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Molecular and Clinical Heterogeneity in CLCN7-dependent Osteopetrosis: Report of 20 Novel Mutations

Alessandra Pangrazio[1,2], Michael Pusch[3], Elena Caldana[1,2], Annalisa Frattini[1], Edoardo Lanino[4], Parag M Tamhankar[5], Shubha Phadke[5], Antonio Gonzalez Meneses Lopez[6], Paul Orchard[7], Ercan Mihci[8], Mario Abinun[9], Michael Wright[10], Kim Vettenranta[11], Ivo Bariæ[12], Daniela Melis[13], Ilhan Tezcan[14], Clarisse Baumann[15], Franco Locatelli[16], Marco Zecca[16], Edwin Horwitz[17], Lamia Sfaihi Ben Mansour[18], Mirjam Van Roij[19], Paolo Vezzoni[1,2], Anna Villa[1,2], and Cristina Sobacchi[1,2]

[1]Institute of Biomedical Technologies, National Research Council, 20090 Segrate, Italy, [2]Istituto Clinico Humanitas IRCCS, 20089 Rozzano, Italy, [3]Institute of Biophysics, National Research Council, 16149 Genova, Italy, [4]Department of Paediatric Haematology-Oncology, IRCCS G. Gaslini, 16147 Genova, Italy, [5]Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India, [6]Dysmorphology Unit, Hospital Universitario Virgen del Rocío, Seville, Spain, [7]Division of Blood and Marrow Transplantation, Department of Paediatrics, University of Minnesota, Minneapolis, USA, [8]Division of Clinical Genetics, Akdeniz University School of Medicine Department of Paediatrics, 07059 Antalya, Turkey, [9]Newcastle General Hospital, Institute for Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK, [10]Northern Genetics Service, Newcastle upon Tyne Hospitals Foundation Trust, Newcastle upon Tyne, UK, [11]Department of Paediatrics, University of Tampere, PO Box 2000, FIN-33521, Finland, [12]Department of Paediatrics, Clinical Hospital Centre Zagreb and School of Medicine, University of Zagreb, Zagreb, Croatia, [13]Department of Paediatrics, Federico II University, Naples, Italy, [14]Division of Paediatric Immunology, Hacettepe University Children's Hospital, Ankara, Turkey, [15]Department of Genetics, Hôpital Robert Debré, Paris, France, [16]Paediatric Haematology/Oncology, University of Pavia, Foundation IRCCS San Matteo, Pavia, Italy, [17]The Children's Hospital of Philadelphia, Abramson Research Center, Philadelphia, PA 19104, USA, [18]Department of Paediatrics, CHU Hedi Chaker, Sfax 3029, Tunisia, [19]Department of Clinical Genetics, VU University Medical Center, 1007 MB Amsterdam, The Netherlands.

*Correspondence to Dr Cristina Sobacchi, Institute of Biomedical Technologies, National Research Council, via Fratelli Cervi 93, 20090 Segrate, Italy. E-mail: cristina.sobacchi@itb.cnr.it, Phone: +390282245164, Fax: +390282245191.

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ABSTRACT: The "Osteopetroses" are genetic diseases whose clinical picture is caused by a defect in bone resorption by osteoclasts. Three main forms can be distinguished on the basis of severity, age of onset and means of inheritance: the dominant benign, the intermediate and the recessive severe form. While several genes have been involved in the pathogenesis of the different types of osteopetroses, the CLCN7 gene has drawn the attention of many researchers, as mutations within this gene are associated with very different phenotypes. We report here the characterization of 25 unpublished patients which has resulted in the identification of 20 novel mutations, including 11 missense mutations, 6 causing premature termination, 1 small deletion and 2 putative splice site defects. Careful analysis of clinical and molecular data led us to several conclusions. First, intermediate osteopetrosis is not homogeneous, since it can comprise both severe dominant forms with an early onset and recessive ones without central nervous system involvement. Second, the

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appropriateness of haematopoietic stem cell transplantation in CLCN7-dependent ARO patients has to be carefully evaluated and exhaustive CNS examination is strongly suggested, as transplantation can almost completely cure the disease in situations where no primary neurological symptoms are present. Finally, the analysis of this largest cohort of CLCN7-dependent ARO patients together with some ADO II families allowed us to draw preliminary genotype-phenotype correlations suggesting that haploinsufficiency is not the mechanism causing ADO II. The availability of biochemical assays to characterize ClC-7 function will help to confirm this hypothesis. ©2009 Wiley-Liss, Inc.

KEY WORDS: osteopetrosis, chloride, CNS defects, transplantation

INTRODUCTION

In the maintenance of bone homeostasis, the regulation of pH in specific intracellular and extracellular compartments of osteoclasts is critical and, besides proton production and secretion, implies the mobilization of other ionic species. In particular, extrusion of chloride anions is required to neutralize the electric current generated by the H^+-ATPase. In this regard, a few years ago it was shown that ClC-7 plays a fundamental role in osteoclast physiology, as demonstrated by the severe osteopetrotic phenotype displayed by the $clc7^{-/-}$ mouse model (Kornak et al., 2001).

CLCN7 (MIM# 602727) is a member of the mammalian ClC gene family (Brandt and Jentsch, 1995). It is expressed in different cell types in vesicles of the endocytotic-lysosomal pathway; in osteoclasts, ClC-7 is localized also in the ruffled border and is involved in the acidification of the resorption lacuna. Recently it has been shown that, as previously suggested by work on bacterial ClC homologues, the ClC-7 protein and other members of the ClC family are not passive chloride ion channels, but rather secondary active chloride/proton antiporters (Accardi & Miller, 2004; Picollo & Pusch, 2005; Scheel et al., 2005; Graves et al., 2008; Zifarelli & Pusch, 2009).

In humans, mutations in the CLCN7 gene give rise to the complete spectrum of osteopetrotic phenotypes, ranging from autosomal recessive osteopetrosis (ARO; MIM# 611490), to intermediate autosomal osteopetrosis (IAO) and even to autosomal dominant osteopetrosis type II (ADO II, Albers-Schönberg disease; MIM# 166600), the latter including also asymptomatic patients.

CLCN7-dependent ARO is usually diagnosed at birth or early in infancy due to generalized osteosclerosis and severe haematological deficits; primary neurological defects, such as cerebral and retinal atrophy, are often present and are displayed also by the knockout mouse (Kornak et al., 2001; Frattini et al., 2003; Kasper et al., 2005). This form is frequently lethal in early life, despite haematological reconstitution by haematopoietic stem cell transplantation (HSCT). Few reports describe this subgroup of ARO patients, since in addition to the cases reported by our group (8 patients in Frattini et al., 2003 and 1 in Shin et al., 2004), only 6 cases have been published (Cleiren et al., 2001; Kornak et al., 2001; Lam et al., 2007 ; Besbas et al., 2009).

CLCN7-associated IAO is an elusive entity; it is generally classified as a form of osteopetrosis milder than ARO, identified in childhood due to fractures after minor trauma, characteristic skeletal radiographic changes found accidentally, mild anaemia and occasional visual impairment. The affected individuals are expected to reach adulthood, in contrast to ARO patients (Campos-Xavier et al., 2003; Frattini et al., 2003).

Finally, the onset of ADO II is usually in late childhood or adolescence, although earlier occurrence has been reported; findings may include a high rate of fractures, osteomyelitis, partial visual loss, and occasional bone marrow failure (Benichou et al., 2000). Although there is some overlap between the clinical severity of IAO and that of ADO II, the dominant means of transmission and the presence of a single mutated allele clearly distinguish this latter form from IAO (Cleiren et al., 2001; Frattini et al., 2003; Del Fattore et al., 2006; Waguespack et al., 2007). The reduced penetrance of the dominant CLCN7 mutations in ADO II has long been recognized, since members of the same affected family with identical mutations in the ClCN7 gene are often symptomatic.

Here we report the clinical and molecular characterization of 25 new CLCN7-dependent patients. Among the mutations identified, 20 are novel. Overall, our data strengthen the concept of the high variability of phenotypes caused by defects in this gene, although the underlying reasons still remain obscure.

MATERIALS AND METHODS

Specimens, including blood and DNA samples, were collected from patients after their parents provided informed consent. Clinical, radiological and laboratory data were collected for genotype-phenotype correlation studies. This research complies with standards established by the local Ethical Committee and the granting agency.

Mutation analysis of the CLCN7 gene (GenBank: AL031600.4) was performed as previously described (Frattini et al., 2003). In the case of new missense mutations, 100 chromosomes from normal unrelated ethnic matched donors were also investigated by direct sequence analysis or enzymatic digestion.

The putative structural consequences of mutations were predicted based on the sequence analysis and multiple sequence alignment information.

RESULTS

CLCN7 gene mutations identified in 22 unrelated families are summarized in Table 1, where the 20 novel mutations are shown in bold. Among them, 2 are predicted to affect splicing, 6 to cause premature termination, 1 is a single amino acid deletion and 11 are missense mutations which have never been found in 100 normal chromosomes.

Amino acid sequence comparison among the ClC-7 homologues of various species and human ClC-6 (which is the member of chloride channel family closest to ClC-7) supports the idea that the new missense mutations identified are not neutral polymorphisms, since the mutated residues are strongly conserved in evolution, except for Leu132. Of note, this amino acid lies in a transmembrane domain where it is likely that the substitution of a highly hydrophobic residue could alter protein function.

Recessive patients and their mutations

Mutations in both alleles were found in probands from 14 families, therefore their disease can be considered recessive in nature. Among them, 7 were homozygous: 5 bore novel mutations (patients 16, 18, 19, 21, 26), while the remaining 2 shared an identical known mutation (patients 17 and 23). Among the homozygous mutations, 3 led to premature translation termination, while 3 were missense (including the one present in two patients).

Thirteen out of 14 mutations identified in the compound heterozygous patients (patients 14, 15, 20, 22, 24, 25, 27) have not been previously described; however p.Arg526Gln in patient 15 was found also in the homozygous patient 26. Three were nonsense, 8 were point mutations leading to amino acid substitutions, 1 was a 3 nucleotide in frame deletion (patient 27) and 1 was a dinucleotide deletion in intron 17 close to the donor splice site of exon 17 (patient 25). The effect of this mutation at the transcript level was verified on the cDNA of the father, who carried the deletion; the sequence analysis showed skipping of exon 17 which was predicted to cause a frameshift and premature termination at the protein level (p.Gly483AspfsX60).

Dominant patients and their mutations

Eleven patients from 8 families showed mutations only in one of the two CLCN7 alleles. The amino acid change p.Gly215Arg, which is the most widespread mutation in ADO II, was found in 2 families (patients 31 and 34), while 3 other previously identified mutations (p.Arg762Gln in pt 28; p.Arg286Gln in pt 30; p.Arg767Trp in the three siblings from family 35) were found in one family each. The remaining 3 families carried novel mutations, 2 were missense (patients 29 and 33), while the third (patient 32) involved the invariant G of the GT donor splice site of intron 23, leading to a hypothetical splicing defect which would affect only the very terminal portion of the molecule.

Interestingly, the large majority of ADO II patients described so far bear mutations that preserve the major structure of the chloride channel, either missense substitutions or disruptive mutations at the very end of the protein (Cleiren et al., 2001; Zhang et al., 2009).

Table 1. Molecular findings in 25 new CLCN7-dependent patients

Patient	Genomic change°	cDNA change†	Protein change/effect§
14	g.18256G>A g.21543G>A	c.1208G>A c.1561G>A	**p.Arg 403Gln** **p.Gly521Arg**
15	g.21559G>A g.23407_23410delCACT	c.1577G>A c.1875_1878delCACT	**p.Arg526Gln*** **p.Thr626ProfsX4**
16	g.23629delC g.23629delC	c.1901delC c.1901delC	**p.Pro634GlnfsX2** **p.Pro634GlnfsX2**
17	g.21558C>T g.21558C>T	c.1576C>T c.1576C>T	p.Arg526Trp p.Arg526Trp
18	g.19239_19240insC g.19239_19240insC	c.1251_1252insC c.1251_1252insC	**p.Val418ArgfsX182** **p.Val418ArgfsX182**
19	g.22797T>C g.22797T>C	c.1646T>C c.1646T>C	**p.Leu549Pro** **p.Leu549Pro**
20	g.24588C>T g.24642G>T	c.2134C>T c.2188G>T	**p.Arg712X** **p.Glu730X**
21	g.14831C>T g.14831C>T	c.811C>T c.811C>T	**p.Arg271X** **p.Arg271X**
22	g.14382G>A g.23680T>C	c.718G>A c.1952T>C	p.Gly240Arg **p.Leu651Pro**
23	g.21558C>T g.21558C>T	c.1576C>T c.1576C>T	p.Arg526Trp p.Arg526Trp
24	g.12955A>G g. 25059G>C	c.641A>G c.2300G>C	**p.Asn214Ser** **p.Arg767Pro***
25	g.21605_21606delTG g.25043C>T	c.1617+6_1617+7delTG c.2284C>T	**r.(spl?)** **p.Arg762Trp***
26	g.21559G>A g.21559G>A	c.1577G>A c.1577G>A	**p.Arg526Gln*** **p.Arg526Gln***
27	g. 11191T>C g.14343_14345delTTG	c.395T>C c.679_681delTTG	**p.Leu132Pro** **p.Leu227del**
28	g.25044G>A	c.2285G>A	p.Arg762Gln
29a/29b	g.16338C>G	c.954C>G	**p.Phe318Leu**
30	g.15924G>A	c.857G>A	p.Arg286Gln
31	g.12957G>A	c.643G>A	p.Gly215Arg
32	g.24705G>A	c.2250+1G>A	**r.spl?**
33	g.25033C>A	c.2274C>A	**p.Phe758Leu**
34	g.12957G>A	c.643G>A	p.Gly215Arg
35a/35b/35c	g.25058C>T	c.2299C>T	p.Arg767Trp

° Accession number genomic sequence of the CLCN7 gene: AL031600.4; † Accession number of the CLCN7 cDNA: AF224741; the numbering used starts with nucleotide +1 for the A of the ATG-translation initiation codon. § New mutations in the CLCN7 gene are in bold. * A different amino acid substitution at the same position has already been reported.

Structural analysis

Unfortunately, ClC-7 is strictly targeted to intracellular membranes in transfected cells (Jentsch, 2008). Thus, standard electrophysiological methods cannot be used to assay the functional impact of the disease-causing mutations. In order to gain some insight into possible disease-causing mechanisms, we mapped the residues listed in Table 1 affected by missense mutation onto the three-dimensional structure of the bacterial ClC-ec1 homologue (Dutzler et al., 2003), for residues residing in the transmembrane part (Fig. 1A), and onto the structure of the C-terminal cytosolic fragment of ClC-5 (Meyer et al., 2007), for C-terminal mutations (Fig. 1B). The dominant mutations are coloured in magenta and the recessive ones in red. In the membrane part, recessive mutations are rather scattered around the structure, none of them however are localized close to the anion binding sites, suggesting indirect effects on protein function and/or stability. Interestingly, in the membrane part the dominant mutations cluster in an intracellularly accessible region relatively close to the subunit interface. It will be interesting to perform functional studies in the future to investigate possible dominant negative effects. In the C-terminus the dominant mutations are distant from the (putative) ATP binding site but are all located in (or shortly after) the α1 helix of CBS2. It can be speculated that this helix is involved in the interaction of the CBS domains with the transmembrane part of the protein.

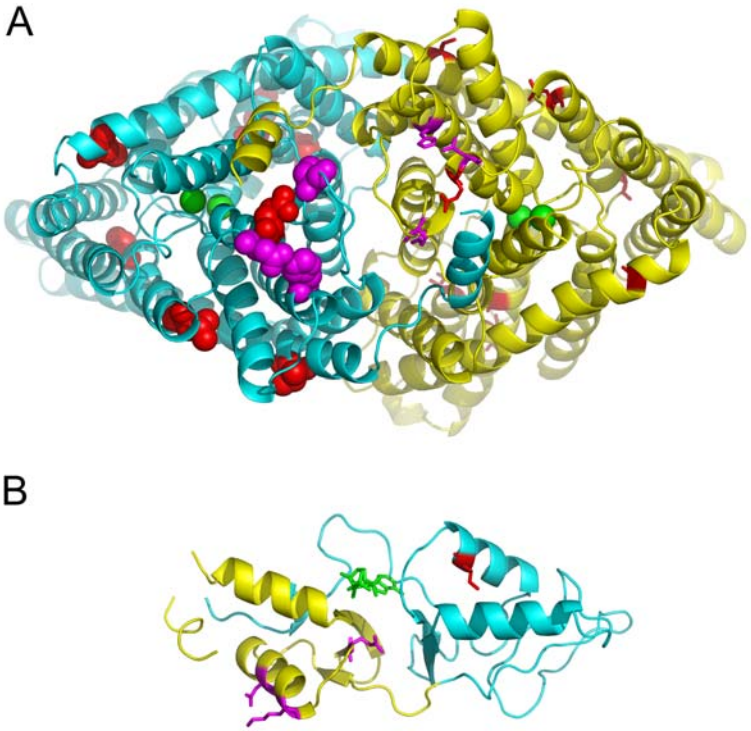

Figure 1. Mutations mapped to the crystal structures of ClC-ec1 and ClC-5-C-terminus. Based on previous works (Dutzler et al., 2002; Estévez and Jentsch, 2002), we defined the residues of the bacterial ClC-ec1 and of the C-terminus of ClC-5 that correspond to the missense mutations listed in Table 1. These residues are highlighted in the graphical representation of the structure of ClC-ec1 (Dutzler et al., 2003) (panel A) and that of the C-terminus of ClC-5 (Meyer et al., 2007) (panel B), respectively. Recessive mutants are shown in red, dominant mutants are shown in magenta. In one of the two (identical) subunits of ClC-ec1 affected residues are shown in space fill, in the latter as sticks. In panel A, the view is approximately from the intracellular side of the membrane and the Cl- ions marking the ion-conducting pore are shown as green spheres. In panel B, the ATP-molecule that is bound between CBS1 (blue) and CBS2 (yellow) is shown in green.

Clinical observations: recessive patients

We previously pointed out the possibility that human CLCN7-dependent ARO bears a particularly poor prognosis due to the primary involvement of the central nervous system (Frattini et al., 2003), in agreement with the findings in mouse (Kornak et al., 2001; Kasper et al., 2005). In order to confirm this original observation, we collected data on the clinical picture of patients (Table 2) and their families and noted several interesting features.

First, primary involvement of the CNS is extremely frequent in CLCN7-dependent ARO; 6 patients (patients 14-18, 22) had severe cerebral atrophy, another 3 displayed severe spasticity (patients 19-21), while patient 23 presented with axial hypotonia and peripheral hypertonia. Patients 24-27 showed only relatively minor neurological deficits probably due to secondary CNS involvement, although visual impairment is found in almost all patients, since it could be of both primary and secondary origin. Therefore, it appears that not all the CLCN7 mutations affect the CNS to the same extent. Indeed, truncating mutations are present on both alleles only in affected individuals with severe CNS involvement. On the other hand, missense mutations could, at least partially, maintain some activity. This is supported by the clinical history of patient 25, who is a compound heterozygote for a missense mutation (p.Arg762Trp) and a two nucleotide deletion next to a splice site, leading to a splicing defect which might allow the production of a small amount of the correctly processed transcript. Interestingly, this patient was the only one with no neurological symptoms, besides blindness.

Second, our group previously observed that HSCT was not successful in a number of CLCN7-dependent ARO with CNS involvement (Frattini et al., 2003). Data collected on the 14 additional ARO patients herein described (Table 2) further support this evidence and show that HSCT is not usually performed when CNS deficit is present, due to the little benefit they would gain (Mazzolari et al., 2009). In this regard, the comparison of the clinical history of patient 15 (previously described in Locatelli et al., 1997) and patient 14 on the one hand, and of patient 25 on the other, is significant. Briefly, at diagnosis (4 months) patient 15 presented with a typical osteopetrotic bone phenotype, mild anaemia and progressive deterioration of visual and neurologic function, together with moderate cortical atrophy. She received a cord blood transplant (CBT) from an unrelated matched donor at 14 months which completely restored the haematological compartment and rescued the bone defect. However, 11 months after transplantation, while bone appearance was almost normal, neurological symptoms were still present and led to death at 3 years of age. Patient 14 showed a similar history, since in spite of a successful transplantation, he died at 16 months of age.

On the other hand, pt 25 presented with blindness, severe anaemia and splenomegaly at 12 months; X-rays performed at 19 months revealed generalized bone sclerosis and the diagnosis of osteopetrosis was drawn. She received a matched unrelated donor (MUD)-BMT at 22 months of age, but the transplant did not engraft; subsequently, at 28 months, she underwent a manipulated, MUD-HSCT with cells obtained from peripheral blood, which completely cured the bone and haematological aspects of the disease. She experienced skin and gut GvHD and received immune suppressive therapy for about two years, without any further complications. About one year later, she developed hypothyroidism, which required hormone replacement therapy. Four years after HSCT, she is alive and well, gaining height and weight, with normal tooth eruption and no neurological symptoms; she attends school with a tutor due to her blindness.

Overall, the clinical history of patient 25, together with those of patients 24, 26 and 27, shows that CLCN7-dependent ARO without CNS defects could constitute a distinct subset, with a longer estimated life and a better response to HSCT even when performed later in life.

Table 2. Clinical findings in 14 new ClCN7-dependent ARO patients

Patient	Age at onset	Bone fractures	Anaemia	Cranial nerve defect	BMT (age)	Outcome	Neural defect
14.	At birth	No	Yes	No at 1 month of age	Yes (2 months)	Dead at 16 months	Cerebral atrophy
15.	4 months	No	Moderate	Progressive visual loss	Yes (14 months)	Dead at 3 years	Cortical atrophy
16. *	Not available	Not available	Not available	Not available	No	Dead	Cortical atrophy
17.	1 month	No	Yes	Blindness	No	Dead at 18 months	Cerebral atrophy
18.	At birth	Yes	Yes	Blindness, deafness	No	Dead in infancy	Severe brain atrophy, ventriculomegaly
19.	2 months	No	Yes	Impaired vision	No	Dead at 3 months	Increased deep tendon reflexes, severe spasticity
20.	2 months	No	Yes	Impaired vision	No	Dead at 5 months	Severe spasticity
21.	At birth	No	Yes	Impaired vision	No	Dead at 16 months	Increased deep tendon reflexes, severe spasticity
22.	<1 month	No	Yes	Blindness, deafness	No	Dead at 1 year	Hypotonia, cerebral atrophy, mental retardation.
23.	3 months	No	Yes	NA	No	Dead at 5 months	Axial hypotonic, peripheral hypertonic
24.	2,5 months	Yes	Moderate	Blindness	Yes (11 months)	Dead 7 years post BMT (GvHD)	Hydrocephalus
25.	12 months	No	Yes	Blindness	1st (22 months) 2nd (28 months)	Alive and well with engraftment	No
26.	7 months	Multiple	Yes	Impaired vision, deafness	Yes (16 months)	Alive without engraftment	Hemiplegic
27.	3 months	Yes	No	Blindness	No	Alive and stable	Hydrocephalus

* Lack of some clinical data is due to the fact that, after sending DNA for analysis, the referring physician could not be contacted anymore
NA: not assessed

Clinical observations: dominant patients

CLCN7-dependent ADO II patients bear a single mutated allele; as already reported (Waguespack et al., 2007), the onset of the disease is not only in adulthood (as in family 35), but also in childhood, as seen in the majority of our patients (from patient 28 to patient 34), most often diagnosed on the basis of fractures. This high presence of patients with early diagnosis could be the result of a bias, since we are a reference centre for ARO but not for ADO II and an early symptom manifestation raises, in the referring physician, the possibility of ARO disease.

A great variability of phenotypes are present, even within families in which the same mutation is carried by several individuals (Cleiren et al., 2001; Frattini et al., 2003; Letizia et al., 2004; Del Fattore et al., 2006; Waguespack et al., 2007; Zhang et al., 2009). In the series of patients here described, the three members with a mutated allele in family 35 had a similar phenotype, while in family 29, with 8 symptomatic members in 4 generations (data not shown), the age of onset ranged from senescence to early childhood (as in the two cousins patients 29a and 29b). Moreover it is likely that asymptomatic members also occurred, as suggested by the presence of two obligate carriers (patient 29b's father and the maternal grandmother of the latter).

Likewise, patient 28's mother was normal at the age of 33 years, on the basis of clinical history, examination, radiological (skeletal survey and bone mineral density by dual energy absorptiometry DEXA scan) and laboratory investigations even though she bore the same mutation as her daughter. On the contrary, clinical symptoms in patient 28 started at 2 months of age (parents first noticed nystagmus) followed by failure to thrive at one year of age and progressive anemia and hepatosplenomegaly at 5 years of age. The diagnosis was made at 7 years due to increased bone density found in X-rays, massive hepatosplenomegaly and optic nerve defect, while no fractures and other neurological dysfunction have been reported so far.

DISCUSSION

Since 2001, mutations in CLCN7 gene have been demonstrated to be involved in the pathogenesis of different forms of osteopetrosis: infantile malignant, intermediate and benign. Here we report 25 new patients from 22 families displaying the whole range of phenotypes, in which we identified 20 novel mutations. A critical evaluation of molecular and clinical findings allows us to draw some definitive conclusions. First, while the classification of patients only on the basis of clinical data can sometimes be questionable (Frattini et al., 2003), the identification of two groups on the basis of the presence of a single or two mutated alleles appears to be an unambiguous criterion. As a consequence, intermediate osteopetrosis is not a homogeneous entity, since it can comprise both severe dominant forms with an early onset and recessive ones without CNS involvement. The first subset pertains to the ADO II form, while the second group is recognized in the present paper for the first time.

Second, the identification of this subset is of great clinical importance since patients in this subgroup have a better prognosis and are candidates for HSCT. As a consequence, the careful evaluation of the neurological compartment in CLCN7-dependent ARO patients is strongly recommended because it has a strong prognostic value and can help to establish the appropriateness of HSCT.

Third, the molecular data obtained in the 25 new patients herein described and those present in prior reports, might suggest that only a subset of mutations have a dominant effect. In fact, there are very few mutations shared by ADO II and ARO, and in this latter case the second allele bears an "ARO-specific" defect, which is likely to be null. Moreover, it can be definitively said that parents of ARO and recessive IAO patients are not only asymptomatic but normal, as are parents of TCIRG1-dependent ARO. These considerations raise the possibility that haploinsufficiency is not the mechanism causing ADO II. When biochemical assays to characterize ClC-7 function are available, this could be tested directly and, more in general, it could be possible to establish clear genotype-phenotype correlations.

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