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Novel *CLCN7* mutation identified in a Han Chinese family with autosomal dominant osteopetrosis-2

Hao Deng, PhD[1,2], Dan He, MS[1], Pengfei Rong, PhD[3], Hongbo Xu, MS[1], Lamei Yuan, MD[1], Liu Li, MS[4], Qian Lu, MS[1] and Yi Guo, PhD[1,5]

Abstract

Osteopetrosis is a heritable bone condition featuring increased bone density due to defective osteoclastic bone resorption. Exome sequencing and Sanger sequencing were conducted in Han Chinese family members, some of whom had typical osteopetrosis, and a novel missense variant c.2350A>T (p.R784W) in the chloride channel 7 gene (*CLCN7*) was identified. This variant cosegregated with the disorder in the family but was not observed in 800 controls. The data indicate that exome sequencing is a powerful and effective molecular diagnostic tool for detecting mutations in osteopetrosis, which is a genetically and clinically heterogeneous disorder. This discovery broadens the *CLCN7* gene mutation spectrum and has important implications for clinical therapeutic regimen decisions, prognosis evaluations, and antenatal diagnoses.

Keywords

Autosomal dominant osteopetrosis-2, exome sequencing, the *CLCN7* gene, mutation

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Introduction

Osteopetrosis is a term used to describe a group of rare heritable conditions, including osteopetrosis, osteopoikilosis, pycnodysostosis, osteomesopyknosis, dysosteosclerosis, osteosclerosis Stanescu type, melorheostosis with osteopoikilosis, and osteopathia striata congenita with cranial stenosis. It is featured by increased bone density on radiographs.[1] Generally, osteopetrosis is a rare monogenic heritable bone condition characterized by increased bone density due to defective osteoclastic bone resorption,[2–4] exhibiting variable clinical signs with an incidence of about 5/100,000.[2,5] It is an autosomal dominant or recessive inherited disorder possibly caused by mutations in the genes involved in the bone formation or resorption.[4,6] The present literature describes at least nine disease-causing genes, including the low-density lipoprotein receptor-related protein 5 gene (*LRP5*, MIM 603506), the chloride channel 7 gene (*CLCN7*, MIM 602727), the T cell immune regulator 1 gene (*TCIRG1*, MIM 604592), the tumor necrosis factor ligand superfamily, member 11 gene (*TNFSF11*, MIM 602642), the carbonic anhydrase II gene (*CA2*, MIM 611492), the osteopetrosis-associated transmembrane protein 1 gene (*OSTM1*, MIM 607649), the pleckstrin homology domain-containing protein, family M, member 1 gene (*PLEKHM1*, MIM 611466), the tumor

[1]Center for Experimental Medicine, the Third Xiangya Hospital, Central South University, Changsha, China
[2]Department of Neurology, the Third Xiangya Hospital, Central South University, Changsha, China
[3]Department of Radiology, the Third Xiangya Hospital, Central South University, Changsha, China
[4]Department of Pediatrics, the Third Xiangya Hospital, Central South University, Changsha, China
[5]Department of Medical Information, Information Security and Big Data Research Institute, Central South University, Changsha, China

Corresponding authors:
Hao Deng, the Third Xiangya Hospital, Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, China.
Email: hdeng008@yahoo.com
Yi Guo, Department of Medical Information, Information Security and Big Data Research Institute, Central South University, 172 Tongzipo Road, Changsha, Hunan 410013, China.
Email: yiguo0816@yahoo.com

necrosis factor receptor superfamily, member 11A gene (*TNFRSF11A*, MIM 603499), and the sorting nexin 10 gene (*SNX10*, MIM 614780).[7,8] Autosomal dominant osteopetrosis-2 (OPTA2, MIM 166600), the most common osteopetrosis, is caused by mutations in the *CLCN7* gene, which play important bone resorption roles.[9,10] The study's aim was to detect the disease-causing gene for a consanguineous Han Chinese family, some of whom suffered from autosomal dominant osteopetrosis featuring bone fracture, bone pain, increased bone mineral density (BMD) in spine and pelvis, and increased alkaline phosphatase (ALP) levels. A novel heterozygous mutation c.2350A>T (p.R784W) in the *CLCN7* gene was identified by exome sequencing and Sanger sequencing.

Materials and methods

Subjects and clinical evaluation

A five-generation, 13-member Han Chinese family, living in Changsha, China, and suffering from osteopetrosis, was enrolled at the Third Xiangya Hospital, Central South University, Changsha, China (Figures 1 and 2). Peripheral blood was collected from six family members including four patients (III:2, IV:1, IV:4, and V:1, Figure 2(a)). Detailed records of clinical manifestations, radiological evidences, and biochemical findings were obtained from all available family members (Table 1). The diagnosis of OPTA2 was made based on the above medical records.[11–13] Peripheral blood samples were also taken from 100 ethnically matched unrelated control volunteers (male-to-female ratio: 50/50; age 32.0 ± 8.2 years), having neither diagnostic features nor family history of osteopetrosis. BMD was examined using a dual-energy X-ray absorptiometry (DXA) densitometer (LUNAR DPX NT+ 74029, General Electric Medical System, USA). Informed written consent was provided by participants or their guardians. A research approval was obtained from the institutional review board of the Third Xiangya Hospital, Central South University, China.

Exome capture

Genomic DNA isolation was performed via phenol-chloroform extraction from the blood samples.[14] Exome sequencing was performed by the Novogene Bioinformatics Institute (Beijing, China). An exome library was established using 1.5 micrograms of DNA from the proband (IV:4, Figure 2(a)). The DNA was sheared into fragments using Covaris sonicator (Covaris Inc., Woburn, MA, USA). Exons were captured using the Agilent SureSelect Human All Exon V5 Kit. After evaluation of DNA quality, pooled samples were intended for sequencing. Following the manufacturer's protocols, sequencing of the library targeting the exome was implemented on the Illumina HiSeq 2500 platform.[15]

Variant analysis

By Burrows-Wheeler Alignment tool (BWA), high-quality paired-end reads were mapped to the human reference

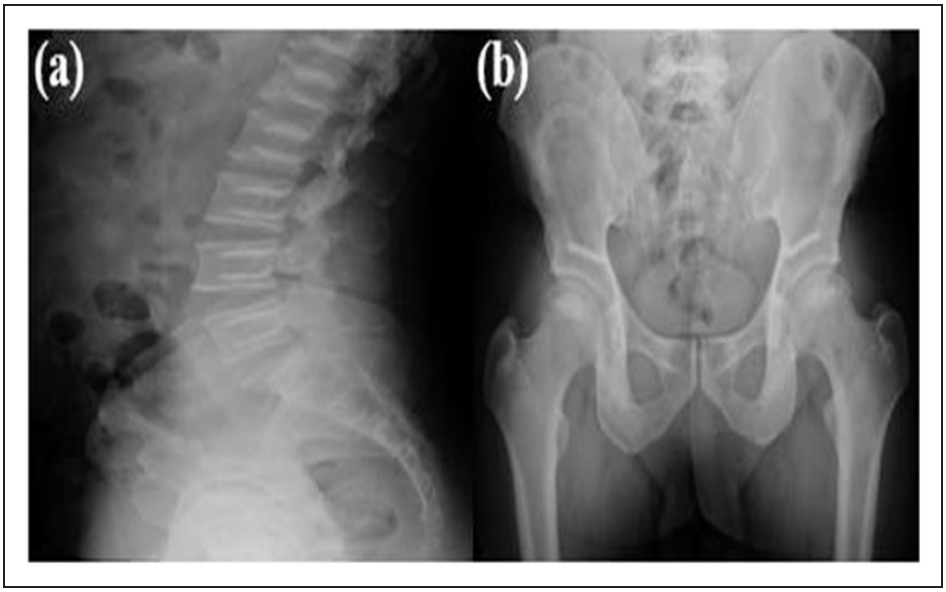

Figure 1. Radiographic signatures of a patient (IV:1) with autosomal dominant osteopetrosis-2. (a) The typical "sandwich" sign of vertebral bodies. (b) The typical "bone-in-bone" sign of iliac wing.

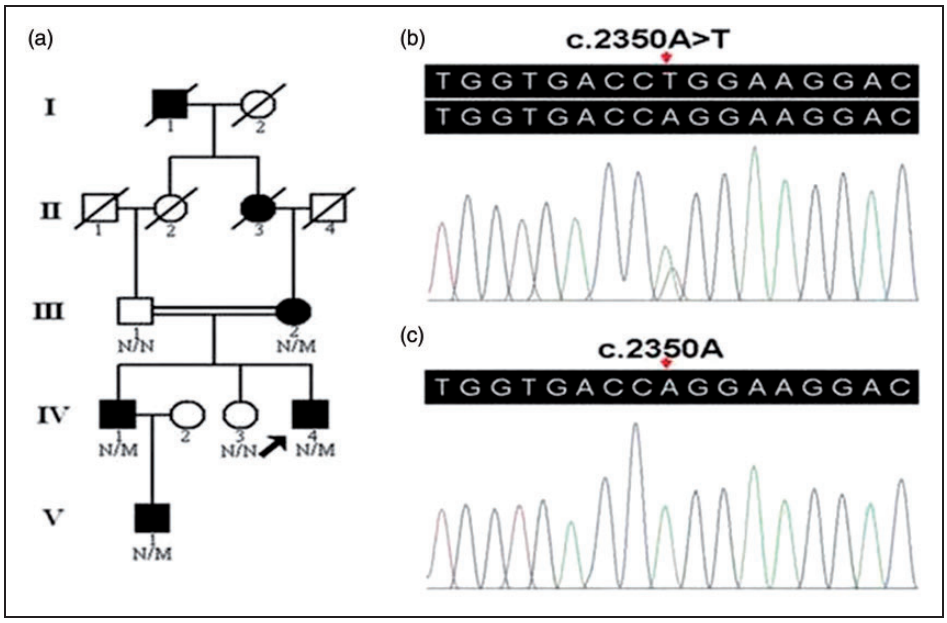

Figure 2. Pedigree and sequence analysis of the family with autosomal dominant osteopetrosis-2 (OPTA2). (a) Pedigree of the OPTA2 family indicating affected family members (fully shaded). N: normal, M: the chloride channel 7 gene (*CLCN7*) c.2350A>T (p.R784W) mutation. Arrow shows the proband. (b) Sequencing analysis reveals the heterozygous *CLCN7* c.2350A>T (p.R784W) mutation in the proband (IV:4). The arrow shows site of mutation. (c) The *CLCN7* gene c.2350A wild type sequence in an unaffected family member (IV:3).

Table 1. Clinical, radiological, and laboratory findings of four autosomal dominant osteopetrosis-2 patients with the chloride channel 7 gene c.2350A>T mutation.

Subjects		III:2	IV:1	IV:4	V:1
Gender		Female	Male	Male	Male
Age (years)		67	46	37	15
Symptoms		Osteoarthritis of knees for five years and backache for 20 years	Clavicle fracture at 11 years old	Diffuse bone pain in the cervical vertebra for four years	Metatarsal fracture at 9 years old
X-rays	Spine	"Sandwich vertebrae" sign	"Sandwich vertebrae" sign	"Sandwich vertebrae" sign	"Sandwich vertebrae" sign
	Pelvis	"Bone-in-bone" sign	"Bone-in-bone" sign	"Bone-in-bone" sign	"Bone-in-bone" sign
BMD	L1–4	↑	↑	↑	↑[a]
	TH	↑	↑	↑	↑[a]
ALP		↑	↑	↑	↑
IP		N	N	↑	N
25-VitD3		N	N	N	↓
25-VitD2		N	N	N	↓
Serum calcium		N	N	N	N
Hemoglobin		N	N	N	N

BMD: bone mineral density; L1–4: lumbar spine 1–4; TH: total hip; IP: inorganic phosphorus; ALP: alkaline phosphatase; 25-VitD3: 25-hydroxy vitamin D3; 25-VitD2: 25-hydroxy vitamin D2; N: normal values; ↑: increased values; ↓: decreased values.

[a]The Z score at L1–4 and total hip was calculated by comparison with the age-specific BMD reference value of Han Chinese children and adolescent.

genome sequence from UCSC database (UCSC hg19, http://genome.ucsc.edu/).[16] To explore single nucleotide polymorphisms (SNPs) and insertions-deletions (indels), high-quality alignment was called to assure variant calling accuracy. The analysis-ready BAM alignment results were procured after removing duplicated reads, conducting local alignment, and recalibrating base quality by Picard (http://sourceforge.net/projects/picard/), Genome Analysis Toolkit and SAMtools. A 100.83× mean sequencing depth provided adequate depth and guaranteed 99% variant calling accuracy of each targeted exome. Given that the variant of interest is rare in the normal population, all variants were filtered against public databases, including 1000 Genomes Project (2012 April release, http://www.1000genomes.org/), database of SNPs build 137 (dbSNP137, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi), and NHLBI-Exome Sequencing Project (ESP) 6500. Variants retained after the filtration of the above databases were ulteriorly filtered by in-house exome database from Novogene Bioinformatics Institute with 700 ethnically matched controls. A prioritization scheme was utilized to for the filtration strategy, similar to those in previous studies.[15-17] Annotate Variation (ANNOVAR) software was used to annotate potential variants.[18] Nonsynonymous SNPs were evaluated using Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/, variant with a score less than 0.05 is predicted to be deleterious) and Polymorphism Phenotyping version 2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/).[19]

Direct Sanger sequencing was performed to identify potential causative variant using an ABI3500 sequencer (Applied Biosystems, Foster City, CA, USA).[14] Primer sequences applied to locus-specific PCR amplification and Sanger sequencing were: 5′-CCCAGCCACACACA AGG-3′ and 5′-AGTGACTCCGGGAGGAAATG-3′. MutationTaster (http://www.mutationtaster.org/) was used to test amino acid substitution impact on protein function. Multiple protein sequence alignment was carried out across different species using the NCBI BLAST (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi).[19]

Results

Clinical characteristics of patients

Table 1 presents detailed clinical, radiological, and laboratory results. Two patients (IV:1 and V:1, Figure 2(a)) had fractures. The other two (III:2 and IV:4, Figure 2(a)) complained of bone pain. One (III:2, Figure 2(a)) was diagnosed with osteoarthritis of the knees. X-rays disclosed that all four patients had high bone density and typical OPTA2 "sandwich" and "bone-in-bone" radiographic signatures (Figure 1). BMD results showed high bone density in all four. All had elevated ALP, but normal serum calcium and hemoglobin. One patient (IV:4, Figure 2(a)) had elevated inorganic phosphorus (IP). The youngest patient (V:1, Figure 2(a)) had low levels of 25-hydroxy vitamin D3 (25-VitD3) and 25-hydroxy vitamin D2 (25-VitD2).

Exome sequencing and identification of pathogenic variants

Exome sequencing generated sequence reads having an average read length of 148.27 bp for a total of 56,208,952. A total of 56,172,518 reads (99.94%) were mapped to human reference genome sequence. A total of 35,234 SNPs, including 16,939 in the exon regions and 1,502 in the splicing sites, were detected. A total of 2,413 indels, including 377 in the exon regions and 177 in the splicing sites, were identified. Variants which have been recorded in 1000 Genomes Project with frequency > 0.5%, dbSNP137 with a minor allele frequency more than 1% and NHLBI-ESP6500, and synonymous variants were excluded. Using a prioritized strategy, potential disease-causing nonsynonymous variants were retained after the bioinformatics predictions by SIFT and PolyPhen-2. Except for a variant c.2350A>T (p.R784W) in the *CLCN7* gene (NCBI reference sequence: NM_001287.5), no variants in known osteopetrosis disease-causing genes were detected.

Sanger sequencing confirmed the heterozygous variant *CLCN7* c.2350A>T in the proband, which was also detected in the other three affected family members (III:2, IV:1, and V:1, Figure 2(a) and (b)) but was absent in the proband's unaffected father and elder sister (III:1 and IV:3, Figure 2(a) and (c)), and the 800 ethnically matched controls (100 normal controls in this study and 700 Chinese controls without OPTA2 from exome sequencing data of Novogene. By MutationTaster, the variant was predicted to be disease-causing with a value close to 1, suggesting a high security of the prediction. The p.Arg784 is shown to be a highly conserved amino acid residue among different species from zebrafish to human according to multiple protein sequence alignment (Figure 3).

Discussion

Osteopetrosis is a rare condition with marked genetic heterogeneity and high clinical variability.[2,20] OPTA2, also known as autosomal dominant osteopetrosis type II (ADO-II) or Albers-Schönberg disease, was first described by Albers Schönberg in 1904 and is generally considered as an adult or adolescent onset condition caused by *CLCN7* mutations.[11,20,21] It occurs in 0.2–1/100,000 adults and generally manifests with osteosclerosis involving the spine, basis cranii, and pelvis,[12,22] exhibiting a highly variable phenotype with penetrance

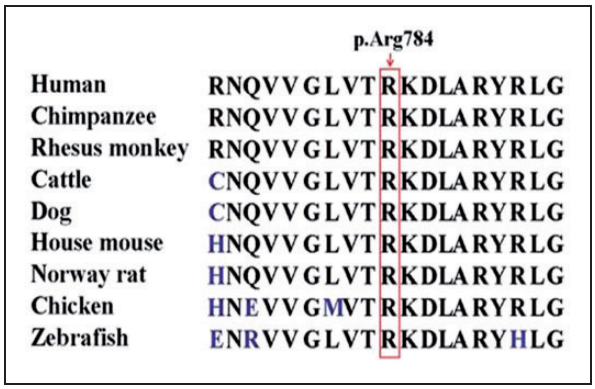

Figure 3. Conservation analysis of chloride channel protein 7 p.Arg784 amino acid residue.

of 56%–90%.[12] Phenotypes can be present either asymptomatically or with symptoms ranging from very mild to severe. The highly variable phenotype, both in presence and severity, may be due to the presence of modifier gene(s) on chromosome 9q21-q22.[21,23] *CLCN7* mutation carriers might present with bone fracture, bone pain, chronic osteomyelitis, osteoarthritis, scoliosis, rickets, deafness, blindness, or be asymptomatic,[11,13,24–26] which may be caused by increased BMD and poor bone quality.[21] Fracture, especially in long bones, is the most likely gender difference-free clinical sequela of OPTA2.[24,27] Other clinical changes may include elevated creatine kinase (CK), CK isoenzyme-MB (CK-MB) and moderate hematological failure.[12,28]

Here, a Han Chinese family, some members of which had hereditary osteosclerosis and a variety of other clinical findings, was studied. Patients were considered to have autosomal dominant osteopetrosis via generation-to-generation transmission. All patients manifested similarly with such conditions as "sandwich" and "bone-in-bone" radiographic signatures, elevated BMD and ALP levels. Bone fracture, bone pain, and osteoarthritis were present in some of these patients. Exome sequencing revealed a novel c.2350A>T (p.R784W) variant in exon 25 of the *CLCN7* gene in the proband. Subsequently, Sanger sequencing disclosed that this variant co-segregated with the disease in this family but was absent in 800 controls. The p.Arg784 is a phylogenetically conserved amino acid residue among various vertebrates, implying its probable importance in structure and function, and bioinformatics predictions suggest the alteration to be pathogenic. All these evidences indicate that the c.2350A>T variant is likely deleterious and may be the pathogenic mutation for OPTA2 in this family.

The *CLCN7* gene consists of 25 exons spanning over 30 kb in the human genome. It encodes the 805-amino-acid chloride channel protein 7 (ClC-7). The ClC-7 protein is a member of the chloride channel family, which is a homodimer having two homologous subunits. Each subunit has eighteen intramembrane α helices, four Cl^- binding sites, and two cystathionine beta synthase (CBS) domains.[11] ClC-7, a $2Cl^-/1H^+$ antiporter, is highly expressed in the osteoclast ruffled membrane, providing the chloride conductance necessary for osteoclast-mediated bone degradation and supporting bone resorption.[10,12,21]

In 2001, using linkage analysis, the OPTA2 disease gene locus was mapped to chromosome 16p13.3 in five French families and one Danish family. Subsequently, several mutations in the *CLCN7* gene were detected in these OPTA2 families.[9,29] Presently, there are more than 30 pathogenic mutations in the *CLCN7* gene identified in OPTA2 patients. The p.G215R, p.P249L, p.R767W, and c.2385_2386delAG in the *CLCN7* gene are considered hotspot mutations.[9,12,13,24,26,27,30–34]

CLCN7 mutations include missense mutations, deletions, insertions, splicing mutations, and repeat variations. At least 85 mutations in the *CLCN7* gene are recorded in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/all.php). The vast majority of mutations are found in patients with OPTA2 and autosomal recessive osteopetrosis type IV (OPTB4), which is another serious type of osteopetrosis.[35]

The mutation p.R784W is located in the CBS2 domain in ClC-7. More than nine mutations have been detected in the CBS2 domain of ClC-7,[9,24,27,31,32,34] which participates in protein sorting.[36] The p.R784W mutant might disturb the protein sorting and then interfere with the protein formation in the ruffled-border formation, which supports lysosomal function and bone resorption.[7,10]

Conclusion

A heterozygous c.2350A>T mutation in the *CLCN7* gene was found to be responsible for OPTA2 in members of a five-generation family. The study revealed that exome sequencing is a powerful and effective strategy to diagnose OPTA2, a heterogeneous disease.[15,20] The findings broaden the *CLCN7* gene mutation spectrum, significantly impact clinical therapeutic regimen decisions, prognosis evaluations, and antenatal diagnoses for OPTA2 family members.[37]

Author Contributions

HD, DH, and YG conceived and designed the experiments. HD, DH, and HX performed the experiments. HD, DH, PR, HX, LY, LL, QL, and YG analyzed the data. HD, DH, PR, LL, and YG contributed reagents/materials/analysis tools. HD, DH, and YG wrote the manuscript. All authors reviewed and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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