Role of FGF23 c.35C>A in Bone Remodeling during Orthodontic Tooth Movement

Xiaoyun Ma, Mengjiao Zhu, Xiaohui Mi and Fengshan Chen

Department of Orthodontics, School of Dentistry, Tongji University, Shanghai Engineering Research Center of Tooth Restoration and Regeneration, Shanghai, P.R. China.

(Accepted for publication, February 14, 2020)

Abstract: Fibroblast growth factor 23 (FGF23), which belongs to FGF family, regulates the serum phosphate concentration and plays an essential role in bone development. Our previous study has reported a mutation of FGF23 c.35C>A in the mandibular prognathism (MP) pedigree. The aim of this article was to determine the effect of FGF23 c.35C>A mutation in alveolar bone remodeling during orthodontic tooth movement (OTM). An orthodontic spring was performed in an OTM mouse model for 7 days. Micro-computed tomography (micro-CT) was used to measure the amount of OTM and observe the periodontal ligament (PDL) after mechanical loading, hematoyxlin-eosin (HE) staining, tartrate-resistant acid phos-}

phatase (TRAP) staining, Runx2 immunostaining. The reverse transcription polymerase chain reaction (RT-qPCR) was also performed to determine mRNA levels. The mutation of FGF23 c.35C>A decreased the OTM, the mRNA expression levels of RANKL, RANK and the count of osteoclasts in compression side (CS). In contrast, the count of osteoblasts in tension side (TS), the mRNA expression levels of COL1A1, OCN were increased when compared to FGF23-WT group after OTM. In summary, we ascertained the obstruction of FGF23 c.35C>A in orthodontic tooth movement, which might due to the stimulation of osteogenesis and the inhibition of bone resorption.

Key words: FGF23 c.35C>A, Bone remodeling, Mechanical loading, Orthodontic tooth movement, Mouse model

Introduction

Mandibular prognathism (MP) is a category of maxillofacial developmental malformation distinguished by the overdevelopment of lower jaw, involving bone, muscle, tooth structure, and dysfunction\(^{\text{1}}\). It may also affect patients’ mental health and confidence. Epidemiology studies suggested that the prevalence of mandibular prognathism is approximately up to 15% in Asian populations and about 1% in Caucasian populations\(^{\text{2,3}}\). Although environment components are linked with this occlusion disorder closely\(^{\text{4}}\), numerous human and animal studies lend support to genetic components in the pathogenesis of MP\(^{\text{5}}\).

Fibroblast growth factor (FGF23), which is mainly synthesized by osteoblasts and osteocytes, is one of the FGF family, and plays a significant role in the systemic circulation\(^{\text{6}}\). FGF23 is reported as an essential moderator of phosphate homeostasis, vitamin D metabolism and highly expressed in bone, which is important for normal skeletal development\(^{\text{7,8}}\). Our previous study obtained a new MP-susceptibility locus through genome-wide linkage analysis and identified the mutation of FGF23 c.35C>A through whole-exome sequencing analysis on an MP pedigree\(^{\text{9}}\). The therapeutic options of MP include early modification of bone growth, orthodontic therapy and combined orthodontic and surgical (or thognathic) therapy\(^{\text{9}}\), and all of them involve bone remodeling. That need us to discover what discrepancy FGF23 c.35C>A would produce in the orthodontic loading process.

In this study, we applied tooth movement mouse model to explore the effect of FGF23 c.35C>A in orthodontic therapy of mandibular prognathism. We have examined that the mutation of FGF23 c.35C>A reduced tooth movement distance and appeared local structure discrepancy compared to control group.

Materials and Methods

Animals

Eight weeks old male FGF23-WT (n=10,22 g per animal) and FGF-23\(^{\text{C35A}}\) (C57BL/6J, n=10,18 g per animal) were employed in this study. The mice were fed soft biscuits and water every day. The room, where the mice lived, was kept constant temperature and humidity, and with a 12-24 h light/dark cycle. The weight and health of each animal were checked every day. The humane endpoint used in the present study was moribund condition including lack of appetite, lethargy, and body weight loss no more than 20%\(^{\text{10}}\). Mice were euthanized by overdose anesthesia by intraperitoneal injection of sodium pentobarbital. All experiments procedures and the care of animals were given ethical approval by Medical Ethics Committee of Tongji University with the following reference number No.2016(002) and followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize animal suffering.

Experiment of orthodontic tooth movement

Animals were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection during the application of orthodontic appliance each time\(^{\text{11}}\). The orthodontic appliance was composed of a coiled...
spring, which consisted of a nickel-titanium wire of diameter 0.2 mm and a coil diameter of 0.75 mm\textsuperscript{11}. The orthodontic spring, which exerted a continued force of 0.35N, was fixed between the maxillary first molar and the incisor by light-cured resin\textsuperscript{11}. The appliance was exclusively fixed on the upper left side.

**Micro-CT Scanning**

Seven days after the mechanical loading, images of the maxillary left molars and the surrounding alveolar bones were acquired by micro-CT scanning (SCANCO Medical AG, Bruttisellen, Switzerland). Images were used to observe that the narrowest interval between the first and second molars. A sagittal view was used to observe the remodeling of tooth attachment tissue of the first molar distobuccal root.

**Measurement of OTM**

To quantify the distance of tooth movement, Adobe Photoshop CS3
Figure 2. Histological changes of the first molar distobuccal root after orthodontic loading. (A) FGF23-WT group, (B) FGF23 c.35C>A group. (bar=100μm)

Figure 3. TRAP-staining images of the first molar distobuccal root. (A) FGF23-WT group, (B) FGF23 c.35C>A group. (bar=100μm). (C) Magnification of the identified area of (A). (D) Magnification of the identified area of (B). (bar=50μm). (E) Quantification of the number of osteoclasts among the groups after orthodontic force loading. Arrows indicate the osteoclasts. Data are presented as the mean ± standard deviation. **P<0.01, OTM, orthodontic tooth movement
(Adobe Systems Incorporated, USA) was used to analyze the images which obtained from Micro-CT scanning. Tooth movement was quantified by the distance between the maxillary first molar and the second molar. Three measurements were conducted for each assessment.

Pathohistological observation

Maxillary bones were collected and fixed in 10% EDTA (pH 7.2) at 4°C, embedded in paraffin and cut into 5-μm sagittal sections. Next, these sections were used for histological examination by hematoxylin-eosin (HE) staining and identifying osteoclasts by tartrate-resistant acid phosphatase (TRAP). The mesial surface of the first molar distobuccal root was used to count osteoclasts.

Immunohistochemistry

For identifying osteoblasts, Runt-related transcription factor 2 (Runx2) immunostaining was performed for the IHC analysis. These sections were incubated with primary antibodies (Runx2, Abcam Inc, Cambridge, MA, USA, 1:500, ab192256) at 4°C overnight, using an immunohistochemistry staining kit (Maixin Bio, Fuzhou, China). Osteoblasts count was measured as a percentage of the alveolar bone surface of the first molar distobuccal root.

RNA extraction and reverse transcription polymerase chain reaction (RT-qPCR)

The periodontal tissues of the maxillary first molar distobuccal root were extracted for isolating total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from RNA using Fast Quant RT kit (KR-106, TIANGEN Biotech, Beijing) at 42°C for 15 min, 95°C for 3 min and 4°C for 5 min, according to the manufacturer’s instructions. Then, relative mRNA analysis was performed by ABI 7500 Real-Time PCR Detection System (Roche Diagnostics, Basel, Switzerland), using SYBR green (SuperReal PreMix Plus, SYBR Green; TIANGEN, Beijing, China). The conditions of reactions were 95°C for 15 min followed 40 cycles of 95°C for 30 sec, 58°C for 20 sec and 72°C for 30 sec, followed by the standard denaturation curve. Primers are listed in Table 1. The 2^−ΔΔCq method was applied to count the mRNA expression levels, the values were run in triplicate.

Statistical analyses

All results were analyzed as the mean ± standard deviation. The difference between the two groups was performed unpaired t test by SPSS software (version 22.0, IBM Corp., Armonk, NY, USA), and P<0.05 was set as statistically significant difference.

Results

The mutation of FGF23 c.35C>A inhibits orthodontic tooth movement.

The Micro-CT and measurement results demonstrated the amount of orthodontic tooth movement in mutant group at 7 d of mechanical loading compared with control group at the same time point (Fig 1). The distance of OTM in control group was significantly increased after 7 days of mechanical loading, about 158.1 ± 17.47 μm. However, the
amount of OTM was significantly less in the FGF23 c.35C>A mutant mice at the same time point, about 66.14 ± 10.32 μm (P < 0.01).

**Histological changes in the periodontium during mechanical loading.**

HE staining revealed the histological variations in the periodontal tissue of the first molar distobuccal root before and after OTM. The mesial sides of the roots experienced compressive stress, while the distal sides of the roots experienced tensile stress during the mechanical loading. Therefore, the periodontal space became narrower in the mesial side and wider on the distal side after mechanical loading. The periodontal space of the maxillary first molar distobuccal root showed relatively few interspaces in mutant mice, when compared to control group at the same time (Fig. 2).

**Mutant FGF23 (c.35C>A) decreased osteoclasts formation during orthodontic force loading.**

TRAP staining was carried out to recognize the histology of osteoclasts before and after orthodontic tooth movement. After mechanical loading, TRAP-positive osteoclasts were mainly observed on the distal area of periodontium and the alveolar bone surface. Therefore, the count of osteoclasts was increased on the mesial area of the first molar distobuccal root at 7 d in all groups after orthodontic force loading (Fig. 3). However, a larger number of osteoclasts were observed in wild type group at the same point (12±1), when compared to the mutant group (7±1, P < 0.01). These results demonstrated that the mutant of FGF23 c.35C>A inhibit bone resorption in the bone remodeling.

**Mutant FGF23 (c.35C>A) increased osteoblasts during orthodontic tooth movement**

Because the Runx2 is one of the important genes in response to osteoblasts in bone modeling and bone homeostasis14), we counted the count of Runx2-positive cells to presume osteoblast number after orthodontic tooth movement. Runx2-positive cells were observed on the tension area of the first molar distobuccal root in the WT group (400 ± 14), but it is less than that in the mutant group (578 ± 41, P< 0.01) at the same time point (Fig. 4). It revealed that the mutant of FGF23 c.35C>A promote bone formation in orthodontic tooth movement.

**Mutant FGF23 (c.35C>A) affects osteoblast and osteoclast markers during orthodontic force loading.**

To investigate the role of FGF23 c.35C>A mutation in the bone remodeling, we characterized the relative gene expression involved in bone formation and resorption. Our date showed that the expression of osteoblast markers (COL1A1, OCN) was significantly up-regulated after mechanical loading, but were higher in mutant group when compared to the FGF23-WT group. However, the expression of osteoblast regulators (RANKL, RANK) were down-regulated in WT group when compared to the mutant group after mechanical loading (Fig. 5).

**Discussion**

It is well known that FGF23, together with FGF19 and FGF21, is a member of mammalian endocrine FGFs15), which can suppress phosphate and 1,25(OH)2D metabolism by reduction of 1α-hydroxylase16). Our previous study has identified FGF23 as one of the causal genes for the observed maxillofacial malformations in the MP pedi-
demonstrated that the mutant of FGF23 c.35C>A might inhibit the OTM which are momentous for bone morphogenesis. FGF23 might be mainly synthesized by osteocytes in the regularly distributed osteocyte lacunar canalicular system(s) of secondary trabeculae establishing after bone remodeling. Described above, FGF23 is closely related to bone remodeling. Therefore, to assess the effect of FGF23 c.35C>A mutation in alveolar bone remodeling after mechanical loading, we used an orthodontic spring to exert a successive force for tooth movement between the maxillary first molar and the incisor, giving an optimal force of 0.35N. In our results, the mutation of FGF23 c.35C>A decreased the amount of orthodontic tooth movement when compared to the WT group, indicating that FGF23 involved in alveolar bone reconstitution.

Tooth movement is a process which involves bone formation and resorption. The reconstitution of tooth attachment tissue responds to orthodontic force loading, which lead to two different opposing sections that were named tension side (TS) and compression (CS). The formation and differentiation of osteoclasts are closely related to the occurrence of bone resorption during bone remodeling, which are the main cells that trigger bone resorption. Previous study reported that after 6d and 10d of OTM, the number of osteoclasts was significantly increased. Our results revealed the decrease of osteoclasts on the pressure area of the first molar root in mutant group during mechanical loading. Therefore, the alveolar bone resorption and the orthodontic tooth movement were inhibited because the mutation of FGF23 c.35C>A.

However, osteoblasts and osteoclasts are required appropriate coupling of deposition and resorption in tension and compression areas of PDL. Previous studies demonstrated that the endosteal region is solely subjected to bone deposition, FGF23-reactive osteocytes might predominate in the endosteal area. In addition, Runx2 is necessary for the differentiation of osteoblasts as well as involved in bone matrix deposition. Previous study also determined osteoblast number through counting Runx2-positive cells. Accordingly, number of Runx2-positive cells was used to determine osteoblasts number. Although Runx2-positive cells were significantly increased after orthodontic force loading in each group, our data shown that the count of Runx2-positive cells was higher in tension regions in the PDL of the mutant group. In agreement, a previous study demonstrated that mutant hFGF23(A12D) stimulated the differentiation of RC cells to osteoblasts. Taken together, the results suggest that the mutation of FG23 c.35C>A inhibited bone remodeling during OTM through the differential responses of osteoblasts and osteoclasts.

Subsequently, we detected related markers of osteoblasts and osteoclasts. Similarly, Higher expression levels of the osteoblast markers (COL1A1, OCN) were observed in TS and CS relative to in mutant mice at the same examined time-point. In contrast, we observed a down-regulation of the expression levels of the osteoclast markers (RANKL, RANK) in the mutant group after mechanical loading. In agreement, down-regulation of the mRNA expression levels of RANKL/RANK might be related to the inhibition of orthodontic tooth movement. These results support a possible explanation of the increased promotion of bone formation in TS and reduced bone resorption in CS in mutant mice.

In conclusion, we demonstrated that the mutation of FGF23 c.35C>A can reduce orthodontic tooth movement due to the increased bone deposition and the reduction of bone resorption under the same force and examined time during mechanical force loading. Further investigation of the mutation of FGF23 c.35C>A may provide better clinical advises and therapeutic developments in the treatment of mandibular prognathism.

Acknowledgments

We are grateful to Mr. Shiqiang Wen for the construction of experimental models and Ms. Wuyi Gong for sample management. Ms. Rongrong Sun involved in the modification of manuscript. This study is supported by the National Natural Science Foundation of China (81371129, 81670973 and 11402175) and the Science and Technology Commission of Shanghai (No.124119a9200).

Conflict of Interest

The authors have declared that no COI exists.

References

1. Van C VA review of the literature on the prevalence of Class III malocclusion and the mandibular prognathic growth hypotheses. Austral Orthod J 12: 23-28, 1991
2. Cua-Benward G B, Dibaj S and Ghassemi B. The prevalence of congenitally missing teeth in class I, II, III malocclusions. J Clin Pediatr Dent 17: 15–17, 1992
3. Litton SF, Ackerman LN, Isaacsom RJ and Shapiro BL. A genetic study of class 3 malocclusion. Am J Orthod 58: 565-577, 1970
4. Yamaguchi T, Park, S B, Narita A, Maki K and Inoue I. Genome-wide linkage analysis of mandibular prognathism in Korean and Japanese patients. J Dent Res 84(3): 255-259, 2005
5. Beenken A and Mohammadi M. The FGF family, biology, pathophysiology and therapy. Nat Rev Drug Discov 8(3): 235-53, 2009
6. Fukumoto S and Yamashita T. FGF23 is a hormone-regulating phosphate metabolism—Unique biological characteristics of FGF23. Bone 40(5): 1190-1195, 2007
7. Sitara D, Razzaque MS, Hesse M, Yoganathan S, Taguchi T, Erben RG, Jäppner H and Lanks B. Homozygous ablation of fibroblast growth factor-23 results in hyperphosphatemia and impaired skeletogenesis, and reverses hypophosphatemia in phex-deficient mice. Matrix Biol 23(7): 421-32, 2004
8. Chen F, Li Q, Gu M, Li X, Yu J and Zhang YB. Identification of a mutation in fgf23 involved in mandibular prognathism. Sci Rep 5: 11250, 2015
9. Ngan P and Moon W. Evolution of Class III treatment in orthodontics. Am J Orthod Dentofacial Orthop 148 (1): 22-36, 2015
10. Stokes W S. Humane endpoints for laboratory animals used in regulatory testing. ILAR J 43 Suppl: S31-38, 2002
11. Taddei SRDA, Mora AP, Andrade J Jr, Garlet GP, Garlet TP, Teixeira M and Silva TAD. Experimental model of tooth movement in mice: A standardized protocol for studying bone remodeling under compression and tensile strains. J Biomech 45(16): 2729-2735, 2012
12. Tanaka M, Miyazawa K, Tabuchi M, T. Yabumoto M, Kadota M. Yoshizako C, Yamane M, Kawatani H, Osada H, Maeda H and Goto S. Effect of Reveromycin A on experimental tooth movement in OPG-/- mice. J Dent Res 91(8): 771-776, 2012
13. Schmittgen TD and Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108, 2008
14. Bronckers AL, Sasaguri K and Engelse MA. Transcription and im-
munolocalization of RUNX2/Cbfa1/Pebp2a A in developing rodent and human craniofacial tissues: further evidence suggesting osteoclasts phagocytose osteocytes. Microsc Res Tech 61: 540–548, 2003

15. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR and Hogenessch JB. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci USA 101(16): 6062-6067, 2004

16. Perwad F, Zhang MY, Tenenhouse HS and Portale AA. Fibroblast growth factor 23 impairs phosphorus and vitamin d metabolism in vivo and suppresses 25-hydroxyvitamin d-1a-hydroxylase expression in vitro. Am J Physiol Renal Physiol 293(5): F1577-1583, 2007

17. Liu S, Gupta A and Quarles LD. Emerging role of fibroblast growth factor 23 in a bone-kidney axis regulating systemic phosphate homeostasis and extracellular matrix mineralization. Curr Opin Nephrol Hypertens 16(4): 329-335, 2007

18. Ubaidus S, Li M, Sultana S, de Freitas PH, Oda K, Maeda T, Takagi R and Amizuka N. FGF23 is mainly synthesized by osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling. J Electron Microsc (Tokyo) 58(6): 381-392, 2009

19. Melsen B. Biological reaction of alveolar bone to orthodontic tooth movement. Angle Orthod 69: 151-158, 1999

20. Garlet TP, Coelho U, Repke CE, Silva J S, Cunha F and Garlet GP. Differential expression of osteoblast and osteoclast chemoattractants in compression and tension sides during orthodontic movement. Cytokine 42(3): 0-335, 2008

21. Yoshimatsu M, Shibata Y, Kitaura H, Chang X, Moriiishi T, Hashimoto F, Yoshida N and Yamaguchi A. Experimental model of tooth movement by orthodontic force in mice and its application to tumor necrosis factor receptor-deficient mice. J Bone Miner Metab 24(1): 20-27, 2006

22. Meikle MC. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. Eur J Orthod 28(3): 221-240 2006

23. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M and Karsenty GA. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes 13: 1025–1036, 1999

24. Niedermair T, Schirner S, Seebröker R, Straub RH and Grässel S. Substance p modulates bone remodeling properties of murine osteoblasts and osteoclasts. Sci Rep 8(1): 9199, 2018

25. Tu Y, Qu T and Chen F. Mutant hFGF23(A12D) stimulates osteoblast differentiation through FGFR3. J Cell Mol Med 23(4): 2933-2942, 2019

26. Taddei SR, Andrade I Jr, Queiroz-Junior C M, Garlet TP, Garlet GP, Garlet G, Cunha F, Teixeira M and Silva TAD. Role of ccr2 in orthodontic tooth movement. Am J Orthod Dentofacial Orthop 141(2): 153-160, 2012
