Indian J Med Res 131, April 2010, pp 508-514

Novel mutations in Indian patients with autosomal recessive infantile malignant osteopetrosis

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Received April 23, 2009

***Background & objectives*: Although clinical reports have described infantile malignant autosomal recessive osteopetrosis (ARO) in Indian patients, no published data are available about the genetic causes of ARO in this population. We investigated the main genetic causes of ARO in eight Indian patients with early postnatal onset and the typical severe clinical course including visual impairment and anaemia.**

***Methods*: Mutation screening in the genes *CLCN7* and *TCIRG1* was done on genomic DNA from 8 affected individuals (diagnosed on the basis of clinical and haematological parameters and characteristic radiological changes of increased bone density) and their parents. In one family, after detection of both mutations in the proband, targeted mutation analysis was also done in chorionic villus samples for prenatal diagnosis.**

***Results*: Six patients had mutations in *TCIRG1* and two patients harboured mutations in *CLCN7* gene. Three of the five different *TCIRG1* mutations identified and both *CLCN7* mutations were novel mutations. Except for the already known mutation p.Ile720del, all *TCIRG1* mutations disrupt conserved splice consensus sequences or lead to premature stop codons. In contrast, both *CLCN7* mutations only lead to missense changes of conserved amino acids. In a foetus harbouring *TCIRG1* mutations osteopetrosis was visible radiologically at 23 wk of gestation.**

***Interpretation & conclusions*: That the *CLCN7* mutations provoke a phenotype as severe as the one caused by *TCIRG1* loss of function suggests the affected residues to be crucial for the function of the ClC-7 chloride channel or chloride/proton-exchanger. Our data also show that ARO can manifest as early as in the second trimester of pregnancy.**

Key words Autosomal recessive - *CLCN7* - India - infantile - malignant osteopetrosis - mutations - *TCIRG1*

Infantile malignant osteopetrosis (arOP; ARO; OMIM 259700) is an autosomal recessive disease manifesting with anaemia, thrombocytopenia, hepatosplenomegaly, visual impairment due to optic atrophy and deafness. Most of the children die during infancy or early childhood without curative treatment by bone marrow transplantation. Though there are no data available about prevalence, it is not a very rare disease in India[1,2]. Osteopetrosis is caused by a defect in osteoclast function. The degradation of the mineralized extracellular matrix of the bone requires acid secretion by the osteoclast ruffled membrane. This

proton transport is driven by a vacuolar (v-) type H^+-ATPase that is anchored to the ruffled membrane by the a3 subunit. Mutations in the gene *TCIRG1* (*ATP6V0A3*) encoding the a3 subunit were found to cause infantile malignant osteopetrosis[3,4]. Other genes mutated in ARO are *CLCN7* and *OSTM1*, that together form a chloride channel or chloride/proton-exchanger which also resides in the ruffled membrane and facilitates acidification[5,6]. Mutations in the *PLEKHM1* gene cause milder forms of autosomal recessive osteopetrosis[7,8]. A mild form of osteopetrosis associated with renal tubular acidosis is caused by carbonic anhydrase II gene[9]. In contrast to all ARO forms mentioned before, a minority of patients has strongly reduced osteoclast numbers. In some of these patients mutations in *TNFSF11*, encoding the osteoclast differentiation factor RANKL, were identified[10]. Mutations in the RANK receptor also cause osteoclast-poor osteopetrosis with additional immunological abnormalities[11]. Here, we report the mutation spectrum in Indian patients with autosomal recessive malignant osteopetrosis.

Material & Methods

Patients: Eight patients diagnosed to have infantile malignant autosomal recessive osteopetrosis (ARO) in the Medical Genetics department of the Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, Uttar Pradesh and the Genetics unit of the Pediatrics department of the All India Institute of Medical Sciences, New Delhi, during 2003 to 2008, were chosen for the study. The diagnosis of ARO was based on typical clinical and haematological parameters and characteristic radiological changes of increased bone density.

Sample collection: This study was conducted as part of a large project ongoing in the Department and ethical clearance was obtained from the Ethic Committee of the Institute (SGPGIMS, Lucknow). Informed consent was obtained from the parents of all affected children. Up to 5 ml EDTA blood was collected through venipuncture. DNA was extracted from 1 ml of this venous EDTA blood using the QIAamp DNA mini kit (Qiagen, Hilden, Gernmay).

Mutation analysis: Patient DNAs were investigated for mutations in the genes *TCIRG1* (*ATP6V0A3*) and *CLCN7* by amplifying all exons and flanking intronic regions by PCR using genomic DNA as a template. PCR conditions and primers have been described previously[4,6,10,12]. Dye terminator sequencing was performed using Big Dye (Applied Biosystems, Fosterville, USA) sequencing mix and an AB 3730 capillary sequencer (Applied Biosystems, Fosterville). Sequences were compared with the reference sequences using the software DNASTAR (DNASTAR, Madison, USA). Reference cDNA sequences were: 1. *CLCN7*: NM_001287, 2. *TCIRG1*: NM_006019. In one family, after detection of both the mutations in the proband, prenatal diagnosis was done three times by mutation testing in DNA from chorionic villi samples. The molecular diagnostic tests were performed in an accredited laboratory (No.: DAP-ML-3869.00 (ISO 15189:2003 and ISO/IEC 17025:2005).

Molecular modeling: The 3D structure of the bacterial CLC chloride channel EcClC was reconstructed by loading the PDB file 1KPK (download under *http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1KPK*) containing the published structure into PyMOL (version pre-1.0) (DeLano Scientific, Palo Alto, CA, USA)[13]. Residue Val297 corresponds to EcClC residue Leu186.

Results

The clinical data and the results of the mutation screening of the patients are given in Table I and Table II, respectively. Three of the patients were Hindu, three were Muslims and two were Sikhs. In two families consanguinity was known. The age at diagnosis was between 3 and 18 months and the mean life expectancy without curative bone marrow transplantation was 3.7 yr. Optic atrophy and visual impairment were major features reported in six patients. In contrast, seizures only appeared in one patient. Blood analysis revealed severe anaemia in all affected children.

Radiographic data were available for all patients and showed a severe generalized osteosclerosis of the long bones, the spine and the skull base (Fig. 1). The long bones were abnormally modeled, had no bone marrow cavity and frequently displayed a bone-within-bone appearance (Fig. 1).

Mutations on both alleles of the genes *TCIRG1* or *CLCN7* were detected in all eight cases. Six patients harboured disease-causing mutations in *TCIRG1* (Table II, Fig. 2). Patients 4, 5 and 6 were homozygous for a mutation: c.2236+1G>T, c.1554+2T>A and c.2160_2162del (Ile721del). DNA was not available for patient 2, but both the parents were found to be heterozygous for the mutation c.2160_2162del (1721del) (Fig. 2). Patients 1 and 3 were compound heterozygous. While patient 1 displayed the mutations c.1554+2T>A and c.2160_2162del (Ile721del) already

Table I. Clinical details of the patients

No.	Patient	Age at diagnosis (months)	Sex	Consanguinity / Religion	Age at death (yr)	Presenting complaint	Other features
1	AR	6	M	No / Muslim	NA	Increasing head size	Obstructive hydrocephalus, optic atrophy
2	MI	6	M	? / Muslim	0.7	Failure to thrive, distension of abdomen	Optic atrophy
3	RT	12	M	No / Hindu	7	Anaemia, distension of abdomen	Optic atrophy
4	AF	18	M	Yes / Muslim	NA	Noisy breathing, prominent eyes, bleeding spots	-
5	KH	6	F	No / Hindu	1	Inability to focus, anaemia	Optic atrophy
6	PR	3	M	No / Hindu	NA	Anaemia, previous sib died of osteopetrosis	-
7	AM	18	M	Yes/ Sikh	5.6	Visual impairment, jaw osteomyelitis	Hypocalcemic seizures, optic atrophy
8	HA	13	F	No/ Sikh	1.6	Anaemia	Facial dysmorphism, optic atrophy

Table II. Results of laboratory investigations and mutation analysis

No.	Patient	Hb (g/dl) (N: 11.5 -15.5)	TLC (X 10^3/ cmm) (N: 5 - 15)	Platelets (Lac/ cmm) (N:1.5 - 4)	Normo-blasts (%.)	Premature cells	ALP (U/l) (N:150 - 420)	Molecular findings	Status
								Mutations in *TCIRG1*	
1	AR	6.1	38.0	0.8	45%	No	386	c.1554+2T>A	Het
								c.2160_2162del (p.Ile721del)	Het
2	MI*	7.4	58.0	0.78	35%	Myelocytes, metamye-locytes	1535	c.2160_2162del (1le721del)	(hom)
3	RT**	7.0	1.04	1.1	+	Yes	1316	c.1684C>T (p.Gln562X)	Het
								c.1653_1654insGTGG (p.Val551fsX670)	Het
4	AF	6.2	-	-	-	-	-	c.2236+1G>T	Hom
5	KH**	7.4	22.7	0.59	+	No	-	c.2160_2162del (p.Ile721del)	Hom
6	PR**	-	-	-	-	-	-	c.1554+2T>A	Hom
								Mutations in *CLCN7*	
7	AM	6.7	12.9	0.99	-	-	2280	c.889G>A (p.Val297Met)	Hom
8	HA	8.2	11.1	0.82	14	-	-	c.1856C>T (p.Pro619Leu)	Hom

*Proband's sample was not available. Both parents were heterozygous for the mutation. **Heterozygosity in the parents was confirmed
N, normal range; TLC, total leucocyte count; ALP, serum alkaline phospha tase

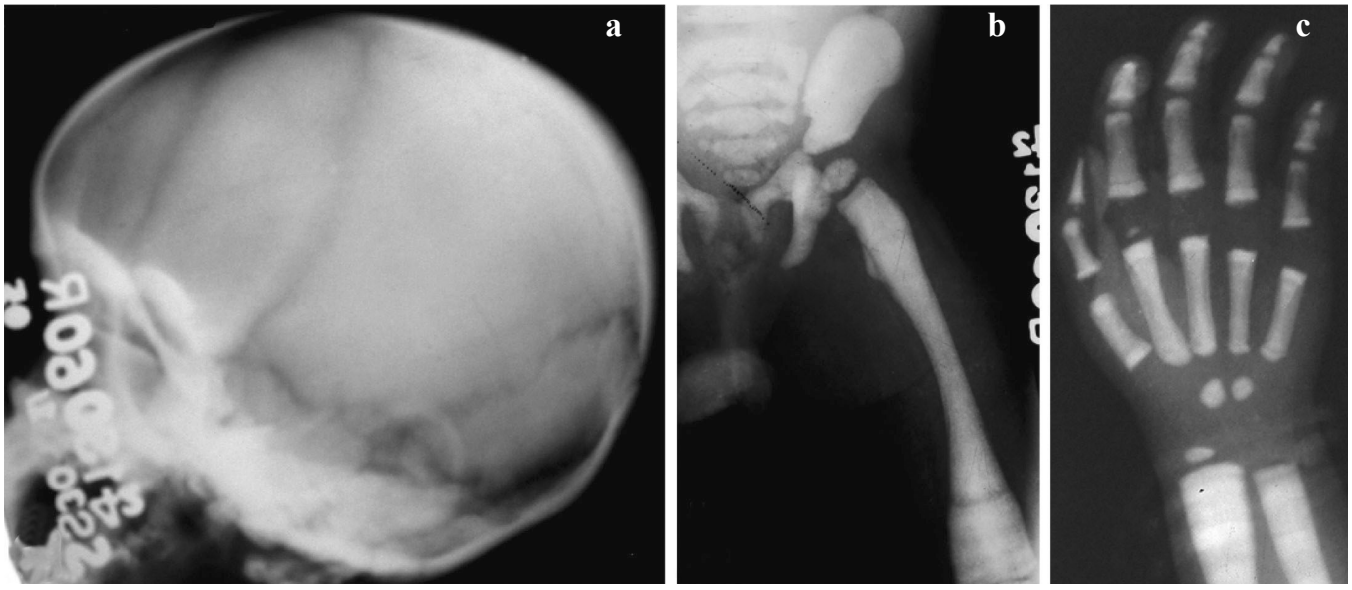

Fig. 1. Radiological phenotype of patient 8 with a *CLCN7* mutation. **(a)** The skull shows the typical sclerosis of the skull base. **(b)** Sclerosis of the vertebral bodies and iliac wings is pronounced. **(c)** The bone-within-bone appearance, especially visible in the metacarpal bones, and the sclerosis and undermodelling of the distal radius and ulna is evident.

found in the other probands, patient 3 harboured the two distinct mutations c.1684C>T (Gln562X) and c.1653_1654insGTGG (Val551fsX670) (Table II).

In the two remaining cases we found novel homozygous mutations in the second most common ARO gene, *CLCN7*: c.889G>A (p.Val297Met) in patient 7 and c.1856C>T (p.Pro619Leu) in patient 8. The possibility of these two novel mutations being

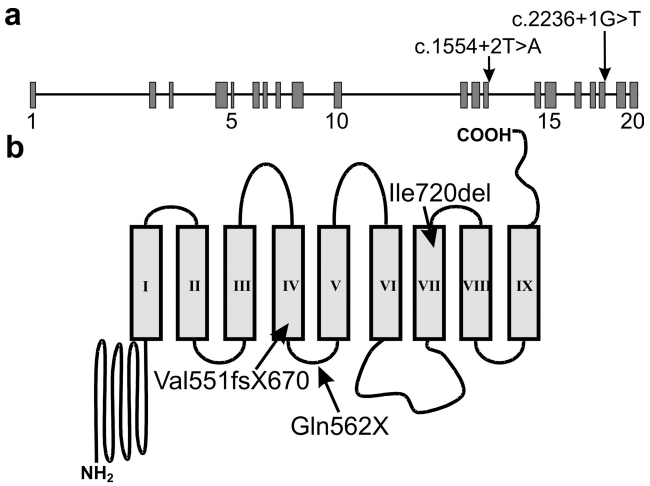

Fig. 2. Mutations in *TCIRG1* (*ATP6V0A3*) identified in Indian osteopetrosis patients. **(a)** The position of splice site mutations is given by arrows in a schematic representation of the genomic organization of the *TCIRG1* gene. The number of every fifth exon is given. **(b)** The position of all mutations not affecting slice sites are indicated in a model of the transmembrane topology of the TCIRG1 protein.

polymorphisms was ruled out by demonstrating their absence in 80 healthy Indian controls (equivalent to 160 alleles). Sequence alignments revealed that both residues are highly conserved in ClC-7 orthologs from different vertebrate species (Fig. 3). Furthermore, Val297 is also conserved in the muscle-specific ClC-1 chloride channel or chloride/proton exchanger indicating a functional importance. Indeed, molecular modeling of the corresponding residue Leu186 in the *Escherichia coli* EcClC indicates that the longer non-polar side chain introduced by a mutation to Met could have an influence on the selectivity filter of the ClC chloride/proton exchanger (Fig. 3).

Patients 1 and 4 had a history of two affected older siblings who had died in infancy and also in the family of patient 6 the next sibling born was affected with osteopetrosis. The mother of patient 3 underwent prenatal diagnosis by chorionic villus sampling in her next three pregnancies. The first prenatal diagnosis showed absence of mutations in the prenatal sample and the pregnancy resulted in the birth of a normal girl. In the second prenatal diagnosis the presence of both *TCIRG1* mutations (p.Gln562X and p.Val551fsX670) in chorionic villus DNA was detected and the pregnancy was terminated at 23 wk of gestation. The radiograph of the foetus showed mildly increased bone density and marrow cavity obstuction (Fig. 4). The third prenatal mutation screening showed normal results; but in the early third trimester the foetus showed

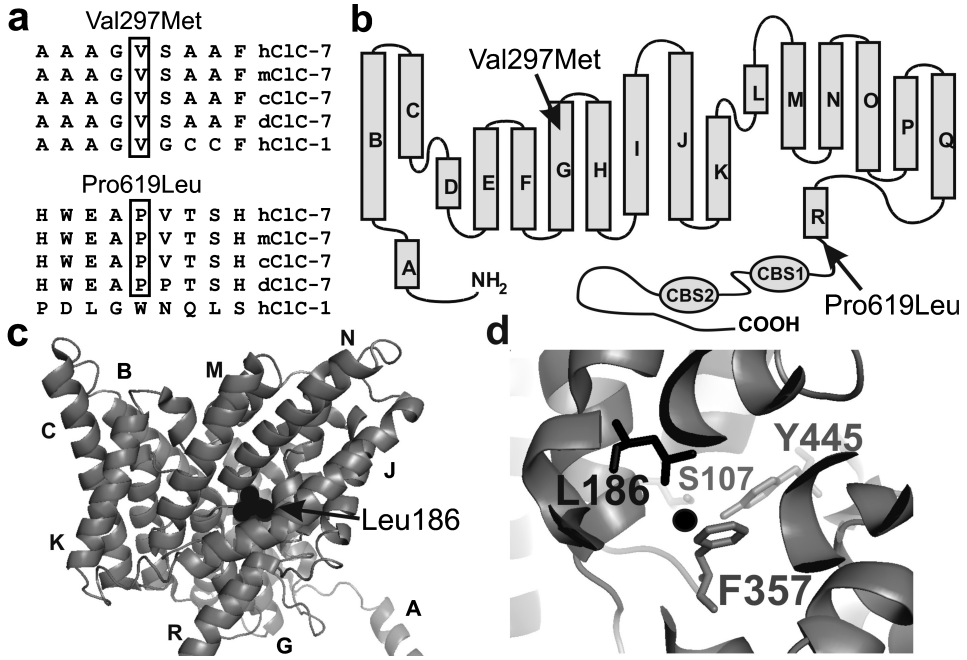

Fig. 3. Mutations identified in the ClC-7 chloride-proton antiporter. **(a)** Multiple alignments of the ClC-7 protein sequences from human (h), mouse (m), chick (c) and zebrafish (d) and of human ClC-1 (hClC-1) around the position of the mutations Val297Met and Pro619Leu. Note complete conservation of the affected residues in all ClC-7 orthologs. While Val297 is also conserved in hClC-1 the region containing Pro619 is very divergent between ClC-7 and ClC-1. **(b)** Position of the two missense mutations in the ClC topology model. While Val297Met resides in helix G Pro619Leu is located after the regulatory helix R. **(c)** 3D modeling of the position of residue Leu186 which corresponds to Val297 in the ClC protein from *E. coli* (EcClC). Although Leu186 is not directly involved in binding of chloride and the formation of the selectivity filter of the chloride/proton exchanger, it lies in proximity to relevant residues. **(d)** Closer modeling of the chloride binding site including residues Ser107, Phe357 and Tyr445. Leu186 is near the chloride ion (represented as black ball, scaling not correct). A further protrusion of the side chain due to mutation of Leu186 to Met could sterically affect the binding site.

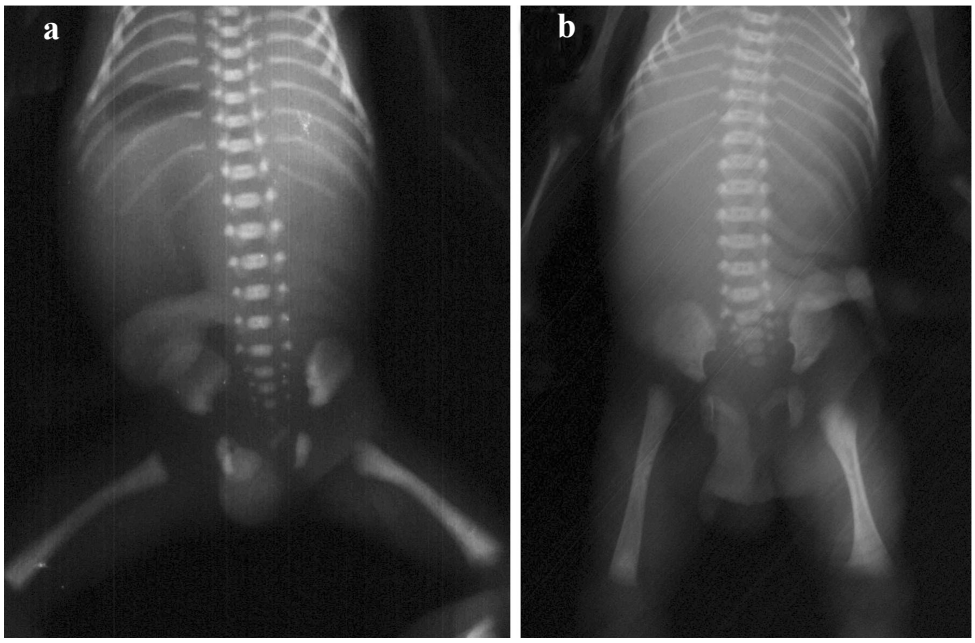

Fig. 4. Post-termination radiograph of an *TCIRG1* mutation-positive **(a)** and an unaffected fetus **(b)** at 23 wk of gestation. **(a)** Note mild increase in general bone density. Obliteration of the bone marrow cavity and mild bone-within-bone appearance is seen in femora. **(b)** Note that there is a clear separation of cortical bone and medullary cavity in the normal foetus.

significant growth retardation. The karyotype of the foetus was normal and ultrasonography did not show any malformation. The outcome of this last pregnancy is not known as the family was lost to follow up.

Discussion

Autosomal recessive malignant osteopetrosis (ARO; arOP) is a serious lethal disorder usually leading to death in infancy and childhood. The only curative treatment is haematopoietic stem cell transplantation[14]. The clinical course of the patients reported here was similar to the commonly seen presentation of ARO[15]. All patients presented with severe anaemia due to constriction of the bone marrow cavity. Only two patients did not show evidence of optic atrophy due to optic nerve encroachment. As was already outlined by Susani *et al*[16], the phenotype caused by *TCIRG1* mutations is relatively uniform. It is, however, striking that the life expectancy differs very much between the individual cases. In our patients, one (patient 2) died at 0.7 yr whereas case 3 survived for 7 yr. The seizures in patient 7 were found to be associated with hypocalcaemia. Since *CLCN7* mutations were identified in this case, it is also possible that the seizures were a sign of neuronopathic changes, which are more frequent if *CLCN7* mutations are present[17].

Mutations in *TCIRG1* gene are the most frequent cause for ARO and are found in approximately 50 per cent of the cases[18]. A total of about 44 different *TCIRG1* mutations have been published[16]. In this study *TCIRG1* mutations were identified in six of the eight patients. Of the five *TCIRG1* mutations identified, c.2160_2162del (Ile721del) and c.1554+2T>A have been reported previously[16]. The splice site mutation in one of the patients, c.2236+1G>T has not been described; but at the same location a G>A mutation has been previously reported[19]. The other two mutations c.1684C>T (Gln562X) and c.1653_1654insGTGG (Val551fsX670) are novel. Since both mutations lead to a loss of several transmembrane helices of the v-type ATPase subunit a3 these clearly entail a loss of function like most of the mutations described so far. Population-specific common mutations for *TCIRG1* gene have been observed in other studies. All nine Costa Rican patients reported by Sobacchi *et al*[19] had either or both of two missense mutations (G405R and R444L). In a study of 55 patients of autosomal recessive osteopetrosis, Susani *et al*[16] reported two mutations namely; c.1674-1G>A (aberrant splicing: r.1674_1884del) and c.2005C>T (protein variation: p.Arg669X), in 17 and 16 alleles, respectively. These two alleles constituted 30 per cent of all *TCIRG1* abnormalities. About 40 per cent of all *TCIRG1* mutations are splice site mutations. This proportion was also found in our patients. Given the high numbers of splice site mutations attempts have been undertaken to prevent abnormal splicing by addition of mutated U1 snRNAs[16].

The second most common cause of ARO are mutations in the gene *CLCN7*[18]. *CLCN7* encodes the chloride channel or chloride/proton exchanger ClC-7 that co-operates with the gene product of *TCIRG1*, the a3 subunit of the v-type H^+-ATPase[20]. Both are necessary for resorptive activity of the osteoclast. On an average 15 per cent of all ARO cases are due to *CLCN7* mutations. In our eight patients we found two *CLCN7* mutations suggesting a higher frequency. Both *CLCN7* mutations are missense mutations of highly conserved residues and have not been previously reported. According to the topology of ClC-7 p.Val297Met resides in helix G and p.Pro619Leu is located after helix R in the C-terminus[13]. Although helix G is not directly involved in chloride ion binding, the side chain of Val297 points towards the ion binding site and it is reasonable to speculate that the substitution by an amino acid like methionine with a longer side chain is able to disturb the architecture of the ion binding site and thus alter the function. Indeed, when the corresponding amino acid Val275 in ClC-1 was mutated to the larger residue Trp the chloride channel function was still measurable, but had changed properties[21].

Our study demonstrates that the ARO phenotype already develops in the second trimester and can in principle be visualized radiologically by an absence of a marrow cavity in the long bones. However, in earlier stages the diagnosis by radiology or ultrasonography can be difficult[22,23]. Therefore, mutation screening is the best means to provide a reliable prenatal diagnosis.

TCIRG1 appears to be an important gene for autosomal recessive malignant osteopetrosis in India, similar to the data for other populations available in the literature. However, the mutational spectrum seems to be different from that in other populations as two of the five *TCIRG1* mutations detected are novel. The previously reported c.1554+2T>A and c.2160_2162del (I721del) appear to be common mutations in Indian patients of infantile osteopetrosis. Data on more patients will be useful in deciding on the strategy for mutation detection in Indian patients.

Acknowledgment

Authors thank Claire Schlack for technical assistance, and acknowledge the OSTEOPETR E-Rare grant from the BMBF (to UK) and the Indian Council of Medical Research, New Delhi for financial support.

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