CLINICAL GENETICS An International Journal of Genetics, Molecular and Personalized Medicine

Clin Genet 2015: 88: 74–79
Printed in Singapore. All rights reserved

CLINICAL GENETICS
doi: 10.1111/cge.12448

Short Report

A founder mutation in the *TCIRG1* gene causes osteopetrosis in the Ashkenazi Jewish population

Anderson SL, Jalas C, Fedick A, Reid KF, Carpenter TO, Chirnomas D, Treff NR, Ekstein J, Rubin BY. A founder mutation in the *TCIRG1* gene causes osteopetrosis in the Ashkenazi Jewish population.
Clin Genet 2015: 88: 74–79. © John Wiley & Sons A/S. Published by John Wiley & Sons Ltd, 2014

Osteopetrosis is a rare and heterogeneous genetic disorder characterized by dense bone mass that is a consequence of defective osteoclast function and/or development. Autosomal recessive osteopetrosis (ARO) is the most severe form and is often fatal within the first years of life; early hematopoietic stem cell transplant (HSCT) remains the only curative treatment for ARO. The majority of the ARO-causing mutations are located in the *TCIRG1* gene. We report here the identification and characterization of an A to T transversion in the fourth base of the intron 2 donor splice site (c.117+4A→T) in *TCIRG1*, a mutation not previously seen in the Ashkenazi Jewish (AJ) population. Analysis of a random sample of individuals of AJ descent revealed a carrier frequency of approximately 1 in 350. Genotyping of five loci adjacent to the c.117+4A→T-containing *TCIRG1* allele revealed that the presence of this mutation in the AJ population is due to a single founder. The identification of this mutation will enable population carrier testing and will facilitate the identification and treatment of individuals homozygous for this mutation.

Conflict of interest

The authors have declared no conflicting interests.

S.L. Anderson[a,†], C. Jalas[b,†], A. Fedick[c], K.F. Reid[a], T.O. Carpenter[d], D. Chirnomas[d], N.R. Treff[c,e], J. Ekstein[f] and B.Y. Rubin[a]

[a]Department of Biological Sciences, Fordham University, Bronx, NY 10458, USA, [b]Bonei Olam, Center for Rare Jewish Genetic Disorders, Brooklyn, NY 11204, USA, [c]Department of Microbiology and Molecular Genetics, Rutgers-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA, [d]Yale University School of Medicine, Departments of Pediatrics (Endocrinology) and Orthopedics and Rehabilitation, New Haven, CT 06520, USA, [e]Reproductive Medicine Associates of New Jersey, Department of Research, Morristown, NJ 07960, USA, and [f]Dor Yeshorim, The Committee for Prevention of Jewish Diseases, Brooklyn, NY 11211, USA

[†]These authors contributed equally to the work.

Key words: Ashkenazi Jewish – founder mutation – osteopetrosis – *TCIRG1*

Corresponding author: B.Y. Rubin, Department of Biological Sciences, Fordham University, 441 E. Fordham Road, Bronx, NY 10458, USA.
Tel.: +1 718 817 3637;
fax: +1 718 817 2792;
e-mail: rubin@fordham.edu

Received 8 May 2014, revised and accepted for publication 19 June 2014

Osteopetrosis is a genetic disorder characterized by dense bone mass that is a consequence of defective osteoclast function and/or development (1). Approximately 70% of patients present with normal or even elevated levels of osteoclasts and are said to have an 'osteoclast-rich' form of osteopetrosis; in the remaining 30%, there is a clear defect in the production and differentiation of these cells and these individuals

are classified as having an 'osteoclast-poor' form of osteopetrosis.

Autosomal recessive ('malignant') osteopetrosis (ARO) is the most severe form of the disease and is often fatal within the first years of life (1, 2). Infants with ARO exhibit abnormal and dense bone structure, susceptibility to fractures, the absence of a bone marrow cavity, anemia and thrombocytopenia. Excessive bone development can cause cranial nerve entrapment. Untreated, ARO patients have a life expectancy of 3–4 years (3).

The majority of the ARO-causing mutations are located in the T-cell, immune regulator 1, ATPase, H+transporting, lysosomal V0 subunit a3 (*TCIRG1*) gene (4, 5). Mammalian V-ATPases consist of at least 13 different subunits, organized into a cytoplasmic V1 domain and a membrane embedded V0 domain. The V1 domain is responsible for the hydrolysis of ATPase and the V0 domain functions as a proton translocator (6). The *a* subunit, which is a component of the V0 domain, exists as four isoforms. The a3 isoform exists primarily in osteoclasts (7). The V-ATPase complex located in the membrane of the ruffled border of the bone-bound osteoclasts releases protons and generates an acidic environment, required for the solubilization of hydroxyapatite, in the resorption area beneath the osteoclast. The osteopetrosis-causing mutations in the *TCIRG1* gene generate a non-functional V-ATPase unable to generate an acidic environment.

As a result of historical founder effects, individuals of Ashkenazi Jewish (AJ) descent are subject to a variety of genetic disorders (8). The success of genetic screening programs for this population has been facilitated by the limited number of disease-causing mutations (9). A recent comprehensive carrier frequency study on individuals of AJ descent has suggested an expansion of the number of disorders that should be routinely tested for and has provided valuable carrier frequency information on 16 diseases prevalent in this population (10).

We show the presence of a founder mutation in the *TCIRG1* gene that is the genetic cause of ARO in three families in which both parents are of AJ descent. Screening of a random population of individuals of AJ descent reveals the carrier frequency of this mutation to be 1 in approximately 350. The identification of this mutation in the AJ population supports its inclusion in carrier screening programs and should facilitate the diagnosis and treatment of ARO in individuals of AJ descent.

Subjects and methods

Human subjects

Blood or cheek swab samples were obtained from the affected children and their parents. Anonymous blood samples from individuals of AJ descent were obtained from the Dor Yeshorim screening program (11). Genomic DNA was purified from blood using QIAamp DNA Blood Kits (Qiagen, Germantown, MD). This work was performed with the approval of the Fordham University institutional review board (IRB).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of fetal cell RNA

RNA was isolated from fetal cells obtained by chorionic villus sampling (CVS) using RNeasy Plus Mini Kits (Qiagen, Germantown, MD) and RT-PCR analysis was performed with OneStep RT-PCR Kits (Qiagen) using TCIRG1-2F and -3R primers (located in exons 2 and 3, respectively) (see Table S1, Supporting Information for primer sequences). The RT-PCR products were analyzed on 2% agarose gels and purified for sequence analysis.

DNA sequencing

DNA sequencing was performed by fluorescent dye terminator detection on an ABI 3730xl Analyzer (GENEWIZ, South Plainfield, NJ).

Genotype analysis for population screening

Genotyping was accomplished via an allelic discrimination assay. Sequence-specific forward and reverse primers were used to amplify a region around the polymorphic sequence, then allelic discrimination was performed utilizing VIC- and FAM-labeled probes to detect the normal and mutant alleles, respectively (see Table S1 for primer and probe sequences).

All DNAs from the individuals identified as being carriers were confirmed to be heterozygous for the c.117+4A→T mutation by PCR amplification and sequencing of a 309 bp fragment surrounding the mutation, using primers located in introns 1 and 2. The sequence of the primers, TCIRG1-int-1 and TCIRG1–int-2, are presented in Table S1.

Haplotype analysis

Microsatellite marker analysis of the region adjacent to the *TCIRG1* gene was performed on the DNA of five affected children and 12 carriers (the three sets of parents of the affected children and the six unrelated individuals identified in the population frequency study). Amplification of the D11S4155, D11S987, D11S1917, D11S1337 and D11S4178 polymorphic microsatellite markers, which are all within 0.4 MB of *TCIRG1*, was performed using the respective primers listed in Table S1. The forward 'F' primer of each pair was labeled on the 5′ end with 6-FAM™ (Fluorescein) dye (IDT, Coralville, IA). The amplified products were run on an ABI 3730xl Analyzer at GENEWIZ (South Plainfield, NJ) and the results analyzed using Peak Scanner v1.0 software (Life Technologies, Grand Island, NY). For each marker analyzed, the smallest PCR product obtained was interpreted as having the least number of repeats; that size PCR product was assigned an arbitrary allele number of '1,' not meant to imply the actual number of repeats, but the smallest relative number for a given marker within

the samples. A PCR product that was, for example, one repeat larger was given an allele number of '2' and so on.

Results

Clinical report

Five patients of AJ descent exhibiting typical signs of ARO were all born to nonconsanguineous AJ parents.

Patient 1 presented at 3 months of age with leukocytosis without fever, hepatosplenomegaly, bulging fontanelle and dysmorphic facial features typical of osteopetrosis. On the basis of phenotypic presentation of osteopetrosis, this child received an unrelated hematopoietic stem cell transplant (HSCT) at 7 months but died from infectious complications.

Patient 2 was noted at 2 months of age to have an increased head circumference as well as a bulging anterior fontanel. No diagnosis was made at the time. At 5 months, the child's head circumference was greater than the upper limit of normal, the bulging anterior fontanel had become more prominent and there was a significant impairment of vision. X rays revealed the presence of dense bones and the child was diagnosed as having osteopetrosis. At 9 months, the child received a HSCT from a fraternal twin brother. The transplantation was successful and the child has continued to develop well.

Patients 3, 4 and 5 were born to a single couple. At 2 months, the parents noted the inability of patient 3 to see. Ocular examination revealed little pupillary response, atrophy of the optic nerve and a flat electroretinogram (ERG). Compression of the optic nerve was noted on a computed tomography (CT) scan. A skeletal survey performed at 5 months revealed dense bones and the child was diagnosed as having osteopetrosis. At 16 months, HSCT was performed with a matched unrelated donor. The child died shortly thereafter from complications associated with anesthesia. Skeletal surveys were performed on patients 4 and 5 shortly after birth and both were determined to have osteopetrosis. Both children received HSCT from unrelated matched donors and both continue to do well.

Identification and characterization of an ARO-causing homozygous mutation in TCIRG1

The suspected diagnosis of osteopetrosis in patient #1 prompted an analysis of the DNA sequence of the patient's *TCIRG1*, *CLCN7* and *OSTM1* genes. For this analysis, the protein-encoding exonic regions with flanking intronic sequences were amplified and sequenced. The homozygous presence of an A to T transversion was detected in the fourth base of the intron 2 donor splice site (c.117+4A→T; NG_007878.1, nt 7377) in the child's *TCIRG1* gene and the parents were determined to be heterozygous for this mutation (Fig. 1a). DNA amplification and sequence analysis revealed that all of the remaining ARO patients were homozygous for the c.117 + 4A → T mutation and all of their parents were heterozygous for this mutation (data not shown).

Definitive demonstration of the disease-causing effect of base changes in the variant positions of donor splice sites is dependent on the ability to detect a pathological effect of these changes. As the surviving individuals homozygous for the c.117+4A→T mutation all received HSCT and therefore no longer had peripheral blood cells bearing this mutation, the impact of the c.117+4A→T mutation was examined in fetal cells homozygous for the mutation (derived from a fetus conceived by the parents of patient 1). DNA sequence analysis of RT-PCR products generated from these cells and from two fetal cell samples not bearing this mutation (derived from unrelated pregnancies), using a primer located at the 5′ end of exon 2 and a primer in exon 3 of the *TCIRG1* gene, revealed that the cells containing the c.117+4A→T mutation generated a smaller transcript (Fig. 1b); sequencing analysis of the RT-PCR products revealed that the smaller transcript was missing the last part of exon 2 (Fig. 1b). This alternative splicing event involved the use of a cryptic splice donor site within exon 2, resulting in a transcript that encoded 14 fewer amino acids in the *TCIRG1*-encoded protein, due to the splicing out of the last 42 nt of the exon (Fig. 1c). It is interesting to note that the homozygous presence of a c.117+4A→T mutation in the *TCIRG1* gene has also been noted in a child with osteopetrosis born to consanguineous parents of Turkish descent. Characterization of the transcript generated from this allele in fibroblast cells also revealed the use of the cryptic splice site in exon 2 (12).

Carrier frequency and haplotype analysis

The identification of six unrelated individuals of AJ descent (the parents of the affected children), who are carriers of the c.117+4A→T mutation, prompted a study of the frequency of this mutation in this population. Genotype analysis was performed using TaqMan technology on 2092 DNA samples collected from a random population of individuals of AJ descent. Two thousand and eighty-eight samples were successfully amplified and six individuals were determined to be heterozygous for the c.117+4A→T bearing allele (Fig. 2). PCR amplification and DNA sequence analysis of a 309 bp fragment encompassing the c.117+4A→T mutation performed on the DNA of the six individuals found to be heterozygous for the mutant allele and on the four samples that failed to amplify in the TaqMan assay confirmed the six individuals to be heterozygous for the c.117+4A→T allele and the four samples that failed to amplify were homozygous for the wildtype allele. The carrier frequency for this mutation was thus determined to be approximately 1 in 350.

To examine whether the presence of this mutation in this population is due to a founder effect, haplotype analysis was performed using five microsatellite markers, D11S4155, D11S987, D11S1917, D11S1337 and D11S4178, located within 0.4 MB of the *TCIRG1* gene. The affected children were observed to be homozygous for the number of repeats at each of the five loci. Microsatellite markers that are 'linked' to a mutation would be expected to bear the same number of repeats

Table 1. The c.117+4A>T allele and its associated haplotype

Individual[a]	c.117 + 4	D11S4155[b]	D11S987[b]	D11S1917[b]	D11S1337[b]	D11S4178[b]
A1	**TT**	**2, 2**	**7, 7**	**3, 3**	**1, 1**	**1, 1**
A2	**TT**	**2, 2**	**7, 7**	**3, 3**	**1, 1**	**1, 1**
A3	**TT**	**2, 2**	**7, 7**	**3, 3**	**1, 1**	**1, 1**
A4	**TT**	**2, 2**	**7, 7**	**3, 3**	**1, 1**	**1, 1**
A5	**TT**	**2, 2**	**7, 7**	**3, 3**	**1, 1**	**1, 1**
C1	**T**A	**2**, 4	5**, 7**	1, **3**	**1, 1**	**1, 1**
C2	**T**A	**2**, 4	4, **7**	**3, 3**	**1**, 6	**1, 1**
C3	**T**A	**2**, 4	5, **7**	**3, 3**	**1**, 5	**1**, 3
C4	**T**A	**2**, 3	2, **7**	1, **3**	**1, 1**	**1, 1**
C5	**T**A	**2**, 3	2, **7**	**3, 3**	**1, 1**	**1, 1**
C6	**T**A	**2**, 4	5, **7**	1, **3**	**1**, 5	**1**, 3
C7	**T**A	**2**, 4	5, **7**	1, **3**	**1, 1**	**1, 1**
C8	**T**A	1, **2**	5, **7**	**3, 3**	**1**, 4	**1, 1**
C9	**T**A	**2**, 4	4, **7**	1, **3**	**1**, 4	**1**, 4
C10	**T**A	**2**, 4	4, **7**	**3**, 5	**1, 1**	**1, 1**
C11	**T**A	**2**, 4	1, **7**	1, **3**	**1, 1**	**1, 1**
C12	**T**A	**2,** 5	2**, 7**	**3, 3**	**1,** 7	**1, 1**

[a]A = affected, C = carrier.
[b]A designation of "1" for the dinucleotide repeat markers is a relative number and represents the least number of repeats seen for a given marker in this cohort of 17 individuals. Bolded letters/numbers represent variants present on the mutant allele.

for each of the markers if the affected child inherited the same allele from each parent. The parents and all other carriers were found to be at least heterozygous for the number of repeats for each of the markers that all of the affected children were homozygous for (Table 1). This finding strongly supports the notion that this mutation is the result of a founder effect.

Discussion

We present the first demonstration of the existence of ARO in the AJ population and demonstrate the disease-causing mutation to be an A to T transversion in the fourth base of the intron 2 donor splice site (c.117+4A→T) of the *TCIRG1* gene. This mutation

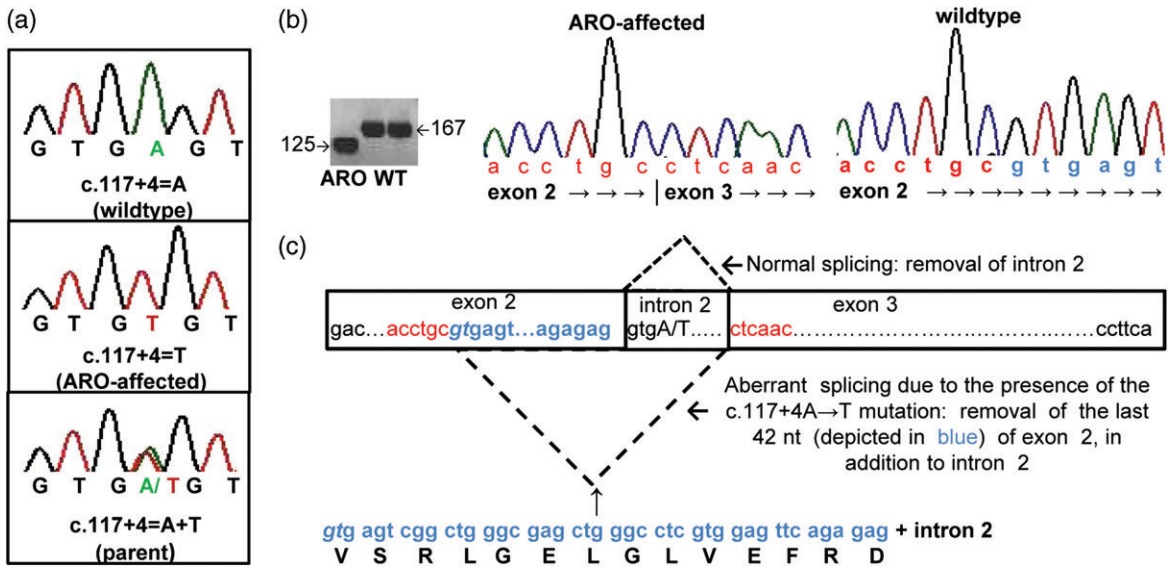

Fig. 1. (**a**) Electropherograms showing sequence of the first six nt of *TCIRG1* intron 2. Polymerase chain reaction (PCR) product from wildtype DNA contains an 'A' (green) in the fourth base in both alleles; from DNA of the autosomal recessive osteopetrosis (ARO)-affected individual (a homozygote), a 'T' (red) in both alleles; from parental (heterozygote) DNA, an 'A' (green) on one allele overlapped with a 'T' (red) from the other allele. (**b**) RNA was subjected to RT-PCR with primers located in exons 2 and 3 with an expected product size of 167 bp. *TCIRG1* transcripts from cells homozygous for the mutation (ARO) generated a smaller product, which sequencing revealed to be 125 bp in size, representing a loss of 42 nucleotides at the end of exon 2, while the products from the unrelated fetal cells (WT) were of the expected size. Electropherograms show nt 74–79 of exon 2 (in red), followed either by the first 6 nt of exon 3 (also in red), due to the exclusion of the last 42 nt of exon 2 (ARO-affected), or nt 80–85 (in blue) of exon 2 (wildtype). (**c**) The c.117+4A→T mutation results in the splicing out of the last 42 nt (in blue) of exon 2, encoding 14 amino acids, in addition to the normally spliced out intron 2. The 42 nt are spliced out of the *TCIRG1* transcript due to the use of a cryptic splice donor site ('gt' in blue, italicized text) when the c.117+4A→T mutation in intron 2 is present. The removal of the 42 nt results in the loss of 14 amino acids (bold, upper-case letters) from the cytoplasmic portion of the *TCIRG1* protein.

Gene	Mutation	Total Tested	Total Results	Homo Wildtype	Hetero Carrier	Homo Affected	Unamplified/ Undetermined
TCIRG1	c.117+4A→T	2092	2088	2082	6	0	4

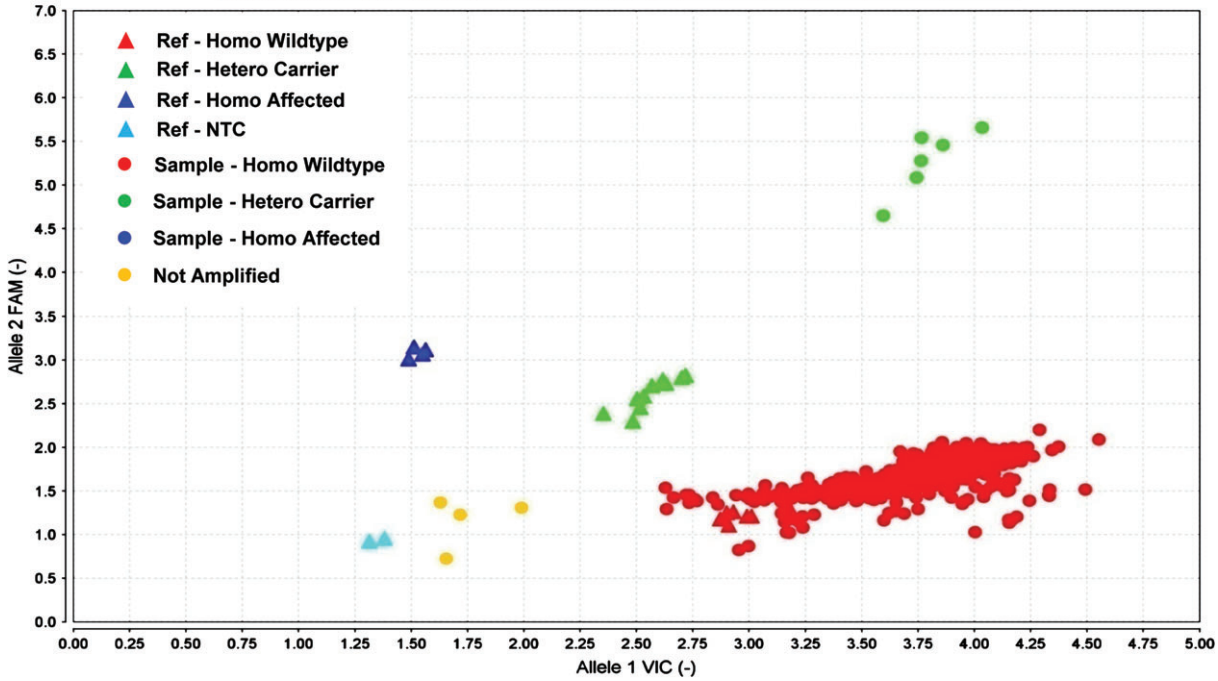

Fig. 2. Carrier frequency determination by allelic discrimination assay. Two thousand and ninety-two DNA samples were assayed for the presence of the c.117+4A→T mutation. Six heterozygotes were discovered among the 2088 that were successfully amplified, demonstrating a carrier frequency of approximately 1 in 350.

alters the splicing of the gene transcript and encodes a protein missing 14 amino acids from the hydrophilic N-terminal, cytoplasmically located portion of the a3 subunit of the V-ATPase.

The *TCIRG1* gene encodes two isoforms of the a3 subunit of V-ATPase: (i) isoform a, which is 830 amino acids long, highly expressed in osteoclasts and essential for their function; and (ii) isoform b, which is transcribed from exon 5 of the *TCIRG1* gene and is missing the first 216 amino acids of isoform a. This latter form is expressed by and essential for activation of T-cells. The fact that the longer isoform a is essential for V-ATPase function in osteoclasts, as evidenced by the ARO-causing mutations in the region of the *TCIRG1* gene that encodes amino acids 1–216 (e.g. c.118–1G→A (13), c.197-1G→A (13), c.480_481insG (13), c.117+4A→T and others), suggests the presence of an essential element(s) within these amino acids, all of which lie within the cytoplasmic domain of the a3 subunit. It is not known at this time what role amino acids 26–39, the portion of the first 216 amino acids of the a3 subunit lost as a consequence of the c.117+4A→T mutation, play in the loss of function of V-ATPase, but it is apparently sufficient to cause severe disease.

The demonstrated presence of this disease-causing mutation in individuals of AJ descent will facilitate the diagnosis of infants who present with osteopetrosis-like symptoms and thereby accelerate the search for suitable hematopoietic stem cell donors.

The homozygous presence of this mutation in five affected children and its heterozygous presence in the six parents of the children and six unrelated individuals, all of whom share the same haplotype as the affected children on at least one allele in the region adjacent to the *TCIRG1* gene, identify the c.117+4A→T mutation as the first founder mutation in *TCIRG1* in the AJ population. In light of its frequency in this population and the significant mortality and medical challenges associated with osteopetrosis, its inclusion in routine carrier testing should be considered.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Acknowledgements

This work was funded by a grant from Bonei Olam, The Center for Rare Jewish Genetic Disorders.

References

1. Tolar J, Teitelbaum SL, Orchard PJ. Osteopetrosis. N Engl J Med 2004: 351: 2839–2849.
2. Mazzolari E, Forino C, Razza A, Porta F, Villa A, Notarangelo LD. A single-center experience in 20 patients with infantile malignant osteopetrosis. Am J Hematol 2009: 84: 473–479.
3. Balemans W, Van Wesenbeeck L, Van Hul W. A clinical and molecular overview of the human osteopetroses. Calcif Tissue Int 2005: 77: 263–274.

4. Frattini A, Orchard PJ, Sobacchi C et al. Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. Nat Genet 2000: 25: 343–346.
5. Sobacchi C, Frattini A, Orchard P et al. The mutational spectrum of human malignant autosomal recessive osteopetrosis. Hum Mol Genet 2001: 10: 1767–1773.
6. Nishi T, Forgac M. The vacuolar (H+)-ATPases – nature's most versatile proton pumps. Nat Rev Mol Cell Biol 2002: 3: 94–103.
7. Manolson MF, Yu H, Chen W et al. The a3 isoform of the 100-kDa V-ATPase subunit is highly but differentially expressed in large (>or=10 nuclei) and small (<or=nuclei) osteoclasts. J Biol Chem 2003: 278: 49271–49278.
8. Slatkin M. A population-genetic test of founder effects and implications for Ashkenazi Jewish diseases. Am J Hum Genet 2004: 75: 282–293.
9. Kaback MM. Population-based genetic screening for reproductive counseling: the Tay-Sachs disease model. Eur J Pediatr 2000: 159 (Suppl. 3): S192–S195.
10. Scott SA, Edelmann L, Liu L, Luo M, Desnick RJ, Kornreich R. Experience with carrier screening and prenatal diagnosis for 16 Ashkenazi Jewish genetic diseases. Hum Mutat 2010: 31: 1240–1250.
11. Ekstein J, Katzenstein H. The Dor Yeshorim story: community-based carrier screening for Tay-Sachs disease. Adv Genet 2001: 44: 297–310.
12. Kornak U, Schulz A, Friedrich W et al. Mutations in the a3 subunit of the vacuolar H(+)-ATPase cause infantile malignant osteopetrosis. Hum Mol Genet 2000: 9: 2059–2063.
13. Pangrazio A, Caldana ME, Iacono NL et al. Autosomal recessive osteopetrosis: report of 41 novel mutations in the TCIRG1 gene and diagnostic implications. Osteoporos Int 2012: 23: 2713–2718.