



ORIGINAL ARTICLE

An *SNX10* mutation causes malignant osteopetrosis of infancy

Memet Aker,^{1,2} Alex Rouvinski,³ Saar Hashavia,⁴ Asaf Ta-Shma,⁴ Avraham Shaag,^{1,2} Shamir Zenvirt,^{1,2} Shoshana Israel,⁵ Michael Weintraub,⁶ Albert Taraboulos,³ Zvi Bar-Shavit,⁷ Orly Elpeleg^{1,2}

¹Monique and Jacques Roboh Department of Genetic Research, Hadassah, Hebrew University Medical Center, Jerusalem, Israel

²Department of Genetic and Metabolic Diseases, Hadassah, Hebrew University Medical Center, Jerusalem, Israel

³Department of Microbiology and Molecular Genetics, IMRIC, The Hebrew

University-Hadassah Medical School, Jerusalem, Israel

⁴Pediatric Department, Hebrew University Medical Center, Jerusalem, Israel

⁵Tissue Typing Unit, Hadassah, Hebrew University Medical Center, Jerusalem, Israel

⁶Department of Pediatric Hematology-Oncology, Hadassah University Hospital, Jerusalem, Israel

⁷Department of Biochemistry and Molecular Biology, IMRIC, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Correspondence to

Professor Orly Elpeleg, Department of Genetic and Metabolic Diseases, Hadassah, Hebrew University Medical Center, Jerusalem 91120, Israel; elpeleg@hadassah.org.il

Received 23 September 2011

Revised 17 February 2012

Accepted 20 February 2012

ABSTRACT

Background Osteopetrosis is a life-threatening, rare disorder typically resulting from osteoclast dysfunction and infrequently from failure to commitment to osteoclast lineage. Patients commonly present in infancy with macrocephaly, feeding difficulties, evolving blindness and deafness, and bone marrow failure. In ~70% of the patients there is a molecularly defined failure to maintain an acid pH at the osteoclast-bone interface (the ruffled border) which is necessary for the bone resorptive activity.

Methods and results In eight patients with infantile osteopetrosis which could be cured by bone marrow transplantation, the study identified by homozygosity mapping in distantly related consanguineous pedigrees a missense mutation in a highly conserved residue in the *SNX10* gene. The mutation segregated with the disease in the families and was carried by one of 211 anonymous individuals of the same ethnicity. In the patients' osteoclasts, the mutant *SNX10* protein was abnormally abundant and its distribution altered. The patients' osteoclasts were fewer and smaller than control cells, their resorptive capacity was markedly deranged, and the endosomal pathway was perturbed as evidenced by the distribution of internalised dextran.

Conclusions *SNX10* was recently shown to interact with vacuolar type H⁺-ATPase (V-ATPase) which pumps protons at the osteoclast-bone interface. Mutations in *TCIRG1*, the gene encoding a subunit of the V-ATPase complex, account for the majority of cases of osteopetrosis. It is speculated that *SNX10* is responsible for the vesicular sorting of V-ATPase from Golgi or for its targeting to the ruffled border. A mutation in *SNX10* may therefore result in 'secondary V-ATPase deficiency' with a failure to acidify the resorption lacuna. Determination of the sequence of the *SNX10* gene is warranted in molecularly undefined patients with recessive 'pure' osteopetrosis of infancy.

INTRODUCTION

Osteopetrosis (OP) is a life threatening disease caused by subnormal osteoclast function, with an incidence of 1 in 250 000 births. The disease usually manifests in the first few months of life with macrocephaly and frontal bossing, resulting in a typical facial appearance. Skull changes—a consequence of defective bone remodelling—result in choanal stenosis, concomitant respiratory problems, and feeding difficulties which are the first clinical manifestations of the disease. The

expanding bones encroach on nerve foramina leading to blindness, deafness, and facial palsy.¹ Complete visual loss occurs invariably in all untreated patients and hearing loss is estimated to affect 78% of individuals with OP.² Tooth eruption defects and severe dental caries are common.³ Calcium feedback haemostasis is impaired and children with OP are at risk of developing hypocalcaemia, with attendant tetanic seizures and secondary hyperparathyroidism. The most severe complication of OP, limiting survival, is bone marrow insufficiency. The abnormal expansion of cortical and trabecular bone physically limits the availability of medullary space for haematopoietic activity leading to life threatening cytopenia and secondary expansion of extramedullary haematopoiesis at sites such as the liver and spleen.

The molecular basis of OP is known in about 70% of the patients.⁴ Most of the genes involved in OP in humans are associated with the maintenance of acid pH at the osteoclast-bone interface which is necessary for bone resorptive activity.

We report on the identification of a new OP associated gene in consanguineous families of Palestinian origin.

PATIENTS AND METHODS

Patients

Four patients (4367, 4321, 261002, 5151), three boys and one girl originating from two consanguineous Palestinian families (figure 1), were the subjects of this study. Their clinical and biochemical data are presented in table 1. Briefly, all four had older relatives with OP and all were suspected of having OP at 1–4 months of age because of macrocephaly or failure to thrive due to feeding problems caused by upper airway. Their neurocognitive development was age appropriate. Physical examination revealed increased head circumference, broad open fontanelle, frontal bossing, and hepatosplenomegaly. Hearing was normal. Functional vision was impaired due to the presence of severe optic atrophy, but the retinæ were intact (table 1). Laboratory investigation revealed anaemia, thrombocytopenia, and high lactate dehydrogenase (LDH). Bone radiographs showed dense bones with no distinction between outer and inner plates due to extensive encroachment of cortical bone into the medullary space. Brain CT scan disclosed narrowed optic and auditory canals with sclerosis of the semicircular canals. The ethmoidal and mastoid air

Figure 1 Simplified family trees showing the relationship between the patients. Not included are, for example, three healthy and five affected, dead sibs of patient 261002 whose DNA were not available. The patients' symbols are filled. Numbered symbols represent individuals whose DNA samples were available for analysis.

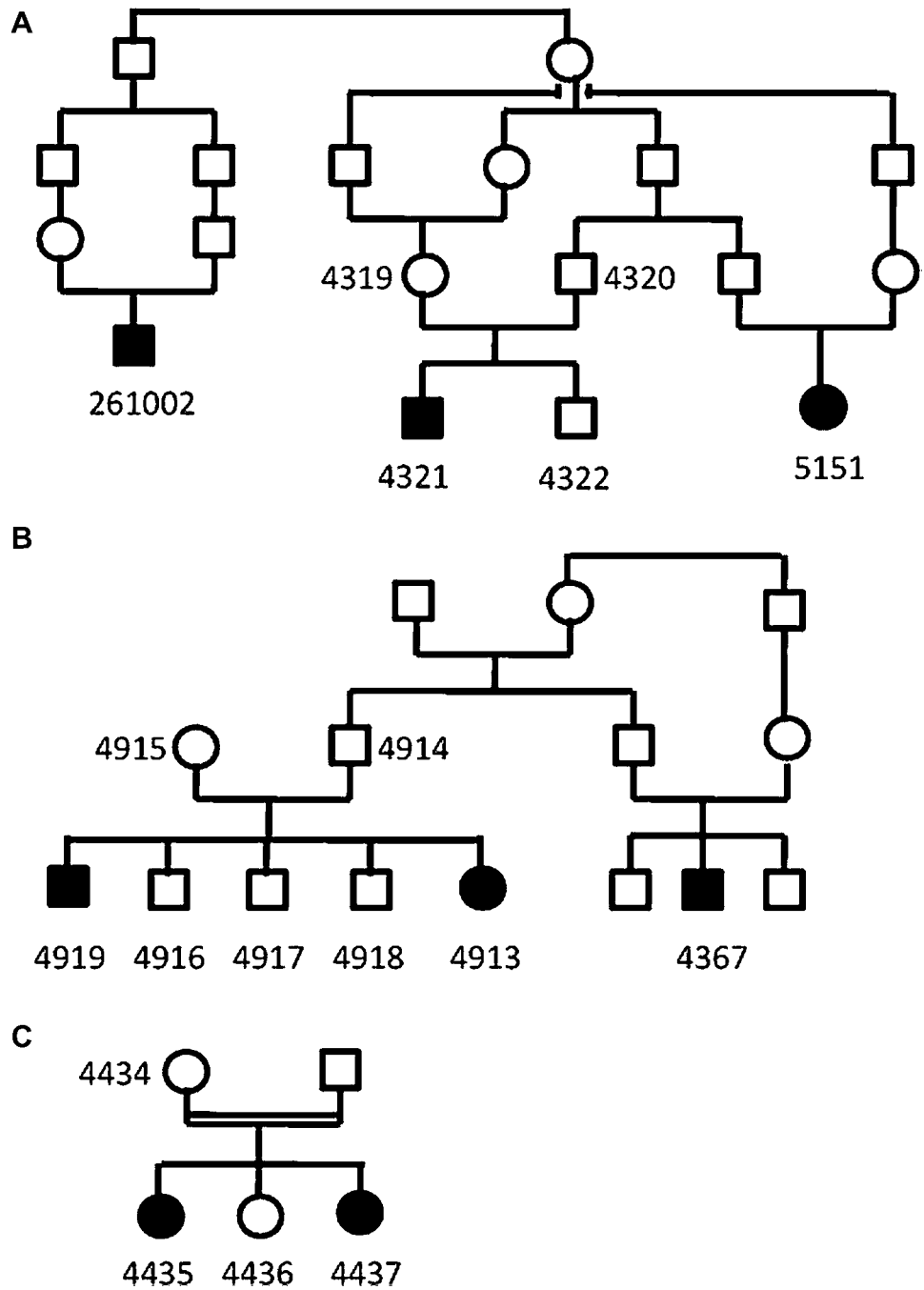


Table 1 Clinical and haematological findings of the patients

Patient	Sex	Age at presentation	Current age (years)	Findings at the time of diagnosis					Course and outcome
				General	Hearing	Eyesight	Haemoglobin (g/dl)	PLT ($\times 10^9/l$)	
261002	M	4 m	6	HSM, MC, FTT	Intact	Unilateral visual loss	6.8	126	Successful BMT at 1 year
4321	M	1 m	4	HSM	Intact	Unilateral visual loss	9.7	54	Successful BMT at 19 months
5151*	F			HSM		Bilateral visual loss	8.8	378	Died at 32 months
4367	M	4 m	10	MC, unilateral microphthalmia	Intact	Unilateral visual loss	10.2	348	V-P shunt for hydrocephaly
4919	M	1 m	18	HSM, MC, facial nerve palsy	Intact	Unilateral visual loss	7.4	153	Successful BMT at 29 months
4913	F	1 year	10	Massive HSM, FTT	Intact	Bilateral visual loss	6.6	39	Left eye perforation, curved humerus and fractures
4437*	F		7	HSM		Bilateral visual loss	Transfusion dependent		Failed BMT at 2 years
4435*	F		3						Successful BMT at 4 months

*These patients were not followed at our centre and information on their presentation and course is sparse.
BMT, bone marrow transplant; FTT, failure to thrive; HSM, hepatosplenomegaly; MC, macrocephaly; PLT, platelets; V-P, ventriculo-peritoneal.

cells and the sphenoidal sinuses were fully ossified. Two patients underwent bone marrow transplantation at 10 and 19 months and on follow-up had normal growth and development.

Methods

Linkage analysis of the diseased gene, using DNA of the four patients, was performed with the GeneChip Human Mapping 250K Nsp Array of Affymetrix, as previously described.⁵ All experiments involving DNA of the patients, their relatives, and healthy controls were approved by the Hadassah Ethical Review Committee.

Osteoclast generation

Human peripheral blood mononuclear leucocytes were separated over Ficoll-Hypaque gradient using standard techniques. The interface layer comprising the peripheral blood mononuclear leucocytes was collected, washed in PBS and resuspended in MEM- α /10% FBS containing 60 ng/ml human M-CSF for 72 h and plated onto 96-well plates (150 000 cells/well) or onto Permanox multi-well slides (used for immunofluorescence and dextran internalisation assay). Monolayers were then washed once and incubated with MEM- α /10% FBS containing 60 ng/ml human M-CSF and 100 ng/ml human RANKL for 14 days. The medium was changed on days 4, 7, and 11. Cells were fixed and stained for TRAP using leucocyte acid phosphatase staining kit (Sigma-Aldrich, St Louis, Missouri, USA).

SNX10 immunostaining

The differentiated osteoclasts were fixed with formaldehyde (4% in PBS, 30 min room temperature), quenched with ammonium chloride (1% w/v in PBS), and permeabilised. They were then blocked with 1% BSA, and stained with an SNX10 antibody

(Santa Cruz, California, USA, catalogue no. sc-104657) followed by an RRX coupled secondary antibody. Cells were then mounted in 5% w/v propyl-gallate in 80% glycerol, 100 mM Tris pH 9.0. Images were obtained with Zeiss Axiovert microscope equipped with epifluorescence using the Metamorph software.

Dextran internalisation in osteoclasts

Differentiated osteoclasts were incubated with 200 μ g/ml aldehyde fixable Alexa 488-dextran (Life Technologies, Grand Island, NY, USA, catalogue no. D22910) for 18 h and then fixed and observed as described above.

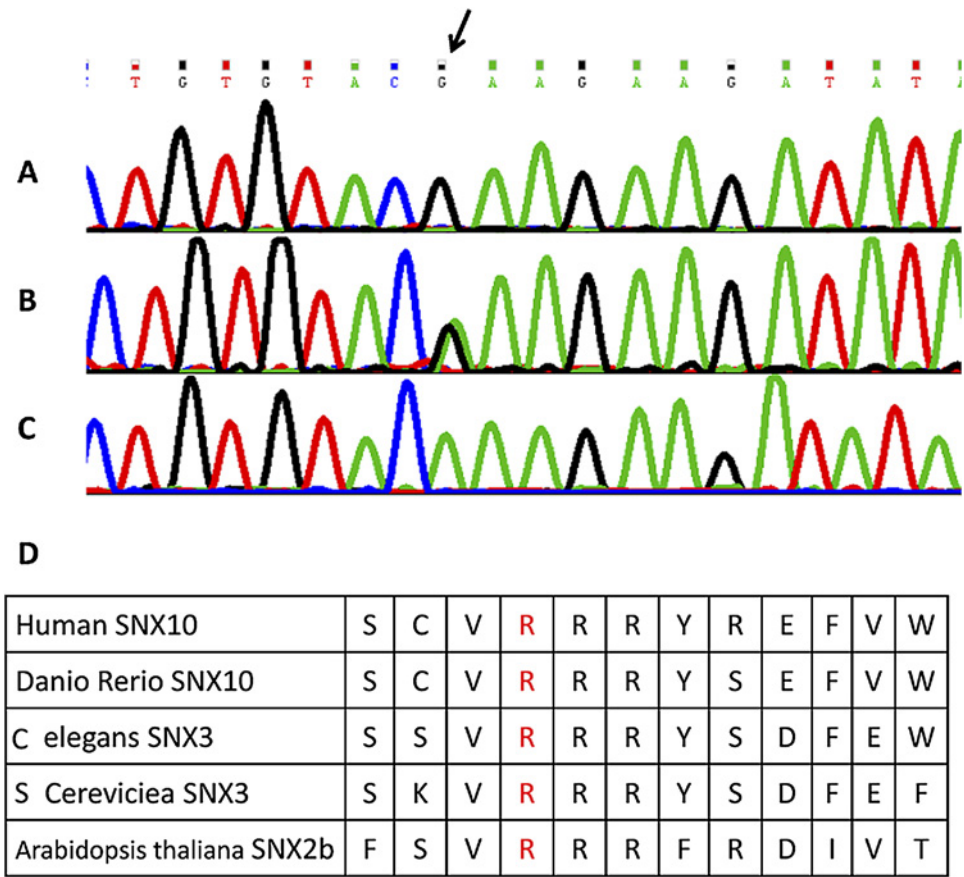
Osteoclastic resorption

Mononuclear fraction of peripheral blood was prepared as for osteoclast generation. Cells were plated onto BD BioCoat Osteologic Bone Cell Culture System chamber slides (150 000 cells/chamber). Monolayers were treated as for osteoclast generation. After 14 days with RANKL the medium was removed, wells were rinsed with water, and a bleach solution (6% NaOCl, 5% NaCl) was added to the wells for 5 min followed by three washes with distilled water. Formation of the resorbed area was examined microscopically; to ensure objectivity the interpretation was performed on unmarked slides.

RESULTS

Because of the similar presentation and the remote consanguinity we searched for shared homozygous regions in the DNA samples of the three pedigree A patients, 5151, 261002, and 4321. The DNA single nucleotide polymorphism (SNP) analysis revealed multiple homozygous regions in each patient, but all three shared a single genomic stretch on chromosome 7:11.46–26.60 Mb (numbering according to Human Genome

Figure 2 The Arg51Gln mutation in the SNX10 gene. DNA sequence of exon 3 in control (A), obligatory carrier (B), and a patient (C). The mutated nucleotide is indicated (arrow). (D) Conserved amino acid sequence around the altered codon (fourth residue). This figure is produced in colour in the online journal—please visit the website to view the colour figure.



build 18). As part of a larger project aiming at the delineation of the molecular basis of OP among Palestinian patients from consanguineous families, we noted that patient 4367, who originated from an unrelated family (pedigree B), had a partly overlapping homozygous region on chromosome 7:25.38–34.26 Mb. Within the common 1.22 Mb genomic stretch, all four patients shared an identical haplotype over the encompassed 128 SNP markers (rs2391129-rs1029561). Five protein coding genes were present within this *NFE2L3*, *HNRNPA2B1*, *CBX3*, *SNX10* and *SKAP2*—and although *SNX10* emerged as a prime candidate, we determined the sequence of all 38 exons and their flanking intronic sequences. No mutation was detected in *NFE2L3*, *HNRNPA2B1*, *CBX3*, and *SKAP2*; however, a missense mutation, c.152 G→A, was identified in exon 3 of *SNX10*. The mutation Arg51Gln (R51Q) was present in a homozygous state in the four patients. We screened both

unrelated and distantly related patients for the mutation and identified an additional four patients (4913, 4919, 4437, and 4435) whose clinical data were integrated into table 1. Altogether, eight patients were homozygous for the mutation, five available parents and four sibs were heterozygous for the mutation, and one sib was homozygous for the normal allele (figure 2A–C). We also screened 211 anonymous individuals of the same ethnicity and identified a single carrier for the R51Q mutation.

Sorting nexin 10 (*SNX10*) belongs to a large and diverse group of 33 proteins of the sorting nexin family. Members of this family share a phosphoinositide binding (PX) domain, and are involved in intracellular processes such as endocytosis, protein sorting, and degradation.⁶ The *SNX10* gene consists of six exons encoding 224 amino acids and the PX domain resides in residues 38–135. Arg51 and its surrounding amino acids are conserved

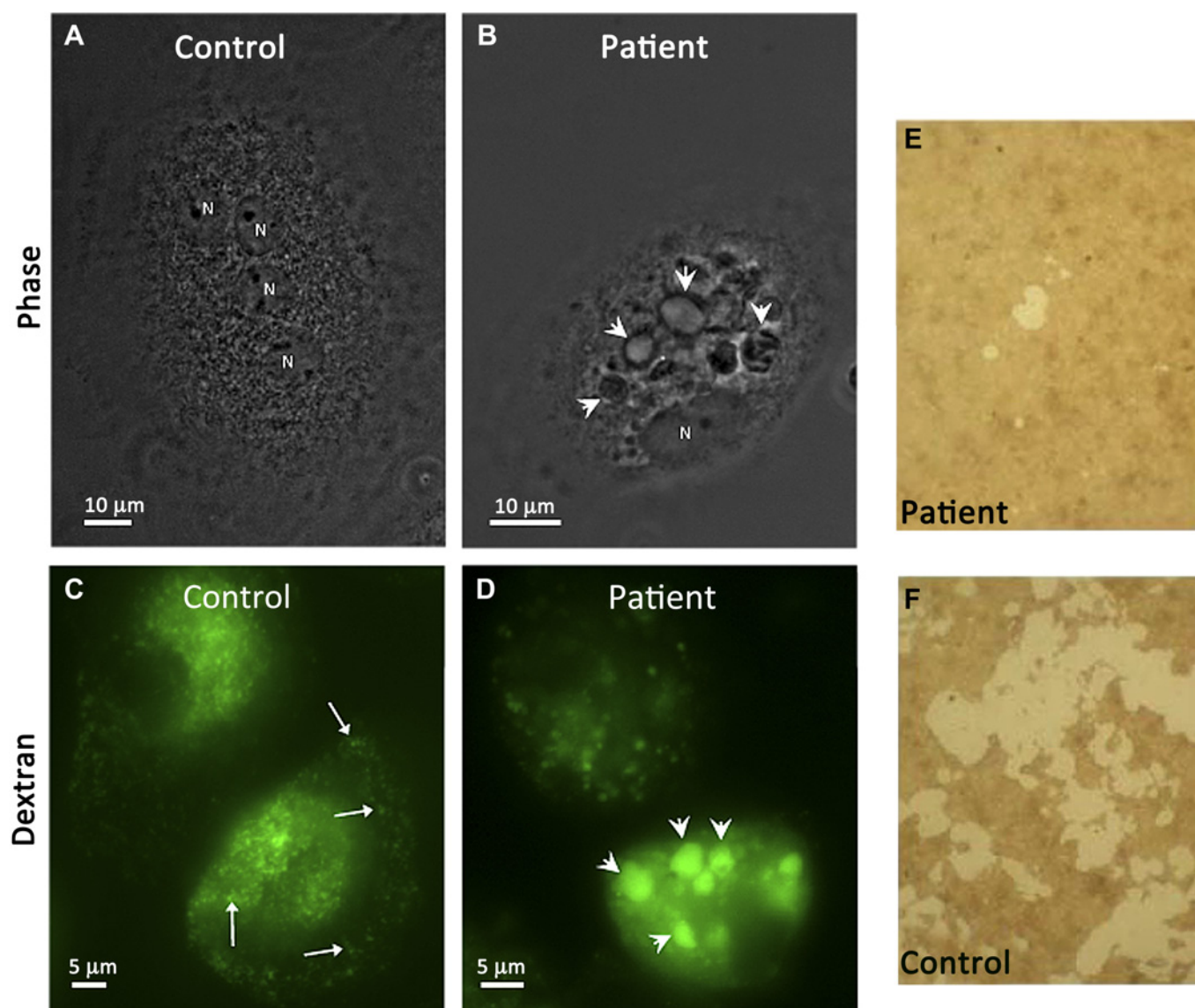


Figure 3 Endosomal and functional abnormalities in osteoclasts derived from mononuclear leukocytes in culture from a patient. Extensive cytoplasmic vacuolation is evident by phase microscopy in patient cells (arrowheads in B) as opposed to control cells (A). These cytoplasmic vacuoles (arrowheads in D) are accessible to internalised dextran Alexa 488 which was used to visualise the endocytic system. Contrasting with these large vacuoles, control cells contain mainly small punctuate staining with dextran (arrows in C). Nuclei are indicated by N in panels A and B. Impaired resorptive function of the patients' cells (E) with fewer and smaller areas cleared from substrate compared to the control (F). Representative fields are shown in the figure.

throughout evolution (figure 2D) as well as among other simplified members of the sorting nexin family, including SNX3 and SNX11. The substitution of arginine by glutamine at position 51 is predicted disease-causing by the pathogenicity prediction software Mutation Taster, SIFT, and PolyPhen.

In order to study the effect of the R51Q mutation in the *SNX10* gene we generated osteoclasts from mononuclear leucocytes of a patient and of an age matched control. Many of the patient cells within the monolayer displayed extensive cytoplasmic vacuolation (figure 3B) which were nearly absent in the control cells (figure 3A). Since *SNX10* was proposed to be involved in endosome homeostasis,⁷ we set out to examine the morphology of the endosomal system in these cells by loading them with fluorescent dextran. This experiment revealed that 25% of the patient cells contained large endosomal vacuoles up to 3–5 μm in diameter (figure 3D). Many of these vacuoles likely corresponded to giant autophagolysosomes since they had a convoluted lumen and accumulated LysoTracker Red (not shown). In control cells (figure 3C) only 2% of the cells displayed these vacuoles, suggesting that mutated *SNX10* is associated with abnormal endosomal morphology.

Next we determined the subcellular distribution of the mutant *SNX10*. Immunostaining of the osteoclasts revealed that the mutant protein in the patient's cells was notably increased. The distribution of the mutant *SNX10* was strikingly different from that of the WT protein. Most prominent were *SNX10* positive punctae which were clustered throughout the cytoplasm (figure 4C–D).

Finally, we studied the resorptive function of the cells using calcium phosphate ceramic thin-film-coated quartz 16-well multitest slides. The patient's cells resorbed far less substrate compared with the control, as revealed by the fewer and smaller areas cleared from substrate (figure 3E–F). It is of note, however, that fewer (~50–60% of control) and smaller osteoclasts were observed in the patient's derived culture than in the control. It is not clear if this is the result of fewer osteoclast precursors in the

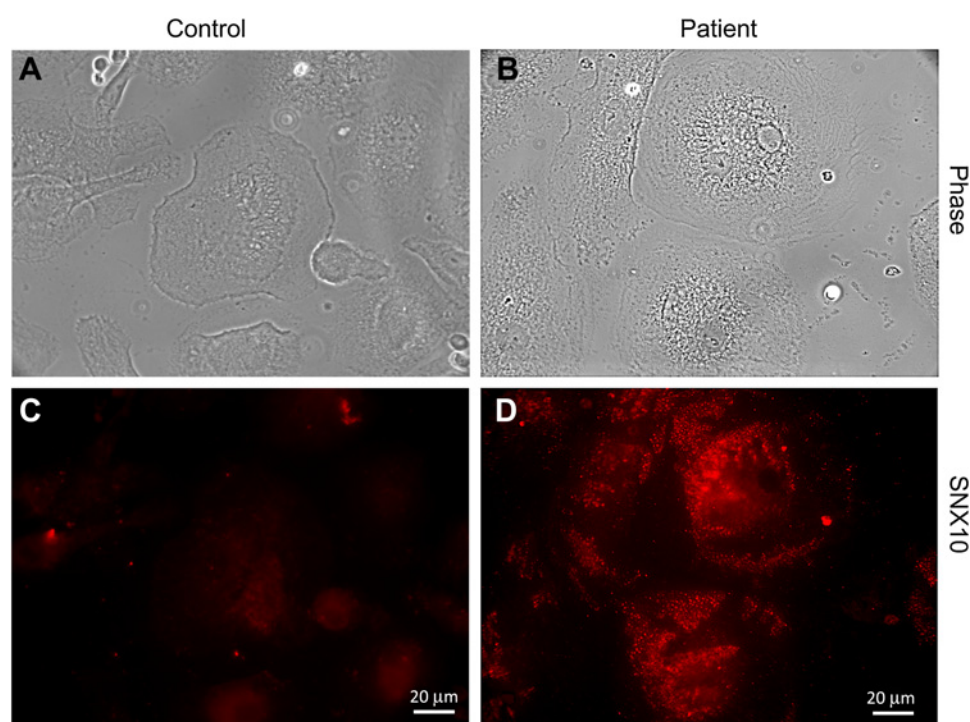
patient's peripheral blood or of an impaired differentiation. Taken together, the results suggest that the R51Q mutation in *SNX10* is associated with dysfunction and possibly impaired differentiation of osteoclasts, likely by interfering with their endosomal pathway.

DISCUSSION

What might be the role of *SNX10* in OP? OP results from failure of osteoclasts to dissolve mineralised bone. Osteoclasts are generated from bone marrow derived monocyte/macrophage precursor cells. An essential signal for initiating osteoclastogenesis is the activation of the receptor activator of nuclear factor- κB (RANK) by its specific ligand, RANKL. RANKL is a haematopoietic factor, carried by osteoblasts and stromal cells, and is involved in the generation of cytosolic Ca^{2+} oscillations. These are important for the expression of the master regulator of osteoclastogenesis, nuclear factor of activated T cells c1 (NFATc1). Osteoclasts function in bone resorption via their ruffled border, a convoluted membrane within the resorption lacuna. This space is acidified by a vacuolar type H^+ -ATPase (V-ATPase), a multi-subunit complex which pumps protons generated by carbonic anhydrase II. Electroneutrality is maintained by the chloride channel *CLCN7* which transports chloride ions into the lacuna as counter ions. *CLCN7* requires transmembrane protein 1 (*OSTM1*) for its proper localisation. Both V-ATPase and *CLCN7* are brought to the ruffled border by late endosome/lysosome, underscoring the importance of vesicular trafficking for osteoclastic resorption. Importantly, *SNX10* was recently shown to co-immunoprecipitate with V-ATPase and regulate its intracellular trafficking.⁸

Mutations in several osteoclast related genes were heretofore associated with OP,⁴ mostly involving the maintenance of acid pH at the osteoclast-bone interface; about half of the patients carry recessive mutations in *TCIRG1* which encode the α -3 subunit of V-ATPase.⁹ An additional 10–15% of the patients harbour mutations in *CLCN7* which encodes the chloride

Figure 4 Strikingly different distribution of *SNX10* in osteoclast enriched mononuclear leucocytes from a patient. Cells were stained with an *SNX10* antibody. In many of the patient's cells (D), *SNX10* accumulated in clustered punctae throughout the cytoplasm (compare with control cells in (C)). (A) and (B): phase microscopy of the fields in (C) and (D).



channel. The clinical manifestations of *CLCN7* mutations are not only OP but also degeneration of the brain and the retina as a result of abnormal lysosomal storage.¹⁰ Rare causes of OP are: mutations in *OSTM1* which interacts with *CLCN7* and also manifests by neonatal neuro-retinal degeneration and microcephaly, contrasting with the presentation in the more common genetic variants¹¹; mutations in *CAII*, which encodes carbonic anhydrase type II, and are characterised by the concomitant occurrence of renal tubular acidosis¹²; and a mutation in Plekstrin homology domain-containing family M member 1 (*PLEKHM1*), which is operative in vesicular transport in osteoclasts via its interaction with Rab7, a Rab GTPase that localises to the late endosome/lysosome,¹³ and causes OP because of the osteoclasts' failure to form a ruffled border.¹⁴ An additional small group of OP patients suffer from defects in osteoclast differentiation, as a result of mutations in either RANK or its ligand RANKL.^{15 16}

We hereby show that a mutation in a conserved residue in the *SNX10* gene is associated with: (1) a strikingly altered cytoplasmic distribution of an excessive amount of the mutant protein; (2) perturbation of the endosomal/lysosomal pathway, demonstrated by the generation of large vacuoles, perhaps autophagolysosomes, which accumulate vast amounts of internalised fluorescent dextran; and (3) abnormally small osteoclasts with reduced resorptive capacity. Since the resorptive capacity of the patients' osteoclasts is decreased and the clinical phenotype is reminiscent of that of patients with *TCIRG1* mutations, we speculate that *SNX10* is responsible for the vesicular sorting of the V-ATPase complex from the Golgi network or for its targeting to the ruffled border. A mutation in *SNX10* would therefore result in a 'secondary V-ATPase deficiency' with a failure to acidify the resorption lacuna. In view of the complete cure achieved by allogeneic bone marrow transplantation, we suggest that the functional impairment caused by *SNX10* mutation is restricted to haematopoietic lineage. Determination of the sequence of the *SNX10* gene is warranted in molecularly undefined patients in order to estimate its relative contribution to OP.

Acknowledgements We thank Judith Melki for linkage analysis and Lital Sheva and Rachel Dahan for excellent technical assistance.

Contributors MA, AT, ZB, OE conceived and designed the experiments; AR, SH, AT, AS, SZ, AT, ZB performed the experiments; MA, AR, AT, AS, SZ, AT, ZB, OE analysed the data; SI, MW contributed reagents/materials/analysis tools; MA, AR, AT, ZB, OE wrote the paper; MA, SH, MV undertook patient management, collection of samples, and delineation of the phenotype.

Funding This study was supported by the Hadassah-Hebrew University Joint Foundation grant to SH.

Competing interests None.

Ethics approval Ethics approval was provided by Hadassah Helsinki committee.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. Steward CG. Neurological aspects of osteopetrosis. *Neuropathol Appl Neurobiol* 2003;**29**:87–97.
2. Dozier TS, Duncan IM, Klein AJ, Lambert PR, Key LL. Otolgic manifestations of malignant osteopetrosis. *Otol Neurotol* 2005;**26**:762–6.
3. Lam DK, Sándor GK, Holmes HI, Carmichael RP, Clokie CM. Marble bone disease: a review of osteopetrosis and its oral health implications for dentists. *J Can Dent Assoc* 2007;**73**:839–43.
4. Villa A, Guerrini MM, Cassani B, Pangrazio A, Sobacchi C. Infantile malignant, autosomal recessive osteopetrosis: the rich and the poor. *Calcif Tissue Int* 2009;**84**:1–12.
5. Edvardson S, Shaag A, Kolesnikova O, Gomori JM, Tarassov I, Einbinder T, Saada A, Elpeleg O. Deleterious mutation in the mitochondrial arginyl-transfer RNA synthetase gene is associated with pontocerebellar hypoplasia. *Am J Hum Genet* 2007;**81**:857–62.
6. Cullen PJ. Endosomal sorting and signalling: an emerging role for sorting nexins. *Nat Rev Mol Cell Biol* 2008;**9**:574–82.
7. Qin B, He M, Chen X, Pei D. Sorting nexin 10 induces giant vacuoles in mammalian cells. *J Biol Chem* 2006;**281**:36891–6.
8. Chen Y, Wu B, Xu L, Li H, Xia J, Yin W, Li Z, Shi D, Li S, Lin S, Shu X, Pei D. A *SNX10*/V-ATPase pathway regulates ciliogenesis in vitro and in vivo. *Cell Res* 2012;**22**:333–45.
9. Frattini A, Orchard PJ, Sobacchi C, Giliani S, Abinun M, Mattsson JP, Keeling DJ, Andersson AK, Wallbrandt P, Zecca L, Notarangelo LD, Vezzoni P, Villa A. Defects in *TCIRG1* subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. *Nat Genet* 2000;**25**:343–6.
10. Kornak U, Kasper D, Bosl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G, Jentsch TJ. Loss of the *ClC-7* chloride channel leads to osteopetrosis in mice and man. *Cell* 2001;**104**:205–15.
11. Chalhoub N, Benachenhou N, Rajapurohitam V, Pata M, Ferron M, Frattini A, Villa A, Vacher J. Grey-lethal mutation induces severe malignant autosomal recessive osteopetrosis in mouse and human. *Nat Med* 2003;**9**:399–406.
12. Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, Tashian RE. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc Natl Acad Sci U S A* 1983;**80**:2752–6.
13. Tabata K, Matsunaga K, Sakane A, Sasaki T, Noda T, Yoshimori T. Rubicon and *PLEKHM1* negatively regulate the endocytic/autophagic pathway via a novel Rab7-binding domain. *Mol Biol Cell* 2010;**21**:4162–72.
14. Van Wesenbeeck L, Odgren PR, Coxon FP, Frattini A, Moens P, Perdu B, MacKay CA, Van Hul E, Timmermans JP, Vanhoenacker F, Jacobs R, Peruzzi B, Teti A, Helfrich MH, Rogers MJ, Villa A, Van Hul W. Involvement of *PLEKHM1* in osteoclastic vesicular transport and osteopetrosis in incisors absent rats and humans. *J Clin Invest* 2007;**117**:919–30.
15. Guerrini MM, Sobacchi C, Cassani B, Abinun M, Kilic SS, Pangrazio A, Moratto D, Mazzolari E, Clayton-Smith J, Orchard P, Coxon FP, Helfrich MH, Crockett JC, Mellis D, Vellodi A, Tezcan I, Notarangelo LD, Rogers MJ, Vezzoni P, Villa A, Frattini A. Human osteoclast-poor osteopetrosis with hypogammaglobulinemia due to *TNFRSF11A* (*RANK*) mutations. *Am J Hum Genet* 2008;**83**:64–76.
16. Sobacchi C, Frattini A, Guerrini MM, Abinun M, Pangrazio A, Susani L, Bredius R, Mancini G, Cant A, Bishop N, Grabowski P, Del Fattore A, Messina C, Errigo G, Coxon FP, Scott DI, Teti A, Rogers MJ, Vezzoni P, Villa A, Helfrich MH. Osteoclast-poor human osteopetrosis due to mutations in the gene encoding *RANKL*. *Nat Genet* 2007;**39**:960–2.