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IMMUNOHISTOCHEMISTRY LOCALISATION OF CNNM4 PROTEIN DURING TOOTH MURINE AMELOGENESIS

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ABSTRACT

Objectives: Homo sapiens cyclin and CBS domain divalent metal cation transport mediator 4 (CNNM4) is a protein that is suggested to play an important role in Mg^{2+} ion transport. Mutations in this gene are associated with Jalili syndrome which consists of cone-rod dystrophy and enamel defects. These results highlighted the importance of the CNNM4 during enamel formation, so our work aimed to study the localisation of CNNM4 during different stages of lower incisor and first molar murine amelogenesis.

Material and Methods: Adult incisors (n: 16) of 4 week mice (C57BL) and pups at PNO, PN4 and PN14 were used to study Cnnm4 expression in different stages of mouse incisor and first molar respectively. The pups were obtained from time mated pregnant mice.

Results: In lower incisor, Cnnm4 was detected in ameloblast only in the maturation stage while in the secretory stage the signals was detected in a very specific way to stratum intermedium (SI). While in lower first molar, Cnnm4 was localised in inner enamel epithelium (pre-ameloblast) and stratum intermedium of the enamel organ at early bell stage (pre-secretory stage) while stellate reticulum and outer enamel epithelium were negative. In addition to that during the secretory stage, Cnnm4 signal was detected in secretory ameloblast under the cusp tip. Dental papilla, odontoblasts and dental follicles were appeared completely negative in both lower incisor and first molar.

Conclusion: CNNM4 has a specific expression pattern in enamel forming cells during different stages of teeth development in mice amelogenesis.

KEYWORDS: CNNM4, Amelogenesis, Development

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INTRODUCTION

Development of the mammalian teeth is a complex process that results from a cross talk between oral ectodermal and the underlying mesenchymal tissues of the first branchial arch (Jussila and Thesleff, 2012). This interaction is completely under the genetic control of many signalling molecules (Tummers and Thesleff, 2009). Amelogenesis or enamel formation is a biomineralization process in which specialised epithelial derived cells, ameloblasts pass through a series of discrete cell morphology and function differentiation states (Smith, 1998).

Amelogenesis is divided mainly into pre-secretory, secretory and maturation stages. In the pre-secretory stage, pre-ameloblasts are separated from the adjacent neural crest cell-derived odontoblasts by a basement membrane that finally degraded before entering of ameloblasts into secretory stage. Once ameloblasts enter their secretory stage, start to secrete a protein-rich, partially mineralised, self-organising enamel matrix. This matrix is mainly composed of a group of extracellular enamel matrix (ECM) proteins, including three main structural proteins amelogenin, enamelin, and ameloblastin and two enamel proteases, matrix metallopeptidase-20 and kallikrein 4. When the matrix reaches full thickness, the secretory stage ends and the ameloblasts enter the maturation stage (Smith, 1998; Moradian-Oldak, 2012). The main function of the maturation stage ameloblasts is to harden the enamel by removal of the organic material from the enamel matrix and the secretion of more calcium and phosphates ions (Smith, 1998; Moradian-Oldak, 2012).

Amelogenesis imperfecta is a condition that affect the clinical appearance of enamel of all, or nearly all the teeth. This condition may be occurs alone or may be associated with morphologic changes elsewhere in the body, but in general it is due to a genomic change (Aldred et al., 2003). Mutations in CNNM4 are associated with Jalili syndrome which consists of cone-rod dystrophy and amelogenesis imperfecta (Parry et al., 2009). The encoded protein suggested to play a role in magnesium (Mg2+) ion transport (Yamazaki et al., 2013). However, how CNNM4 dysfunction impairs these two developmentally different organs remain to be determined although it was attributed to magnesium homeostasis disturbances.

In this study we are trying to investigate Cnnm4 protein expression in different stage of murine incisor and molar amelogenesis using Immunohistochemical technique.

MATERIAL AND METHODS

Animals:

Adult male (n=20) C57BL mice strains were used in this study. Animals were housed in a temperature-controlled room under artificial illumination with access to food and water ad libitum (Clinical Science Building, St. James's University Hospital-Leeds University-UK). Eight Jaws containing lower incisors were obtained from adult mice aged 4 weeks to describe different stages of the process of enamel formation (pre-secretory, secretory and maturation stages). For molar amelogenesis, three embryos were used at PNO, PN4, and PN14 developmental stages to describe different amelogenesis stages of mouse molar. The embryos were obtained from time mated pregnant mice and the embryonic day 0 (E0) was designated as the day on which a vaginal plug was confirmed.

Sections preparation and immune-staining:

Immediately after scarification of the pregnant mothers at the right embryological time, the heads of puppies were fixed with 4% paraformaldehyde (PFA) in 0.01M phosphate buffer saline (PBS, pH 7.4), processed and embedded in paraffin as usual. Jaw of the adult mice and heads of puppies at PN4, and PN14 were then decalcified in 8% formic acids for two weeks before they were processed and embedded in paraffin. Serial sections were carried

out on all the paraffin blocks at a thickness of 5 μm and stained with Haematoxylin and eosin (H & E) to get the right orientation of different stages of molar enamel formation. Selected sections were used for CNNM4 immune-staining.

Sections were de-paraffined in two changes of xylene for 20 minutes each, and then placed in 2 changes of absolute alcohol for 5 minutes each. The endogenous peroxidase activity was blocked by immersing the slides in 2% hydrogen peroxide/ methanol solution for 10 minutes. Monoclonal Cnnm4 primary antibody was obtained commercially (Sigma-UK), loaded in 1/100 dilution and incubated for 1 hour at room temperature. EnVision kit was used as developing system according to the manufacture instructions (Dako EnVision K5007).

RESULTS

Expression pattern of CNNM4 in adult mouse incisor amelogenesis:

In the secretory stage of enamel formation, expression of CNNM4 was detected in the stratum intermedium (SI) cell layer that is located over the secretory ameloblasts. However, secretory ameloblasts itself did not show any CNNM4 signals (Figure1. AII). While during the maturation stage of enamel formation, CNNM4 expression was detected in a strong way in both of the maturation ameloblasts and the papillary cells (Figur1. BII). CNNM4 expression was completely negative in dental follicle in different stages of incisor amelogenesis (Figure1).

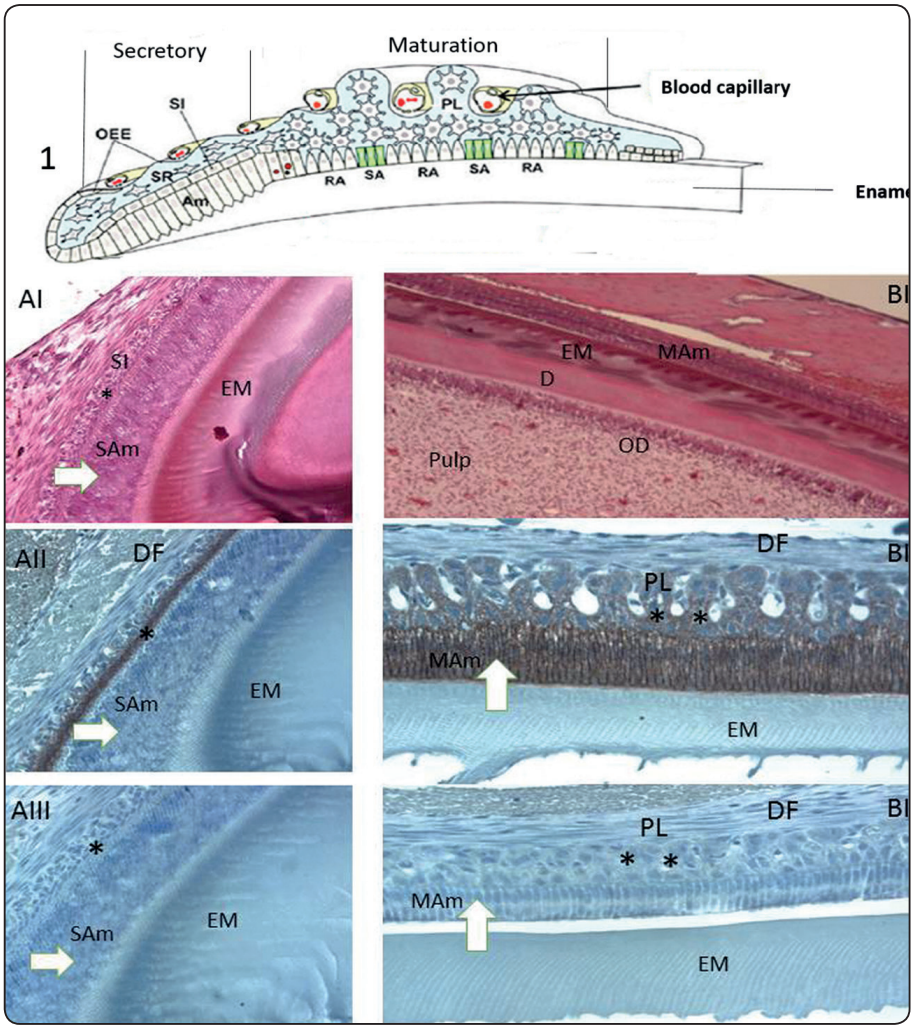

Fig. (1) CNNM4 protein localization in murine incisor amelogenesis

AI, AII and AIII show the mouse lower incisor at secretory stage. AI; H&E staining sections for orientation of the incisor secretory stage. AII; Cnnm4 expression was detected in the stratum intermedium (SI. asterisk), secretory ameloblast (SAm. White arrow) has negative expression of CNNM4. AIII; Cnnm4 expression was detected in the maturation stage ameloblast (MAm. White arrow), Cnnm4 signals was also detected in the papillary layer (PL. asterisk). BIII; Cnnm4 negative control. 1; Dental follicle (DF. Was always negative for Cnnm4 expression. The diagram for murine lower central incisor describing the different stages of amelogenesis (modified and edited from Robinson, 2013).

Expression pattern of CNNM4 in mouse molars amelogenesis:

CNNM4 expression was traced and localised in sections of lower first molar at embryonic days PN0, PN4, and PN14. At PN0. At birth (PN0), the molar tooth germ appeared to be at late cap stage and beginning of the early bell stage, where all cells of the enamel organ could be easily seen (Figure 2). Ameloblasts and the rest of the enamel organ were in pre-secretory stage of enamel formation. CNNM4 expression was detected in the pre- secretory ameloblast and stratum intermedium; however it was very weak in the stellate reticulum (SR) and outer enamel epithelium (OEE) of the enamel organ (Figure 2A). At PN4 all the ameloblasts became in secretory stage which start to secret the enamel matrix, CNNM4 was not detected in secretory ameloblasts cells except in those cells under the cusp tip (Figure 2B). At PN14 that represent late stage of enamel formation (maturation stage), CNNM4 has again strong expression to CNNM4 similar to the maturation stage of lower incisor (Figure2C). Dental papilla and odontoblast were completely negatives (Figure2).

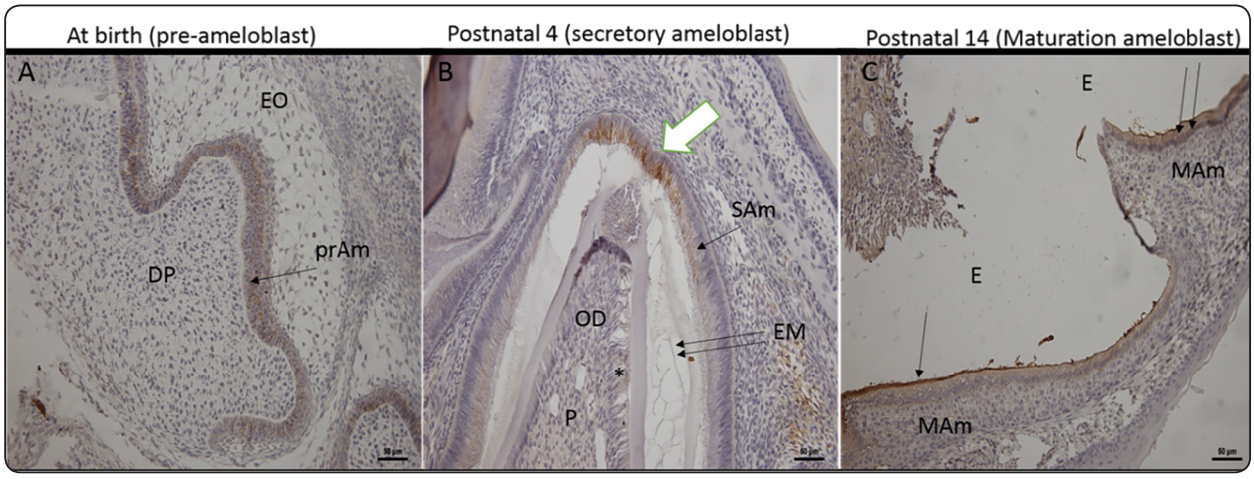

Fig. (2) Cnnm4 protein localization in murine first molar amelogenesis

A, B and C show the mouse molar at persecutory, secretory, and Maturation stages amelogenesis respectively. A; Cnnm4 expression was detected in the persecutory ameloblasts (PreAm-single arrow). B; Cnnm4 was negatively expressed in the secretory ameloblasts (SAm- single arrow) but was detected under the cusp tip (white arrow). C; Cnnm4 expression was detected in the maturation ameloblasts (MAm-single and double arrows). Dental papilla (DP) and pulp showed negative expression for Cnnm4.

DISCUSSION

In humans, obtaining developing teeth in different developmental stages is almost impossible. This issue is considered one of the most challenges in studying and clarifying the underlying molecular mechanisms of amelogenesis. Unlike humans, rodents have two type of teeth; the incisor teeth erupt continuously throughout life and offer access to all stages of amelogenesis and the molar teeth are not continuously erupting during the life of the animal, so it is more close to human teeth development (Baron et al., 2010). Therefore, the mouse is an excellent model organism to investigate the fundamental events of amelogenesis.

Mutations in CNNM4 have been reported to cause Jalili syndrome which consists of cone-rod dystrophy and enamel defects (Parry et al., 2009; Polok et al., 2009). CNNM4 is a protein that is suggested to play an important role in Mg^{2+} ion transport during enamel crystals formation. It was also shown that CNNM4 is expressed by ameloblast in the maturation and secretory stage using only

rodent incisor in two different reports (Parry et al., 2009, Polok et al., 2009). In another study, CNNM4 was found to be expressed in maturation stage ameloblast with high intensity while in the secretory stage it was restricted only to the stratum intermedium (Yamazaki et al., 2013).

In our study much attention have been given to describe the expression pattern of CNNM4 in pre-secretory- secretory and maturation stages using rodent incisor and first molar amelogenesis as well. Our study have confirmed CNNM4 expression in the maturation stage ameloblasts and stratum intermedium cells only during secretory stage of mouse lower incisor, so we support both previous studies by Parry et.al., 2009 and Yamazaki et al., 2013. In addition to that, Cnnm4 was always localised in the cells of papillary layer of the enamel organ in the secretory and maturation stages of amelogenesis in mouse incisor.

Our study also showed some subtle Cnnm4 expression differences between lower incisor and lower first molar as the expression of Cnnm4 was detected in inner enamel epithelium (pre-ameloblast) and stratum intermedium before enamel formation (Figure 2A). Secretory ameloblast also showed some Cnnm4 expression under the cusp tip of the firs molar that may be due to enamel rod structural specification at the cusp region.

Although mature enamel crystals is hydroxy-apatite (HAP) (Ca10 [PO4]6[OH]2), it is believed that the mineral phase that starts at the mineralisation front as amorphous calcium phosphate (ACP) which latter changes to hydroxyapatite (Beniash et al., 2009). During this conversion between different enamel crystals phase, enamel crystals can incorporate other ions such as carbon (as carbonate), magnesium and fluoride (Aoba, 1996). These trace elements are well controlled by ameloblasts and the rest of enamel organ cells, as these elements can alter the physical properties of the mature enamel.

Incorporation of Mg^{2+} ions during enamel formation render its mineralisation. It was found that the amount of incorporated Mg^{2+} ions during the maturation stage is higher than that incorporated in the enamel during the secretory stage (Jalevik B et al., 2001). Our study showed that maturation stage ameloblasts express Cnnm4 in a higher intensity than secretory ameloblast which confirms other studies in the litature. This results actually fit with the level of Mg^{2+} during enamel formation indicating that Cnnm4 play a pivotal role in removing Mg^{2+}ions from developing enamel and also most of the Mg^{2+} incorporated with enamel crystals during enamel formation are removed during the maturation stage.

In our study we have shown that the expression pattern in rodent incisor and first molar teeth are similar. Moreover we have shown that the expression pattern of CNNM4 is concomitant with the level of Mg^{2+} during enamel formation. This study beside the other studies in the literature highlight the role of Cnnm4 in Mg^{2+} ion control during enamel formation. No doubt that CNNM4 is an exciting and important protein for enamel formation and more studies need to be done to investigate the role and the function of CNNM4 in enamel formation on the molecular level.

REFERENCES

1. Jussila, M. and Thesleff, I. (2012). Signaling networks regulating tooth organogenesis and regeneration, and the specification of dental mesenchymal and epithelial cell lineages. Cold Spring Harb. Perspect. Biol. 4, a008425.
2. Moradian-Oldak J: Protein-mediated enamel mineralization. Front Biosci 2012; 17: 1996– 2023.
3. Smith CE: Cellular and chemical events during enamel maturation. Crit Rev Oral Biol Med 1998; 9:128–161.
4. Aldred, M.J., R. Savarirayan, P.J. Crawford (2003) Amelogenesis imperfecta: a classification and catalogue for the 21st century. Oral Dis 9: 19–23.
1. Parry, D.A., Mighell, A.J., El-Sayed, W., Shore, R.C., Jalili, I.K., Dollfus, H., Bloch-Zupan, A., Carlos, R., Carr, I.M., Downey, L.M., et al. (2009). Mutations in CNNM4 Cause Jalili Syndrome, Consisting of Autosomal-Recessive Cone-Rod Dystrophy and Amelogenesis Imperfecta. American Journal of Human Genetics 84, 266-273.

2. Yamazaki D, et al. Basolateral Mg2+ extrusion via CNNM4 mediates transcellular Mg2+ transport across epithelia: a mouse model. PLoS Genet. 2013; 9(12):e1003983.
3. Tummers, M. and Thesleff, I. 2009. The importance of signal pathway modulation in all aspects of tooth development. J Exp Zool B Mol Dev Evol, 312B, 309-19.
4. Barron M J, Brookes SJ, Kirkham J*, Shore RC, Hunt C, Mironov A, Kingswell NJ, Maycock J, Shuttleworth CA and Dixon MJ. A mutation in the mouse Amelx tri-tyrosyl domain results in impaired secretion of amelogenin and phenocopies human X-linked amelogenesis imperfecta. Human Molecular Genetics doi:10.1093/hmg/ddq001 (2010)
5. Polok B, Escher P, Ambresin A, Chouery E, Bolay S, Meunier I et al. Mutations in CNNM4 cause recessive cone rod dystrophy with amelogenesis imperfecta. Am J Hum Genet 2009; 84: 259–265
6. Beniash, E., Metzler, R. A., Lam, R. S. & Gilbert, P. U. 2009. Transient amorphous calcium phosphate in forming enamel. J Struct Biol, 166, 133-43.
7. Aoba, T. 1996. Recent observations on enamel crystal formation during mammalian amelogenesis. Anat Rec, 245, 208-18.
8. Jalevik B, Odelius H, Dietz W, Nore´n J (2001) Secondary ion mass spectrometry and X-ray microanalysis of hypomineralized enamel in human permanent first molars. Arch Oral Biol 46: 239–247.