Identification of *TCIRG1* and *CLCN7* gene mutations in a patient with autosomal recessive osteopetrosis

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Abstract. Osteopetrosis is a heritable bone disorder that exhibits highly clinical and genetical heterogeneity, and is caused by defective osteoclastic resorption. The three main forms are the autosomal recessive severe (ARO), the intermediate autosomal and the autosomal dominant benign osteopetrosis forms. In the present study, the clinical, biochemical and radiological manifestations were described in a patient with osteopetrosis. Sequence analysis identified the compound heterozygous mutations, c.909C>A (p.Tyr303X) and c.2008C>T (p.Arg670X), in *TCIRG1*, and a heterozygous splicing mutation, c.1798-1G>T, in the chloride channel 7 gene (*CLCN7*). Two aberrant forms of the *CLCN7* transcripts, c.1798_1883 (exon 20) deletion predicted to cause p.Leu601GlyfsX13, and the c.1798_1821 deletion, the first 24 bp of the exon 20, predicted to cause p.Gly600_Gln607del, were detected by further analysis of the splicing patterns in the leukocytes. The patient's asymptomatic mother carried the *TCIRG1* c.909C>A (p.Tyr303X) and *CLCN7* c.1798-1G>T mutations, while the asymptomatic father carried the *TCIRG1* c.2008C>T (p.Arg670X) mutation only. The patient was finally diagnosed with ARO on the basis of clinical and biochemical parameters, radiological changes and genetic defects. To the best of our knowledge, this is the first reported case of a patient with osteopetrosis who carries *TCIRG1* and *CLCN7* mutations. In addition, among the three mutations, *TCIRG1* c.909C>A and *CLCN7* c.1798-1G>T were novel mutations.

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Introduction

Bone development and homeostasis is achieved by a strict balance between bone formation by osteoblasts and resorption by osteoclasts. Defective bone resorption results in osteopetrosis, an inherited disorder encompassing a clinical and genetical heterogenous group of conditions. Three main forms are classified on the basis of inheritance patterns, age of onset and severity. These are the autosomal recessive severe (ARO; MIM 259700), intermediate autosomal (IAO) and autosomal dominant benign osteopetrosis (ADO) forms (1). All these forms are characterized by an increased bone density, which may result in various phenotypical features, including fractures, osteomyelitis, deformity, dental abnormalities, bone marrow impairment and cranial nerve compression. (2). Osteopetrosis is a rare condition, and the estimated incidence of ARO is 1 in 250,000 births, while that of ADO is 1 in 20,000 births (3,4).

Mutations in at least 10 genes have been identified as causative in various types of osteopetrosis cases in humans (2). However, the majority of the osteopetrosis cases reported thus far are caused by defects in gene products involved in the regulation of the intra- and extracellular pH of osteoclasts. Two significant molecules that are involved in the acidification machinery are the proton pump, vacuolar ATPase (V-ATPase), and the chloride-specific ion channel, CLCN7. Defects in *TCIRG1*, which encodes the V-ATPase a3 subunit, have been reported to be responsible for ARO in >50% of patients (5-7). However, mutations in the *CLCN7* gene give rise to the complete spectrum of osteopetrosis, underlying ~15% of all ARO cases, almost all known cases of IAO and 75% of ADO type II cases (ADO II; Albers-Schönberg disease; MIM 166600) (8-10).

Materials and methods

Patient. A 21-month-old male was admitted to Shanghai Children's Medical Center (Shanghai, China) due to persistent

anemia and a lack of dentition. The patient was full-term at birth, with a normal weight and length, and a subsequent weight and length of 12 kg (50th percentile) and 84.8 cm (between 25th and 50th percentile), respectively, at 21 months. A prominent forehead, enlarged abdomen with moderate hepatosplenomegaly and visual disturbance were noted during the physical examination. Laboratory tests revealed that the patient had moderate anemia, elevated levels of serum alkaline phosphatase, parathyroid hormone, creatine kinase and MB isoenzyme, and decreased serum Ca^{2+} levels. The levels of phosphonium, 1,25-dihydroxy vitamin D3, lactate dehydrogenase, thyroid hormone and thyroid stimulating hormone were within the normal ranges. Imaging examinations consisting of computerized tomography (CT) scans (LightSpeed 16 Slice CT; GE Healthcare, Fairfield, CT, USA) and X-rays (AMX IV Plus Portable X-Ray, GE Healthcare) revealed a general increase of bone density involving the skull, vertebrae and limbs (Fig. 1). The diagnosis of osteopetrosis was based on the skeletal radiographs along with the clinical and laboratory data. The patient was scheduled to undergo a bone marrow transplant; however, died of an infection following intensive chemotherapy. The patient was the only son of a non-related couple. No clinical abnormalities were noted in the parents, and further biochemical and radiological examinations of the patient's mother also appeared normal.

Molecular analysis. Ethylenediaminetetraacetic acid-peripheral blood samples were obtained from the patient, his parents and 100 healthy individuals. Genomic DNA was isolated from the peripheral blood leukocytes using the QIAmp DNA Blood kit (Qiagen, Hilden, Germany). All the exons and exon-intron boundaries of the *TCIRG1* and *CLCN7* genes from the patient's genomic DNA were amplified by PCR, and the primer sequences are listed in Tables I and II. The PCR products were analyzed by direct DNA sequencing on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). Only genomic fragments containing the mutations identified in the patient were amplified and sequenced for the parents and the normal controls. Several polymorphisms, including single-nucleotide polymorphisms (SNPs), rs12926089, rs12926669 and rs960467, and the variable number tandem repeat (VNTR) in intron 8 of *CLCN7* were also analyzed in the pedigree.

Reverse transcription-polymerase chain reaction (RT-PCR). The total RNA of the patient and the parents was isolated from their peripheral leukocytes using the QIAmp RNA Blood kit (Qiagen). The first-strand cDNA was synthesized using random primers, oligo primers and PrimeScript Reverse Transcriptase (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China). The synthesized products were amplified with primer P1, 5'-TACGGGCTCACGGTGTCTG-3', which is located in exon 17, and primer P2, 5'-GGTAGGCGTCTCGGAAGTC-3', which is located in exon 23 of *CLCN7*. The PCR products were further amplified using semi-nested oligonucleotide primers P1 and P3, 5'-TTGTGCTTTAGGAGAACGA-3', which is located in exon 22 of *CLCN7*. The products were cloned into the pMD 18-T vector (Takara Biotechnology (Dalian) Co., Ltd.) and 30 clones were selected and sequenced.

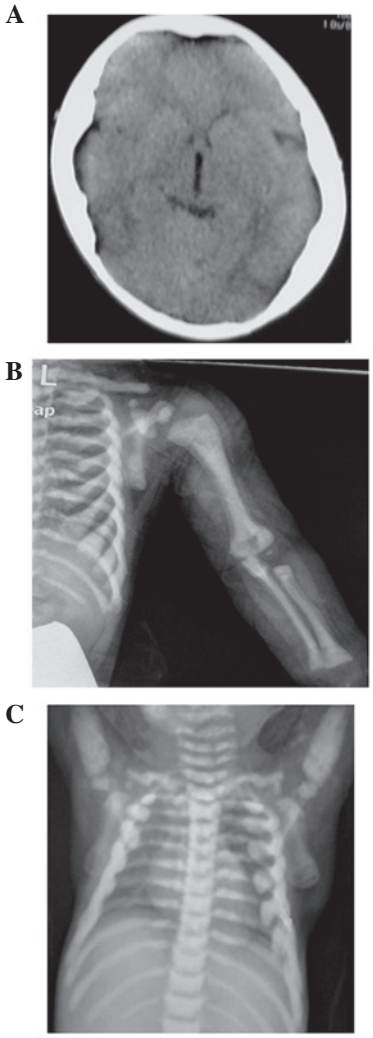

Figure 1. Imaging examinations consisting of a CT scan and X-rays of the proband. A generalized increase in bone density involving (A) the skull, (B) the limbs and (C) the vertebrae was observed. CT, computed tomography.

Ethics statement. The study was approved by the Ethics Committee of Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from the parents and the 100 healthy individuals prior to blood sampling and DNA analysis.

Results

Molecular analysis of the TCIRG1 and CLCN7 genes. Direct genomic DNA sequencing of the patient revealed compound heterozygous mutations, c.909C>A (p.Tyr303X) and c.2008C>T (p.Arg670X), in *TCIRG1* and a heterozygous splice site mutation, c.1798-1G>T, in the *CLCN7* gene (Fig. 2). The patient's mother carried the *TCIRG1* c.909C>A (p.Tyr303X) and *CLCN7* c.1798-1G>T mutations, while the patient's father carried the *TCIRG1* c.2008C>T (p.Arg670X) mutation. The *CLCN7* c.1798-1G>T variation was not detected in the 100 healthy individuals. In addition, several SNPs, including rs12926089, rs12926669 and rs960467, and the intron 8 VNTR in the *CLCN7* gene were investigated. However, no differences in the investigated polymorphisms were identified between the patient and his mother (Table III).

Table I. PCR primers used for amplification of *TCIRG1* gene.

Primer	Sequence (5' to 3')	Size (bp)
EXON1 F	TCAACCTCTCCCAGACTTCC	320
EXON1 R	CTGAGCTGCATTCACGGAG	
EXON2 F	TCAGTGAGTGAAGGTGCACAG	319
EXON2 R	GTTCAAATGGGGCCAGG	
EXON3 F	TCCACACCTTTCTGGAGGAG	266
EXON3 R	TTTCAGATCAAACTTGGCCC	
EXON4+EXON5 F	GAGTTTGGGGCAGCAGG	564
EXON4+EXON5 R	CACTGGACAAGGAGTCGGAG	
EXON6+EXON7 F	GAGGCCTCCTGCCTTCC	561
EXON6+EXON7 R	GGCCAGAAGGACACAGCTAC	
EXON8 F	CCTATCGTGACTCCTCCCC	262
EXON8 R	ACCTCCTGCACCCACCTC	
EXON9 F	GAGGTGGGTGCAGGAGG	372
EXON9 R	CTGGAAGTGAGGCAGAAACG	
EXON10 F	ATCTCCAGCTGGGCCTG	301
EXON10 R	CCTCAGGCTCACACCCAC	
EXON11+EXON12 F	AAGTGATGGGTTCTTGACTGC	570
EXON11+EXON12 R	AGGAATGCATCACTGCGG	
EXON13 F	AGTCTGGCTGGAGGTGAGG	291
EXON13 R	CACACAGGAGTGCTCAGCG	
EXON14+EXON15 F	GGGAAAACAGGGTGGTGAG	597
EXON14+EXON15 R	GATCTTGCAGCTCCCAGTG	
EXON16+EXON17 F	CGTGACTGCTGTGACTCAGG	604
EXON16+EXON17 R	GCAGAACTCGATGGTGTGG	
EXON18 F	GCCTGGATGATGAAGAGGAG	307
EXON18 R	AACTGAGGCCCAGAGAGAAG	
EXON19+EXON20 F	AAGTGGGACTGTCCAAGGAG	613
EXON19+EXON20 R	TCCCAGATCCTACACCATGC	

PCR, polymerase chain reaction; F, forward; R, reverse.

Transcription experiment of the CLCN7 c.1798-1G>T mutation. RT-PCR performed on the RNA extracted from the father enabled the detection of one band of the expected 560 bp (from exon 17 to exon 22 of the *CLCN7* gene; Fig. 3A, lane 1). However, the same amplification conducted using RNA extracted from the mother and the patient, resulted in two different PCR fragments (Fig. 3A; lanes 2 and 3). In the patient, cloning and sequencing revealed the presence of the normal transcript corresponding to the 560-bp fragment and of two aberrant forms of the transcripts. The majority of transcripts resulted from the entire deletion of exon 20 (c.1798_1883del; Fig. 3B), which was detected in 11 out of the 30 clones selected. The minority of transcripts resulted from the deletion of the first 24 bp of exon 20 (c.1798_1821del; Fig. 3C), which was detected in three out of the 30 clones picked. Similar deletions were also identified in the patient's mother, while no aberrant transcripts were detected in the father.

Discussion

In the present study, the case of a patient with osteopetrosis was investigated. Molecular analysis of the patient revealed compound heterozygous nonsense mutations in *TCIRG1* and a heterozygous splice site mutation in *CLCN7*. Among the three mutations, *TCIRG1* c.909C>A (p.Tyr303X) and *CLCN7* c.1798-1G>T were novel mutations. *CLCN7* encodes the chloride-specific ion channel, CLCN7, which cooperates with the gene product of *TCIRG1*, the a3 subunit of V-ATPase (11). CLCN7 is essential for efficient proton pumping due to its role in neutralizing current, and is involved in the secretion of acid into the resorption lacuna, a specialized acidic compartment for mineral bone matrix degradation (12,13). To the best of our knowledge, this is the first reported case of osteopetrosis that carried the *TCIRG1* and *CLCN7* gene mutations.

In order to determine the effect of *CLCN7* c.1798-1G>T, a transcription experiment was subsequently conducted. The mutations that affect mRNA splicing have been revealed to account for a number of hereditary disorders (14-16). Three splicing mutations of *CLCN7* have been reported in osteopetrosis thus far, including c.916+57A>T, c.1617+6_1617+7delTG and c.2250+1G>A (9,17). The splicing mutation, *CLCN7* c.1798-1G>A, reported in the present study has eradicated the invariant G of the AG splice acceptor site of intron 19.

Table II. PCR primers used for amplification of *CLCN7* gene.

Primer	Sequence (5' to 3')	Size (bp)
Promoter F (rs960467)	GGAAGCCTCCACTCCGACCC	475
Promoter R (rs960467)	GTGATGAGCGACGGCGACCA	
Exon1 F	CGTTGCAGGTCACATGGTC	470
Exon1 R	GCCTCCGAAGACTCCAGAC	
Exon2 F	CGGATCAGTTCTGCTTCCAG	511
Exon2 R	CATGCTGTCACTGCTGTCCT	
Exon3+Exon4 F	TGCTGGGATTGTAGGTGTCA	629
Exon3+Exon4 R	GAGCAGCCTTCTTGGTTACG	
Exon5+Exon6 F	CACACTGGGCCCTTCATAAT	810
Exon5+Exon6 R	TCTGCTCCTCCTGAGGTTGT	
Exon7 F	GTGTCTGCTGCTCTCCTCAG	243
Exon7 R	GCTCCTGAACCAGCAAAGAG	
Exon8+Exon9 F (VNTR in intron 8)	GCTTGGCTGCTGTTTAGCTC	764
Exon8+Exon9 R (VNTR in intron 8)	AAGCCCATCTCCCTGAGTG	
Exon10+Exon11 F	GTGCTGACCCTGCTGTCTCT	797
Exon10+Exon11 R	AGGACCAAGGCCTGACAGA	
Exon12 F	CACTGGCAAGTCCAGAGAGG	559
Exon12 R	GCAGCAACTGTGTGACATCC	
Exon13 F	CCAGTGTGTTTCTCCCCTGT	443
Exon13 R	CTGTGGTTTTTGCCAACAGA	
Exon14 F	ATTGCTCTGCTGGACACCTT	551
Exon14 R	GCAGGGCCTCACTTCCTAC	
Exon15 F (rs12926089, rs12926669)	CAGTGTCCTCCATCAGGGACT	401
Exon15 R (rs12926089, rs12926669)	CTCTGAGATCTGGGTGGACAG	
Exon16 F	CTCCCAACGTGTGCTCTCTC	306
Exon16 R	ATCCTCCTGCCTTGGTCTCT	
Exon17 F	TGAGAACAGGGAGCCTTCTG	432
Exon17 R	AGGTGCGACACTTTTGTCCT	
Exon18+Exon19 F	GGTGACTGTGCCCTCTGC	730
Exon18+Exon19 R	CCCAGAAACCCTGAGCCTAC	
Exon20+Exon21 F	CTGTGAGCCTCCAAACAGC	717
Exon20+Exon21 R	GTCCACACAGCCCTCCAT	
Exon22+Exon23 F	AGGCTGGTGTGAGCAGGTAG	638
Exon22+Exon23 R	GCCCCTTGACTTCAGCTCTA	
Exon24+Exon25 F	CTGAAGTCAAGGGGCTGAGG	806
Exon24+Exon25 R	AGACCACTGCCCACAACAG	

PCR, polymerase chain reaction; CLCN7, chloride channel 7 gene; F, forward; R, reverse.

The most common consequence of splicing mutations is exon skipping, followed by the activation of aberrant splice sites and intron retention (18,19). In the present study, two aberrant patterns of transcripts were detected with different proportions. The most common transcript was the skipping of exon 20 (c.1798_1883), which was predicted to cause a frameshift and a premature termination codon (p.Leu601GlyfsX13). The least common was the activation of aberrant splice sites (c.1798_1821 deletion), which was predicted to cause the in-frame deletion of eight amino acid residues (p.Gly600_Gln607del). Notably, aberrant splicing is predicted to affect only the C-terminal cytosolic portion of the ClCN7 protein and not its transmembrane portion.

Mutations in the *CLCN7* gene have been demonstrated to be involved in the pathogenesis of various forms of osteopetrosis since 2001 (12). Heterozygous mutations in *CLCN7* may lead to ADO II, which is associated with less severe clinical features and late onset. To further investigate whether the *CLCN7* c.1798-1G>T mutation was pathogenic, a thorough biochemical and radiological examination was performed on the 29-year-old mother who harbored the heterozygous *CLCN7* splicing and *TCIRG1* p.Tyr303X mutations. However, no abnormal clinical, biochemical or radiological manifestations were observed. Previous studies have reported several polymorphisms in the *CLCN7* gene, including rs960467,

Table III. Molecular analysis of the *TCIRG1* and *CLCN7* genes.

Subject	Nucleotide change of *TCIRG1*[a]	Nucleotide change of *CLCN7*[b]	Genotypes[c]			
			rs960467	rs12926089	rs12926669	Intron 8 VNTR
Patient	c.909C>A c.2008C>T	c.1798-1G>T	G/A	G	T	Three repeat units
Mother	c.909C>A	c.1798-1G>T	G/A	G	T	Three repeat units
Father	c.2008C>T	-[d]	G	G	T	Three repeat units

[a]Accession number of the *TCIRG1* cDNA, NM_006019.3. The numbering used starts with nucleotide +1 for the A of the ATG-translation initiation codon. [b]Accession number of the *CLCN7* cDNA, NM_001287.5. The numbering used starts with nucleotide +1 for the A of the ATG-translation initiation codon. [c]rs960467, *CLCN7* NG_007567.1:g.4907G>A; rs12926089, *CLCN7* NM_001287.5:c. 1252G>A, NP_001278.1:p.Val418Met; rs12926669, *CLCN7* NM_001287.5:c.1245T>C, NP_001278.1:p.Ile415=; Intron 8 VNTR, a 50-bp VNTR residing in intron 8 of *CLCN7*. [d]No nucleotide change. VNTR, variable number tandem repeat.

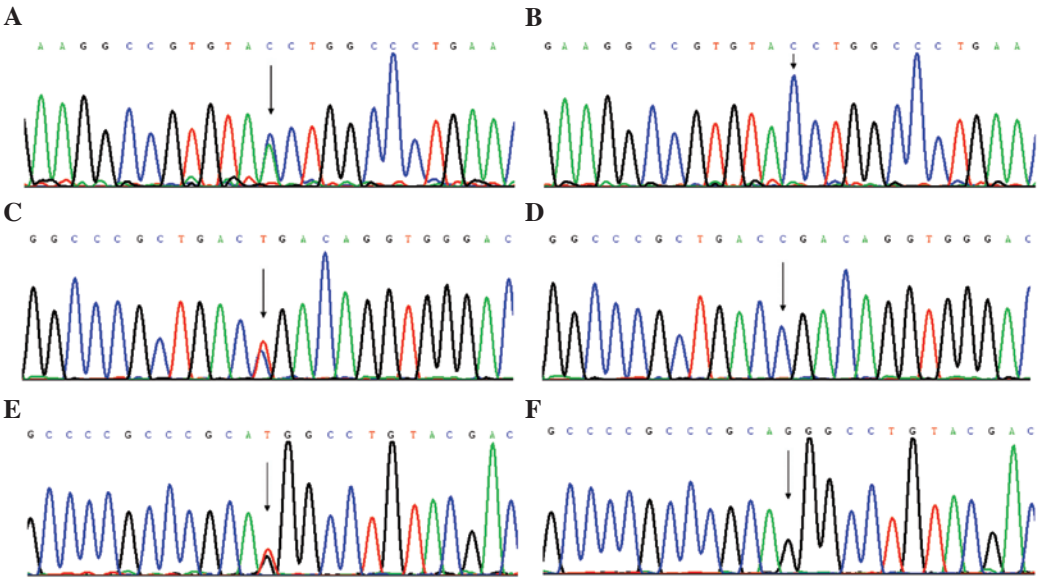

Figure 2. Molecular analysis of the *TCIRG1* and *CLCN7* genes. A genetic analysis showing (A) the *TCIRG1* c.909C>A (p.Y303X), (C) *TCIRG1* c.2008C>T (p.R670X) and (E) *CLCN7* c.1798-1G>T mutations in the proband and (B, D , and F) the corresponding sequencing of the normal control. The arrow indicates the site of heterozygous mutation.

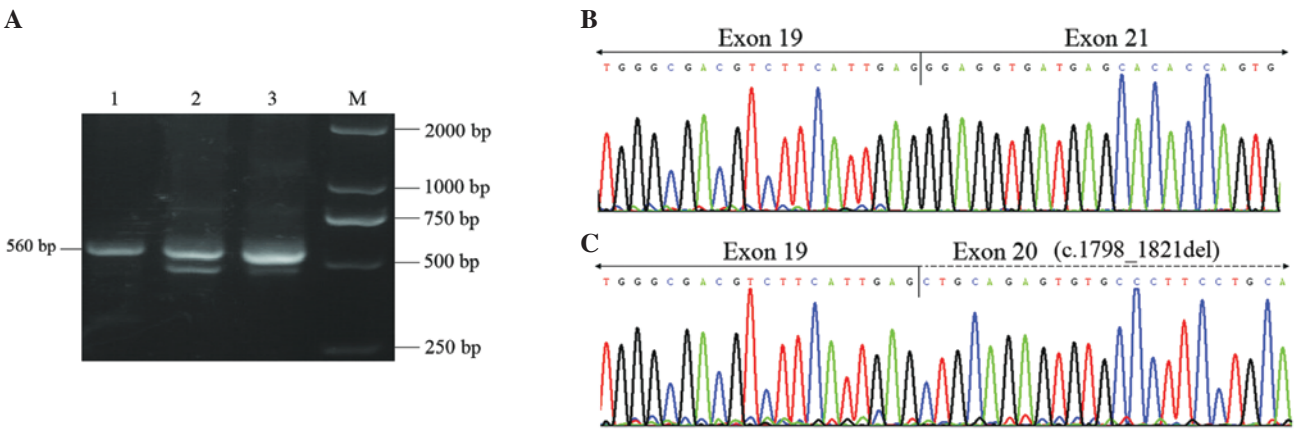

Figure 3. Transcription experiment of the *CLCN7* c.1798-1G>T mutation (A) agarose-gel (1%) electrophoresis of *CLCN7* RT-PCR products. RT-PCR performed on the RNA extracted from the father allowed detection of one band of the expected 560 bp, from exon 17 to exon 22 of the *CLCN7* gene (lane 1). The same amplification conducted using RNA extracted from the mother (lane 2) and the proband (lane 3) resulted in two different PCR fragments. M, 2,000-bp DNA ladder. Cloning and sequencing of the pMD 18-T vectors containing *CLCN7* RT-PCR products revealed two aberrant splicing forms, including (B) c.1798_1883 (exon 20) deletion and (C) c.1798_1821, the first 24 bp of the exon 20 deletion. The horizontal solid and dashed arrows indicate the areas of the two different exons. RT-PCR, reverse transcription polymerase chain reaction.

rs12926089 (Val418Met) and rs12926669, and a VNTR in intron 8, which were associated with the penetrance of the ADO phenotype and the variation in bone mineral density (20-22). To determine whether these polymorphisms were associated with the different severities of the osteopetrosis in the pedigree, the SNPs and the VNTR were genotyped. No difference was revealed between the patient and the mother. The *CLCN7* c.1798-1G>T mutation appeared to be a non-pathogenic variation in the present case, although it was associated with aberrant splicing. However, completely excluding the pathogenicity of *CLCN7* c.1798-1G>T is inappropriate due to the incomplete penetrance of ADO II. The factors involved in the phenotypic variability remain unknown.

According to previous studies, the compound heterozygous nonsense mutations of *TCIRG1* were enough to cause malignant osteopetrosis (5-7). Biallelic mutations in the *TCIRG1* gene are well known to be responsible for ARO, and the absence of symptoms in the mother together with the characteristic ARO phenotype (early postnatal onset, generalized increased bone density and severe clinical course, including anemia, hypodontia and visual impairment) of the patient may indicate a diagnosis of classic *TCIRG1*-dependent ARO in this case.

In conclusion, a patient with ARO was studied. The compound heterozygous mutations, c.909C>A (p.Tyr303X) and c.2008C>T (p.Arg670X), in *TCIRG1* and a heterozygous splicing mutation, c.1798-1G>T, in the *CLCN7* gene were identified in the patient. Among the three mutations, *TCIRG1* c.909C>A (p.Tyr303X) and *CLCN7* c.1798-1G>T were novel mutations. This study highlights that *TCIRG1* and *CLCN7* should be sequenced in order to gain a comprehensive molecular diagnosis of osteopetrosis.

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