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SHORT COMMUNICATION

Novel mutation of *TCIRG1* and clinical pictures of two infantile malignant osteopetrosis patients

Ping Yuan · Zhihui Yue · Liangzhong Sun · Weijun Huang · Bin Hu · Zhiyun Yang · Yuelin Hu · Hua Xiao · Hui Shi · Qing Zhou · Yiming Wang

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Abstract Infantile malignant osteopetrosis (IMO) (OMIM 259700) is a lethal autosomal recessive disease. The underlying gene in most IMO patients is *TCIRG1*. This codes for the TCIRG1 protein involved in the cellular proton pump, which is highly expressed on surfaces of osteoclasts. We have characterized a family comprising two affected siblings born to healthy parents. The sister and her younger brother both presented classical X-ray images of IMO at 17 h and 16 weeks, respectively, after birth, and both died after the appearance of fever and flu-like symptoms months later. Radiographs revealed normal bone density in both parents. Mutation detection of the *TCIRG1* gene was performed in the boy and the parents. The novel mutation c.242delC (p.Pro81ArgfsX85) and the known mutation c.1114C>T (p.Gln372X) were both identified in the boy. Both mutations are predicted to introduce premature stop codons, with deletion of 666 amino acids from the C terminus of the TCIRG1 protein of one allele and 459 from the other. Both mutations involve loss of part or the whole of the ATPase V0-complex domain of the protein. The father carries the c.242delC (p.Pro81ArgfsX85) mutation and the mother the c.1114C>T (p.Gln372X). Our findings provide new data for pre- and post-natal genetic diagnosis and identification of heterozygous carriers of the disease.

Keywords Osteopetrosis · Infantile malignant osteopetrosis · *TCIRG1* · Novel mutation · Proton pump

P. Yuan · W. Huang · B. Hu · Y. Wang (✉)
Department of Medical Genetics, Center for Genome Research, Zhongshan School of Medicine, Sun Yat-Sen University, 74 Zhongshan Road II, Guangzhou 510080, People's Republic of China
e-mail: ywzhong@hotmail.com

Z. Yue · L. Sun
Department of Paediatrics, First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, People's Republic of China

Z. Yang
Department of Radiology, First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, People's Republic of China

Y. Hu
Department of Radiology, Guangzhou Women and Children's Medical Center, Guangzhou 510623, People's Republic of China

H. Xiao
Department of Neurological Rehabilitation, Guangzhou Women and Children's Medical Center, Guangzhou 510120, People's Republic of China

H. Shi
Department of Paediatrics, Guangzhou Women and Children's Medical Center, Guangzhou 510623, People's Republic of China

Q. Zhou
Department of Paediatrics, The People's Hospital of Yudu County, Ganzhou 342300, People's Republic of China

Introduction

Infantile malignant osteopetrosis (IMO) (OMIM 259700) is a rare, autosomal recessively inherited disorder and the most severe type of osteopetrosis. Affected individuals usually die in infancy or the first decade of life [1], bone marrow failure and subsequent complications usually being the direct causes of death [2]. To date, seven genes are believed to be independently responsible for non-syndromic forms of autosomal recessive osteopetrosis (ARO) [3–10], three of which, *TCIRG1*, *CLCN7*, and *OSTM1*, are reported to underlie this form of the disease [3, 4, 7, 8]. Errors in the T-cell immune regulator 1 gene (*TCIRG1*; *ATP6i*; Entrez

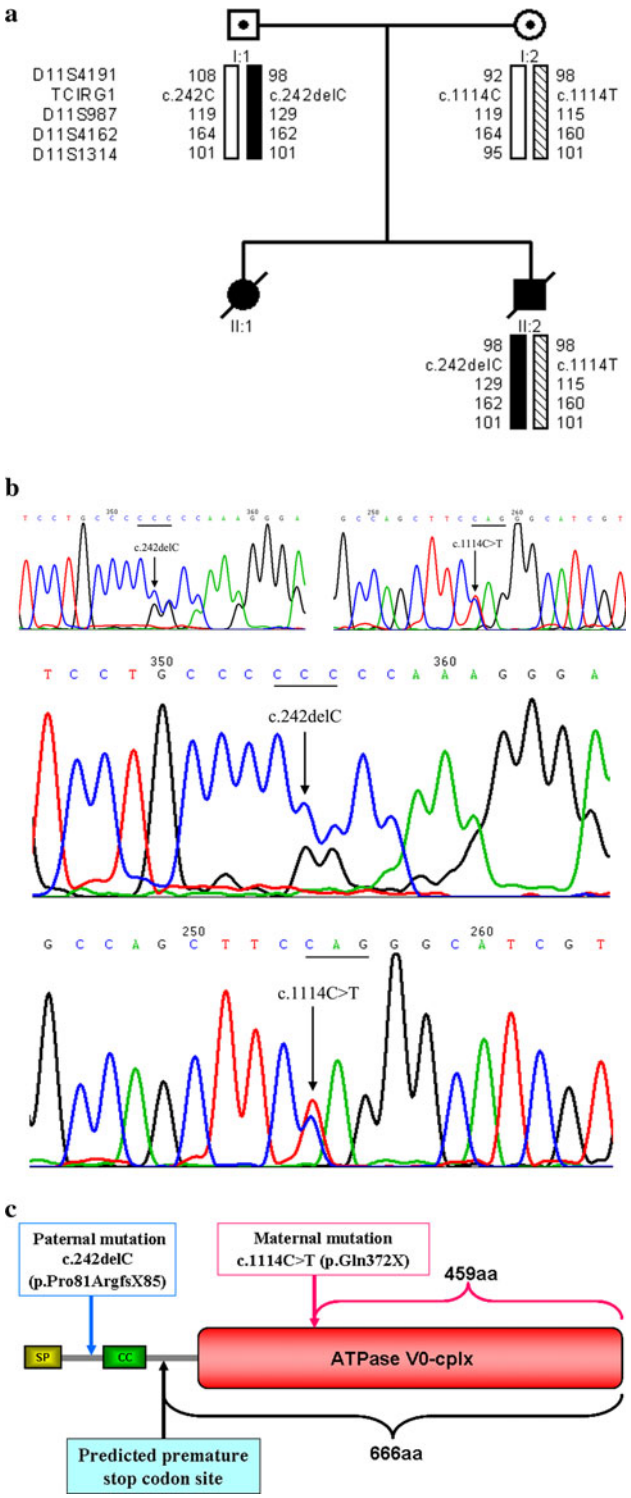

Fig. 1 Genetic Analyses of the family. **a** Pedigree of the family and haplotype analysis. Markers used in haplotype construction are listed at the *upper left*. *Black bars* depict the paternal disease haplotype and *hatched bars* the disease haplotype of the mother. The son (II:2) inherited both mutations from his parents and hence the heterozygous compound mutation. **b** Partial nucleotide sequences of the *TCIRG1* gene. *Top panel*: Forward sequences in the boy (II:2). *Arrows*: Point to the c.242delC (p.Pro81ArgfsX85) (*left*) and c.1114C>T (p.Gln372X) (*right*) mutations. *Middle panel*: Forward sequence in the father (I:1); the *arrow* indicates the c.242delC (p.Pro81ArgfsX85) mutation. *Bottom panel*: Forward sequence in the mother (I:2), the *arrow* depicts the c.1114C>T (p.Gln372X) mutation. Mutated codons are *underlined*. **c** The wild-type TCIRG1 protein has 830 amino acids and contains a signal peptide motif (SP), a coiled coil motif (CC), and an ATPase V0-complex domain (ATPase V0-cplx). *Arrows* indicate the locations of paternal and maternal mutations and the premature stop codon introduced by the paternal mutation. The paternal mutation, c.242delC (p.Pro81ArgfsX85), is predicted to delete 666 amino acids from the C terminus of the TCIRG1 protein. The maternal mutation, c.1114C>T (p.Gln372X), occurred in the ATPase V0-complex domain and is predicted to delete 459 amino acids from the C terminus

Gene ID: 10312) is believed to be responsible for about half of IMO patients [3, 4, 11–13]. This gene codes for the human a3 subunit of the vacuolar-type H^+-adenosine triphosphatase (V-ATPase) (Swiss-Prot ID: Q13488). It is composed of two functional domains: a water-soluble V1 and a membrane-embedded V0 subunit, which direct ATP hydrolysis, driving the proton pump and subsequent acidification of the extracellular compartment [14, 15]. The a3 isoform, encoded by the *TCIRG1* gene, is part of the V0 complex and highly expressed on the surfaces of osteoclasts [15, 16]. The enzyme V-ATPase plays an important role in bone remodeling and development [15]. Apart from a case involving cord blood transplantation reported from Taiwan [17], there have been no data regarding *TCIRG1* mutations from the Chinese population. In this paper, we report our clinical and genetic findings of a family from China with two infantile malignant osteopetrosis patients.

Subjects and methods

The family, from Guangdong Province China, is comprised of two patients and their unaffected parents from a non-consanguineous marriage (Fig. 1a). Detailed clinical and laboratory examinations were performed on the patients, including X-ray examinations. IMO was diagnosed independently by two pediatricians and two radiologists according to the criteria defined by Stark et al. [18]. Physical examination and hand X-rays were also performed on the parents. Fifty healthy, unrelated volunteers were recruited as controls. Mutation of the *TCIRG1* gene was detected by sequencing the polymerase chain reaction (PCR) products of all coding exons (exons 2–20) and their flanking intronic regions, using primers designed by Oligo 6.0 (http://www.oligo.net/downloads.html). (Primer sequences and PCR conditions are available on request.) The results were compared with those retrieved from the UCSC Genome Browser on Human Mar 2006 Assembly (http://genome.ucsc.edu). Mutation nomenclature recommended by den Dunnen and Antonarakis (http://www.hgvs.org/mutnomen/) [19] was

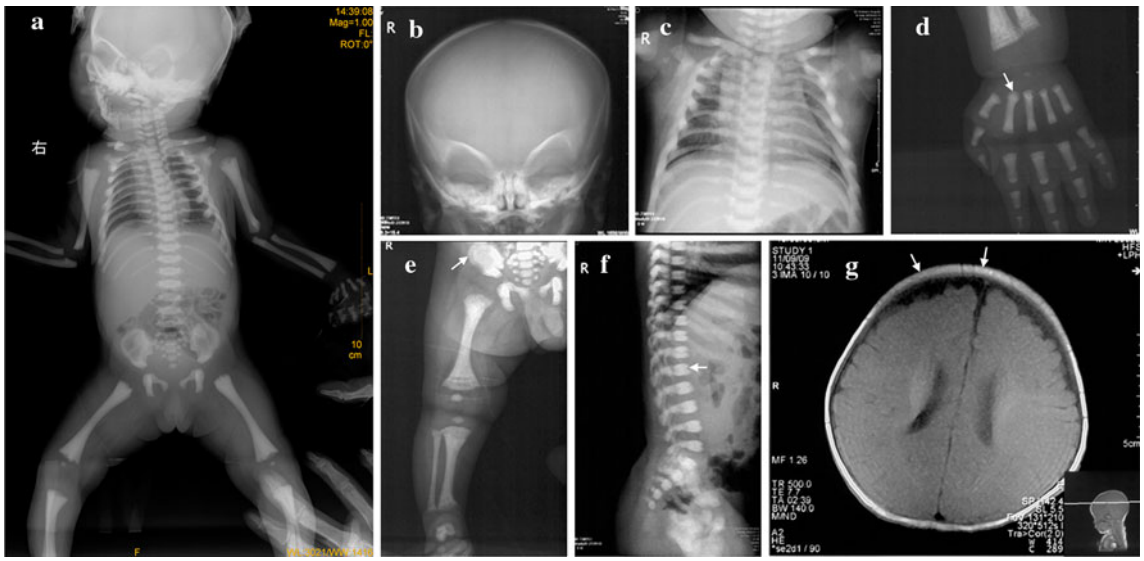

Fig. 2 **a** Skeleton of the sister showing increased bone density. Skull (**b**) and chest (**c**) of the brother showing sclerotic changes in the base of the skull, dense mastoid and small pituitary fossa, the clavicle, ribs, and scapula bones. X-ray film of the right hand (**d**) and leg (**e**) of the brother showing sclerosis of carpal bones, phalanges, and long bones resulting in obliteration of the marrow cavity and the "bone in bone" appearance of the metacarpal bones (*arrow*) and iliac wings (*arrow*). **f** The brother's vertebral column showing sclerosis of vertebral endplates resulting in a "sandwich vertebrae" appearance (*arrow*). **g** Skull MRI of the brother showing the subarachnoid space of fronto-temporal region and inter-hemispheric broadening (*arrows*)

adopted with +1 corresponding to the A of the ATG translation initiation codon of the GenBank cDNA sequence NM_006019. Haplotypes were constructed with microsatellite markers (D11S4191, D11S987, D11S4162, and D11S1314; Applied Biosystems, Foster City, CA) spanning the *TCIRG1* locus and the mutations identified in the family members by Cyrillic 2.1 (http://www.cyrillicsoftware.com/). CLUSTAL X (1.81) [20] was used to compare the human TCIRG1 amino acid sequence (*Homo sapiens* NP_006010) with five orthologues. The NCBI Open Reading Frame Finder (ORF Finder; http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify the consequent changes of c.242delC in the osteoclast-specific transcript (OC116) (NP_006010). The information for motifs and domains of the TCIRG1 wild protein was obtained from the Interpro (http://www.ebi.ac.uk/interpro/) and Human Protein Reference Database [21].

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Sun Yat-Sen University.

Results

Clinical characteristics of patients

The sister

The sister was born by full-term vaginal delivery. Her Apgar scores were 3, 4, and 7 at 1, 5, and 20 min, respectively. The girl was referred to the hospital 17 h after birth because of groaning, shortness breath, and low response immediately after birth. Physical examination on admission demonstrated irritation, anterior fontanelle flatness, and softness (1.5 × 1.0 cm), hypotonia with decreased tone in the cervical muscle and limbs, and rough breathing sounds bilaterally with no rales. The liver margin was 0.5 cm below the right costae, and the spleen was not palpable at physical examination. A complete blood cell count revealed no significant abnormality. Serum calcium was very low at 0.47 mmol/l. X-ray examination revealed whole skeleton sclerosis and obliteration of marrow cavities, especially in the long bones (Fig. 2a). Thoracic and lumbar vertebrae were very radio dense and had the "sandwich vertebra" appearance. Chest X-ray revealed pneumonia. Infantile malignant osteopetrosis, neonatal aspiration pneumonia, hypocalcemia, and hypoxic-ischemic encephalopathy were diagnosed. Antibiotics, diuretics, and calcium were administrated. However, the parents gave up treatment 3 days later. She subsequently developed fever and flu-like symptoms, which lasted a week, and the patient died at the end of the week at the age of 24 weeks. Unfortunately, no blood sample was retained for genetic analysis.

The brother

The brother was born 2 years later by cesarean section at full term. The boy was referred to the hospital at the age of 16 weeks due to pallor, failure to thrive for 2 weeks, and

bulging of the anterior fontanelle for 5 days. Physical examination on admission revealed growth retardation (height 48, 51, and 56.5 cm at 0, 1, and 3 months, respectively), bulging anterior fontanelle (3 × 3 cm), and hypotonia with decreased tone in cervical muscle and limbs. The liver margin was 1 cm below the right costae, and the spleen was not palpable at physical examination. He showed no inclination to laugh or any interest in watching people and did not react to sound stimulations. Hematological tests showed slight anemia (hemoglobin 101 g/l; normal range: 110–120 g/l) [22], an increased percentage of reticulocyte (6.12%; normal range: 0.5–1.5%) [22], thrombocytopenia (platelet 97 × 10^9/l; normal range: 100 × 10^9–300 × 10^9/l) [22], and marginal leukocyte count (13 × 10^9/l; normal range: 11 × 10^9/l–12 × 10^9/l) [22]. The blood calcium level and visual evoked potentials were not assessed due to parental non-compliance. Skeletal X-ray examinations revealed generally increased bone density, sclerosis, and bone marrow cavity obliteration, especially in the long bones (Fig. 2b–f). X-ray images of metacarpal and iliac bones revealed surrounding, unevenly thickened, and thinned bone tissue, which gave a "bone-in-bone" appearance (Fig. 2d, e). Thoracic and lumbar vertebrae were very radio dense and showed alternating bands ("sandwich vertebrae") (Fig. 2f). Auditory evoked potentials in the brain stem indicated mild abnormal hearing in the right ear. Abdominal ultrasound showed the liver and spleen to be of normal size, but examination of the skull by ultrasound and magnetic resonance imaging revealed hydrocephalus (Fig. 2g). The electroencephalogram was normal. Infantile malignant osteopetrosis complicated with hydrocephalus and growth retardation was diagnosed. The parents refused admission, and the boy was treated at the out-patient department with furosemide to reduce the intracranial pressure. He started laughing out loud after 5 days of treatment and watching people after 7 days of treatment. The anterior fontanelle became flat (3 × 3 cm). However, hypotonia with decreased tone in the cervical muscle and limbs and the non-responsiveness to sound stimulation continued. After 9 days of management, the parents refused further treatment. The boy developed fever and flu-like symptoms, and died at 19 weeks of age.

Physical and X-ray examinations showed both parents were healthy, with normal bone density in the hands.

Mutation detection, haplotype analysis, and bioinformatics analysis

Mutation of *TCIRG1* was screened for in the brother and both parents. Direct sequencing of both DNA strands in the boy revealed the compound heterozygous mutations of c.242delC (p.Pro81ArgfsX85) and c.1114C>T (p.Gln372X) (Fig. 1b). The former, c.242delC (p.Pro81ArgfsX85), is a novel mutation, but c.1114C>T (p.Gln372X) has been reported previously [11]. Sequencing of the parental DNA showed that the father was the carrier of c.242delC (p.Pro81ArgfsX85) and the mother of c.1114C>T (p.Gln372X) (Fig. 1b). Haplotype construction revealed that the boy had inherited the c.242delC mutation and the haplotype from his father and the c.1114C>T mutation and the haplotype from his mother (Fig. 1a, b). The paternal mutation, c.242delC (p.Pro81ArgfsX85), occurred in exon 4, causing a reading frameshift and is predicted to introduce a premature stop codon at position 165, truncating 666 amino acids at the C terminus of the TCIRG1 protein (Fig. 1c). As the truncation wipes out the entire ATPase V0-complex domain of the protein, which is involved in the pumping of protons across membranes, this mutation is predicted to result in total loss of proton pumping function. The maternal mutation, c.1114C>T (p.Gln372X), occurred in exon 10 of the ATPase V0-complex domain itself. This mutation introduces a premature stop codon at the site and results in loss of the 459 C-terminal amino acids from the TCIRG1 protein, deleting part of the ATPase V0-complex domain (Fig. 1c). Alignment of the TCIRG1 protein across species showed highly conserved proline and glutamine at the 81st and 372nd positions, respectively, in human, chimpanzee, dog, rat, mouse, and zebrafish (data not shown). Both mutations were absent from 100 control human chromosomes, suggesting they are not normal polymorphisms.

Discussion

Clinical presentations of osteopetrosis are very heterogeneous, with infantile malignant osteopetrosis the most severe subtype. Clinically, the disease is marked by increased bone density coupled with multisystem manifestations originating from dysfunction of the proton pump activities of osteoclasts. Reportedly most patients die of infection because of obliteration of the bone marrow in infancy or early childhood [2, 8, 23]. In the present study, both patients exhibited classical pictures of osteopetrosis on X-ray examination. X-ray examination performed immediately after birth of the sister revealed bone marrow obliteration, although her blood count was normal at the time. This may be explained by the blood test being too early to detect changes in marrow function. Her serum calcium level (0.47 mmol/l) was remarkably low (1.75–3 mmol/l), but she did not show hyperspasmia. The involvement of both respiratory and nervous systems was obvious. The patient finally died of manifestations indicating infection at 24 weeks. The younger brother's symptoms were first noticed by the parents 14 weeks after

birth. A complete blood count showed slight anemia and thrombocytopenia, and an increased percentage of reticulocyte, which probably reflected the impairment of medullary hematopoiesis because of sclerosis, and obliteration of bone marrow cavities, as well as the beginning of extramedullary hematopoiesis at that time. The boy also presented with remarkable symptoms of the nervous system and had growth retardation and hydrocephalus. Unfortunately, the parents refused further treatment. At 19 weeks of age, he also died of symptoms indicating infection.

Genetically osteopetrosis is very heterogeneous, with autosomal dominant, autosomal recessive, and X-linked modes of inheritance. Infantile malignant osteopetrosis shows an autosomal recessive inheritance pattern for the *TCIRG1* gene responsible in most IMO patients. *TCIRG1* is known to have two variant transcripts: OC116 (NP_006010), consisting of all 20 exons, but the translation starts from exon 2; and TIRC7 (NP_006044), which is spliced from exon 5 to exon 20, but with retention of the entirety of intron 5 [24, 25]. OC116 is predominantly expressed in osteoclasts, whereas TIRC7 is expressed in various tissues and thought to be essential in T-cell activation [24, 25]. In the presented cases, OC116 is the transcript expected to be most affected by the compound heterozygous mutations.

Although mutations in *TCIRG1* are generally considered to be recessive, heterozygous mutations have been reported both in patients and healthy carriers [3, 11, 13], a discrepancy previously explained as due to failure to detect an additional mutation in affected individuals [3, 11, 13]. In our family, the boy carried the heterozygous compound mutations, whereas the parents are each heterozygous carriers of one or the other of the mutations. The parents are both apparently healthy, and X-ray examination of their hands showed normal bone density. These observations are consistent with recessive causation of the disease. Although no genetic data are available for the deceased sister, it is reasonable to assume she probably had the same compound heterozygous mutations as her brother. Both mutations identified in the family are predicted to delete part or the whole of the ATPase V0-complex domain and retain the 5′ transcripts. The partial 5′ transcripts are supposedly degraded by the mechanism of "nonsense mediated mRNA decay" [26]. The mutations, therefore, wipe out the entire protein product and result in the total loss of the proton pumping function of the gene.

Chloride channel 7 gene (*CLCN7*) is another gene responsible for IMO. The encoded chloride channel 7 protein (CLC-7) (Swiss-Prot ID: P51798) is believed to mediate chloride conductance across the ruffled border of the osteoclast and membranes of late endosomes and lysosomes, thereby contributing to the acidification of the microenvironment [2, 7, 27]. A whole spectrum of *CLCN7*-related phenotypes of osteopetrosis have been reported, from IMO, the most severe subtype to an intermediate phenotype to an apparently normal phenotype, with the modes of transmission being autosomal dominant or recessive, respectively [7, 28, 29]. Also from the Chinese population, Zhang et al. [30] reported a family in which the Glu798FS mutation caused typical type II autosomal dominant osteopetrosis in one family member, but it was completely asymptomatic in four other family members. Despite the heterogeneity in the phenotype-genotype correlation, *CLCN7*-related IMO remains autosomal recessive [7, 29], which implies the dosage effects of the gene. The likely explanation for the diversity of the *CLCN7*-related phenotypes may lie in epistasis, i.e., interactions of genes and/or the existence of modifier genes, apart from the differences in the mutation itself [2, 30, 31].

In summary we report two IMO patients from China. The patients exhibited typical bone changes of osteopetrosis, predominantly neural symptoms, and died of manifestations indicating infection. One novel and one known mutations have been identified in the *TCIRG1* gene. Our findings provide new data for pre- and post-natal diagnosis of the disease and for identification of heterozygous carriers.

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