SHORT COMMUNICATION

Decreased osteogenic activity and mineralization of alveolar bone cells from a patient with amelogenesis imperfecta and FAM83H 1261G > T mutation

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Abstract  FAM83H mutations lead to autosomal dominant hypocalcified amelogenesis imperfecta (ADHCAI). However, the biological role of FAM83H remains unclear. The present study aimed to characterize the alveolar bone cells isolated from a patient with ADHCAI having the mutation, c.1261G > T, p.E421*, in FAM83H. We showed that FAM83H mutant cells had proliferation ability and morphology similar to the controls. The F-actin staining revealed that FAM83H mutant cells were remained in the earlier stages of cell spreading compared to the controls at 30 min, but their spreading was advanced comparable to the controls at later stages. After osteogenic induction, a significant decrease in mRNA levels of RUNX2 and ALP was observed in FAM83H mutant cells at day 7 compared with day 3 while their expressions were increased in the controls. The OPN levels in FAM83H mutant cells were not significantly changed at day 7 compared to day 3 while the controls showed a significant increase. After 14 days, the mineral deposition of FAM83H mutant cells was slightly lower than that of the controls. In conclusion, we identify that FAM83H bone cells have lower expression of osteogenic
marker genes and mineralization while they maintain their morphology, proliferation, and spreading. Consistent with previous studies in the ameloblasts and periodontal ligamental cells, these evidences propose that FAM83H influences osteogenic differentiation across different cell types in oral cavity.

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Introduction

Family with sequence similarity 83 member H (FAM83H; OMIM * 611927) is a member of FAM83 family. Mutations in FAM83H are the major cause of autosomal dominant hypocalcified amelogenesis imperfecta (ADHCAI) presenting abnormal enamel mineralization. Recent studies have demonstrated that the periodontal ligament is the major cause of ADHCAI. 

In oral cavity, FAM83H expression was observed in the ameloblasts, odontoblasts, and alveolar bone. Murine ameloblast cell line transfected with Fam83h mutant cDNA (c.1186C>T) exhibited a significant decrease in expression of osteogenic marker genes, namely Runx2, Alp, and Ocn, corresponding with the reduction of ALP activity. 10 Our previous study demonstrated that the periodontal ligament cells isolated from the ADHCAI patient with FAM83H mutation (p.E421*) showed impaired proliferation and down-regulation of OCN, BSP, and COL1 levels after mineralization induction. The teeth affected with ADHCAI also showed reduced mineral density. These lines of evidence show that the FAM83H mutant cells had impaired functions related to osteogenic differentiation and mineralization. The present study for the first time investigated the characteristics and mineralization ability of alveolar bone cells obtained from a patient with the heterozygous nonsense mutation, c.1261G>T, p.E421*, in the FAM83H gene.

Materials and methods

Subject enrollment

The study was approved by Institutional Review Board (IRB No. 163/61), Faculty of Medicine, Chulalongkorn University and complied with the Declaration of Helsinki. Informed consents were obtained from all participants in this study. Genetic mutation was analyzed by whole exome sequencing as described in our previous study. The identified variant was validated in the proband and other family members by Sanger sequencing.

Cell isolation

Bone chips were obtained from the buccal bone plate of a patient affected with ADHCAI during surgical extraction of impacted third molars. Cell isolation was performed by explanting method. Briefly, bone chips were maintained in Dulbecco’s modified eagle medium (DMEM) (Gibco, USA) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Cells were subcultured upon reaching confluence. These cells were considered as passage 1. The present study utilized cells at passages 3–6. The controls were alveolar bone cells at similar passage from healthy subjects.

Cell proliferation assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was employed to determine cell proliferation. Cells (12,500 cells) were plated in 24 well-plate and maintained in growth medium. Cells were incubated with MTT solution (1 mg/mL) for 10 min. The precipitated formazans crystals were solubilized using dimethylsulfoxide solution and glycine buffer. The absorbance was monitored at 570 nm.

Immunofluorescence staining

Cells were incubated with 3% glutaraldehyde (Fluka Analytical, USA) for 10 mins. After washing with PBS, samples were treated with 0.1% Triton-X100 (Sigma–Aldrich, USA). Samples were incubated with rhodamine-phalloidin antibody (Invitrogen, USA) in 10% horse serum and stained with DAPI.

Scanning electron microscopic (SEM)

Cells were fixed with 3% glutaraldehyde and processed through ethanol dehydration steps. Subsequently, cells were critically point dried, sputter-coated with carbon, and examined by SEM (Quanta 250, FEI, Hillsboro, OR, USA). Cell spreading was categorized into 4 stages according to previous publication.
Osteogenic differentiation

Cells were seeded at the density of 50,000 cells/well in a 24-well plate. After confluency, the medium was changed to osteogenic induction medium supplemented with dexamethasone (100 nM), L-ascorbic acid (50 μg/mL), and β-glycerophosphate (10 mM). The cells were stained with alizarin red S to determine mineralization. Staining was solubilized in cetylpyridium chloride solution and the absorbance was measured at 570 nm.

Polymerase chain reaction

Total RNA was extracted by RiboEx solution (GeneAll, Korea). Reverse transcriptase kit (Promega, Madison, WI, USA) was employed to convert RNA (1 μg) to cDNA. Real-time polymerase chain reaction (RT-PCR) was performed to determine gene expression levels using FastStart® Essential DNA Green Master (Roche, USA) and MiniOpticon system (Bio-rad). Melting curve analysis was examined to ensure product specificity. Expression value was calculated using $2^{-ΔΔCt}$ method. Primer sequences are shown in Supplementary Table 1.

Statistical analyses

The statistical analysis was performed using Prism7 (GraphPad Software, CA, USA). The significant difference between two groups was determined using Mann Whitney U test. Kruskal Wallis test followed by pairwise comparison was utilized for three or more group comparison. The statistically significant difference was considered when p value less than 0.05.

Results

Clinical and molecular characterisation of a patient with ADHCAI and FAM83H mutation

The proband, a Thai female at 21 years of age, presented with generalized yellowish porous enamel. The radiopacity of her enamel was reduced (Fig. 1A–C). Whole exome sequencing identified the heterozygous nonsense mutation (c.1261G>T, p.E421*) in exon 5 of FAM83H (NM_198488.3) leading to ADHCAI in the proband. This mutation was located in the frequent mutation sites between amino acid 287-694 of FAM83H (Fig. 2A). The mutation was also detected in her father and brother having the same tooth condition but not in her unaffected mother by Sanger sequencing (Fig. 2B). Clinical and radiographic features of the proband were described in our previous study.11 Bone chips were obtained from the proband during osteoplasty according to orthodontic treatment plan.

Proliferation and spreading of FAM83H mutant cells

MTT assay showed that the cells obtained from the alveolar bone of the proband (called FAM83H mutant cells in this study) showed an increase in cell number at day 7 compared to day 1 (Fig. 1D). At day 7, their number was comparable to the controls (Fig. 1D). These show that FAM83H mutant cells had an ability to proliferate in vitro.

At early time points, SEM revealed that FAM83H mutant cells demonstrated a round shape with small filopodia and/or lamellodia which were less extended than those of the controls (Fig. 3A–F). Later, at 6 and 24-hour culture, both FAM83H mutant and control cells showed flatten shape, suggesting a complete cell spreading (Fig. 3C–H). Stages of cell spreading was determined according to previous publication.11 At 30 min, the majority of FAM83H mutant cells were categorized into stage 1 and 2 while the controls were in stages 2 and 3 (Fig. 3I). The FAM83H mutant cells progressed into stage 2, 3, and 4 at 2 h and their spreading was comparable to the controls at 6 and 24 h.

F-actin staining showed the unspecific organization of actin in the cytoplasm of both FAM83H mutant and control cells at 30 min (Fig. 4A–C). At 2 and 6 h, the orientation of F-actin was present around the edge of cell membrane.
Figure 2  Mutation analysis. The schematic diagram of *FAM83H* gene (NM_198488.3) showed the location of c.1261G>T, p.E421* mutation. The structural domains of FAM83H (NP_940890.3) illustrated the previously reported mutations spanning between amino acid 287 and 694 (A). Electropherograms identified the heterozygous missense mutation, c.1261G>T, p.E421*, in the proband and her affected father and brother, but not in her mother (B).

Figure 3  Cell spreading of *FAM83H* mutant cells. Morphology and cell spreading were evaluated by scanning electron microscope at 30 min (mins), 2 h (h), 6h and 24h after seeding. Representative images of *FAM83H* mutant cells (A–D) and control cells (E–H). The percentage of cell spreading stage (stage 1–4) measured at 30 mins and 2h (I).
The stress fibers were obviously detected in all groups at 24 h (Fig. 4J-L). There was no marked difference in F-actin organization between FAM83H mutant and control cells during this observation period.

Osteogenic differentiation of FAM83H mutant cells

The mRNA level of FAM83H was downregulated in FAM83H mutant cells (Supplementary Fig. 1). The expression of osteogenic markers was evaluated using RT-PCR after cells were maintained in osteogenic medium for 3 and 7 days. We observed that FAM83H mutant cells exhibited a significant decrease of RUNX2 and ALP expression at day 7 compared with day 3 while those markers were increased in the controls (Fig. 5A,B). While the expression levels of OPN in FAM83H mutant cells were not significantly different between day 7 and day 3, the significant upregulation was found in the controls (Fig. 5C). Both FAM83H mutant and control cells showed significant increase of DSPP and OCN levels at day 7 compared with day 3 (Fig. 5D,E). FAM83H mutant cells exhibited mineral deposition but slightly lower than the controls after culturing in osteogenic induction medium for 14 days (Fig. 5F and Supplementary Fig. 2).

Discussion

FAM83H has been shown to involve in mineralization process. This study investigated the characteristics of cells obtained from the alveolar bone of a patient affected with ADHCAI and the truncating mutation (p.E421*) in FAM83H. We observed that FAM83H mutant cells had comparable morphology, proliferation, spreading, and cytoskeletal arrangement to the controls. After osteogenic induction, FAM83H mutant cells exhibited a slight reduction in mineral deposition and a significant decrease in the expression of osteogenic marker genes compared to the controls.

The effects of FAM83H on cell proliferation have been reported in several cell types. Deletion of FAM83H resulted in reduced proliferation and induction of G0/G1 cell cycle arrest of hepatocellular carcinoma cells. Impaired cell proliferative ability was observed in human periodontal ligament cells with p.E421* mutation in FAM83H. This study detected a significant reduction in the number of FAM83H mutant cells compared to control2 at day 3, however, a significant difference was not observed at day 7. These suggest that FAM83H mutation do not alter the proliferation ability of human alveolar bone cells and the mutation might variably influence cell proliferation depending on cell types.
Previous report demonstrated that mouse ameloblast cells with Fam83h mutation (c.1186C>T, p.Q396*) exhibited lower expression of Runx2, Alp, and Ocn and ALP activity than the controls after osteogenic induction. Those expression levels were rescued by Wnt signaling inhibitor. In humans, the periodontal ligamental cells with FAM83H mutation exhibited a decrease in BSP, COL1, OCN mRNA expression. Consistently, this study detected that human alveolar bone cells with FAM83H mutation had a significant decrease in RUNX2 and ALP and unchanged OPN expression at day 7 compared with day 3 while the controls showed a significant upregulation of those markers. The expression of DSPP and OCN in FAM83H mutant and control cells was upregulated at day 7 comparing with day 3 in which FAM83H mutant cells showed lower fold changes. Taken together, these suggest that multiple types of cells with FAM83H mutation had compromised ability in osteogenic differentiation.

We found that the mineral deposition of FAM83H mutant cells was slightly lower than the controls. An in vitro mineralization has been shown to be associated with osteogenic differentiation potency but the regulatory mechanisms of each can be independent. For example, inorganic phosphate regulation has been shown to independently promote mineralization and differentiation. The control of local crystal nucleation and growth is crucial for mineralization process but may not directly associate with osteogenic differentiation. Hence, the roles of FAM83H in mineralization process across different types of cells should be further elucidated.

In conclusion, we show that FAM83H alveolar bone cells have reduced expression of osteogenic markers and slight decrease in mineral deposition, suggesting their compromised ability in osteogenic differentiation. The morphology, proliferation, spreading, and cytoskeleton of FAM83H bone cells are comparable to those of the controls. Consistent with previous studies in the ameloblasts and periodontal ligamental cells, our findings propose an influence of FAM83H on the osteogenic differentiation across cell types in oral cavity.

Conflict of interest
None.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2019.07.005.

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