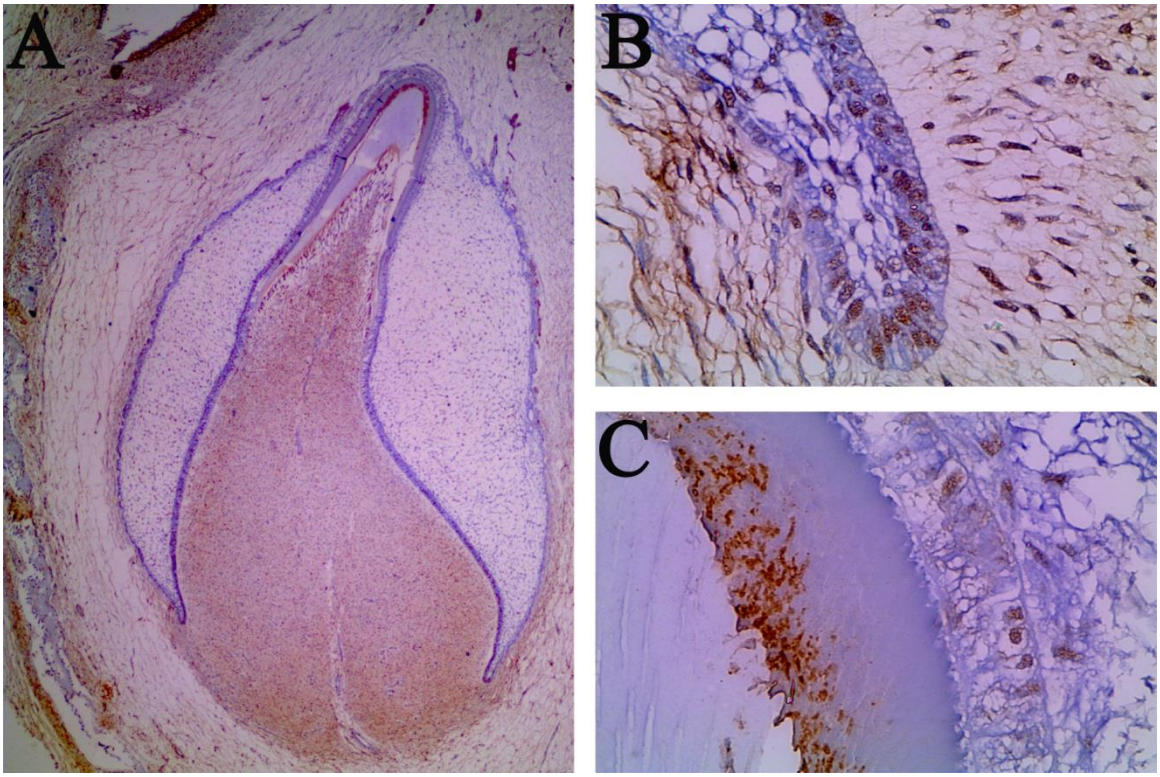

Figure-10. A. Shows expression of Notch-1 in LBS, x20. B. Shows intense nuclear staining of Notch-1 in the cervical loop of enamel organ, x400. C. Shows reduced number of positively stained cells with intense reaction in the inner part of enamel matrix, x400.

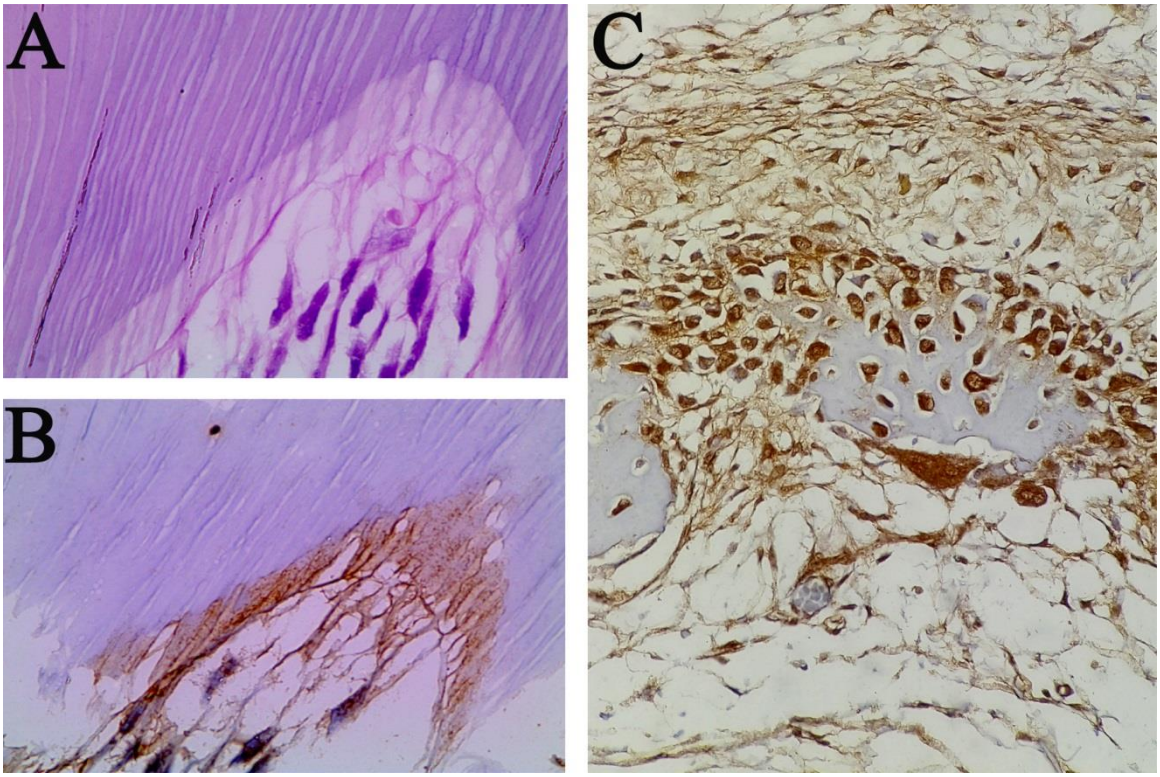

Figure-11. A. Shows pre-dentin and odontoblasts which showed positive reaction to Notch-1as shown in B, x400. C. Shows intense reaction of Notch-1 in osteoblasts, osteoclasts and osteoid matrix, x400.

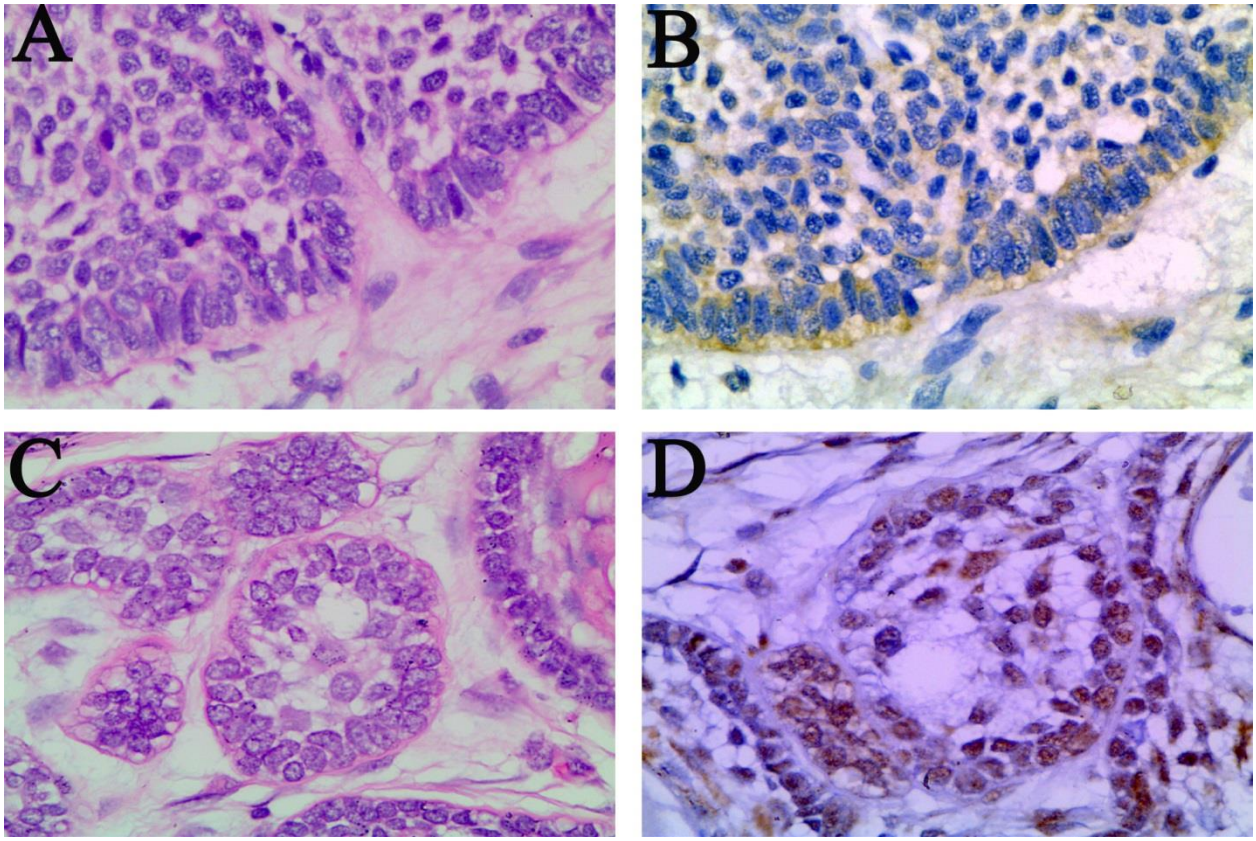

Figure-12. Shows expression of Notch-1 in ameloblastoma. A. Shows tumor islands with peripheral and central cells which reacted positively to Notch-1 (cytoplasm) as shown in B, x400. C. Shows tumor islands with peripheral and central cells which reacted positively to Notch-1 (cytoplasm and nucleus) as shown in D, x400.

DISCUSSION

Information regarding expression of ameloblastin and Notch in human tooth germ is limited or negligible.[6,12,13,5] The present study evaluated the expression of ameloblastin and Notch-1 by immunohistochemistry in the human tooth germ and ameloblastoma to ascertain the correlation between them, and also to identify the stem cell pool in the enamel organ.

The description and stages of human tooth germs are well described in the standard histology textbooks. Briefly, the enamel organ is composed of OEE, IEE, SR and SI. The dental papilla is composed of undifferentiated ectomesenchymal cells during early stages (early bell stage) and preodontoblasts and/or odontoblasts at a later stage (late bell stage) of differentiation. Under the light microscope, an acellular but fibrillar zone separates the enamel organ from the dental papilla **(Figure 1 & 2)**. Under the electron microscope, the equivalent of acellular zone is defined as a highly specialized lamina fibroreticularis of the basement membrane that supports the IEE.[29] The cell processes of the dental papilla are inserted to the fibronectin-rich filaments of the lamina fibroreticularis, thereby linking the dental papilla cells to the basement membrane of the enamel organ.[29] This arrangement is considered to be crucial for the differentiation of the odontoblasts in the developing tooth germs.[29] In addition, the development of tooth germ involves morpho- and histo-differentiation characterized by sequential reciprocal signaling between the enamel organ and the ectomesenchyme (dental papilla and follicle) necessary for the elaboration of the functional products of the respective tissues.[6,10,11]

I. Immunohistochemical expression of ameloblastin in the human tooth germ.

The present study shows that although the undifferentiated cells of the dental papilla, preodontoblasts and odontoblasts are unreactive to ameloblastin, a distinct pattern of positive reaction occurred in the acellular zone of the dental papilla in relation to the IEE lineage cells. This pattern of positive reaction in the acellular zone of the dental papilla was evident till ameloblastin was first expressed in the transitional PSA and in the PSA during the EBS and LBS, respectively (**Figure 3 & 4**). These observations indicate that ameloblastin is expressed in a stage specific manner in the IEE lineage cells, which occurred only when the expression is excluded in the acellular zone of the dental papilla.

The present observations are consistent with a previous study where the expression of ameloblastin appeared in the dental papilla and preodontoblasts and disappeared before it was expressed in the IEE lineage cells.[10] Nevertheless, unlike the previous study ameloblastin did not react with the undifferentiated cells of the dental papillae and preodontoblasts or odontoblasts. In the enamel organ, however, the expression of ameloblastin may be detected in the presecretory stage of ameloblasts, but it is secreted in amounts too small to be detected immunohistochemically until after the formation of mantle dentin had started.[10,11] This finding is consistent with the present observations.

Review of the literature reveals that ameloblastin is expressed in both the secretory ameloblasts and enamel matrix with intense distribution at the DEJ in human tooth germs.[6,13] In the rat incisor and molar tooth germs, expression of ameloblastin begins from the presecretory stage and continues till the late maturation stage in the rat incisor and molar tooth germs.[6,11] However, in the human tooth germs employed in the present study, ameloblastin reaction was initially less intense and then became negative in the SA but

stained diffusely the bulk of the enamel matrix with a patchy to linear distribution at the DEJ **(Figure 5).** This finding is consistent with ameloblastin expression in human tooth germs.[6,13] Similar findings were also described by an earlier study in rat incisor and molar tooth germs, where secretory and post secretory ameloblasts stained weakly than the enamel matrix.[10]

In odontogenesis, the reciprocal epithelial and mesenchymal signaling during tooth morphogenesis is manifested by an inverted pattern of expression of matrix proteins by epithelial and ectomesenchymal cells as they differentiate.[10,11] Therefore, the inverted pattern of expression of ameloblastin, noted in the present study, between the acellular zone of the dental papilla and the IEE lineage cells, and the sequential but fluctuating levels of expression within the IEE lineage cells suggests that ameloblastin may first have a role in cell signaling necessary for the differentiation and/or maturation process required for the synthesis of matrix proteins (enamel and dentin) and then in mineralization of the enamel matrix.[10] The latter supposition adds credibility to our observation that once enamel matrix was formed the expression of ameloblastin in the SA becomes weak or undetectable (**Figure 5**).[6,12,13]

In contrast, it was shown that lack of expression of ameloblastin in SA of null-mice cause detachment of the enamel matrix from the SA, which also proliferate into multilayers by adopting early phenotypes of IEE lineage cells.[1] This indicates that expression of ameloblastin in the SA was not only required for inhibition of proliferation of ameloblasts, but also to maintain the differentiated state of the SA by preventing the separation of enamel matrix from the plasma membrane of the ameloblasts.[8,1] However, in the present study neither detachment nor proliferation of ameloblasts occurred when the SA reacted negatively to ameloblastin once the enamel matrix was formed **(Figure 5).** This shows that in advanced

stages ameloblastin is more likely to play a role in enamel mineralization than cell-matrix adhesions.[11,1,2] However, as found in this study, it seems plausible that expression of ameloblastin in the acellular zone of the dental papillae during the early differentiation stages might be required for the maintenance of cell-matrix adhesions required for the differentiation of both odontoblasts and ameloblasts.[29,1]

In the present study ameloblastin was also expressed in the early osteoid and chondroid matrix, but becomes negative during maturation (**Figure 6**). Our findings are consistent with the previous reports.[2,20] Further, it has been suggested that the expression of ameloblastin in immature bone and cartilage functions in anchoring the bone-forming cells on the surface of the newly secreted bone matrix and its loss in the mature bone indicate that it has a role in cell signaling or differentiation.[2,20]

II. Immunohistochemical expression of ameloblastin in ameloblastoma.

The literature reveals that ameloblastin was not detected by immunohistochemistry either in the tumor cells or the stroma of conventional ameloblastoma whereas it has been reported that ameloblastin positively stained the tumor cells in peripheral variant.[12,22,24] In contrast, in the present study, the expression of ameloblastin in ameloblastoma shows reaction pattern similar to the human tooth germs with positive staining in the transitional PSA morphology and negative reaction in the IEE and SA morphology (**Figure 7 & 8**). The reaction pattern in the stroma surrounding the tumor component was also similar to the expression pattern found in the human tooth germs. The differences between the present observation and previous reports may well be due to the selection of cases and variation in the immunohistochemical procedures.

III. Immunohistochemical expression of Notch-1 in the human tooth germ.

Notch is a transmembrane protein receptor considered to play significant role in cell fate decisions.[30] Notch-1, Notch-2 and Notch-3 expression have been well addressed in rodent tooth development during normal odontogenesis by in situ hybridization, immunohistochemical techniques and in experimental situations.[30,32,3,5,4,37] In general, these studies reveal that Notch is expressed in the epithelial component of the tooth where dividing cells reside. In the rat incisor tooth germ, it is located in a small region of the cervical loop (or apical bud) which comprises OEE, IEE and SR. However, the expression of the Notch is excluded in the epithelial cells that contact the ectomesenchyme. These observations indicate that Notch maintains the cells at the cervical loop in an immature state and its exclusion is necessary for the beginning of differentiation process which is thought to be regulated by the signals from the ectomesenchyme. In addition, it has shown from Dil tracer analysis that some stratum intermedium cells diverge from the IEE.[4]

The literature reveals that the various types of Notch are expressed in an overlapping but defined regions of the tooth germ. Pertinently, expression of Notch-1 is restricted to the SI, Notch-2 in SR and OEE and Notch-3 in all the cell layers of the enamel organ.[30,32,3,5,4] However, between EBS and LBS.[32] During the EBS, in the molar tooth germ of the rat, Notch was expressed in the cells of enamel organ but was excluded in the differentiating IEE lineage cells. In the dental papilla, Notch-1 was restricted only to the blood vessels and Notch-2 and 3 can be found in the undifferentiated cells of the dental papilla.[32] In contrast, during LBS, Notch-1 was not detected in the enamel organ but all three types of Notch were expressed in the ectomesenchyme other than the odontoblasts.[30,32]

It is clear from the foregoing that Notch-1 is only restricted to the cervical loop and SI of the enamel organ during EBS.[32] On the other hand, Notch-1 is expressed in the dental papilla other than the odontoblasts, but not in the enamel organ during LBS.[32]

In the present study, Notch-1 was employed to determine the dividing cell pool and to specifically mark the stratum intermedium, but our results shows that Notch-1 was expressed in both the enamel organ and ectomesenchyme (dental papilla and follicle) with varying degrees of intensity during the EBS and LBS **(Figure 9 & 10),** which is inconsistent with the observations in the rat tooth germs.[32] However, in the only published human study, Notch-1 was expressed in all the cell layers of enamel organ other than the IEE lineage and in the dental papilla, which is partly in consonance with our findings.[5] These findings suggest that Notch-1 could neither identify dividing cell pool nor SI unequivocally in the present study using human tooth germs. Nevertheless, it may have a role in the differentiation of IEE lineage based on the reduced intensity and decrease in the number of positively stained cells during the LBS compared to the EBS.

In addition, although it was not our primary intention, our results show that Notch-1 was also detected in the enamel matrix at the interface with dentin. The latter, however, was negative while staining was detected in the predentin **(Figure 10C & 11A&B).** It is not clear whether the expression of Notch-1 linked to enamel mineralization? However, it has been shown that delay in enamel mineralization with consequent alterations in the enamel microstructure occurred in adult mice when injected with blocking antibodies against Notch-1, Notch-2, Jag-1 and Jag-2, either alone or in combinations for 6-12 days.[44]

The literature reveals, from cell culture study using MC3T3-E1 or C3H10T1/2 mouse cell lines by Northern and Western blot analysis, and transfection and luciferase assay, that Notch-1 induces osteoblastic differentiation and formation of calcified nodules.[34] In a similar study using human third molar dental follicle cell culture, it has been shown that Notch-1 inhibits osteoblastic differentiation of the dental follicle cells.[43] It has been reported that Notch-1 was expressed in the osteoblasts lining the calvarial bones of mouse embryos by immunohistochemistry.[31] The present study shows that Notch-1 was expressed in the formative and resorptive cells of immature bone, but not in the mature bone around the human tooth germ **(Figure 11C).** The results of the present study are consistent with the previous literature with regard to the expression of Notch in osteoblasts.[31]

IV. Immunohistochemical expression of Notch-1 in ameloblastoma

The literature reveals that Notch-1 was expressed in the peripheral cells and in the central SR-like cells of ameloblastoma in a cytoplasmic/nuclear/membranous staining pattern.[5,36,39-41] Our observation is in accordance with the previous reports. The literature shows that expression of Notch in ameloblastoma has been attributed to cell differentiation or tumorogenesis. However, our results show it is difficult to determine cell proliferation and differentiation based on the pattern of Notch-1 as its expression in ameloblastoma parallels the epithelial component of the tooth germ **(Figure - 12).**

LIMITATIONS OF THE STUDY:

As ameloblastin and Notch are research markers, a number of dilution threshold is required to determine ideal dilutions. This will invariably result in shortage of the marker of the study.

AMELOBLASTIN:

In the present study, the reaction at various dilutions are noted as follows,

- At dilution 1:50, 1:100, 1:150 in PBS, the expression was evident throughout the enamel organ from the inner enamel epithelium, preameloblast, transitional presecretory ameloblasts, presecretory ameloblasts, secretory ameloblasts and enamel matrix as well as in the stratum intermedium, stellate reticulum and outer enamel epithelium with the cytoplasmic staining pattern although the intensity of the staining decreases when the dilution increases. 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 1:250 in PBS, the expression was evident only in the enamel matrix with less background staining.
- At dilution 1:300, 1:350, 1:400, 1:450, 1:500 in PBS, the expression was not detected in any of the cells throughout the enamel organ.

NOTCH:

In the present study, the reaction at various dilutions are noted as follows,

- At dilution 1:25 in PBS, the expression was evident throughout the enamel organ from the inner enamel epithelium, preameloblast, transitional presecretory ameloblasts, presecretory ameloblasts, secretory ameloblasts and as well as stratum intermedium, stellate reticulum and outer enamel epithelium with mild cytoplasmic staining although the intensity of staining increased with 1:50 dilution with both nuclear and cytoplasmic staining. Expression was also noted in the odontoblasts, dental papilla cells and dental follicle cells with minimal background staining.

SUMMARY AND CONCLUSION

SUMMARY

The objective of the present study was to compare the expression pattern of ameloblastin and Notch-1 in human tooth germ and associated structures and in ameloblastoma to determine cytodifferentiation between human tooth germ and ameloblastoma and to determine the stem cell pool in the enamel organ. The present study included 11 tooth germs (EBS-4 and LBS-7) from 7 unclaimed fetuses (20-26 weeks old) and 6 tissue samples of ameloblastoma from the archival samples. The expression of ameloblastin was detected in the acellular zone with fluctuating reaction in the corresponding IEE lineage in the human tooth germ that parallels the similar type of IEE lineage cells within the ameloblastoma. Notch-1 was expressed in all cell layers of the enamel organ in both EBS (cytoplasm and nucleus) and LBS (nucleus) of human tooth germ but reduced in intensity during differentiation. The Notch-1 staining in the ameloblastoma parallels the reaction pattern in the EBS and LBS of human tooth germ. Notch-1 neither identifies stem cell pool in the human tooth germ nor restricted to SI. Ameloblastin and Notch-1 was expressed in the immature osteoid and chondroid matrix but not in the mature bone.

CONCLUSION:

1. Ameloblastin in the acellular zone of the dental papilla during early differentiation might be required for the maintenance of cell-matrix adhesions required for the differentiation of odontoblasts and ameloblasts.
2. The expression of ameoblastin in human tooth germ and ameloblastoma showed similar reaction pattern related to the common cytodifferentiation occurring among them.
3. Notch-1may have a role in the differentiation of IEE lineage based on the reduced intensity and decrease in the number of positively stained cells in LBS compared to EBS.
4. The expression of Notch-1 in ameloblastoma parallels the reaction pattern in the EBS and LBS of human tooth germ but difficult to determine the cytodifferentiation within the tumor.
5. Notch-1 neither detects stem cell pool in the enamel organ of human tooth germ nor remains specific to SI rather have a role in enamel and bone formation.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Fukomoto S, Kiba T, Hall B et al. Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of amelblasts. J Cell Biol 2004;5:973-983.

2. Spahr A, Lyngstadaas SP, Slaby I et al. Ameloblastin expression during craniofacial bone formation in rats. Eur J Oral Sci 2006;114:504-511.

3. Harada H, Kettunen P, Jung H-S et al. Localization of putative stem cells in Dental epithelium and their association with Notch and FGF signaling. J Cell Biol. 1999;147:105-120.

4. Harada H, Ichimori Y, Yokohama-Tamaki T et al. Startum intermedium lineage diverges from ameloblast lineage via Notch signaling. Biochem Biophys Res Commun 2006;340: 611-616.

5. Kumamoto H, Ohki K. Detection of Notch signaling molecules in ameloblastomas. J Oral Pathol Med 2008;37:228-234.

6. Lee SK, Krebsbach PH, Matsuki Y et al. Ameloblastin expression in rat incisors and human tooth germs. Int J Dev Biol 1996;40:1141-1150.

7. Krebsbach PH, Lee SK, Matsuki Y et al. Full-length sequence, localization and chromosomal mapping of Ameloblastin. A novel tooth-specific gene. J Biol Chem 1996; 8:4431-4435.

8. Uchida T, Murakami C, Dohi N et al. Synthesis, Secretion, Degradation and Fate of Ameloblastin During Matrix Formation Stage of the Rat Incisor as shown by Immunocytochemistry and Immunochemistry using Region Specific Antibodies 1997;45:1329-1340.

9. Begue-Kirn, Krebsbach PH, Bartlett JD et al. Dentin sialoprotein, Dentin phosphoprotein, enamelysin and ameloblastin: Tooth specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sci 1998;106:963-70.

10. Fong CD, Cerny R, Hammarstrom L et al. Sequential expression of an amelin gene in mesenchymal and epithelial cells during odontogenesis in rats. Eur J Oral Sci 1998;106:324-330.

11. Nanci A, Zalzal S, Lavoie P et al. Comparative Immunochemical Analyses of the Developmental Expression and Distribution of Ameoblastin and Amelogenin in Rat Icisors. J Histochem Cytochem 1998;46:911-934.

12. Takata T, Zhoa M, Uchida T et al. Immunohistochemical demonstration of an enamel sheath protein, Sheathelin, in odontogenic tumors. Virchows Arch 2000;436:324-329.

13. MacDougall M, Simmons D, Gu TT et al. Cloning, characterization and immunolocalization of human ameloblastin. Eur J Oral Sci 2000;108:303-310.

14. Nagano T, Odia S, Ando H. Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. J Dent Res 2003;82:982-986.

15. Lee SK, Kim SM, Lee YJ. The structure of the rat ameloblastin tooth germ and its expression in amelogenesis. Mol Cells 2003;15:216-225.

16. Perdigao PF, Gomez RS, Pimenta FJGS et al. Ameloblastin gene mutations associated with epithelial odontogenic tumors. Oral Oncol 2004;40:841-846.

17. Quintana MAT, Gaete M, Hernandez M et al. Ameloblastin and amelogenin expression in post natal developing mouse molars. J Oral Sci 2005;47:27-34.

18. Wazen RM, Moffatt P, Zalzal SF et al. A mouse model expressing a truncated form of ameloblastin exhibits dental and junctional epithelium defects. Matrix Biol 2009;28:292-303.

19. Smith CE, Wazen R, Hu Y et al. Consequences for enamel development and mineralization resulting from loss of function of ameloblastin or enamelin. Eur J Oral Sci 2009;117:485-497.

20. Tamburstuen MV, Reseland JE, Spahr A et al. Ameloblastin expression and putative autoregulation in mesenchymal cells suggest a role in early bone formation and repair. Bone 2011;48:406-413.

21. Zhang Y, Zhang X, Lu X et al. Ameloblastin regulates cell attachment and proliferation through RhoA and p27. Eur J Oral Sci 2011;119:280-285.

22. Crivelini MM, Felipini RC, Miyahara GI 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.

23. Hirose N, Shimazu A, Watanabe M et al. Ameloblastin in Hertwig‟s epithelial root sheath regulates tooth root formation and development, PLoS ONE 2013; 8:e54449.

24. Kim YS, Lee SK. Different Protein Expressions Between Peripheral Amelobastoma and Oral Basal Cell Carcinoma Occurred at the Same Mandibular Molar Area. Korean J Pathol 2014;48:151-158.

25. Slavkin M, Bringas P. The fine structure of the extracellular matrix during epithelia-mesenchymal interactions in the rabbit embryonic incisor. Anat Rec;1969:237-256.

26. Sisca RF, Proenza DV. Initial dentin formation in human deciduous teeth. An electron microscopic study. Calc Tiss Res 1972;9:1-16.

27. Sawada T. Expression of basement membrane component in the Dental papilla mesenchyme of monkey tooth germs – An immunohistochemical study. Connect Tissue Res 1995;32:55-61.

28. Reith EJ. The early stage of amelogenesis as observed in molar teeth of young rats. J Ultrastruct Res 1996;17:503-526.

29. Sawada T, Inoue S. Ultrastructural verification of anchoring role of lamina fibroreticularis of dental basement membrane in odontogenesis. J Electron Microsc 1999;48:919-928

30. Mitsiadis TA, Lardelli M, Lendahl V et al. Expression of Notch 1,2 and 3 is regulated by epithelial-mesenchymal interactions and retinoic acid in the developing mouse tooth and associated with determination of cell fate. J Cell Biol 1995;130:407-418.

31. Luo B, Aster JC, Hasserjian RP et al. Isolation and functional analysis of a cDNA for human Jagged 2, a gene encoding a ligand for the Notch 1 receptor. Mol Cell Biol 1997;17:6057-6067.

32. Mitsiadis TA, Hirsinger E, Lendahl U et al. Delta-Notch signaling in odontogenesis: Correlation with cytodifferentiation and evidence for feedback regulation. Dev Biol 1998;204:420-431.

33. Kawano S, Morotomi T, Toyomo T et al. Establishment of Dental Epithelial Cell line (HAT-7) and the cell differentiation dependent on Notch signaling pathway. Connect Tissue Res 2002;43:409-412.

34. Tezuka K-I, Yasuda M, Watanabe N et al. Stimulation of osteoblastic cell differentiation by Notch. J Bone Miner Res 2002;17:231-239.

35. Nakano K, Siar CH, Tsujigiwa H et al. Notch signaling in benign and malignant ameloblastic neoplasms. Eur J Med Res 2008;13:476-480.

36. Borkosky SS, Nagatsuka H, Orita Y et a. Sequential expression of Notch 1, Jagged 2 and Math 1 in molar tooth germ of mouse. Bio Cell 2008;32:251-257.

37. He F, Yang Z, Tan Y et al. Effects of Notch Ligand Delta 1 on the proliferation and differentiation of human dental pulp stem cells in vitro. Arch Oral Biol 2009;54:216-222.

38. Siar CH, Nakano K, Han PP et al. Differential expression of Notch receptors and their ligands in desmoplastic ameloblastoma. J Oral Pathol Med 2010;39:552-558.

39. Siar CH, Chuah KS, Nakano K et al. Notch 4 overexpression in ameloblastoma correlates with the solid/multicystic phenotype. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:224-233.

40. Muraki E, Nakano K, Maeda H et al. Immunohistochemical localization of Notch signaling in ameloblastomas. Eur J Med Res 2011;16:253-257.

41. Chen X, Li Y, Alawi F et al. An amelogenin mutation leads to disruption of the odonogenic apparatus and aberrant expression of Notch-1. J Oral Pathol Med 2011;40:235-242.

42. Viale-Bouroncle S, Gosau M, Morsczeck C. Notch-1 signaling regulates the BMP2/DLX3 directed osteogenic differentiation of dental follicle cells. Biochem Biophys Res Commun 2014;443:500-504.

43. Jheon AH, Prochazkova M, Meng B et al. Inhibition of Notch signaling during mouse incisor renewal leads to enamel defects. J Bone Miner Res 2016;31:152-162.

44. Sharief M. Expression of E-cadherin, cytokeratin-14 and cytokeratin-19 in tooth germ and ameloblastoma (dissertation). Chennai (TN): The Tamil Nadu Dr.M.G.R. Medical University; 2017.