台灣口腔醫學衛生科學雜誌
ISSN 1560-1587

造釉細胞瘤的運鈣蛋白質與牙釉質形成素之表現：與正常牙釉質形成的關係

陳重仁[1] 黃純德[2] 黃逸岳[3] Pinkney H. Gilchrist[4] Baldev Singh[4] James L. Borke [4]

1 義守大學 生物科技學系

2 高雄醫學大學口腔醫學院 口腔衛生科學研究所

3 高雄醫學大學口腔醫學院 牙醫學系

4 Department of Oral Biology and Maxillofacial Pathology, Medical College of Georgia, Augusta, Georgia, U.S.A.

正常牙釉質形成的過程包含表皮造釉母細胞逐漸成熟分化及牙釉基質的形成與基質之鈣化。此過程有一部分是受牙釉質形成素（amelogenin）的調節。 造釉母細胞的牙釉質形成素蛋白質與運鈣蛋白質的表現與基質形成及鈣化大體上是一致的。 齒源性腫瘤與正常牙胚在某些構造與部位在形態上或誘發關係上有某種程度類似的發育過程。在正常牙釉質形成過程中， 牙釉質形成素，漿膜鈣離子幫浦 （PMCA），鈣結合素（calbindin）在造釉母細胞的分泌前期階段只有微量的表現。但在牙釉質基質形成與開始鈣化時，才逐漸明顯增加。然而，造釉細胞瘤並不會形成基質或有鈣化現象。為了進一步瞭解造釉細胞瘤於牙釉基質的形成及鈣化過程的調節機制與正常牙胚的差異，本研究利用免疫組織化學法，在十個造釉細胞瘤的病例，發現在柵狀排列的周邊類造釉細胞中都有牙釉質形成素、漿膜鈣離子幫浦及鈣結合素的表現。利用 von Kossa 切片染色， 我們亦發現所有造釉細胞瘤都有鈣離子散佈的現象。因此我們的研究指出造釉細胞瘤的分化階段雖然類似但是卻不同於分泌前期階段的造釉母細胞。我們的研究顯示造釉細胞瘤之細胞外基質形成和鈣化的現象有異常的脫節現象。

關鍵詞：牙釉質形成素, 造釉細胞瘤, 鈣離子幫浦, 鈣結合素

聯絡人姓名：Dr. James L. Borke
通訊處：Medical College of Georgia, School of Dentistry Augusta, Georgia 30912-1129, USA
電話：（706）721-2032
傳真：（706）721-6252

受文日期：民國九十四年七月一日
接受刊載：民國九十四年九月十一日

Taiwan J Oral Med Health Sci 2005; 21:85-94

Calcium Transport Protein and Amelogenin Expression in Ameloblastoma: Relationship to Normal Amelogenesis

Jung-Ren Chen[1], Shun-Te Huang[2], Yueh Huang[3], Pinkney H. Gilchrist[4], Baldev Singh[4], James L. Borke[4]

[1] Department of Biological Science and Technology, I-Shou University, Kaohsiung, Taiwan
[2] Graduate Institute of Oral Health Science, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[3] Faculty of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[4] Department of Oral Biology and Maxillofacial Pathology, Medical College of Georgia, Augusta, Georgia, U.S.A.

The progressive maturation of the ameloblast epithelium, formation of enamel matrix, and the mineralization of that matrix characterize normal amelogenesis. This process is regulated, in part, by the amelogenins. The expression by ameloblasts of amelogenin proteins as well as calcium transport proteins parallels matrix formation and mineralization. Odontogenic tumors reproduce, to varying degrees, the morphological and inductive relationships found among various parts of normal tooth germ. In normal amelogenesis, amelogenin, plasma membrane Ca^{++}-pump（PMCA）, and calbindin-28KDa（calbindin）proteins are minimally expressed in presecretory ameloblasts, but progressively increase in expression later as the enamel matrix forms and begins to mineralize. Ameloblastomas, however, do not form matrix or mineralize. In the present study, we demonstrate by immunohistochemistry, that amelogenin, PMCA and calbindin proteins are all expressed in the ameloblast-like palisading peripheral epithelial cells of ten cases of ameloblastoma. In serial sections processed by the von Kossa technique, we also found diffuse calcium in all tumors studied. Our study suggests that ameloblastomas recapitulate a stage of differentiation beyond the presecretory

stage and that the mechanisms of extracellular matrix formation and mineralization associated with normal amelogenesis may be uncoupled in ameloblastomas.

Key words: amelogenin, ameloblastoma, Ca^{++}-pump, calbindin

Correspondence: Dr. James L. Borke
Address: Medical College of Georgia, School of Dentistry Augusta, Georgia 30912-1129, USA
TEL:（706）721-2032
FAX:（706）721-6252
Submitted: July, 1, 2005
Accepted: September, 11, 2005

Taiwan J Oral Med Health Sci 2005; 21:85-94

Introduction

In normal oral epithelium, ameloblasts are largely responsible for the formation of enamel. In this process, ameloblasts lay down an enamel matrix followed by mineralization of the matrix through the massive influx of large quantities of calcium and phosphate.

The formation of enamel occurs in stages with changes in both protein composition and mineral content occurring at each stage [1]. The amelogenins represent over 90% of the enamel extracellular matrix proteins. Extracellular proteolytic processing of amelogenins has been shown to be associated with the change in protein composition of the matrix observed at each stage [2]. Amelogenins are proteins of 22-30 KDa that have a composition rich in proline, glutamine and histidine residues [3]. They are the primary constituent of the enamel matrix, and are presumed to play a major role in mineralization and the overall structural organization of enamel[4]. In the mineralization process, amelogenins are believed to regulate the size and morphology of the developing enamel crystals [3]. The presence of amelogenin mRNA and protein has been demonstrated in presecretory ameloblasts of the rat incisor, but increased amelogenin expression parallels enamel formation and increases greatly in abundance during the later stages of enamel formation [5].

In addition to the control of matrix formation and mineralization exerted by amelogenin, the control of the mineralization process involves the regulated transport of calcium ions across the ameloblast epithelial cell layer [6]. The mechanism responsible for regulated calcium movement into the enamel matrix may involve two calcium transport proteins, the plasma membrane Ca^{++}-pump (PMCA) and vitamin D-dependent calbindin-28kDa (calbindin), both of which are expressed by normal ameloblasts. PMCA and calbindin are expressed in ameloblasts at the onset of mineralization, and are known to transport large amounts of calcium in other tissues[7]. While both PMCA and calbindin are expressed in many cell types, in the enamel organ, calbindin is expressed exclusively by ameloblasts [8].

Ameloblastomas are benign soft tumors displaying characteristics of the primitive enamel organ[9]. Ameloblastomas differ from ameloblasts in that they are unable to form enamel matrix, and they do not mineralize [10,11]. In our study, we use immunohistochemistry to determine if the expression of amelogenin, PMCA and calbindin in ameloblastomas mimics their expression in the presecretory or maturing enamel organ.

Materials and Methods

Ten odontogenic conventional (solid) ameloblastoma specimens were selected from paraffin embedded specimens in the tissue archives of the Oral Biology and Maxillofacial Pathology Department at the Medical College of Georgia. Sagittal sections of normal rat mandibles showing the progression of enamel formation and the stages of differentiation of

ameloblasts were also processed along with the ameloblastoma tissues for comparison of stages and as a positive control for immunohistochemical and von Kossa staining. Tissue blocks were sectioned（5 μm）and stained for immunohistochemistry using the avidin-biotin-peroxidase technique of Hsu et al [12]. Briefly, tissue sections on glass slides were deparaffinized in limonene and rehydrated through a graded series of ethanol to water. Sections were covered with a 3% hydrogen peroxide solution to quench endogenous peroxidase activity followed by a 10 mg/ml solution of bovine serum albumin in phosphate buffered saline（PBS）to block non-specific interaction of the antibodies with tissue proteins. Monoclonal antibody 5F10 specific for multiple isoforms of the plasma membrane Ca^{++}-pump（PMCA）at a dilution of 1:750 and monoclonal antibody CL300 specific for calbindin-28kDa at a dilution of 1:1000 were used to detect the calcium transport proteins [13]. Polyclonal anti-amelogenin antibody prepared against the full-length recombinant M179 mouse amelogenin and which cross-reacts with all known amelogenin isoforms, was used at a dilution of 1:750 to detect amelogenin protein expression. The binding of the specific antibodies was detected by incubating the sections with biotinylated horse anti-mouse Ig secondary antibody（for PMCA and calbindin）or biotinylated goat anti-rabbit Ig secondary antibody（for amelogenin). After washing in PBS, the sections were incubated in an avidin-biotin-peroxidase complex solution. The proteins were visualized as a brown precipitate over the sites of peroxidase-conjugated antibody binding when exposed to diaminobenzidine tetrahydrochloride in the presence of H2O2.

Rat mandibles and ameloblastomas were also processed for calcium localization by the von Kossa technique[14]. With this technique, tissue sections are exposed to sunlight in the presence of 1% silver nitrate. The calcium salts precipitate with the silver nitrate to form a brown-black reaction product, which is fixed in sodium thiosulfate and visualized on the sections.

Antibodies prepared against PMCA（5F10）and calbindin（CL300）were obtained from Sigma, St. Louis, MO. Antibody M179 specific for amelogenin was a gift from Dr. Alan Fincham at USC in Los Angeles. Reagents for the avidin-biotin-peroxidase procedure were obtained from Vector Inc., Burlingame, CA. All other reagents were obtained from Sigma, and were of the highest quality available.

Results

Longitudinal sections of normal rat mandibles were examined for the presence of PMCA, calbindin, amelogenin, and calcium for comparison to 10 cases of conventional（solid）ameloblastoma.

Figure 1A shows the progressive expression（++ to ++++）of PMCA in the plasma membranes of the secretory ameloblasts of rat incisor enamel organ by immunohistochemistry.

Taiwan J Oral Med Health Sci 2005; 21:85-94

Figure 2A shows similar positive immunohistochemical localization of calbindin (++++) in the secretory ameloblasts of the rat incisor enamel organ, but in the cytoplasm of these cells. Figure 3A also shows progressive expression (++) of amelogenin in the enamel matrix and ameloblast cells of rat incisor enamel organ beginning beyond the presecretory stage.

Localization of calcium deposition was also studied in normal rat mandibles using the von Kossa technique [14, 15]. Enamel and dentin in the rat mandibles were both found to stain intensely with the von Kossa technique. Figure 4A shows the localization of calcium (black-brown) in the rat mandible after staining by the von Kossa technique. This figure shows positive ++++ histochemical localization of calcium in secretory ameloblasts, the site of forming enamel and in dentin.

In all ameloblastoma tumors examined, both stellate reticulum-like cells and rounded columnar cells show positive immunoreactivity

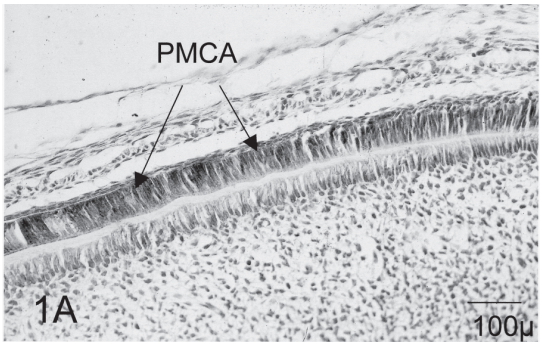

Fig 1A. Section of rat mandible showing the onset of expression and the progressive immunohistochemical localization of PMCA in the secretory ameloblasts of the rat incisor enamel organ.

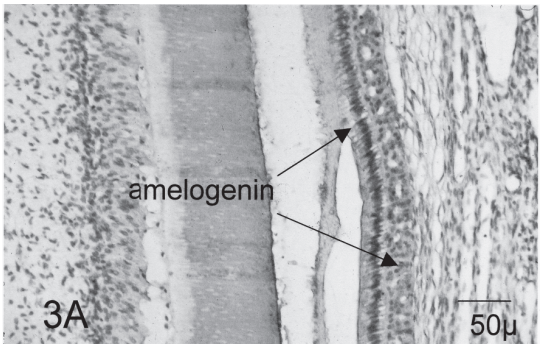

Fig 3A. Section of rat mandible showing expression of amelogenin in the enamel matrix and ameloblast cells of the rat incisor enamel organ.

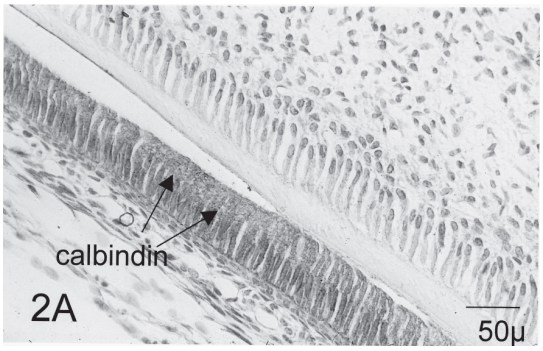

Fig 2A. Section of rat mandible showing immunohistochemical localization of calbindin in the secretory ameloblasts of the rat incisor enamel organ.

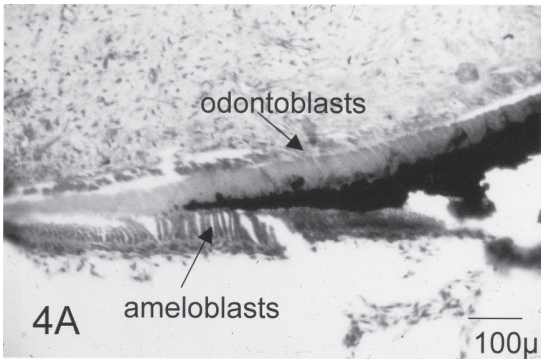

Fig 4A. Section of rat mandible showing secretory ameloblasts and odontoblasts of the rat mandible after staining by the von Kossa technique. Calcium localization appears as a black-brown precipitate.

with specific antibodies to all PMCA, calbindin, and amelogenin proteins. For these tumor sections, staining intensity ranged from ++ to ++++. Figure 1B shows positive (++++) immunohistochemical staining for PMCA in a representative section of an ameloblastoma suggesting differentiation beyond the presecretory stage in which PMCA protein is not expressed[13]. Figure 2B also shows positive immunohistochemical staining for calbindin (+++) in a representative section of an ameloblastoma also suggestive of differentiation beyond the presecretory stage.

The presence of two Ca^{++} transport proteins, PMCA and calbindin in ameloblastomas, although present in a wide range of tissues, is significant because these proteins are found together in normal ameloblasts and other Ca^{++} transport tissues in large abundance, and yet ameloblastomas also do not mineralize.

Figure 3B shows positive (++) immunoreactivity with an antibody to amelogenin in a representative section of ameloblastoma also

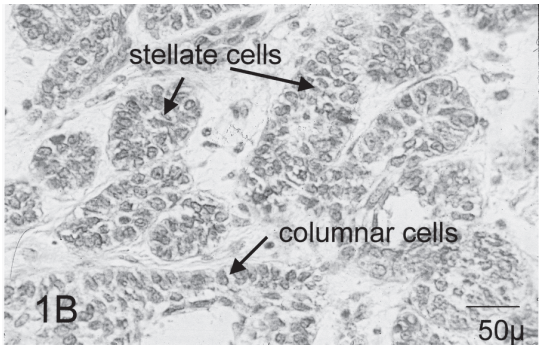

Fig 1B. Histological section of a representative ameloblastoma showing positive immunohistochemical localization of PMCA, suggestive of enamel organ differentiation beyond the presecretory stage.

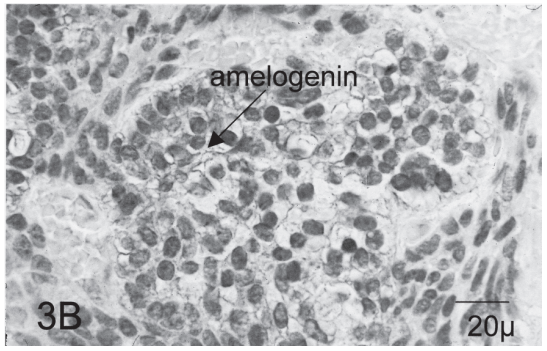

Fig 3B. Histological section of a representative ameloblastoma showing positive immunoreactivity for amelogenin and suggesting ameloblast-like differentiation beyond the presecretory stage.

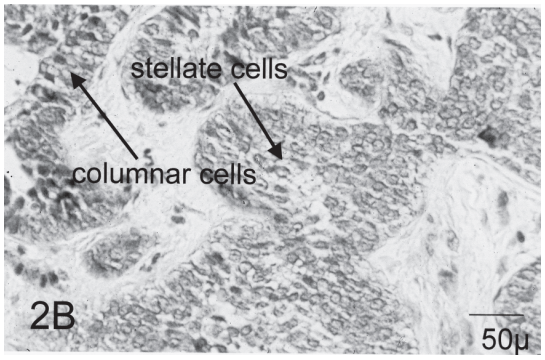

Fig 2B. Histological section of a representative ameloblastoma showing positive immunohistochemical localization of calbindin, suggestive of enamel organ differentiation beyond the presecretory stage.

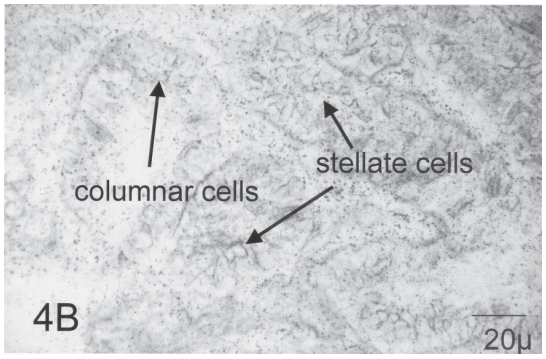

Fig 4B. Representative section of an ameloblastoma stained by the von Kossa technique and showing weak staining for calcium.

suggesting differentiation beyond the presecretory stage. Localization of amelogenin, a major protein in the enamel matrix is interesting because ameoblastomas also do not produce an extracellular matrix [9].

Figure 4B is a representative section of an ameloblastoma stained for Ca^{++} using the von Kossa technique. The diffuse positive staining suggests that despite the presence of Ca^{++}, mineralization of this tumor has not occurred.

Discussion

The continuously erupting rat incisor represents a unique model for the study of amelogenesis. Sectioning of the rat mandible in the sagittal plane yields specimens for histology and immunohistochemistry demonstrating presecretory, secretory, transitional and maturation ameloblasts in a single section. In our previous work, we have used this model to place in a temporal relationship the onset of expression of the calcium transport proteins (calbindin and PMCA), the formation of enamel extracellular matrix, and the mineralization of that matrix, all of which increase greatly after the presecretory phase of amelogenesis. In our present study, we have used this model to compare the characteristics of the ameloblasts during normal amelogenesis with the ameloblast-like characteristics manifest in human ameloblastomas.

Amelogenins are the major proteins in the enamel extracellular matrix. These consist of a heterogeneous group of alternatively spliced forms derived from multiple exons [16]. The complexity of the matrix is increased by the presence of other proteins in low abundance and multiple degredative pathways increasing still further the heterogeneity. Amelogenin processing, and interaction of the various amelogenin products with the immediately surrounding mineral environment are key to the regulation of formation of the large apatite crystals of the enamel [3]. Recent studies by Snead et al. (1992) [9] suggest that ameloblastoma cells contain antisense mRNA, presumably transcribed from the opposite strand of an amelogenin gene. Antisense RNA has the ability to bind to the sense strand of the mRNA preventing it from being translated to protein. This may account for the inability of these tumors to produce matrix. In our study, however, we show that amelogenin-specific antibody binds to the cytoplasm of the palisading epithelial cells of the ameloblastomas as well as to the cytoplasm of normal ameloblasts in the rat mandible. This suggests that some matrix protein may be formed but that it cannot be processed or released appropriately to promote mineralization.

To remain viable, Ca^{++} transport tissues such as the ameloblast epithelium must maintain an intracellular Ca^{++} concentration of $\sim 10^{-7}$M while moving large amounts of Ca^{++} across the cell[6]. This is accomplished through the action of calcium binding proteins such as calbindin, which may act as Ca^{++} shuttles to transport Ca^{++} across the cytoplasm. Also

necessary in these tissues are Ca^{++} efflux mechanisms capable of moving the Ca^{++} out of the cell against steep concentration and electrical gradients in a vectorial manner. In the ameloblast, this may be accomplished at least in part through the actions of PMCA.

In the odontogenic epithelium of rats and humans, calbindin is localized exclusively in ameloblasts of the enamel organ[8]. PMCA is also present at high levels in these cells [13]. The onset of expression of these proteins in the rat incisor enamel organ corresponds to the transition between presecretory and secretory ameloblasts, the first detection of matrix formation, and the onset of mineralization[13]. Both of calbindin and PMCA were detected in the ten ameloblastoma tissues examined.

The von Kossa technique is used for the localization of calcium in tissue sections. Deposition of a brown-black precipitate after using this procedure localizes the distribution of calcium in a manner roughly proportional to the amount of calcium present. Our rat mandible sections showed high concentrations of precipitate in areas of bone, dentin, and enamel. We also found diffuse precipitate deposition corresponding to calcium localization in and around the ameloblastoma tumors in all cases examined. Negative control sections did not show this deposition. Concentration of calcium in the microenvironment near the mineralization front of normal enamel is a necessary prelude to mineral deposition and crystal formation [6]. Our observation of calcium transport proteins and diffuse calcium distribution surrounding ameloblastoma cells suggests that ameloblastomas retain the mechanisms necessary for the delivery of calcium. Our study also suggests that the delivered calcium finds either no matrix or an unsuitable matrix on which to form crystals.

Taken together, our studies suggest that the calcium transport mechanism may be sufficiently developed in palisading epithelial cells in ameloblastoma tumors to deliver calcium to the microenvironment surrounding the cells. Our findings also suggest that the absence of a suitable matrix is the primary reason that ameloblastomas do not calcify.

Acknowledgments

The authors would like to thank the Medical College of Georgia Dental Foundation（JLB）and National Science Council（NSC 93-2314-B-21-001, NSC 94-2314-B-21-007 to JRC）for support, and Ms. Michelle Burnside for her careful work and helpful suggestions in the preparation of this manuscript.

References

1. Sato K, Hattori M, Aoba T. Disturbed Enamel Mineralization in a Rat Incisor Model. Adv Dent Res. 1996; 10(2): 216-224.
2. Brookes SJ, Robinson C, Kirkham J, Bonass WA. Biochemistry and Molecular Biology of Amelogenin Proteins of Developing Dental Enamel. Arch Oral Biol. 1995; 40: 1-14.
3. Robinson C, Brookes SJ, Kirkham J, Bonass

WA, Shore, RC. Crystal Growth in Dental Enamel: The Role of Amelogenins and Albumin. Adv Dent Res. 1996; 10(2): 173-180.

4. Eisenmann DR Amelogenesis and Enamel Structure. In: Oral Histology, editor, Ten Cate, AR. 4th ed, St. Louis: Mosby; 1994. p. 218-256.
5. Nanci A, Bendayan M, Slavkin HC. Enamel Protein Biosynthesis and Secretion in Mouse Incisor Secretory Ameloblasts as Revealed by High Resolution Immunocytochemistry. J Histochem Cytochem. 1985; 33: 1153-1160.
6. Bawden JW. Calcium Transport During Mineralization. Anat. Rec. 1989; 224: 226-233.
7. Carafoli E. Calcium Pump of the Plasma Membrane. Physiolog. Rev. 1991; 71: 129-153.
8. Berdal A, Hotton D, Pike TW, Mathieu H, Dupret JM. Cell- and Stage-specific Expression of Vitamin D Receptor and Calbindin Genes in Rat Incisor: Regulation by 1,25-dihydroxyvitamin D3. Dev Biol. 1993; 155: 172-179.
9. Snead ML, Luo W, Hsu DD, Melrose RJ, Lau EC, Stenman G Human Amelobastoma Tumors Express the Amelogenin Gene. Oral Surg. Oral Med. Oral Pathol. 1992; 74: 64-72.
10. Le Charpentier Y Classification of odontogenic tumors, 2nd edition（WHO, 1992）Ann Pathol. 1994;14(1):55-7.
11. Waldron CA, El-Mofty SK. A Histopathologic Study of 116 Ameloblastomas with Special Reference to the Desmoplastic Variant. Oral Surg Oral Med Oral Pathol. 1987; 63: 441-446.
12. Hsu S, Raine L, Fanger H. Use of Avidin-Biotin-Peroxidase Complex（ABC）in Immunoperoxidase Techniques: A Comparison Between ABC and Unlabeled Antibody（PAP）Procedures. J Histochem Cytochem. 1981; 29: 577-580.
13. Borke JL, Zaki AE, Eisenmann DR, Ashrafi SH, Ashrafi SS, Penniston JT. Expression of Plasma Membrane Ca^{++} Pump Epitopes Parallels the Progression of Enamel and Dentin Mineralization in Rat Incisor. J Histochem Cytochem. 1993; 41: 175-181.
14. Kossa J von. Beitr. z. path. Anat. u. z. allg. Path. 1901; 29: 163.
15. Sheehan DC Hrapchak BB: Theory and Practice of Histotechnology; 1980, 227.
16. Gibson CW, Golub E, Ding W, Shimakawa H, Young M, Termine J, Rosenbloom J. Identification of the leucine-rich ameloginin peptide（LRAP）as the transcription product of an alternatively spliced transcript. Biochem Biophys Res Commun. 1991; 174: 1306-1312.