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Functional outcome of a novel *SLC29A3* mutation identified in a patient with H syndrome

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ABSTRACT

The H syndrome (OMIM 612391) is an autosomal recessive disorder characterized by hyperpigmentation, hypertrichosis, histiocytosis and short stature. It is caused by mutations in the *SLC29A3* gene, which encodes for the equilibrative nucleoside transporter 3 protein (ENT3), of still uncertain subcellular localisation. Here we report a new case of H syndrome with the novel mutation c.243delA, which has been concomitantly described by others [A. Bolze, A. Abhyankar, A.V. Grant, B. Patel, R. Yadav, M. Byun, D. Caillez, J.F. Emile, M. Pastor-Anglada, L. Abel, A. Puel, R. Govindarajan, L. de Pontual, J.L. Casanova, A mild form of SLC29A3 disorder: a frameshift deletion leads to the paradoxical translation of an otherwise noncoding mRNA splice variant, PLoS ONE 7 (2012) e29708]. Patient-derived primary skin fibroblasts and B-lymphoblastoid cell lines (B-LCL) were obtained and, although no differences were found in mRNA levels of ENT3, a significant increase in plasma membrane equilibrative transport activity was found in fibroblasts from the patient.

Loss of function of key proteins implicated in nucleoside metabolism can lead to mitochondrial DNA (mtDNA) depletion syndromes (MDS). Measurement of respiratory chain complex activity revealed that mitochondrial function was unaltered. Neither fibroblasts nor B-LCL showed mtDNA depletion when compared with controls. Fibroblasts and B-LCL from the patient were not particularly protected when mitochondrial damage was induced using nucleoside-derived drugs susceptible to being transported by ENT3. Analysis of mtDNA amounts in tissues obtained at autopsy proved inconclusive with respect to mitochondrial involvement in the pathogenesis of this syndrome. Overall, the data do not support the inclusion of H syndrome among the MDS and these findings are compatible with its recent inclusion among the lysosomal storage diseases.

1. Introduction

The H syndrome (OMIM 612391) is a recently described syndrome caused by mutations in the *SLC29A3* gene that encodes for the human equilibrative nucleoside transporter 3 (hENT3). It is an autosomal recessive disorder that is characterised by hyperpigmentation, histiocytosis, hypertrichosis and short stature. Other symptoms such as diabetes and hearing loss have also been related to this syndrome [1,2].

ENT3 is involved in nucleoside transport and was first described in 2001 [3]. In contrast to the SLC29 gene family members ENT1 and ENT2, ENT3 has been mostly found in intracellular structures. Indeed, ENT3 was initially localised in lysosomes but it was later shown that it is more likely to be expressed in mitochondria, where it may play a role in the mitochondrial toxicity of certain nucleoside-derived drugs [4].

Different *SLC29A3* mutations have been described. Molho-Pessach et al. described c.1279G>A, c.1309G>A and c.1045del mutations [5], and later reported two patients with different mutations that, however, affected the same amino acid [6]. Cliffe et al. have also described c.347T>G, c.940delT, c.1309G>A, c.1330G>T and c.1346C>G mutations [1], while Priya et al. have recently described the c.400C>T and the p.G437R mutations [7].

Abbreviation: MDS, mtDNA depletion syndrome.

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Loss of function of key proteins implicated in nucleoside metabolism, leading to unbalanced deoxyribonucleotide pools, has been recognised as a common cause of a group of mitochondrial disorders called mitochondrial DNA (mtDNA) depletion syndromes (MDS), which are characterised by severe reduction of the mtDNA copy number [8]. Here we report a novel case of H syndrome, caused by mutations in the gene encoding a nucleoside transporter. The biochemical and physiological consequences of this mutation have been explored, as a way to determine whether signs of mitochondrial involvement can be found. This patient died shortly after the molecular diagnosis and consequently this is the first time that tissue necropsies from an H syndrome patient have become available for research.

2. Methods

2.1. Mutational analysis

The *SLC29A3* gene was analyzed by PCR amplification from genomic DNA extracted from blood samples using intronic oligonucleotide primers, as described previously [5], and sequenced using BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA). The nomenclature of the variations refers to the *SLC29A3* cDNA sequence (GenBank accession number NM_018344.5) with 1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

2.2. Cell culture and lymphocyte transformation

Skin fibroblasts from the patient were obtained by skin biopsy and cultured in DMEM supplemented with 10% fetal bovine serum (v/v), 50 units/ml penicillin, 50 μg/ml streptomycin, 100 μM non-essential amino acids and 2 mM L-glutamine.

B-lymphoblastoid cell lines (B-LCL) were generated from the patient and two healthy controls through Epstein–Barr virus (EBV) infection as follows: Peripheral blood mononuclear cells were obtained through Ficoll gradient centrifugation and cultured in 3.2 ml of complete medium (RPMI-1640, Invitrogen, Carlsbad, CA; 15% dialysed heat-inactivated FBS, Invitrogen; 10 mM HEPES; 2 mM L-glutamine; 1 U/l penicillin; 1 U/l streptomycin; 50 μg/ml gentamicin), supplemented with 1.8 ml of EBV-containing supernatant from cultured B95-8 marmoset cells. One microgram per milliliter of anti-CD3 OKT3 (eBioscience, San Diego, CA) was added to avoid T-cell response.

2.3. Assessment of mitochondrial respiratory chain activities

Cell homogenates from cultured skin fibroblasts and B-LCL were obtained as described previously [9]. Mitochondrial respiratory chain complex (II + III) and complex IV activities, as well as the Krebs cycle citrate synthase (CS) activity, were determined following described methods [9] Complex (II + III) and complex IV activities were also referred to CS activity to normalise for the mitochondrial content.

2.4. Nucleoside screening in urine

Random urine from the patient and six healthy controls was diluted 1:20 with phosphate-buffered saline, treated with perchloric acid and centrifuged. Five microliters of the supernatant was analysed by high-performance liquid chromatography to detect nucleosides and other UV-absorbing compounds, as described elsewhere [10]. This method has been set up in our laboratory to positively detect and quantify the nucleosides thymidine, deoxyuridine, uridine adenosine and the base thymine.

2.5. RNA isolation and RT-PCR

Total RNA was isolated by using TriPure reagent (Roche), following the manufacturer's instructions. One microgram of RNA was used to generate cDNA, which was further used for subsequent RT-PCR with the Applied Biosystems 7500 Real-time PCR system. The primers and probes used to amplify nucleoside transporter cDNA were the same as described previously [11]. As for ENT3, we used the FAM-labelled probe ATGGCTCCTCCTGATATC and primers 5′-GACCGGCTCCTTTCCTATGAG-3′ and 5′-GCCACGGCGCTGAC-3′ to amplify a region of exon 4. GAPDH and 18S were used as housekeeping genes.

2.6. Nucleoside uptake assays and quantification of mtDNA copy number

Transport activity measurements were performed as previously described [12]. For mtDNA quantification, plates were seeded at 20,000 cells/cm^2. Twenty-four hours after seeding, cells were treated with zidovudine (AZT; Sigma), zalcitabine (ddC; Sigma), gemcitabine (dFdC; generously provided by Lilly S.A., Madrid, Spain) or ribavirin (Sigma) for 72 h. After treatment, total DNA was extracted with Qiamp (Qiagen, Hilden, Germany). mtDNA copy number was measured by quantitative real-time PCR using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with Taqman probes. For mtDNA copy number assessment, the *MT-RNR1* gene (encoding 12S rRNA) was amplified and normalised with nuclear DNA copy number by simultaneous measurement of the single copy nuclear gene *RPP30* (encoding RNAse P), as described elsewhere [13].

2.7. MTT cytotoxicity assay

Twenty-four hours after seeding, cultures were exposed for 72 h to increasing concentrations of the drug. Viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data were fitted to a dose–response curve, using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA) to obtain the IC_{50}.

3. Results

3.1. Case report

We report a male patient of Moroccan origin who at the time of molecular diagnosis was 19 years old. He had suffered poorly controlled type I diabetes mellitus and slow growth since childhood and had a short stature, a final height of 127 cm and a weight of 32 kg. He had indurated cutaneous patches with hyperpigmentation and discrete hypertrichosis involving the legs and pubis. Clinical examination revealed inguinal and axillary adenopathy, hepatosplenomegaly, micropenis, hallux valgus and bilateral camptodactyly of the fingers of both hands. Further studies revealed hypogonadism and enlarged atria with insufficiency of the tricuspid and pulmonary valves. A cutaneous biopsy revealed the histological changes described in H syndrome. The patient died at the age of 20, probably because of brain edema, although the exact cause of death remains unclear.

Analysis of the patient's urine did not reveal any differences in thymidine, deoxyuridine, uridine, adenosine or thymine excretion levels as compared with controls.

3.2. Molecular genetic analysis of the SLC29A3 gene

Screening for mutations in the *SLC29A3* gene identified a single-base deletion mutation in position 243 (c.243delA) by exon

amplification and further sequencing, this mutation being present at the homozygous state (Fig. 1). We also found the following common polymorphisms in the *SLC29A3* gene (all at the homozygous state): missense variations R18G (A52G; rs2277257), S158F (C473T; rs780668), V239I (TG714/715CA; rs2252996) and I326V (A976G; rs2487068) and a conservative change in amino acid position 336 (G336G; T1008C) (Fig. 1B). Of these four missense variations only R18G and S158F result in non-conservative amino acid changes. None of the *SLC29A3* mutations previously associated with H-syndrome [1,5,14,15] were found in this patient, but we concur with others [16] regarding the detection of this novel single-base deletion mutation in position 243 (c.243delA). In the absence of pedigree data, it is hard to determine whether the alteration segregates with disease.

3.3. mRNA expression and activity of nucleoside transporters

Bolze et al. [16] have very recently demonstrated that the frameshift deletion c.243delA allows the translation, expression and function of an otherwise non-coding, out-of-frame mRNA splice variant lacking exon 3 that is eliminated by nonsense-mediated mRNA decay in healthy individuals. The mutated isoform differs from the wild-type hENT3 by the modification of 20 amino acids (in exon 2) and the removal of 28 other amino acids (exon 3), including the second transmembrane domain. As a result, this new isoform still displays some minor functional activity when expressed in *Xenopus* oocytes [16].

Since there is some overlapping activity among SLC29 members [17], we first decided to measure the expression and activity of other SLC29 family members in an attempt to identify putative compensatory mechanisms. Analysis of ENT1, ENT2 and ENT3 mRNA expression was performed by RT-PCR in H syndrome and control fibroblasts and revealed no difference in the ENT-type expression pattern, with even ENT3 mRNA levels being similar, when amplified with primers around exon 4. However, there was a striking difference in the plasma membrane transport activity measured as the uptake of [^{3}H]uridine. As shown in Fig. 2, control fibroblasts exhibited a total ENT-related activity of 16.5 ± 8.2 pmol uridine/mg protein.min, of which ENT1 accounted for 5.00 ± 7.8 pmol uridine/mg protein.min. In contrast the patient's fibroblasts showed a transport rate of 208.2 ± 11.2 pmol uridine/mg protein.min, which could not be inhibited by NBTI and was only partially inhibited by dipyridamole.

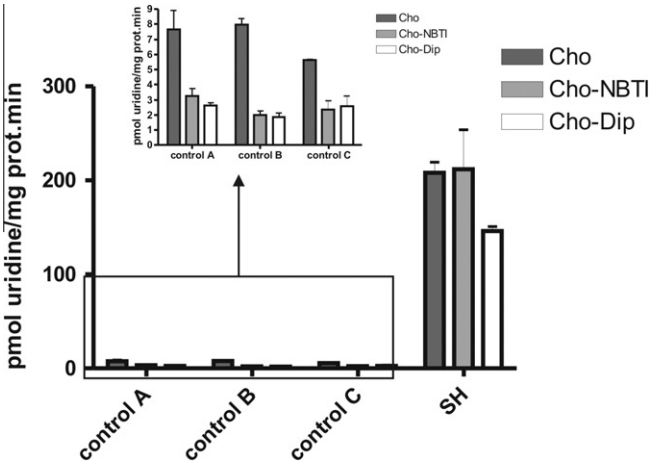

Fig. 2. Endogenous activity of nucleoside transporters in fibroblasts. Uridine uptake was measured with 1 μM [^{3}H]uridine at 1 min. 1 μM NBTI was used to discriminate ENT1 (NBTI-sensitive), whereas 10 μM dipyridamole was used to discriminate the ENT1 + ENT2 fraction. Results are the mean of two independent experiments performed in triplicate or quadruplicate.

Since differences might be tissue specific, we also determined both mRNA and activity levels in B-LCL, but results were variable and inconclusive among the samples.

3.4. Mitochondrial respiratory chain activity and mtDNA copy number

ENT3 has been previously described as a mitochondrial carrier [4]. Thus, we considered the possibility of ENT3 dysfunction causing mitochondrial toxicity, thereby affecting the enzyme activity of the respiratory chain complexes. However, as shown in Table 1, activities of complexes II, III and IV were not altered in the H syndrome-derived samples.

We also evaluated whether there were differences in mtDNA copy number. We observed that neither fibroblasts nor B-LCL had mtDNA depletion when compared with controls. At this point, the patient died, and pathologists provided us with autopsy specimens of the brain, heart, muscle and skin embedded in formol, from which we obtained genomic DNA. mtDNA was also measured in four control samples. High variability was detected among controls and, more importantly, relatively high Ct's were detected for

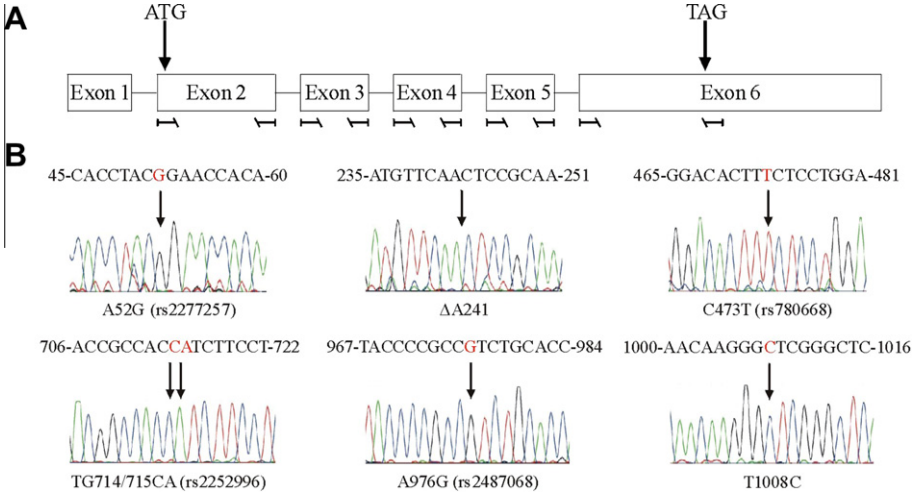

Fig. 1. Identification of *SLC29A3* sequence variations. (A) Schematic representation of exons present in the *SLC29A3* gene and primers used for exon amplification. Start and stop codon localisation is indicated. (B) Sequence chromatograms of exons 2, 4, 5 and 6. Sequence variations are shown (*vertical arrows*) in a DNA sample of a single H syndrome patient.

Table 1
Measurement of respiratory chain complex activity.

	Complex IV (a)	CS (a)	Complex (II + III) (a)	C IV/CS (b)	C(II + III)/CS (b)
Primary skin fibroblasts					
Patient	94.2	65.3	48.0	1.44	0.74
Control 1	59.2	41.5	30.8	1.43	0.74
Control 2	52.8	81.7	40.5	0.65	0.50
B-Lymphoblastoid cell lines (B-LCL)					
Patient	140	195	29.9	0.72	0.15
Control 3	160	192	47.1	0.84	0.25
Control 4	152	228	40.5	0.67	0.18

(a): nmoles/min/mg protein.
(b): U/U citrate synthase.

the patient, which could be partly related to the occurrence of DNA degradation. Based upon these observations, no relevant differences could be detected among controls and the patient's samples.

3.5. Effect of nucleoside analogue treatment on mtDNA copy number

We hypothesised that although there was no basal mtDNA depletion in fibroblasts and B-LCL, if hENT3 is a mitochondrial transporter as reported, cells obtained from the patient should somehow be protected from nucleoside-derived drug-induced mitochondrial toxicity. Thus, we decided to evaluate the effect of gemcitabine (dFdC), zidovudine (AZT), zalcitabine (ddC) and ribavirin on mtDNA copy number. Two drug doses were used for this purpose – a low-toxicity dose, which corresponded to the IC_{25} drug concentration, and a high cytotoxic dose, around IC_{75}, both previously determined by MTT assays. After drug treatment, H syndrome fibroblasts and B-LCL cells did not show any significant difference in mtDNA copy number when compared with control cells (Fig. 3). An apparent change was observed in dFdC-treated fibroblasts, since those from the H syndrome patient appeared to be protected in some way against dFdC-induced mtDNA depletion (Fig. 3A); nevertheless, values did not reach statistical significance.

3.6. Cytotoxicity assay

We followed up the previous observation by performing MTT viability assays for both control and H syndrome fibroblasts after dFdC treatment. IC_{75} values for dFdC were lower in control fibroblasts (16.6 nM) than in H syndrome fibroblasts (40 nM) (Fig. 4A). At 30 nM of dFdC, a statistically significant difference ($p < 0.05$) was found (Fig. 4B). No significant differences were found for the other drugs tested.

4. Discussion

Here we report a new case of H syndrome caused by a novel mutation within the *SLC29A3* gene (c.243delA), which has recently also been identified by Bolze et al. [16] in two siblings of Moroccan origin. Interestingly, although these patients bear the same mutation within the *SLC29A3* gene, the clinical phenotype of our patient was dramatically more severe than that of the patients reported by Bolze and colleagues. The differences in our patient included poorly controlled type I diabetes mellitus and slow growth associated with hypogonadism and hepatosplenomegaly. Bolze and colleagues have accurately described how this novel mutation (a 1-bp deletion) allows for the expression and function of an alternatively spliced mRNA variant that is normally non-coding. This mutated variant encodes a truncated protein that appears to show some basal transport activity, and this feature offers a possible explanation for a mild phenotype when compared to other patients bearing mutations associated with non-functional transporters.

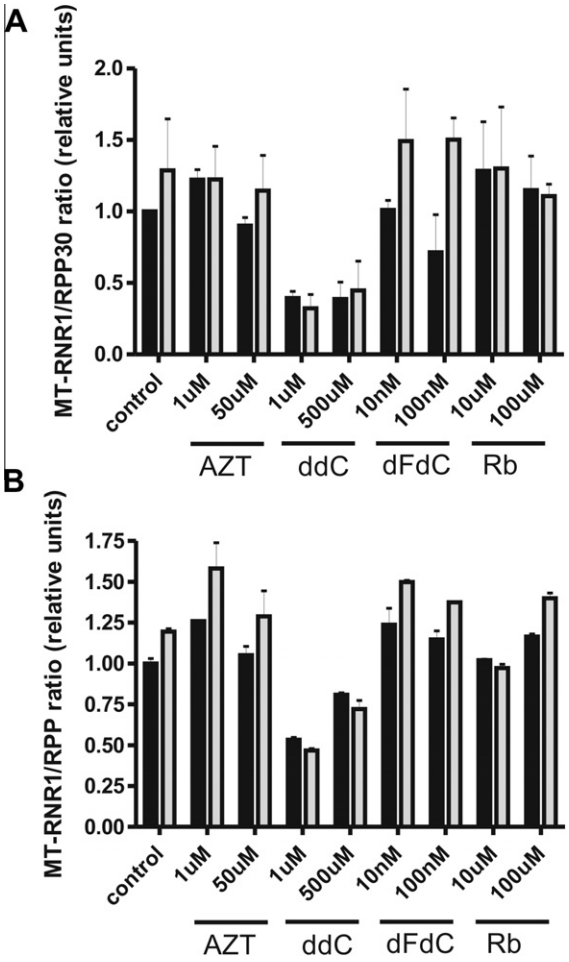

Fig. 3. Control (*black bars*) and H syndrome (*grey bars*) total DNA was extracted and fragments of a mitochondrial (*MT-RNR1*) and a nuclear (*RPP30*) gene amplified by real-time PCR. mtDNA copy number is expressed as the ratio between the mitochondrial and the nuclear gene. Measurements were performed in cultured fibroblasts (A) and B-LCL (B).

Nevertheless, even for this particular deletion, heterogeneity in the clinical phenotype is evident, which suggests that other mechanisms contribute to the pathogenesis.

In this study, we have used patient-derived cells and tissues to investigate some biochemical features that might shed light on the pathogenesis of the disease. The lack of differences between patient and control fibroblasts when amplifying exon 4 is in concordance with Bolze et al. [16]. Nevertheless, a high rate of hENT2-type activity and the occurrence of an equilibrative transporter activity of unknown nature that was insensitive to NBTI

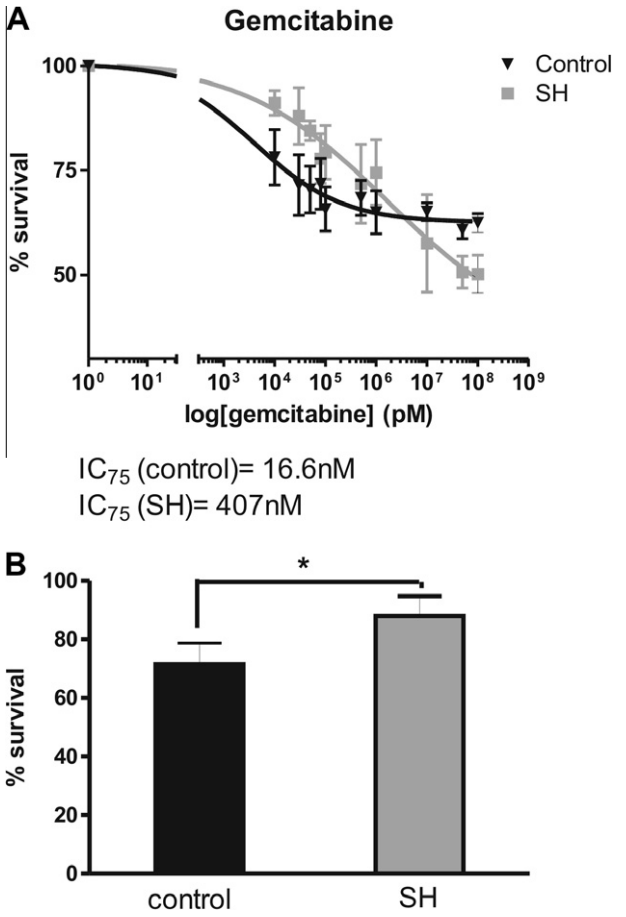

Fig. 4. Viability assay in fibroblasts. (A) Control and H syndrome fibroblasts were treated with dFdC and viability measured by MTT assay. Results are the mean of four independent experiments performed in quadruplicate. (B) At 30 nM dFdC, a statistically significant difference was found between control and H syndrome fibroblasts (*$p < 0.05$).

and dipyridamole was observed in the patient's fibroblasts. In concordance with our data, a recent study has demonstrated that ENT3 deficiency in murine macrophages results in the intracellular accumulation of adenosine, mainly but probably not exclusively attributable to the accumulation of nucleosides in lysosomes [18]. Nucleoside intracellular accumulation in total cell lysates was far higher than in isolated lysosomes, indicating that impaired ENT3 lysosomal function is compatible with increased nucleoside uptake activity at the plasma membrane.

As already mentioned, it has been suggested that ENT3 is mainly located in intracellular structures, such as lysosomes and mitochondria. It has previously been reported that truncation or mutation of the hydrophilic N-terminal region of the protein causes the protein to be relocated to the cell surface [17]. However, plasma membrane localisation of ENT3 has also been reported in some placental cell lines and tissues and immunostaining analysis for hENT3 identified its presence in the cytoplasm of hepatocytes. Overall, subcellular localisation of native hENT3 appears to be variable and cell type dependent, with expression in mitochondria, lysosomes and/or the plasma membrane [4].

Bolze et al. have demonstrated that c.243delA leads to the expression of mutated mRNA transcript variant 3, which is originally subject to nonsense-mediated mRNA decay and has more activity than variant 3 *wt* but significantly less than variant 1 *wt*, and whose predicted protein structure putatively has the N-terminal segment at the external part of the membrane [16]. Thus, whereas *wt* ENT3 usually localizes at intracellular structures, either mitochondria or lysosomes [17], ENT3 c.243delA may have a different subcellular localization. Insertion of ENT3 c.243delA at the plasma membrane may account for the increased NBTI/dipyridamole-insensitive transport rates detected in H syndrome fibroblasts.

ENT3-mediated mitochondrial transport has been suggested to play an important role in basal mitochondrial metabolism [4]. We therefore decided to examine mitochondrial function by measuring both the activity of mitochondrial respiratory chain complexes and the mtDNA copy number. None of these parameters was significantly altered in H-syndrome cultured fibroblasts and transformed lymphocytes when compared to their control counterparts. Nevertheless, mtDNA depletion syndromes are characterized by tissue-specific loss of mtDNA copy number and clinical heterogeneity [19], making the co-existence of mtDNA depletion in some tissues and normal mtDNA levels in fibroblasts and B-LCL perfectly plausible. In fact, no reductions in mtDNA copy number are observed in primary skin fibroblasts from patients with mutations in *TYMP* or *TK2* [20,21]. Analysis of the mtDNA content in tissues which could show mtDNA depletion also proved inconclusive.

ENT3 may be also implicated in the mitochondrial toxicity of nucleoside drugs, including the anti-HIV dideoxynucleoside analogues [4]. When we incubated fibroblasts with different drugs that cause mitochondrial toxicity, mtDNA copy number decreased slightly in dFdC-treated cells. These effects were consistent with MTT viability assays, showing H syndrome fibroblasts to be less sensitive to dFdC but not to the other drugs tested, which did not exert any effect on mtDNA content in either fibroblasts or B-CLC. Whether ENT3 c.243delA is mislocalised or has altered kinetics (i.e. decreased affinity) for dFdC that could explain its reduced toxicity is an issue that requires further investigation. Moreover, increased nucleoside uptake through ENT2 at the plasma membrane of H syndrome fibroblasts could be masking differences in mitochondrial toxicity for some drugs, since ENT2 can transport AZT and ddC but not dFdC, for which ENT2 has a high K_m of around 740 μM [22]. However, ENT3 does not show a particular preference for dFdC as substrate, the dFdC transport rates via ENT3 being similar to those described for ribavirin and two-fold lower than those reported for ddC [4]. This would argue against ENT3 playing a major role in the ability of nucleoside-derived drugs to promote mtDNA depletion and cytotoxicity.

Overall, these observations suggest that the biology of ENT3 is complex. This is an issue that will require comprehensive work in the near future to achieve a better understanding of the pathogenesis of this syndrome. In this context, Hsu et al. [18] recently generated and characterised an ENT3 null mouse model which turned out to show a macrophage-dominated histiocytosis and marked lysosomal disturbances in macrophages, thus compromising the immune response to infection. Macrophage lysosomes from ENT3−/− mice showed increased intracellular adenosine levels and altered pH regulation. Under physiological conditions, ENT3 would favour the recycling of nucleosides, taking advantage of its H^+ sensitivity in a subcellular context of acidic pH. In conclusion, this contribution describes a mutation within the *SLC29A3* gene (c.243delA), recently identified by others in two siblings who showed a milder phenotype than our patient. Functional data and mtDNA analysis are consistent with the view that ENT3 function may not be a major determinant of mtDNA stability. Although the occurrence of ENT3 in mitochondria is still compatible with the data provided here, based upon these observations we would suggest that the pathogenesis of H syndrome is not associated with clear mitochondrial toxicity, in agreement with the recent observations by Hsu et al. [18] in ENT3 null mice.

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