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Clinical Vignette

Characterization of a Novel Alu-Alu Recombination-Mediated Genomic Deletion in the *TCIRG1* Gene in Five Osteopetrotic Patients

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ABSTRACT: Human malignant autosomal recessive osteopetrosis (ARO) is a genetically heterogeneous disorder caused by reduced bone resorption by osteoclasts. Biallelic mutations in the *TCIRG1* gene, encoding the a3 subunit of the vacuolar proton pump, are responsible for more than one half of ARO patients. However, a few patients with monoallelic mutations have been described, raising the possibility of a dominant-like *TCIRG1*-dependent osteopetrosis, of a digenic disease, or of peculiar mutations difficult to detect with standard methods. We describe here a novel genomic deletion in the *TCIRG1* gene explaining why, in some patients, mutations in only one allele have previously been found. The analysis of a proband from a consanguineous Turkish family allowed us to define the deletion boundaries encompassing introns 10 and 13 and occurring within *AluSx* repeat sequences, suggesting *Alu*-mediated homologous recombination as a mechanism. An identical genomic deletion at the heterozygous level was found in four unrelated Italian families in whom only a single mutated allele has previously been found. *TCIRG1* haplotype analysis in these five families suggests a possible common ancestral origin for this large deletion. In summary, we describe the identification of a novel genomic deletion in the *TCIRG1* gene that is of clinical relevance, especially in prenatal diagnosis.
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Key words: osteopetrosis, autosomal recessive osteopetrosis, *TCIRG1*, genomic deletion, *Alu* repeats

INTRODUCTION

Infantile malignant autosomal recessive osteopetrosis (ARO; OMIM 259700) is a severe bone disease resulting from a deficit in bone resorption caused by osteoclast malfunction or formation.[1] It presents early in life with extreme sclerosis of the skeleton and is associated with a number of severe clinical manifestations, including macrocephaly, cranial nerve dysfunction such as deafness and blindness, pancytopenia, and hepatosplenomegaly caused by severe bone marrow failure beginning in early infancy or in fetal life. Deafness and blindness are generally thought to be secondary to pressure on cranial nerves exerted by the overgrowth of bone. The disease is often lethal within the first decade of life because of anemia and secondary infections. The only effective treatment for this disease is bone marrow transplantation (BMT) because the osteoclasts are from hematopoietic cell origin.[2] Although at least five genes have been found mutated in ARO,[3–7] more than one half of ARO patients bear mutations in the *TCIRG1* gene.[3,8–13] This form is associated to a classic osteopetrotic phenotype with secondary nervous system involvement (hydrocephalus, Arnold-Chiari malformation, and cranial nerve defects) and can be partially rescued by HLA-matched hematopoietic stem cell transplantation.[14,15] Thus far, 61 different mutations have been found in our cohort of >100 *TCIRG1*-dependent ARO patients (unpublished data). These include missense (24%), nonsense (20%), small deletions (22%), small insertions (5%), and splice site mutations (29%). Although most of the patients have mutations in both alleles, the occurrence of patients with only a single mutation has been recognized from the beginning.[8,9] Analysis of 1 kb of the *TCIRG1* promoter region in these patients failed to detect abnormalities.

The authors state that they have no conflicts of interest.

MATERIALS AND METHODS

Samples and mutation analysis

Clinical diagnosis of severe osteopetrosis was based on the following criteria at presentation: early diagnosis (before 1 yr of age), radiographic evidence of markedly increased BMD, pancytopenia, hepatosplenomegaly, and cranial nerves defects. DNA samples were obtained from all

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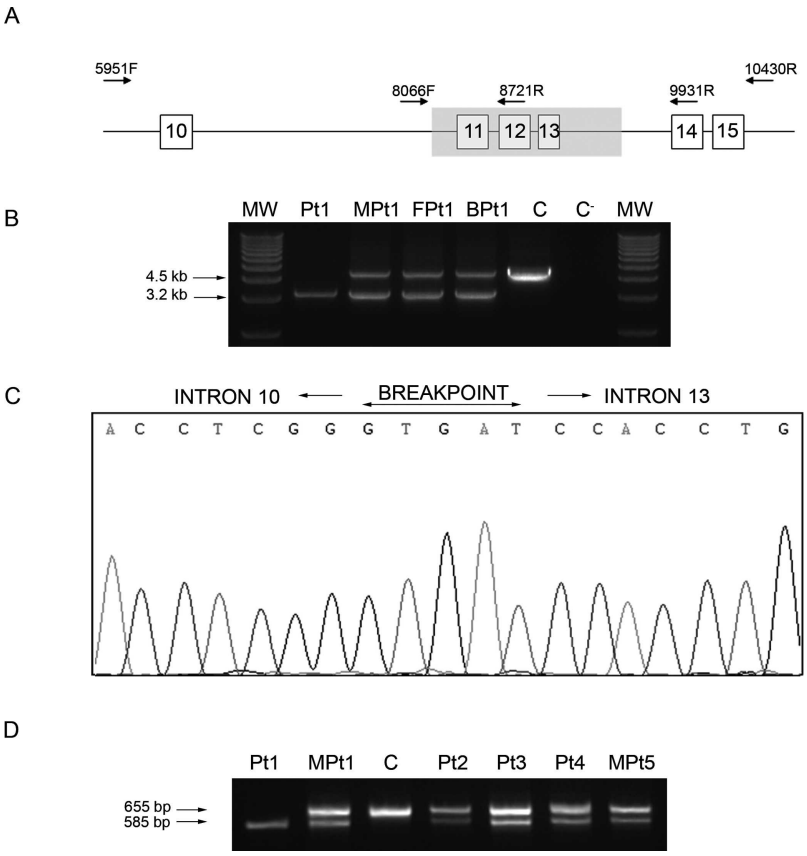

FIG. 1. Characterization of the 1.3-kb deletion (g.8280_9560del) in *TCIRG1*. (A) Schematic representation of the *TCIRG1* genomic region spanning exons 10–15. The 1.3-kb deletion mutation is depicted by the shaded area. Primers 5951F and 10430R were used to amplify a 4.5-kb region with a long-range PCR protocol. Primers 8066F and 9931R were used in PCR to refine the location of the breakpoint. Primers 8066F, 8721R, and 9931R were used for rapid diagnostic detection of the deletion. (B) Detection of the genomic deletion carried out with long-range PCR. A product of 3.2 kb instead of the expected 4.5-kb amplicon was obtained with genomic DNA from patient 1 (Pt1); both products were obtained with DNAs from his heterozygous parents (MPt1 and FPt1) and from his healthy brother (BPt1); only the expected 4.5-kb band was obtained from an unrelated healthy control (C); MW: 1-kb DNA ladder (Invitrogen). (C) Sequencing of the 585-bp PCR product obtained in Pt1 with primers 8066F and 9931R. The precise breakpoint lies in the 5 bp shared by both intron 10 and intron 13. (D) Rapid screening of mutation g.8280_9560del. A single band of 585 bp was visualized in patients 1 (Pt1), whereas two bands of 585 and 655 bp were observed in patients 2–4 (Pt2–Pt4), in the mother of patient 5 (MPt5), and in the healthy carrier from family 1 (MPt1), used as heterozygous control. The unaffected control (C) generated only the 655-bp band.

individuals in the study after receipt of informed consent. Mutation analysis of the *TCIRG1* gene was performed using direct DNA sequencing of PCR-amplified exons as previously described.[3,9]

Characterization of the deletion

The detection of the large genomic deletion was carried out with long-range PCR, performed using the Expand PCR System (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions and with forward primer in intron 9 (5951F; 5′-CACCGTTCTGGTCTGTCTCT-3′) and reverse primer in intron 15 (10430R; 5′-GGGCCTGCATCCCCGTTTCT-3′) of the *TCIRG1* gene (nucleotide numbers are referred to GenBank AF033033). A series of forward and reverse primers, mapping within this region, were used in the amplification to obtain a product for direct sequencing and to refine the location of the breakpoints. Sequence analysis of a short product obtained with a forward primer in intron 10 (8066F; 5′-CTTCGCTCTGTCGCGCAGG-3′) and a reverse primer in exon 14 (9931R; 5′-CAAGTGGTTGGCAGCCAGGC-3′) allowed us to identify the precise breakpoints of the deletion.

PCR detection of the deletion

After the exact positions of the breakpoints had been confirmed by sequence within introns 10 and 13 (see the Results section), three primers were used for rapid diagnostic detection of the deletion mutation: the forward primer in intron 10 and the reverse primer in exon 14, previously described, and an additional reverse primer (8721R; 5′-GGCCCATAAGCAGGAGCAG-3′) located in exon 12. These three primers were used in a single PCR reaction, with standard conditions and annealing temperature at 65°C.

RESULTS

Characterization of a 1.3-kb deletion in a Turkish ARO patient

The third child of unrelated parents from the same Turkish village was referred to us with a diagnosis of ARO. He showed a classical phenotype with "bone in bone" signs, anemia, hepatosplenomegaly, and optic nerve involvement. We obtained DNA from the patient, his parents, and his healthy brother but not from his affected sister who died after BMT. Amplification of the *TCIRG1* gene in the proband (Pt1) gave the expected normal products from all the genomic segments[9] except for a fragment including exons 11–13, whereas the expected amplicon for this region was obtained from his parents' and healthy brother's DNA. This raised the possibility of the presence of a genomic deletion spanning exons 11–13. To confirm the suspected deletion at the genomic level, we used long-range PCR using a forward primer in intron 9 (primer 5951F) and a reverse primer in intron 15 (primer 10430R; Fig. 1A). A frag-

Table 1. Clinical Features of *TCIRG1*-Dependent Osteopetrotic Patients

Patient	*Age at onset (mo)*	*Bone biopsy (no. of osteoclasts)*	*Bone fractures*	*Anemia/hepato-splenomegalia*	*Cranial nerve defect*	*BMT (age)*	*Outcome*	*CNS defect*
1	1	Not done	No	Yes	Impaired vision	No	Alive	No
2	6	Yes (normal)	No	Yes	No	9 mo	Alive	No
3	6	Yes (normal)	Yes (femur)	Yes	No	11 mo	Alive with engraftment	Macrocephaly
4	3	Not Done	No	Yes	Impaired vision, deafness	No	Dead at 10 mo	Hydrocephalus, Arnold_Chiari 1
5	4	Yes (normal)	No	No	Impaired vision	No	Dead at 1 yr	Hydrocephalus, nystagmus

ment of ~3.2 kb, instead of the ~4.5-kb wildtype PCR product, was obtained with DNA from the patient, whereas both products were obtained with DNA from his heterozygous parents and his healthy brother (Fig. 1B). To identify the deletion breakpoints, primers 8066F and 9931R were used to amplify across the deletion (Fig. 1A). A fragment of 585 bp, instead of the ~1.9-kb wildtype PCR product, was obtained in Pt1. Sequencing of the 585-bp fragment resulted in the identification of the 5′ and 3′ breakpoints within introns 10 and 13, respectively (g.8280_9560del; Fig. 1C).

Detection of the 1.3-kb deletion in four unrelated Italian patients

The identification of the exact breakpoints allowed us to develop an easy and rapid test for detecting this mutation at both homozygous and heterozygous state, with a standard PCR reaction using three primers, two of them being positioned outside the deletion (primers 8066F and 9931R), and the third one placed within the deletion (primer 8721R) (Fig. 1A). In the presence of the normal allele, a 655-bp fragment is generated by amplification using primers 8066F and 8721R, whereas in these amplification conditions, the expected ~1.9 kb amplicon spanning the region of the deletion is not generated with primers 8066F and 9931R. In the presence of the deleted allele a 585-bp product is amplified with primers 8066F and 9931R.

We re-examined 15 patients in whom only a single *TCIRG1* allele was detected, and we amplified a diagnostic 585-bp band in 4 of them (Fig. 1D). Their clinical data are summarized in Table 1. One of these (Pt2) has been previously reported, patient 52 in Susani et al.,[13] as a compound heterozygote bearing a nonsense mutation (p.Y512X) and a cDNA deletion spanning exons 11–13, which introduces a frameshift starting at codon 389 with a premature termination of translation 151 codons downstream (p.Ala389AspfsX151). Genomic analysis at that time was unable to identify the basis of the abnormal transcript and the uncertainty between splice site defect or genomic deletion remained unresolved. Standard analysis in Pt3 showed a mutation on one allele only, as confirmed by parent DNA analysis. Patient 3 and his healthy father were heterozygous for a mutation (previously identified in other patients) at the donor splice site of intron 6 (c.630 + 2T>C) leading to the skipping of exon 7 (c.631_713del), whereas no mutation was detected in his mother with standard analysis. Patient 4 was apparently homozygous for a single base pair substitution in exon 13, which resulted in the same nonsense mutation of patient 2 (p.Y512X). However, the proband's mother did not carry this mutation. Parental disomy was excluded because of the presence of heterozygous *TCIRG1* polymorphisms in the patient. This suggested that the proband had inherited a genomic deletion spanning exon 13 from his mother, thus reducing the paternal nonsense mutation to hemizygosity. For patient 5, no DNA was available, and the analysis was carried out on her parents. The father was found to be heterozygous for a two nucleotides base pair insertion in exon 16 (g.10691_2 ins AG) that disrupts the reading frame (p.Val631ArgfsX57), whereas no mutation was found in the mother.

As shown in Fig. 1D, diagnostic PCR generated a single band of 585 bp in patient 1, whereas two bands of 585 and 655 bp were observed in patients 2, 3, and 4 and in the mother of patient 5, as well as in the mother of patient 1, used as heterozygous control. Finally, a single band of 655 bp was observed in a healthy control, indicating the absence of the deletion. The deletion breakpoints were determined in all four cases by sequencing of the 585-bp PCR products generated by primers 8066F and 9931R and found to be identical to those in Pt1.

Alu-mediated homologous recombination

Comparing the intronic sequences spanning the deletion breakpoints, in introns 10 and 13, we found stretches of >300 bp with ~83% homology. Further in silico analysis[16] identified these as *Alu* sequences of the *Alu-Sx* subfamily (Fig. 2A). The precise site of the recombination event has been narrowed to a 5-bp sequence, (GTGAT), which shows 100% homology in the two *AluSx*-repeat regions involved (Fig. 2B). The fact that one copy of these tracts was deleted in its entirety, in the junction sequence, provides strong evidence for *Alu*-mediated homologous recombination as the mechanism involved.

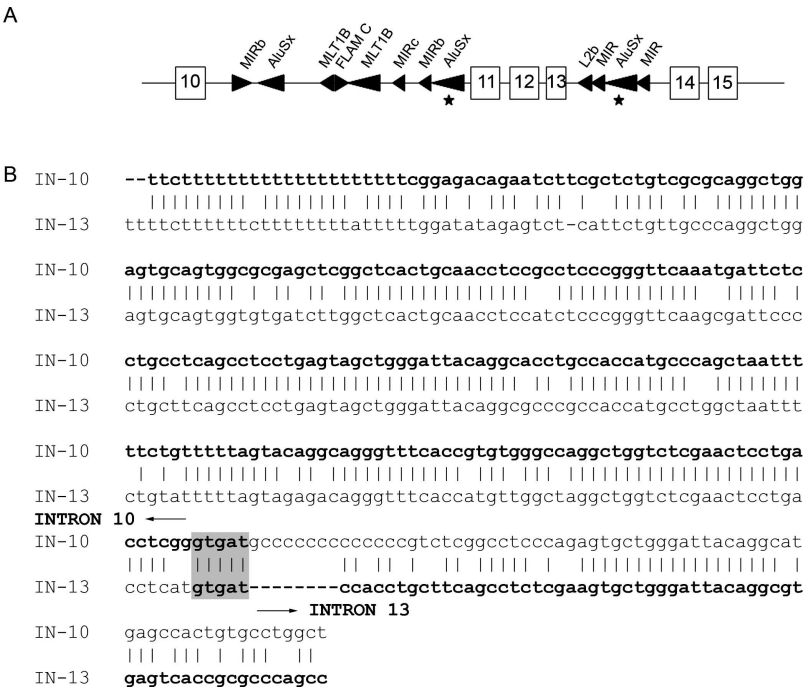

FIG. 2. Abundance of repetitive elements within introns 10 and 13 of *TCIRG1*. (A) Schematic representation of the genomic region spanning exons 10–15. Orientation and type of the repetitive sequence elements comprised in this region are indicated by arrowheads, the size of which is proportional to the length of the corresponding repeat. The two *AluSx* sequences involved in the 1.3-kb deletion are indicated by asterisks. (B) Sequence alignment between the *AluSx* repeats, including a 5-bp sequence containing the deletion breakpoints (shaded). Bold represents the sequence of the *TCIRG1* mutated allele that results from the recombination event.

Haplotype analysis

The presence of this large genomic deletion in six alleles of five patients, four of them from the South of Italy, could reflect the presence of a founder effect or, alternatively, it could be caused by a recurring event. We examined nine SNP markers within the gene in all the five families. The findings in Pt1, homozygous for the deletion, allowed us to define the haplotype (A-A-T-C-C-A-G-A-C) associated with the deletion (Table 2). Analysis of Pt2, Pt3, Pt4, and haplotype reconstruction based on their parents' studies confirmed the presence, in all of them, of the deletion-associated haplotype defined in Pt1. For family 5, genotyping was carried out on the mother, who was found to be homozygous for the haplotype. These data, showing that the 1.3-kb deletion occurred in the same genetic background in all patients, together with the fact that all the patients shared identical breakpoints, could suggest that the mutation arose only once in the Mediterranean area, because continuous gene exchange occurred in this geographical region.

DISCUSSION

In contrast to that observed in the *ClCN7* gene, whose mutations seem to be responsible for a continuum of symptoms and cause both classical ADOII and intermediate/severe osteopetrosis,[4,17–20] *TCIRG1* mutations have been reported thus far only in classical severe ARO.[3,8–13] However, a few patients with a single mutated allele have been described by us and others,[8,9] raising the possibility of the existence of *TCIRG1*-dependent dominant osteopetrosis. Nonetheless, the identification of the same mutation in the healthy parents ruled out this hypothesis.[9] An alternative explanation could be that an additional mutation in a second gene contributed to the osteopetrotic phenotype, but for our patients with a single *TCIRG1* mutated allele, we excluded the presence of mutations in the other genes known to cause ARO, although the contribution of other unknown genes cannot be ruled out. The data reported in this paper strengthen our original hypothesis that the failure in detecting the second mutation in our patients was caused by our PCR-based strategy, which did not allow the identification of relatively large lesions.[9] Finding this large deletion in 4 of the 15 patients in whom only a single *TCIRG1* allele has been detected tends toward the exclusion of the possibility of a digenic disease and suggests that even larger deletions could be responsible for other undetected *TCIRG1* abnormalities.

In the case of the deletion reported here, we designed a PCR-based test that is easy to perform, making PCR the test of choice even in heterozygous patients. Theoretically, the deletion could also be detected by fluorescent in situ hybridization (FISH), although the size of the deletion does not allow the design of large probes usually needed for this technique. On the other hand, larger heterozygous deletions (such as those that may be present in the remaining 11 patients) could be successfully investigated by FISH. Unfortunately, this cytogenetic technique requires fresh cells, which were not available in our cases.

Many disease-causing extensive deletions are caused by recombination between *Alu* or other transposable elements.[21] Some of these rearrangements arose independently multiple times in the human population, as clearly illustrated by the high number of *Alu*-mediated deletions, for example, in the LDL receptor locus, resulting in familial hypocholesterolemia, and by the rearrangements involving the MLL locus, a frequent factor in acute myeloid leukemias.[21] The genes showing high levels of *Alu-Alu* recom-

TABLE 2. HAPLOTYPE ANALYSIS

	Introns 10–13	*Promoter*		*Intron 2*	*Exon 3*	*Exon 4*	*Intron 4*	*Exon 14*	*Intron 15*	*Intron 15*
		g.-1082G/A	*g.-899G/A*	*g.2497T/C*	*g.2827C/T*	*g.3856C/T*	*g.3900A/G*	*g.10022G/C*	*g.10495A/G*	*g.10504C/T*
	g.8280_9560 del	*H* = 0,496*	*H* = 0,495*	—	*H* = 0,184*	*H* = 0,025*	—	*H* = 0,025*	*H* = 0,438*	—
Pt 1	Homozygous	A/A	A/A	T/T	C/C	C/C	A/A	G/G	A/A	C/C
Pt 2	Heterozygous	A/A	A/A	T/T	C/T	C/C	A/A	G/G	A/A	C/C
Pt 2 mother	Heterozygous	—	—	—	C/C	—	—	—	—	—
Pt 3	Heterozygous	G/A	A/A	T/C	C/C	C/T	A/G	G/G	A/G	C/T
Pt 3 mother	Heterozygous	A/A	—	T/C	—	C/C	A/G	—	A/G	C/C
Pt 3 father	No deletion	—	—	C/C	—	—	G/G	—	G/G	—
Pt 4	Heterozygous	A/A	A/A	T/T	C/T	C/C	A/A	G/G	A/A	C/C
Pt 4 mother	Heterozygous	—	—	—	C/C	—	—	—	—	—
Pt 5 mother	Heterozygous	A/A	A/A	T/T	C/C	C/C	A/A	G/G	A/A	C/C
Deletion-associated haplotype		A	A	T	C	C	A	G	A	C

* Heterozygosity reported by SNP database (http://www.ncbi.nlm.nih.gov/SNP/).

bination tend to have a large number of *Alu* sequences. Therefore, *Alu* density may help to contribute toward this recombination as recently shown by the analysis of 20 genes disrupted in human diseases showing a significant statistical correlation between the content of transposable elements of the same subfamily and the frequency of large gene deletions.[22] Computational analysis of the entire *TCIRG1* gene performed by the RepeatMasker Documentation program[16] showed a high content of repetitive elements in intron 10 and intron 13 (accounting for 55% and 68% of the intronic length, respectively; Fig. 2A), suggesting that we should screen for other rearrangements involving introns 10 and 13 of the *TCIRG1* gene. Our analysis using long-range PCR (spanning from nucleotide 5951 to 10,430) confirmed that, in our patients, the only possible rearrangement in this region involved the described breakpoints.

An important implication in the failure in detecting large allelic deletions is that the identification of a single variant sequence may be erroneously interpreted as a homozygous mutation if masked by a coincident exonic deletion. Accurate molecular diagnosis is important not only for patient management, but also for genetic counseling of family members, and, in particular, the identification of mutations in both alleles is essential for a correct prenatal diagnosis.

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