Chinese Medical Journal®

Promotion effect of FGF23 on osteopenia in congenital scoliosis through FGFr3/TNAP/OPN pathway

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Abstract

Background: Congenital scoliosis (CS) is a complex spinal malformation of unknown etiology with abnormal bone metabolism. Fibroblast growth factor 23 (FGF23), secreted by osteoblasts and osteocytes, can inhibit bone formation and mineralization. This research aims to investigate the relationship between CS and FGF23.

Methods: We collected peripheral blood from two pairs of identical twins for methylation sequencing of the target region. FGF23 mRNA levels in the peripheral blood of CS patients and age-matched controls were measured. Receiver operator characteristic (ROC) curve analyses were conducted to evaluate the specificity and sensitivity of FGF23. The expression levels of FGF23 and its downstream factors fibroblast growth factor receptor 3 (FGFr3)/tissue non-specific alkaline phosphatase (TNAP)/osteopontin (OPN) in primary osteoblasts from CS patients (CS-Ob) and controls (CT-Ob) were detected. In addition, the osteogenic abilities of FGF23-knockdown or FGF23-overexpressing Ob were examined.

Results: DNA methylation of the *FGF23* gene in CS patients was decreased compared to that of their identical twins, accompanied by increased mRNA levels. CS patients had increased peripheral blood FGF23 mRNA levels and decreased computed tomography (CT) values compared with controls. The FGF23 mRNA levels were negatively correlated with the CT value of the spine, and ROCs of FGF23 mRNA levels showed high sensitivity and specificity for CS. Additionally, significantly increased levels of FGF23, FGFr3, OPN, impaired osteogenic mineralization and lower TNAP levels were observed in CS-Ob. Moreover, FGF23 overexpression in CT-Ob increased FGFr3 and OPN levels and decreased TNAP levels, while FGF23 knockdown induced downregulation of FGFr3 and OPN but upregulation of TNAP in CS-Ob. Mineralization of CS-Ob was rescued after FGF23 knockdown.

Conclusions: Our results suggested increased peripheral blood FGF23 levels, decreased bone mineral density in CS patients, and a good predictive ability of CS by peripheral blood FGF23 levels. FGF23 may contribute to osteopenia in CS patients through FGFr3/TNAP/OPN pathway.

Keywords: Scoliosis; Fibroblast growth factor 23; Fibroblast growth factor receptor 3; Osteopenia

Introduction

Congenital scoliosis (CS) is a spinal deformity caused by abnormal vertebral development and has a worldwide incidence of 0.5‰–1‰.[1,2] Hemivertebra, or segmentation failure, is the most common manifestation of CS.[3] In addition, smaller vertebrae and low bone mineral density (BMD) have been reported.[4] The etiology of CS is still unclear, but it is widely believed that some genetic defects, such as the Notch pathway and *TBX6* (T-box transcription factor 6) mutations, and environmental factors, such as gestational diabetes and anoxia, can lead to CS.[5] Specifically, the influence of environmental factors can contribute to phenotypic differences through epigenetic changes, the mechanisms of which mainly include DNA methylation, histone modification, and non-coding RNAs.[6]

DNA methylation, one of the most important epigenetic mechanisms, has recently been shown to play a role in the development of CS. By examining genome-wide DNA methylation of the hemivertebra and normal spinal process from patients with CS with whole-genome bisulfite sequencing (WGBS), Liu *et al*[7] identified 343 genes with hyper-differentially methylated regions (hyper-DMRs) and 222 genes with hypo-DMRs, which might be associated with the formation of hemivertebrae and CS.

In 1967, Peterson *et al*[8] at the Mayo Clinic in the United States reported a set of identical twins, one with congenital spinal deformity and the other with normal spinal morphology. Similar cases have subsequently been

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000002690

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Chinese Medical Journal 2023;136(12)
Received: 09-11-2022; **Online:** 16-05-2023 **Edited by:** Xiuyuan Hao

reported many times.[9] In identical twins, because both individuals arise from the same oosperm, their appearance, blood type, physical characteristics, and disease susceptibility are similar.[10] Although recent studies have revealed that identical twins do not share a completely identical set of genes, it is generally assumed that identical twins have identical genetic DNA sequences, and their physical differences are attributed mainly to environmental factors.[11]

Bone metabolism is an important factor in bone development and plays a significant role in scoliosis. In a study involving 66 people, Zhu *et al*[4] found that there was a reduction in bone mass in patients with CS. The low bone mass in patients with CS was not limited to the spine but was also observed in the bones of the limbs, including the femur. In mouse models of congenital vertebral abnormalities induced by anoxia during pregnancy, the hemivertebrae of CS mice grew more slowly than those of normal vertebrae.[12] Murakami *et al*[13] identified *OLFML1* (olfactomedin-like 1) gene variation in CS patients and verified it in a mouse model, indicating that this mutation could lead to a differentiation disorder in mouse osteoblasts and abnormal bone tissue development. These studies suggest that CS patients have a bone metabolism disorder; however, the specific mechanism of bone mass reduction caused by the bone metabolism disorder in CS remains unclear.

Fibroblast growth factor 23 (FGF23) was a bone-derived phosphaturic hormone secreted by osteoblasts and osteocytes, which was first reported by Yamashita *et al*[14] in 2000. Rafaelsen *et al*[15] found that FGF23 played an important role in serum phosphate balance and excessive FGF23 could lead to impaired bone matrix mineralization, rickets, and osteomalacia.[16,17] In addition, Rupp *et al*[18] also reported that FGF23 levels had a significant negative logarithmic correlation with trabeculae, and high FGF23 levels were associated with impaired trabecular bone microarchitecture in osteoporosis patients.

In this study, we first analyzed the differences in DNA methylation between two CS patients and their identical twins and subsequently explore the relationship between the changes in the FGF23 gene expression levels and the decreases in BMD in CS patients.

Methods

Ethical approval

This study adhered to the principles of the *Declaration of Helsinki* and was approved by the medical ethics committee of Xiangya Hospital, Central South University (No. 201703359). Written informed consent was acquired from each of the patients (or their parents and legal guardians) to authorize treatment, imageology findings, and photographic documentation.

DNA methylation sequencing

Two pairs of identical twins were included in the methylation sequencing phase of the experiment. The first pair was male, aged 15 years. The phenotype of the CS patient was T10/T11 butterfly vertebra, T12/L1/L2 fusion, classified by International Consortium for Vertebral Anomalies and Scoliosis (ICVAS) as multiple defects of the vertebrae (M-SDV) type, labeled as CS1, and the twin with a normal spine was labeled as N1 [Figure 1A, B]. The second pair was female, aged 4 years. The phenotype of the CS patient was T11/T12 fusion, classified as M-SDV and labeled as CS2, and the twin with a normal spine was labeled as N2 [Figure 1C, D]. We collected peripheral blood from the two pairs of identical twins and extracted DNA. After quality testing, targeted bisulfite sequencing was performed to detect the DNA methylation levels of CpG islands, gene promoters, and other significant methylated regions. The sequencing work was carried out by Wuhan BGI Company (China), and the specific steps are available on the company's official website (http://www.genomics.cn).

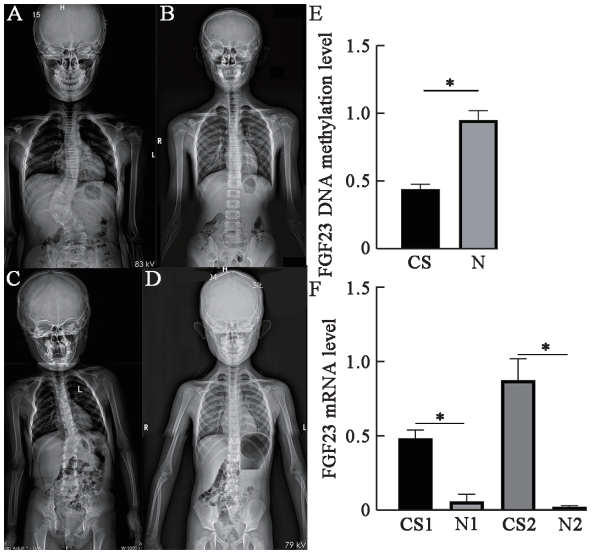

Figure 1: DNA methylation of the *FGF23* gene was decreased in the identical twins with CS, and its mRNA levels were increased. (A–D) X-rays of two sets of identical twins. (A) CS1. (B) N1. (C) CS2. (D) N2. (E) *FGF23* DNA methylation levels of two sets of identical twins. (F) FGF23 mRNA levels of two sets of identical twins (each sample was repeated three times). $^{*}P$ <0.001. CS: Congenital scoliosis; N: Control; FGF23: Fibroblast growth factor 23.

Study design and population

Blood was collected from 20 patients with CS and 23 controls aged 3–37 years at Xiangya Hospital between 2018 and 2020. Moreover, the facet joints of eight CS patients and four controls scheduled to be removed during a posterior approach surgery were carefully and harmlessly collected. All patients and controls were fully characterized based on their clinical manifestations, X-ray, computed tomography (CT), magnetic resonance imaging (MRI) results, etc. CT values of the patients' spine were used to assess BMD, as described in the references.[19] The controls included patients with herniated discs and spinal injuries, and individuals with neuromuscular or genetic diseases and those who used hormones or immune inhibitors were excluded from this study.

Blood collection and RNA extraction

After the enrollment of the patients was confirmed, 3 mL of venous blood was drawn by nurses. Then, 400 μL of fresh blood was mixed with 1200 μL of TRIzol reagent (CWBio, Beijing, China). A total of 320 μL of chloroform was then added and vortexed thoroughly. Centrifugation was performed at 12,000 × *g* at 4°C for 15 min. Then, 400 μL of the supernatant was taken, and 400 μL of isopropyl alcohol was added to the mixture and centrifuged, after which the precipitated RNA was washed with 75% ethanol. After being dried thoroughly, the RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water. RNA quality was tested by electrophoresis in 1% agarose gel, and RNA was stored at –80°C for later use.

Real-time polymerase chain reaction (RT-PCR)

RNA from each sample was reversely transcribed into cDNA using a HiFiScript cDNA Synthesis Kit (CWBio, Beijing, China), and RT-PCR was performed with specific primers to the targeted mRNAs using NovoStart® Probe qPCR Super Mix (Novoprotein, Shanghai, China). The expression levels in the samples were quantified using GAPDH as the internal control. The relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used are described in Supplementary Table 1, http://links.lww.com/CM9/B552.

Extraction of primary osteoblasts and cell culture

The facet joint tissues of CS patients and the control were washed with a phosphate buffer solution (PBS) containing 1% penicillin–streptomycin, and the cancellous bone was then carefully dissected and chopped. The chopped bone was successively digested at 37°C with 0.25% trypsin for 30 min and with 0.1% collagenase I for 8 h. The cell pellets obtained after screening and centrifugation were resuspended in a complete culture medium and cultured, and the expanded cultured P3 cells were used for subsequent experiments. Primary osteoblasts were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (vivacell, Shanghai, China) and 1% penicillin–streptomycin at 37°C/5% CO_2.

Western blotting

Primary osteoblasts were ultrasonically lysed in radioimmunoprecipitation assay lysis buffer (CWBio) to obtain whole-cell lysates. The obtained proteins were normalized and fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels (20 mg/well) and transferred to polyvinylidene difluoride membranes (Immobilon, Darmstadt, Germany). Immunoblots were incubated overnight at 4°C with polyclonal rabbit anti-FGF23 (1: 1000, Bioss, Beijing, China) and monoclonal mouse anti-β actin (1: 5000, Abcam, Cambridge, UK) in primer antibody diluent (Beyotime, Shanghai, China). After being washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK). The protein bands were visualized with an enhanced chemiluminescence kit (Beyotime) and were quantified with Quantity One 4.6 software (Bio-Rad, California, USA).

Transfection of FGF23 with siRNA and plasmids

Human FGF23 siRNA and normal control (NC) siRNA were purchased from RiboBio Company (Guangzhou, China) and transfected into primary osteoblasts extracted from CS patients by a riboFECT CP Transfection Kit (RiboBio) following the manufacturer's instructions. The human FGF23 plasmid was produced by Hanbio Company (Shanghai, China) and transfected into primary osteoblasts of control by Lipo8000™ Transfection Reagent (Beyotime). RT-PCR and Western blotting were used to measure the transfection efficiency.

Osteogenic differentiation and alizarin red staining

Osteoblasts were seeded in 6-well plates and incubated to 75% confluence. After 24 h following passaging, the maintenance medium was replaced with osteogenic induction differentiation medium (Cyagen, California, USA). The medium was replaced every 3 days. The cells were fixed with 10% formaldehyde for 30 min and stained with 0.1% alizarin red for 1 h at 37°C to stain calcified nodules after differentiation.

Statistical analysis

All results were shown as the mean ± standard error of the mean (SEM). Student's *t*-test was used to compare two independent sample groups; analysis of variance (ANOVA) was used for more than two groups. Pearson's correlation analysis was used to assess the correlation between the two groups. Receiver operating characteristic (ROC) curve analysis was used for CS prediction and typing with related gene expression levels. Differences were considered statistically significant when $P < 0.05$. All data analyses were performed using IBM SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL, USA).

Results

DNA methylation of the FGF23 gene was decreased in identical twins with CS, and its expression levels were increased

The CS patients CS1 and CS2 were considered the patient (CS) group, and N1 and N2 were used as the control group. Regional methylation differences were compared between the two groups. In this sequencing study, a total of 75 genes with different methylation levels were screened out, including 24 regions with increased methylation levels in the CS group and 51 regions with decreased methylation levels in the CS group. Considering that both CS patients had low BMD while their identical twins had normal bone mass, we limited the gene function to osteogenesis and screened out six genes with different methylation levels [Supplementary Table 2, 3, http://links.lww.com/CM9/B552]. A negative regu-

lator of osteogenesis, FGF23, with the largest difference in methylation, was selected for the subsequent experiments. RT-PCR revealed that FGF23 mRNA levels were increased in the twins with CS, consistent with their low bone mass [Figure 1E, F].

General physiological features of patients with CS and the control group

Blood was collected from 43 patients at an outpatient clinic for mRNA expression level measurement. As shown in Table 1, the CS group contained 8 males and 12 females with an average age of 11.6 ± 3.1 years and 15.2 ± 9.4 years, respectively, while the control group contained 10 males and 13 females with an average age of 15.1 ± 4.5 years and 16.5 ± 5.1 year, respectively. There was no significant difference in age or sex between the CS group and the control group (P >0.05). The major curvature of the spine in the CS males (CS-M) and CS females (CS-F) was 48.6 ± 23.6° and 50.2 ± 12.3°, respectively. In addition, according to the ICVAS typing, 9 patients had single defect of the vertebrae (S-SDV) (3 males, 6 females), and 11 patients had M-SDV (5 males, 6 females).

Alteration of FGF23 mRNA levels in patients with CS

To explore the relationship between FGF23 mRNA levels in the blood and CS incidence or phenotype, total RNA was extracted from the collected blood and quantified by RT-PCR using the housekeeping gene GAPDH for analysis. RT-PCR showed that the blood mRNA levels of FGF23 were significantly increased in the CS patient group (0.573 ± 0.694) compared with the control group (0.061 ± 0.093) [Figure 2A]. The values of spinal CT in the CS patients were significantly lower than those in the control group (167.3 ± 47.2 HU *vs.* 267.2 ± 74.3 HU, P <0.001) [Figure 2B]. Further, Spearman analyses showed a negative correlation between the mRNA levels of FGF23 in peripheral blood and the CT value of the patients [Figure 2C]. Unfortunately, Spearman analyses suggested no significant correlation between FGF23 mRNA levels and other clinical symptoms, such as the number of malformed vertebrae and local Cobb angle, in patients with CS [Figure 2D, E].

To evaluate the predictive capability of FGF23 mRNA levels for CS, ROC curve analysis was carried out. The best cutoff was 0.929 based on an area under the curve (AUC) of 0.902 (95% confidence interval [CI], 0.810–0.995) [Figure 2F], and the blood FGF23 concentration exhibited a satisfactory predictive capability (P <0.001).

Briefly, these data suggested that compared to controls, patients with CS had much higher blood FGF23 levels. There was a negative correlation between FGF23 mRNA levels and CT values. Moreover, the FGF23 levels in blood showed outstanding predictive accuracy for CS.

Association of sex with FGF23 mRNA levels in CS patients and controls

Although the high prevalence of idiopathic scoliosis in females has been confirmed, the difference in the incidence of CS between males and females is not clear, and the relationship between FGF23 and sex remains unclear. Therefore, we analyzed blood FGF23 levels by sex. First, we compared blood FGF23 levels between males (CT-M) and females (CT-F) in the control group. As shown in Figure 3A, there was no significant difference in blood FGF23 levels between CT-M and CT-F. However, among patients with CS, blood FGF23 levels in males (CS-M) were higher than those in females (CS-F) [Figure 3B].

ROC analyses were conducted by sex. The best cutoff calculated by the ROC curves for CS-M and CT-M was

Table 1: Baseline characteristics of study populations of CS and CT.

Characteristics	CS (N = 20)		CT (N = 23)		t/χ^2	P-value
	Male	Female	Male	Female		
Case (n)	8	12	10	13	0.053*	0.911
Age (years)	11.6 ± 3.1	15.2 ± 9.4	15.1 ± 4.5	16.5 ± 5.1	–0.819†	0.421P1
					0.674†	0.573P2
					–1.409†	0.166P3
Curve angle (°)	48.6 ± 23.6	50.2 ± 12.3	–	–	0.329†	0.747
CT value (HU)	155.6 ± 30.3	179.1 ± 57.1	255.7 ± 81.2	276.4 ± 66.9	0.963†	0.352P1
					–0.558†	0.584P2
					–4.473†	<0.001P3
Type						
S-SDV	3	6	–	–	–	–
M-SDV	5	6	–	–	–	–

Data were shown as mean ± standard deviation. CS: Congenital scoliosis (CS-F female CS patients, CS-M male CS patients); CT: Control (CT-F female CT patients, CT-M male CT patients); CT value: Computed tomography value; HU: Hounsfield unit; M-SDV: Multiple defects of the vertebrae; P1: Comparison of CS-M and CS-F; P2: Comparison of CT-M and CT-F; P3: Comparison of CS and CT; S-SDV: Single defect of the vertebrae; –: Not applicable. *χ^2 value. †t value.

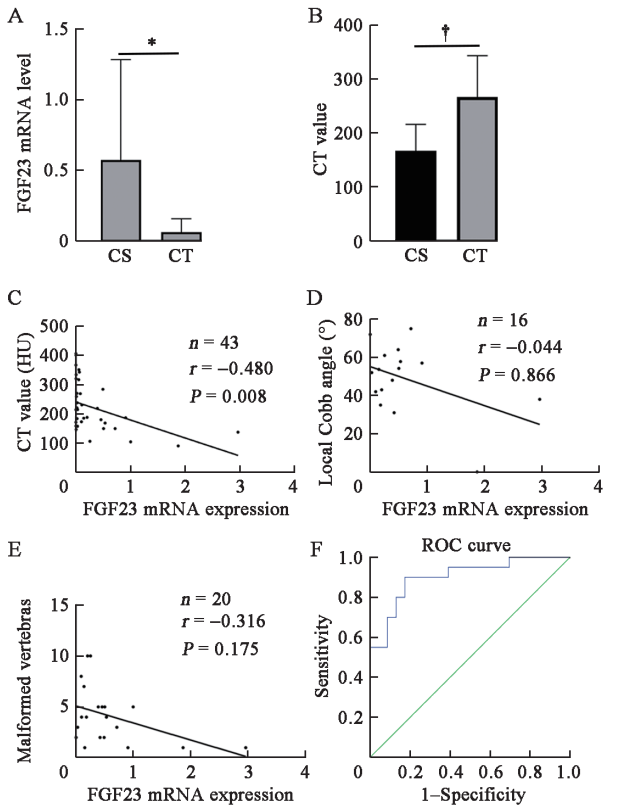

Figure 2: Changes of FGF23 mRNA levels in blood from patients with CS. (A) The mRNA levels of FGF23 in patients with CS (n = 20) and controls (n = 23). (B) Spine CT value of patients with CS (n = 20) and controls (n = 23). (C) Correlation of mRNA levels of FGF23 with CT value. (D) Correlation of mRNA levels of FGF23 in CS patients with local Cobb angels (missing image data of 4 patients). (E) Correlation of mRNA levels of FGF23 in CS patients with number of malformed vertebras. (F) ROC curves for mRNA levels of FGF23 discriminating between patients with CS (n = 20) and controls (n = 23). $^{*}P$ <0.01; $^{\dagger}P$ <0.001. CS: Congenital scoliosis; CT: control; CT value: Computerized tomography value; FGF23: Fibroblast growth factor 23; ROC: Receiver operating characteristic.

0.104, based on an AUC of 0.975 (95% CI, 0.913–1.000). The best cutoff between CS-F and CT-F was 0.929, based on an AUC of 0.872 (95% CI, 0.726–1.000) [Figure 3C, D].

These data showed that the blood FGF23 mRNA levels were increased in the CS-M group compared with the CS-F group.

Association of the ICVAS classification with FGF23 mRNA levels in patients with CS and controls

According to the ICVAS classification, we further divided the CS groups into individuals with an S-SDV or M-SDV. As shown in Figure 3E, compared with controls, individuals in both the M-SDV and S-SDV groups showed an increase in the concentration of blood FGF23 mRNA. Moreover, in the CS group, we found that patients in the S-SDV group showed significantly higher blood FGF23 mRNA levels than those in the M-SDV group.

Similarly, the ROC curves for mRNA levels of FGF23 discriminating between patients with different ICVAS types are shown in Figure 3F–H. The best cutoff between the M-SDV group and controls was 0.093, based on an AUC of 0.905 (95% CI, 0.805–1.000). The best cutoff between the S-SDV group and controls for FGF23 mRNA levels was 0.132, based on an AUC of 0.889 (95% CI, 0.751–1.000). Furthermore, we also performed a ROC curve analysis between the M-SDV and S-SDV groups, and the best cutoff was 0.813, based on an AUC of 0.677 (95% CI, 0.423–0.930).

These data suggested that the levels of FGF23 mRNA in peripheral blood had a good diagnostic ability for both M-SDV and S-SDV CS patients.

Alteration of FGF23 levels and osteogenesis in osteoblasts from patients with CS

As a regulator of calcium–phosphorus levels secreted by bone cells, FGF23 has been proven to play an important role in osteogenesis and mineralization. Therefore, we performed experiments to verify whether FGF23 played a role in BMD reduction in patients with CS. First, we extracted and cultured osteoblasts from the inferior facet joint of CS patients (CS-Ob) and the control group (CT-Ob) and extracted total RNA and protein for RT-PCR and Western blot detection. As shown in Figure 4A–C, both RT-PCR and Western blot proved that the expression levels of FGF23 in CS-Ob were increased compared with those in CT-Ob. The mRNA levels of FGFr3 (fibroblast growth factor receptor 3), an FGF23 receptor, and osteopontin (OPN) were also elevated in CS-Ob [Figure 4D,E]. In addition, we examined the expression of osteogenic markers in CS-Ob and CT-Ob. The results showed that TNAP and the Osteoprotegerin/Receptor Activator of Nuclear Factor-κB Ligand (OPG/RANKL) ratio in CS-Ob were significantly decreased compared with those in CT-Ob [Figure 4F, G]. Alizarin red staining also demonstrated that CS-Ob had reduced osteogenic mineralization compared with CT-Ob [Figure 4H].

These data indicated that the levels of FGF23 and its receptor FGFr3 were increased in CS-Ob, while its osteogenesis-related molecules such as OPN, TNAP, and OPG/RANKL, and mineralization capacity were decreased compared with CT-Ob.

Changes caused by FGF23 overexpression in primary osteoblasts from controls

To verify the effect of elevated levels of FGF23 on osteoblasts, we overexpressed FGF23 in CT-Ob. RT-PCR and WB showed that the FGF23 levels in the FGF23-overexpressing group were significantly higher than those in the control group, confirming the successful transfection of the FGF23 plasmid [Figure 5A–C]. FGF23 overexpression led to an increase in FGFr3 and a decrease in its downstream factor TNAP [Figure 5D]. Additionally, the levels of OPN, a mineralization inhibitor regulated by TNAP, were also increased in the FGF23 overexpression group [Figure 5E, F]. However, FGF23 overexpression did not cause significant changes in the OPG/RANKL ratio [Figure 5G].

Effects of FGF23 knockdown in primary osteoblasts from patients with CS

Considering that the expression levels of FGF23 were elevated in CS-Ob, we knocked down FGF23 in CS-Ob

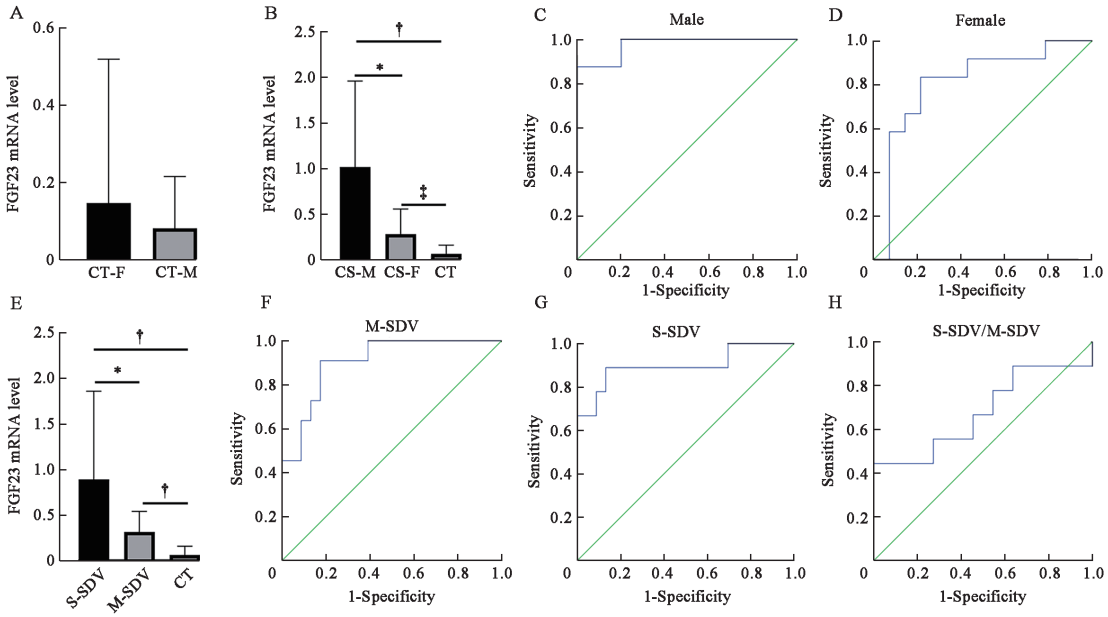

Figure 3: Association of sex and ICVAS classification with FGF23 mRNA levels in CS patients and controls. (A) The mRNA levels of FGF23 in controls compared according to sex. (B) The mRNA levels of FGF23 in CS patients compared according to sex. (C) ROC curves for mRNA levels discriminating between CS patients and controls in males. (D) ROC curves for mRNA levels discriminating between CS patients and controls in females. (E) The mRNA levels of FGF23 in controls compared according to ICVAS. (F, G) ROC curves for mRNA levels of FGF23 discriminating between S-SDV/M-SDV patients and controls. (H) ROC curves for mRNA levels of FGF23 discriminating between S-SDV and M-SDV patients. $^{*}P$ <0.05; $^{\dagger}P$ <0.001; $^{\ddagger}P$ <0.01. CS: Congenital scoliosis; CS-F: Female patients with CS; CS-M: Male patients with CS; CT: control; CT-F: Female controls; CT-M: Male controls; FGF23: Fibroblast growth factor 23; ICVAS: International Consortium for Vertebral Anomalies and Scoliosis; M-SDV: Multiple defects of the vertebrae; ROC: Receiver operating characteristic; S-SDV: Single defect of the vertebrae.

to demonstrate its effect. Successful knockdown of FGF23 was observed in CS-Ob [Figure 6A–C]. Knockdown of FGF23 resulted in decreased FGFr3 expression levels and upregulated TNAP in CS-Ob [Figure 6D]. OPN was also found to be significantly reduced in the FGF23 siRNA group [Figure 6E, F]. Similarly, FGF23 knockdown did not significantly change the OPG/RANKL ratio [Figure 6G]. At the same time, the bone mineralization ability of FGF23-knockdown CS-Ob was increased compared with that of control CS-Ob [Figure 6H].

Altogether, our data showed that FGF23 levels were significantly higher in patients with CS than in controls and could assist with the diagnosis of CS, and FGF23 could regulate the mineralization of osteoblasts through the FGFr3/TNAP/OPN pathway.

Discussion

To date, due to the unknown etiology and factors that influence CS, there has been no essential progress in the treatment of patients with CS, which mainly includes observation, bracing, and surgery.[20] However, although surgery is the only curative tool, it is very risky and costly.[3,21] Thus, it is important to reveal the pathology and mechanism of CS to explore better treatment options.

In the mammalian genome, DNA methylation, a major type of epigenetic modification mostly associated with transcriptional repression, involves the transfer of a methyl group onto the C5 position of a cytosine to form 5-methylcytosine.[22] Epigenetics, including DNA methylation, is an important environmental factor that plays a vital role in CS development.[23] We found reduced *FGF23* gene DNA methylation levels in CS patients compared with their identical twins by targeted-region methylation sequencing, and CS patients had increased mRNA levels of FGF23, consistent with the clinical symptoms of their low BMD.

In 2000, Yamashita *et al*[14] first isolated a newly reported secreted protein named FGF23 from mice. Subsequently, *FGF23* was shown to be mutated in autosomal dominant hypophosphatemic rickets (ADHR) and to cause osteogenic disorders.[24] Moreover, FGF23 has been found to negatively regulate osteogenesis and mineralization, and several pathways, such as the OPG/RANKL, ERK1/2 (extracellular signal-related kinases 1 and 2), and OPN pathways, are associated with this role.[25-27] Considering the characteristics of CS patients, such as disordered osteogenesis and low BMD, we suspected that FGF23 might play a role in CS.

To further clarify the expression of FGF23 in CS patients and controls and its correlation with the clinical characteristics of CS, we expanded the clinical sample and found that the levels of FGF23 mRNA in the peripheral blood of CS patients were significantly higher than those of the control group. Moreover, a significant correlation between the CT value of the spine and FGF23 mRNA levels in the peripheral blood of patients was observed. Since 2011, Hounsfield units (HUs) from clinical CT scanning have been widely recommended to assess BMD.[28] HU values of lumbar vertebrae from L1 to L5 have excellent reliability, a significant correlation with Dual Energy X-ray Absorptiometry *T*-scores, and good

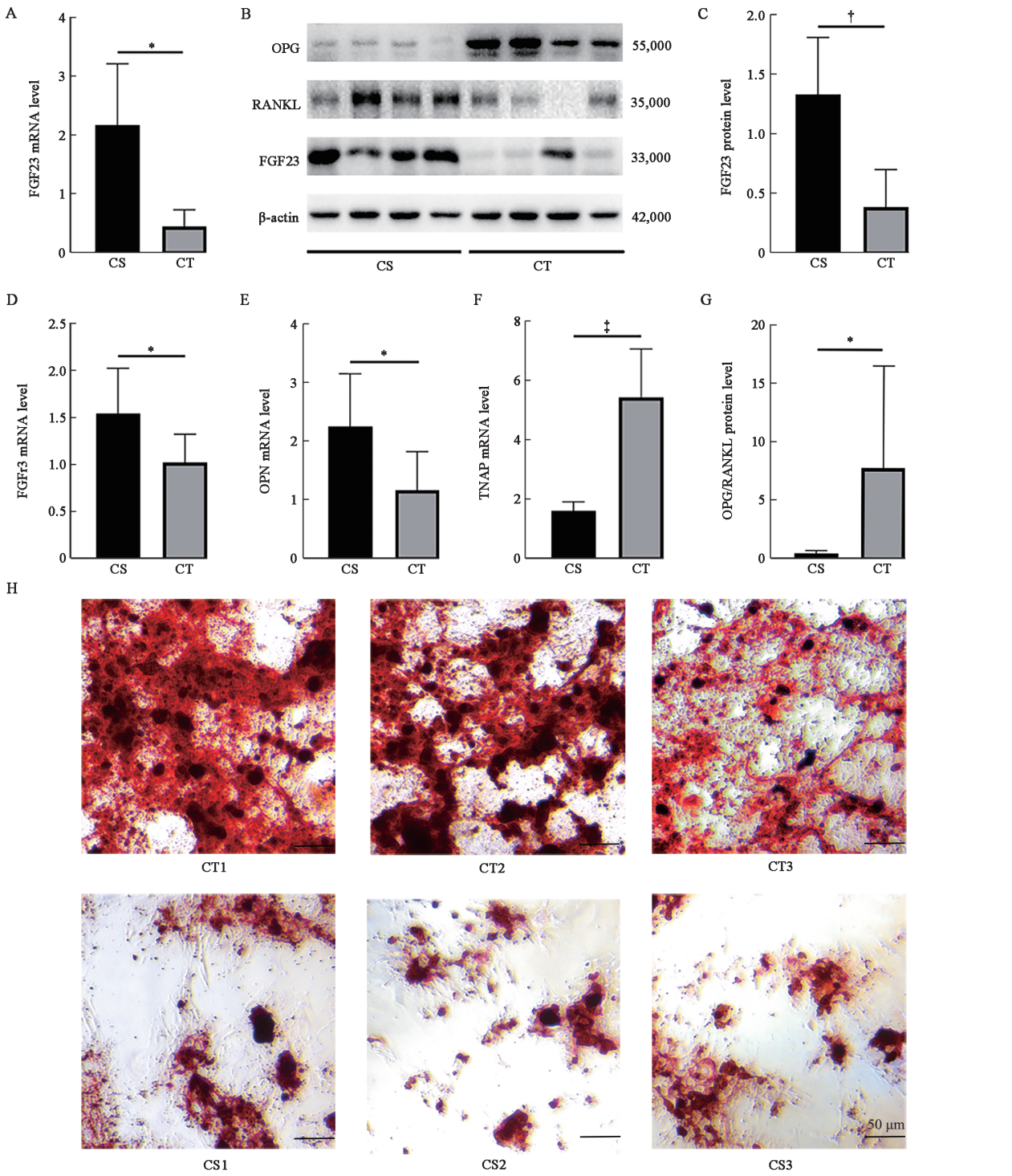

Figure 4: Changes of FGF23 related protein levels in CS patients and controls. (A) mRNA levels of FGF23 in primary osteoblast cells extracted from facet joint tissues of CS patients and controls. (B-C) The related protein levels of FGF23 in primary osteoblast form patients with CS and controls. (D) mRNA levels of FGFr3 in primary osteoblast cells. (E) mRNA levels of OPN in primary osteoblast cells. (F) mRNA levels of TNAP in primary osteoblast cells. (G) The protein levels of OPG/RANKL in primary osteoblast form patients with CS and controls. (H) Alizarin red staining for osteogenic differentiation of primary osteoblasts (14 days after differentiation). Scale bar, 50 μm. $^{*}P$ <0.05; $^{\dagger}P$ <0.01; $^{\ddagger}P$ <0.001. CS: Congenital scoliosis; CT: Control; FGF23: Fibroblast growth factor 23; mRNA: messenger ribonucleic acid; OPG/RANKL: Osteoprotegerin/Receptor Activator of Nuclear Factor-κB Ligand; OPN: Osteopontin; TNAP: tissue non-specific alkaline phosphatase.

performance in diagnosing osteoporosis.[19,29] Given that many patients lacked DXA (Dual-emission X-ray absorptiometry) results, vertebral CT values were used to assess BMD in our study, and we found that the CT value of the control group was higher than that of the CS group. Using micro-CT scanning, Zhu *et al*[4] also found that BMD in CS patients was significantly lower than that in the control group, and the whole body, from the spine to the limbs, had decreased BMD, indicating disordered bone metabolism in CS patients.

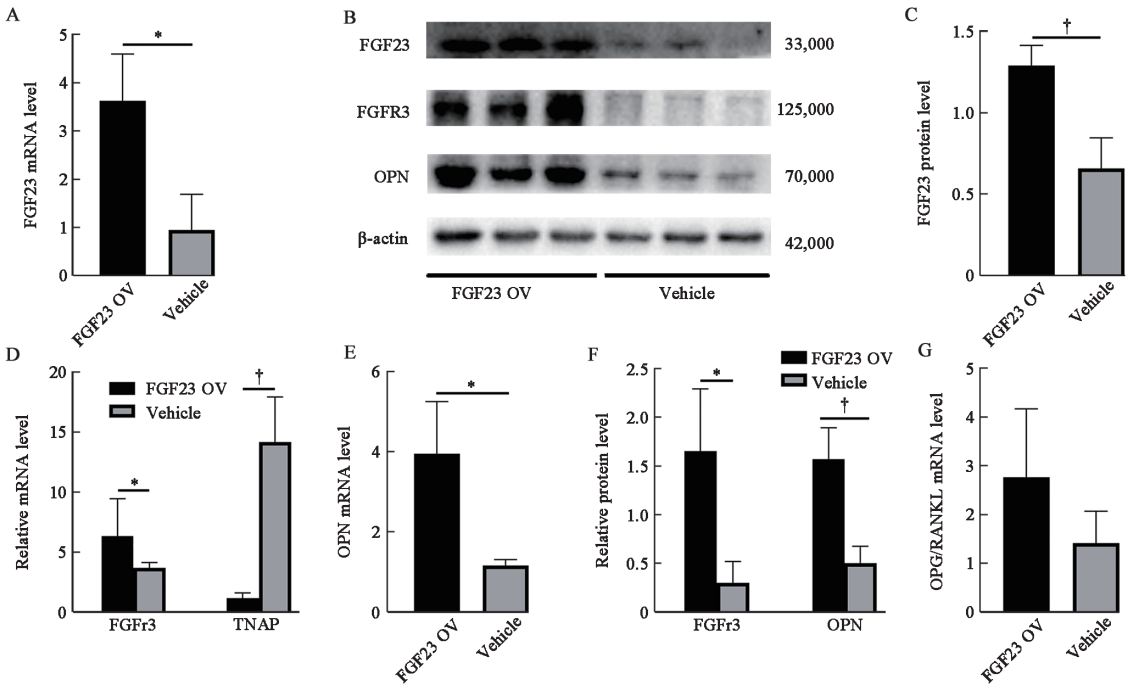

Figure 5: Changes of FGFr3/TNAP/OPN pathway in FGF23-overexpressing primary osteoblasts from control group (CT-Ob). (A) The mRNA levels of FGF23 in FGF23-overexpressing CT-Ob, $n = 6$ independent transfection samples. (B, C) The related protein levels of FGF23 in FGF23-overexpressing CT-Ob, $n = 6$ independent transfection samples. (D) The mRNA levels of FGFr3 and TNAP in FGF23-overexpressing CT-Ob.(E) The mRNA levels of OPN in FGF23-overexpressing CT-Ob. (F) The protein levels of FGFr3 and OPN in FGF23-overexpressing CT-Ob. (G) The ratio of OPG/RANKL mRNA levels in FGF23-overexpressing CT-Ob. $^{*}P < 0.05$; $^{\dagger}P < 0.01$. CT-Ob: Osteoblasts from control group; FGF23: Fibroblast growth factor 23; FGF23 OV: FGF23 overexpression; FGFr3: Fibroblast growth factor receptor 3; OPN: Osteopontin.

In the ROC analysis, peripheral blood FGF23 mRNA levels could assist with the diagnosis of CS, suggesting that peripheral blood FGF23 mRNA could be considered a biomarker for CS. Considering that fetal vertebral malformation arises in the early stage of pregnancy, but X-ray irradiation and other examination methods cannot be used at this stage of pregnancy due to radiation risks and other reasons, it is of great clinical significance to develop an accurate and easy marker for the early diagnosis of CS.[30,31] However, due to the lack of maternal peripheral blood or umbilical cord blood samples taken during pregnancy, it is impossible to know at this point whether the levels of FGF23 mRNA in the maternal blood change during pregnancy when CS is present in the fetus. Therefore, whether the levels of FGF23 mRNA can be used for the early diagnosis of patients with CS needs further study.

In our subsequent analysis, we found that there was no significant difference in the expression levels of FGF23 mRNA between males and females in the non-CS control group, but the levels of FGF23 mRNA in the peripheral blood of CS-M were significantly higher than those of CS-F. These data may be consistent with epidemiological studies from Korea and the Middle East, which observed more male patients with CS than females, although another study found that the incidence of intraspinal malformations in patients with CS was not significantly correlated with sex.[32–34]

Next, we further detected the expression of FGF23 and related proteins in primary osteoblasts of CS patients. Despite the decreased ratio of the osteogenic marker OPG/RANKL in primary osteoblasts of CS patients, neither FGF23 overexpression nor knockdown in primary osteoblasts caused changes in the OPG/RANKL ratio. Meanwhile, changes in the FGFr3/TNAP/OPN pathway were observed not only in primary osteoblasts of CS patients but also in FGF23-overexpressing CT-Ob and FGF23-knockdown CS-Ob. TNAP, an ectoenzyme, is responsible for the local production of inorganic phosphate for mineralization by hydrolyzing pyrophosphate (PPi) in the extracellular matrix. TNAP dysfunction mutants are characterized by impaired bone mineralization through the accumulation of PPi.[35] TNAP-deficient osteoblasts cannot be mineralized *in vitro*, suggesting the critical importance of TNAP for bone mineralization.[36] PPi accumulation caused by TNAP defects can also stimulate OPN secretion.[37] OPN, also known as bone sialoprotein I, is a protein identified in osteoblasts. *OPN*$^{-/-}$ mice display higher bone mineral content and mineral crystallinity than wild-type mice.[38] In mice, FGF23 inhibits TNAP expression through its receptor FGFr3, leading to phosphate accumulation and upregulation of OPN, resulting in impaired osteoblast mineralization.[39,40] Therefore, we speculate that FGF23 may regulate osteogenesis in CS patients through the FGFr3/TNAP/OPN pathway rather than OPG/RANKL.

In this study, we found lower DNA methylation of the *FGF23* gene in CS patients from twins, accompanied by increased mRNA levels. Given that DNA methylation is reported to inhibit gene expression,[41] we speculated that increased peripheral blood FGF23 mRNA levels might

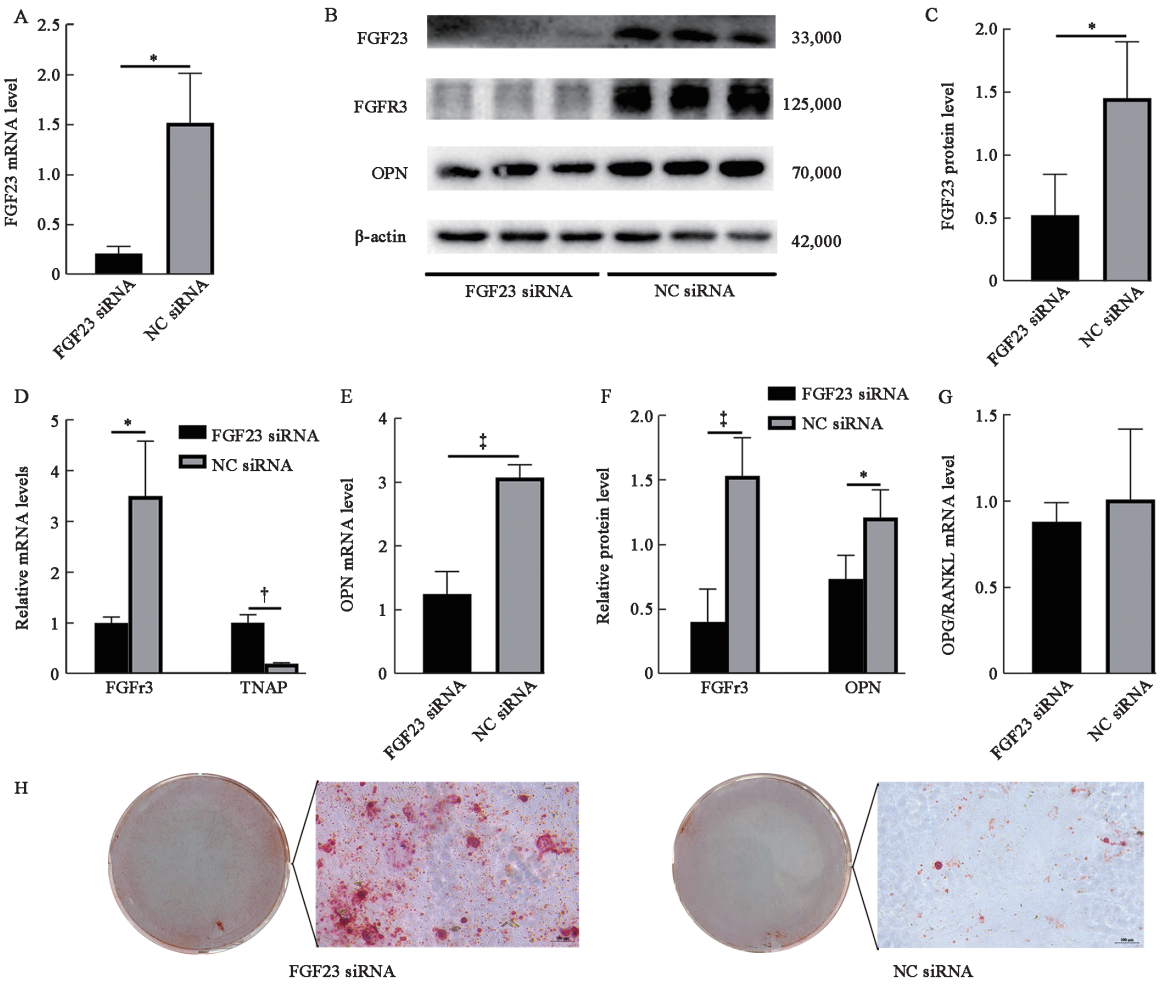

Figure 6: Changes of FGFr3/TNAP/OPN pathway in FGF23-knockdown primary osteoblasts from CS patients (CS-Ob). (A) The mRNA levels of FGF23 in FGF23- knockdown CS-Ob, $n =$ 6 independent transfection samples. (B,C) The related protein levels of FGF23 in FGF23-knockdown CS-Ob, $n = 6$ independent transfection cells. (D) The mRNA levels of FGFr3 and TNAP in FGF23-knockdown CS-Ob. (E) The mRNA levels of OPN in FGF23-knockdown CS-Ob. (F) The protein levels of FGFr3 and OPN in FGF23-knockdown CS-Ob. (G) The ratio of OPG/RANKL mRNA levels in FGF23-knockdown CS-Ob. (H) Alizarin red staining for osteogenic differentiation of FGF23 knockdown and control CS-Ob (7 days after differentiation). Scale bar, 100 μm. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$. CS: Congenital scoliosis; CS-Ob: Osteoblasts from CS patients; FGF23: Fibroblast growth factor 23; NC: Negative control; OPN: Osteopontin; siRNA: small interfering ribonucleic acid.

be associated with lower levels of DNA methylation. However, the relationship between higher FGF23 levels in CS-Ob and lower FGF23 DNA methylation is unclear, since FGF23 methylation levels were not measured in Ob in our study. Although DNA methylation is a tissue-dependent epigenetic modification, we observed upregulation of FGF23 in both the peripheral blood and Ob of CS patients. Therefore, we thought that FGF23 DNA methylation levels may be not only decreased in the blood. However, further investigation is necessary to prove this hypothesis.

In conclusion, our results demonstrated increased peripheral blood FGF23 levels and decreased BMD in CS patients and a negative correlation of peripheral blood FGF23 levels with spinal CT values. Meanwhile, peripheral blood FGF23 levels could assist with the diagnosis of CS. Increased FGF23 levels in the osteoblasts of CS patients could inhibit mineralization through the FGFr3/TNAP/OPN pathway, which might contribute to osteopenia in CS patients.

Funding

This study was funded by the National Natural Science Foundation of China (No. 82072390) and the Natural Science Foundation of Hunan, China (No. 2020JJ4873).

Conflicts of interest

None.

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How to cite this article: Zhang HQ, Xiang G, Li J, He SH, Wang YJ, Deng A, Wang YX, Guo CF. Promotion effect of FGF23 on osteopenia in congenital scoliosis through FGFr3/TNAP/OPN pathway. Chin Med J 2023;136:1468–1477. doi: 10.1097/CM9.0000000000002690