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A Homozygous Mutation in 5′ Untranslated Region of *TNFRSF11A* Leading to Molecular Diagnosis of Osteopetrosis Coinheritance With Wiskott-Aldrich Syndrome

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Summary: Wiskott-Aldrich syndrome (WAS) and osteopetrosis are 2 different, rare hereditary diseases. Here we report clinical and molecular genetics investigations on an infant patient with persistent thrombocytopenia and prolonged fever. He was clinical diagnosed as osteopetrosis according to clinical presentation, radiologic skeletal features, and bone biopsy results. Gene sequencing demonstrated a de novo homozygous mutation in 5′-untranslated region of *TNFRSF11A*, c.−45A > G, which is relating to osteopetrosis. Meanwhile, a hemizygous transition mutation in WAS gene, c.400G > A diagnosed the infant with WAS. This is the first clinical report for the diagnosis of osteopetrosis coinheritance with WAS in a single patient.

Key Words: Wiskott-Aldrich syndrome, osteopetrosis, next-generation sequencing, *TNFRSF11A*, untranslated region

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BACKGROUND

Osteopetrosis is a genetic disorder characterized by an increased bone mass due to defects in osteoclast formation and function. It mainly includes 2 forms: autosomal recessive osteopetrosis (ARO), also known as infantile malignant osteopetrosis for its early onset and high mortality; and autosomal dominant osteopetrosis.[1] The genetic basis of osteopetrosis has now been extensively studied: mutations in *TCIRG1*, *CLCN7*, *OSTM1*, *SNX10*, and *PLEKHM1* induce osteoclast-rich ARO (in which osteoclasts are abundant but functionally impaired), whereas mutations in *TNFSF11* and *TNFRSF11A* induce osteoclast-poor ARO.[2] *TNFSF11* encodes tumor necrosis factor ligand superfamily member 11 (better known as RANKL), produced mainly by osteoblasts and stromal. *TNFRSF11A* encodes tumor necrosis factor receptor superfamily member 11A (RANKL cognate receptor, RANK) mainly expressed by osteoclast precursors and mature osteoclasts. It is now well established that loss-of-function mutations in *TNFRSF11A* and *TNFSF11* involved in the RANK/RANKL signaling pathway lead to ARO.[3]

Wiskott-Aldrich syndrome (WAS), a rare X-linked recessive disease, is caused by mutations of the WAS protein (WASP) gene and characterized by microthrombocytopenia, eczema, recurrent infections, autoimmune phenomena, and increased incidence of malignancy.[4] As an important regulator of the actin cytoskeleton, WASP, expressed by all hematopoietic cell lineages and precursor cells, plays an important role in hematopoietic and immune cell functions including effective migration, phagocytosis, and immune synapse formation. Loss of WASP activity leads to immunodeficiency, autoimmunity, and microthrombocytopenia.[5] To date, over 300 kinds of mutations involved with WAS gene have been described. Most missense mutations are located in exons 1 to 4, whereas splice-site mutations predominantly in introns 6 to 10. Typical WAS was diagnosed depending on the clinical presentations and gene mutation analysis.[4,6]

Although the rare genetic diseases osteopetrosis and WAS have been occasionally described, the coinheritance of both osteopetrosis and WAS in 1 patient, to our best knowledge, has never been reported before. In this report, a case was radiologically and clinically diagnosed as infant osteopetrosis correlated with a single nucleotide transition in the 5′ untranslated region (5′ UTR) of the *TNFRSF11A* gene. Furthermore, this infant was genetically diagnosed with WAS, a missense mutation in exon 4 of *WAS* gene detected by the next-generation sequencing (NGS) analysis. Our results indicate that autosomal recessive and X-linked recessive diseases can occur simultaneously. These clinical findings together with the results of exome sequencing throw some light on the diagnosis of rare diseases.

Case Presentation

Clinical Data

A 1.5-month-old male infant was admitted to our hospital due to persistent thrombocytopenia and prolonged fever for 10 days. The patient had been physically examined at birth and reported as grossly normal. His platelet counts

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Informed consent was obtained from the patient's parents according to the Helsinki Declaration. The study protocols were approved by the Ethics Committee of Anhui Provincial Children's Hospital.

We confirm that the patient (families) have given their consent for the publication of this case report and any accompanying images.

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TABLE 1. Blood Examinations

	Routine Blood Test				Immunoglobulin			Biochemical Examination					
Items	**WBC (10^9/L)**	**Hb (g/L)**	**PLT (10^9/L)**	**MPV (fL)**	**IgM (g/L)**	**IgA (g/L)**	**IgG (g/L)**	**AST (U/L)**	**ALT (IU/L)**	**LDH (IU/L)**	**GGT (IU/L)**	**Ca^{2+} (mmol/L)**	**CRP (mg/L)**
Value	18.59	74	50	10.8	0.14	0.46	16.48	105	134	367	709	2.17	59.2
Age-matched normal values	8-12.5	120-170	100-300	7-11	0.23-0.91	0.13-0.35	0.23-0.91	0-60	0-60	80-285	0-50	2.2-2.6	0-8
Date of test	October 14, 2016				October 14, 2016			October 14, 2016					

ALT indicates alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein; GGT, gamma-glutamyltransfera; HB, hemoglobin; IG, immunoglobulin; LDH, lactate dehydrogenase; MPV, mean platelet volume; PLT, Platele; WBC, white blood cell.

and mean platelet volume (MPV) was normal at birth. Furthermore, his parents denied any positive family history for bleeding disorders or hematologic malignancies.

To evaluate the disease severity and to identify the causes, comprehensive physical examination and necessary laboratory tests were performed. The infant's physical examination was unremarkable except for rales in the right middle lobe and the skull deformities of cephalus quadratus. Neither obvious hearing difficulty and visual disturbances nor eczematous skin lesions were detected. His routine blood test revealed leukocytosis, anemia, and thrombocytopenia with normal MPV (Table 1). Then the bone marrow aspirate was performed to exclude the possibility of leukemia (Fig. 1A). Skeletal radiography was carried out as the skull deformities of cephalus quadratus were usually associated with the dry tap of bone marrow aspiration.[7] Radiographs showed a generalized increase in bone mass density, a typical marker of osteopetrosis (Figs. 1B, C). In addition, bone biopsy shows a significant decrease in osteoclasts and an increased number of cancellous substances in the iliac crest of this patient (Figs. 1D, E).

Serologic tests for immunoglobulins (Igs) revealed a decrease in IgM and an increase in IgG and IgA concentration (Table 1). Immunophenotyping of lymphocytes from peripheral blood showed a low level of both $CD3^-CD16^+CD56^+$ NK and

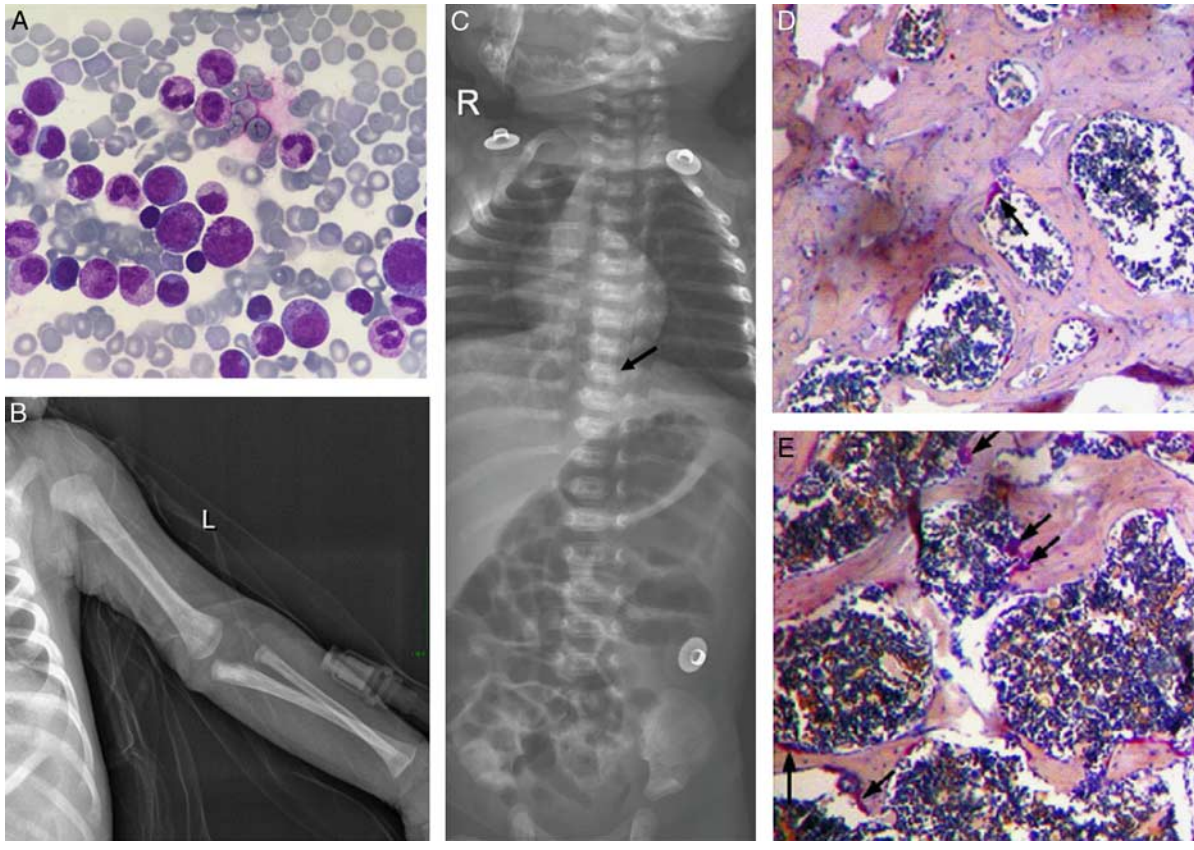

FIGURE 1. Skeletal radiographs, bone marrow smear, and bone biopsy. A and B, radiographs show a diffuse increase in bone density of vertebrae and ribs, and vertebral endplates are thickened (sandwich vertebrae sign, arrowhead). These reveal the classical osteopetrotic phenotype in this patient. C, The bone marrow cytologically shows myeloproliferation is active, megakaryocytes are scarce, and no blasts are observed. D and E, Bone biopsy demonstrated that osteoclasts (arrowhead) were significantly reduced, and cancellous substance increased in the iliac crest in this patient (D), as compared with the healthy control (E).

TABLE 2. Immunophenotyping Results

Cell Type	CD (Specify Markers)	Percentage	Age-matched Normal Percentage Values	Absolute Values ($\times 10^9$/L)
B-lineage	$CD3^-/CD19^+$	2.4	9.02-14.1	0.17
Lymphocyte (total)	$CD3^+$	81.7	61.7-77	6.01
Helper/inducer	$CD3^+CD4^+$	33.7	25.8-41.6	2.48
Cytoxic/suppressor	$CD3^+CD4^+/CD3^+CD8^+$	1.5	0.9-1.9	0.11
Natural killer cells	$CD3^-CD16^+CD56^+$	6.7	10.4-19.78	0.49

$CD3^-CD19^+$ B lymphocytes by Flow Cytometry (FC500) (Table 2, Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JPHO/A360). Comprehensive analysis of metabolic disease ruled out common metabolic conditions. The further biochemic investigation suggested that hypocalcemia and subsequent liver defects were established (Table 2). Additional etiologic diagnosis of cytomegalovirus (CMV) infection was confirmed by the positive results of CMV-antibodies and CMV-DNA (3.22×10^4 copies).

On the basis of the clinical presentation and ancillary findings, this patient was primarily diagnosed with osteopetrosis, thrombocytopenia, bronchopneumonia, CMV infection, and common variable immunodeficiency. Meanwhile, genetic analysis for the detection of inherited platelet disorders and osteopetrosis was investigated immediately using NGS. Because of the normal platelet volume and no skin changes, WAS was not initially taken into consideration. Initial treatment included effective anti-infection therapy and usual supportive care. Infection was rapidly controlled as the body temperature decreased back to normal and rales disappeared. Intravenous immunoglobulin (1 g/kg for 2 d) and platelet transfusions were given for the persistent thrombocytopenia. After hospital discharge, the patient had recurrent infections and gastrointestinal bleed and died of pneumonia when he was still on the transplant waiting list at the age of 3 months.

Investigations

Considering that this infant presented with typical radiologic skeletal features of osteopetrosis, 7 identified pathogenicity genes of osteopetrosis was analyzed by the NGS as previously recommended.[2] Because of the patient's persistent thrombocytopenia and no response to conventional therapy, 71 candidate genes related to inherited platelet disorders were analyzed.[8] His parent's blood samples were also sent to confirm the sources of mutation.

NGS and DNA sequence analysis was used in this case. Briefly, the sheared genomic DNA, sheared by sonication, was then hybridized with a NimbleGen probe capture array. The array covered about 400 genes including all the thrombocytopenia and osteopetrosis related genes from the OMIM database (www.omim.org) (Joy Orient, China). The libraries were first tested for enrichment by quantitative polymerase chain reaction (PCR) and for size distribution and concentration using the Agilent Bioanalyzer 2100. The samples were then sequenced on an Illumina Hiseq. 2500. Raw image files were processed by the BclToFastq (Illumina) for base calling and the raw data generating. Sanger sequencing was used to confirm the mutation in *WAS* and *TNFRSF11A* gene of the proband (Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/JPHO/A361, list The PCR primers and length of PCR product).

In the first place, NGS analysis showed a pathogenic variant in accordance with guidelines,[9] consisting of a hemizygous transition mutation in WAS gene, c.400G > A, causing substitution of alanine for threonine at amino acid position 134 (p.A134T, NM_000377). His mother was confirmed to be a carrier of this WAS mutation by Sanger sequencing (Fig. 2A). This nucleotide transition, located in Exon 4 of the *WAS* gene, had been identified as the mutation responsible for the X-chromosome-linked recessive WAS.[10] Judging from the genetic result and his clinical

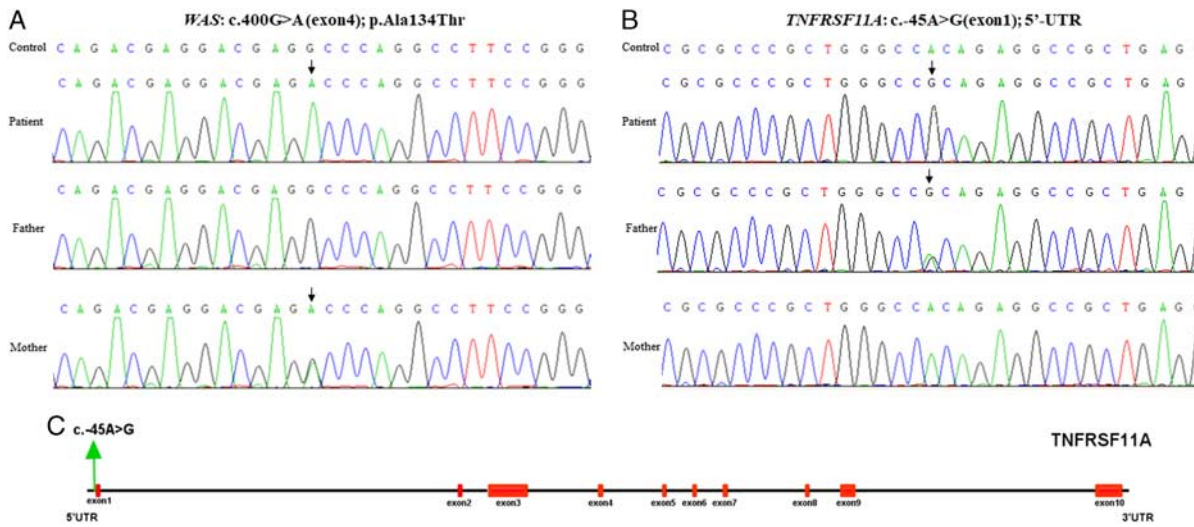

FIGURE 2. Molecular genetic analysis. A, Validation by Sanger sequencing of the c.400G > A mutation in Wiskott-Aldrich syndrome (WAS). The arrow indicates the position of the mutated base. The propositus was hemizygous and the mother heterozygous for the mutation. B, Sequencing of *TNFRSF11A* revealed a homozygous single nucleotide transition (c.−45A > G). C, This mutation is in the upstream of exon 1, at the 5′ untranslated region (5 UTR) of *TNFRSF11A* gene. 3′ UTR indicates untranslated region.

presentation, the infant was diagnosed with WAS with a clinical score 3.[11]

To further study the genetic evidence underlying the clinical finding of osteopetrosis, the molecular analysis of genes known to be responsible for the different types of osteopetrosis (*TCIRG1*, *CLCN7*, *PLEKHM1*, *RANKL*, *RANK*, and *SNX10*) was performed by NGS of exons and intron-exon boundaries as previously described.[12] As a result, a homozygous single nucleotide transitions, c.−45A > G (Fig. 2B), in the 5′ UTR of *TNFRSF11A* gene had been uncovered (Fig. 2C). The infant's father was found to be heterozygous for this mutation. This variant has not been recorded in any of the publicly available single nucleotide polymorphism database (1000Genomes, ExAC, ESP, HGMD, Clinvar) and inhouse database in Chinese Han people. To date, no literature or other genetic database records have reported that this 5′ UTR of *TNFRSF11A* gene leads directly to the pathophysiology of osteopetrosis. On the basis of the clinical presentation and genetic analysis, we identified a likely pathogenic variant according to the guidelines.[9]

DISCUSSION AND CONCLUSION

In the present case, WAS was not initially considered as the priority diagnosis due to normal MPV and the absence of eczema. In fact, a number of cases diagnosed with WAS have been described with normal or even increased MPV.[13,14] Thus, the refractory thrombocytopenia to first-line therapy and immune deficiency may be the common features despite the distinctive atypical clinical presentation in this inherited thrombocytopenia. It's concluded that both normal platelet size and the absence of eczema should not exclude the diagnosis of WAS, and the high-throughput sequencing will help to disclose the genetic basis.

In the past few years, exome sequencing has greatly promoted the discovery of the genetic defects of osteopetrosis underlying Mendelian disorders.[15,16] Mutations in the coding region of the *TNFRSF11A* gene may lead to a defect in osteoclast formation and result in clinical manifestations.[17] However, current sets of probes for exome capture target not only coding regions but also 5′ UTR and 3′ untranslated regions and stretches of intronic regions. Recently, several cases have demonstrated that the mutations in the noncoding region even in the intronic region can contribute to inherited diseases including ARO.[12,18–20] In the present case, with a clinical diagnosis of osteopetrosis based on radiologic findings and hematologic defects, a single nucleotide mutation in the 5′ UTR of *TNFRSF11A* gene were identified. These findings provide a de novo likely pathogenic mutation involved in osteoclast-poor formatted recessive osteopetrosis correlated with hereditary. Although mutations in 5′ UTR usually have significant effects on transcription at the level of mRNA, it is difficult to decide whether 5′ UTR mutation is also significant in this case as the infant died.

In conclusion, the present case reported a de novo single nucleotide transitions in the 5′ UTR of *TNFRSF11A* as the basis of molecular diagnosis of osteopetrosis. The findings reported here are the first to identify the coinheritance of 2 rare inherent diseases, ARO, and X-linked recessive WAS. Further studies are needed to elucidate the molecular pathophysiology of this mutation in 5′ UTR and additional families are needed for the full description of the phenotypic manifestations.

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