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The effect of four mutations on the expression of iduronate-2-sulfatase in mucopolysaccharidosis type II

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Abstract

Mucopolysaccharidosis type II (Hunter syndrome; OMIM 309900) is a rare X-linked recessive lysosomal storage disorder caused by the deficiency of the enzyme iduronate-2-sulfatase (IDS; EC 3.1.6.13). Different alterations at the IDS locus, mostly missense mutations, have been demonstrated, by expression study, as deleterious, causing significant consequences on the enzyme function or stability. In the present study we report on the results of the transient expression of the novel K347T, 533delTT, N265I and the already described 473delTCC (previously named ΔS117) mutations in the COS 7 cells proving their functional consequence on IDS activity. This type of information is potentially useful for genotype–phenotype correlation, prognosis and possible therapeutic intervention.

Keywords: Iduronate-2-sulfatase; Mucopolysaccharidosis type II; Hunter syndrome; Transient expression; COS cell; Western blot

1. Introduction

Mucopolysaccharidosis type II (Hunter syndrome; OMIM 309900) is a rare X-linked recessive lysosomal storage disorder caused by the deficiency of the enzyme iduronate-2-sulfatase (IDS; EC 3.1.6.13), responsible for heparan sulfate and dermatan sulfate degradation. A broad spectrum of clinical phenotypes has been observed ranging from the mild form with late onset and absent or moderate mental retardation to the severe neuronopathic form with early death [1]. The IDS gene spans approx. 24 kb, contains nine exons and is located on the Xq27/28 boundary of the long arm of the X-chromosome (GenBank accession No. U66082) [2,3]. The IDS full-length cDNA encodes a polypeptide of 550 amino acids [4]. The biosynthesis and processing of the IDS protein have been studied in overexpressing transfected fibroblasts; the enzyme is synthesized as two precursor forms of 76 and 90 kDa that are converted, through a 62 kDa intermediate, into 55 and 45 kDa mature polypeptides [5,6]. To date, more than 200 different mutations have been identified at the IDS locus; among these, some mutations, mostly missense, have been demonstrated, by expression study, as deleterious, causing significant consequences on the enzyme function or stability [7–10]. Three novel mutations (K347T, 533delTT, N265I), as well as the previously described 473delTCC (namely

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ΔS117) [7], identified in four out of 28 Italian patients were chosen to investigate in vitro their effect on enzyme expression. In the present study we report these results and discuss the functional consequence of the considered mutations on IDS activity and maturation.

2. Materials and methods

2.1. Patients

The K347T, 533delTT, N265I, 473delTCC mutations were identified during the mutational analysis of a panel of Italian patients [11]. The MPS II diagnosis was based on the high excretion of heparan and dermatan sulfate in urine and the IDS deficiency in leukocytes and/or cell lines (fibroblasts or lymphoblasts). Details about patients' phenotypes and genotypes are reported in Table 1.

2.2. Mutational analysis

Total RNA and DNA samples were extracted from cultured fibroblasts or lymphoblasts or peripheral lymphocytes using the standard protocols or suitable kits (Qiagen).

Genomic DNA amplification was carried out by sets of specific primers flanking each exon. The PCR amplification conditions were those reported elsewhere [12]. Non-isotopic single strand conformation polymorphism (SSCP) was used for exon screening. The samples, selected on the basis of mobility shift, were sequenced. To confirm the existence of the mutations, restriction analysis was performed (Table 1). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using MLV reverse transcriptase (BRL) and random hexanucleotide primers (Promega) at 42°C for 60 min followed by 5 min at 65°C, in a total volume of 20 ml. The first strand cDNA was PCR-amplified with the specific primers (exon 1F 5′-ATGCCGCCACCCGGACCGGCC and exon 9R 5′-TCAAGGCATCAACAACTGGAAA). RT-PCR products were cloned in pCR2.1 (Invitrogen) and sequenced. Sequence analysis was performed by an ABI 373A DNA automated sequencer with a dye terminator cycle sequencing kit (Applied Biosystems).

2.3. Site directed mutagenesis

IDS expression plasmid pCAGGS-IDS [13] was mutagenized using the commercially available Transformer II Kit (Clontech). Briefly, the constructs were generated using the pCAGGS vector containing the normal IDS cDNA and two oligonucleotides: the mutagenic primer, that contains the considered mutation, and the selection primer (Sel-*Xho*I) that mutates the unique *Sal*I site in pCAGGS to a *Xho*I site, according to the method described by Deng and Nickoloff [14]. The mutant DNA strand was synthesized and the parental DNA was linearized by digestion with *Sal*I for 2 h. By electroporation (one shot at 2500 V, 25 mF, 200 W), the *Escherichia coli mutS* cells were transformed using 2 μg of the reaction mixture; then, to increase the content of mutant plasmids, the *E. coli* cells were grown in 5 ml LB medium overnight in the presence of ampicillin. Miniprep DNA, isolated by the Plasmid Mini Kit (Qiagen), after a second round of digestion with *Sal*I, was electroporated into *E. coli* cells and grown on plates overnight. The resulting colonies were selected by restriction digestion analysis on the basis of the absence of the *Sal*I site and then sequenced to verify the presence of the required mutations. The sequences of the oligonucleotides used to introduce the mutations into wild-type IDS cDNA and of the selection primer are listed in Table 2.

Table 1
Patients' data and details about the corresponding mutations

Phenotype	Genotype	Mutation description	Exon	Nucleotide change	ER[a] site +/−	Protein alteration
Severe	K347T	missense	VIII	AAA > ACA	+*Mae*III	Lys > Thr
Intermediate	N265I	missense	VI	AAC > ATC	+*Fok*I	Asn > Ile
Severe	473delTCC	in-frame deletion	III	TCC > —	+*Hph*I	Loss of a serine at codon 117
Severe	533delTT	not in-frame deletion	III	TTT > –T	+*Alw*26I	Chain termination

[a]ER, enzyme restriction.

Table 2
Oligonucleotides used for PCR directed site mutagenesis

Mutations	Oligonucleotide sequences
K347T	5′-GGAGAATGGGCCACATACAGCAATTTTG-3′
473delTCC	5′-GCTGGAAACTTC___ACCATCCCCCAG-3′
533delTT	5′-GTGGGAAAAGTC__TCACCCTGGGAT-3′
N265I	5′-GTGGCCTACATCCCCTGGATGG-3′
Sel-*Xho*I	5′-GTGCCACCTGGCTCGAGATTGATTATTGA-3′

2.4. COS cell transfection procedure

COS 7 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin at 37°C in a 5% CO_2/air atmosphere. Ten micrograms of wild-type and mutant cDNA expression vector constructs were introduced into COS 7 cells by electroporation. The experimental conditions were: $4–5\times10^6$ cells in a final volume of 800 μl PBS without Ca^{2+}/Mg^{2+}, 10 μg of plasmid DNA, one shot at 1200 V, 25 μF, in a Bio-Rad Gene Pulser apparatus. The cells, cultured in 100 mm^2 petri dishes were harvested 48 h after transfection, washed twice with 150 mM NaCl, resuspended in the same buffer and sonicated. All transfections were performed in triplicate. Each triplicate underwent the Lowry assay [15] and the IDS activity test, according to the method described as follows.

2.5. Enzyme analysis

The tritiated disaccharide substrate, *O*-(a-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-3*H*-mannitol-6-sulfate, is hydrolyzed by iduronate sulfates to inorganic sulfate and the monosulfated disaccharide product, *O*-(a-L-idopyranosyluronic acid)-(1→4)-2,5-anhydro-D-3*H*-mannitol-6-sulfate.
To test for IDS activity, the cellular extracts were dialyzed overnight against 4 l of distilled water. IDS assay was performed by incubating 30 μg of proteins for 2 h with the radiolabeled disaccharide substrate. To measure the activity of the enzyme in the samples, the radioactive products were separated from the substrate on an anion-exchange resin column [1].

2.6. Immunoblot analysis

Twenty micrograms of the total soluble cellular extract were boiled 5 min in Laemmli buffer, electrophoresed through 10% SDS–PAGE and electroblotted onto nitrocellulose membranes (Gelman Sciences). The membranes were treated with 5% dry milk in TTBS (20 mM Tris–HCl pH 7, 50 mM NaCl, 0,1% Tween 20) to inhibit non-specific binding. Anti-IDS polyclonal antiserum was used at a 1/1000 dilution in TTBS [10]. Visualization of antibody binding was carried out with ECL (Amersham), according to the manufacturer's instructions.

3. Results

3.1. Mutational analysis

Three novel mutations (K347T, 533delTT and N265I) and a previously described mutation (473delTCC) [7] were identified during the mutational analysis of a panel of Italian MPS II patients with phenotypes ranging from the mild to the severe form [11]. The A to C transversion was found in a patient with the severe form of the disease, at cDNA position 1164 (exon VIII) resulting in the replacement of lysine for threonine at codon 347 (K347T). Another transversion (A to T), detected in a patient affected by the intermediate clinical form, occurred at cDNA position 918 (exon VI) causing the consequent replacement of asparagine for isoleucine at codon 265 (N265I). The 533delTT was a microdeletion of two nucleotides at codon 137 causing a frameshift and premature termination of translation at codon 143. The lesion was detected in IDS exon III of a severely affected patient. The in-frame deletion of three nucleotides at cDNA position 473 caused the loss of the serine amino acid at codon 117 in a patient with severe clinical manifestation of the disease. Patients' genotypes and phenotypes are summarized in Table 1. All mutations, identified both by sequencing of the cDNA and the genomic DNA, were also confirmed by restriction endonuclease analysis.

3.2. Expression study

The functional consequence of the K347T,

Table 3
IDS activity in COS 7 transfected with wild-type and mutant cDNAs

Constructs	IDS activity[a] (U/mg protein)
K347T	12.7
473delTCC	12.2
533delTT	13
N265I	29.6
Wild-type	394.8
Untransfected cells	12.3

[a]IDS activity values are the average of three different transfections.

533delTT, N265I and 473delTCC mutations on IDS enzyme were evaluated by in vitro expression of the related mutant proteins and studied through activity measurements and Western blot analysis. After transfection of COS 7 with the wild-type and mutant plasmids, cells transfected with wild-type IDS cDNA had an activity of 394.8 U/mg protein, resulting in a 32-fold increase in comparison with untransfected cells that showed an IDS activity of 12.3 U/mg protein. No significant IDS activity was found in cells expressing K347T, 533delTT and 473delTCC mutations (Table 3).

In the cells expressing the normal IDS cDNA the immunoblot analysis showed the 55 kDa band corresponding to the mature form of the enzyme, whereas neither the 90 kDa precursor nor the 62 kDa intermediate forms were detectable [5,10]. The most likely explanation is that a batch of antibody different from that used previously [10] could be responsible for the misdetection of the 90 kDa and 62 kDa forms. Indeed, pulse-chase experiments of COS cells expressing normal cDNA confirmed that this new batch of antibody was not able to recognize the precursor form (data not shown). The cells transfected with the K347T, 473delTCC and N265I mutations presented similar results, even though the band of 55 kDa was less intense with respect to the wild-type enzyme. In contrast, no protein form was revealed in cells transfected with the cDNA containing the 533delTT mutation (Fig. 1).

4. Discussion

An important question arises as to whether or not a single base pair change is indeed the pathological lesion causing disease. The major evidence of causality for novel mutations should involve in vitro expression of the mutant gene to verify the enzymatic defect. To date, however, in vitro expression experiments are rarely applied, probably because of technical difficulty. As reviewed recently [16], some expression studies utilized Western blot analysis showing the presence of the precursor but not of the mature IDS form; some other studies, based on pulse-chase labeling experiments, resulted in the presence of a normal amount of precursor; finally, only subcellular fractionation studies demonstrated a generalized high proportion of IDS polypeptide content in an early vacuolar compartment, although Western blot and pulse-chase experiments resulted apparently normal [10]. In this study, in order to evaluate the functional consequence of the K347T, 533delTT, N265I and 473delTCC mutations on IDS enzyme, related mutant proteins were expressed in vitro and studied through activity measurements and Western blot analysis.

Our data proved the deleterious nature of the K347T, N265I, 473delTTC and 533delTT mutations. Both K347T and N265I are missense mutations occurring in residues not evolutionary conserved [17].

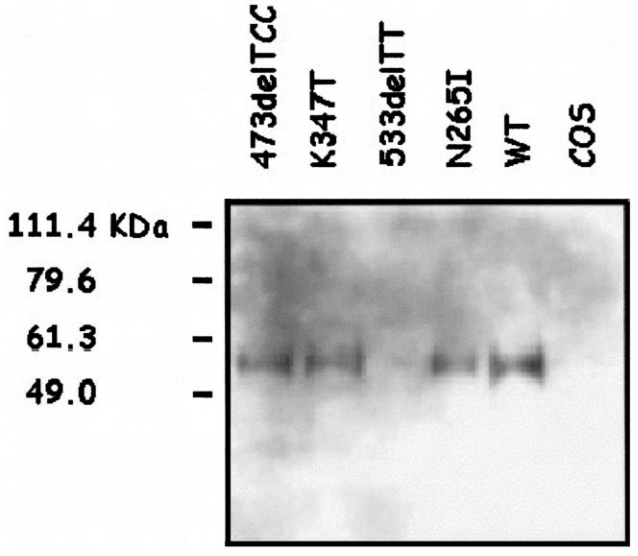

Fig. 1. Immunoblot analysis of COS 7 cells transiently transfected with mutants and wild-type (WT) constructs. After transfection and cell lysis, an equal loading of protein (20 μg) was used for each sample. The last lane (COS) is referred to untransfected COS cells. Weights of the molecular mass markers (in kDa) are shown on the left.

However, the K347T construct expressed an IDS activity completely overlapping that of untransfected COS cells, correlating with the severe clinical manifestations of the patient. On the other hand, the N265I construct expressed a residual activity (7.5% of the wild-type activity) probably related to the intermediate form of the disease. Both the 473delTCC and 533delTT microdeletions, in-frame and not in-frame, respectively, presented no significant IDS activity. Intriguingly, when the mutant constructs underwent Western blot analysis, the K347T, 473delTCC and N265I mutations yielded the 55 kDa mature form, as the wild-type construct, suggesting that alterations in these positions do not inhibit formation of the mature 55 kDa polypeptide, whereas no cross-reacting material was revealed in case of the 533delTT mutation, suggesting that the predicted premature stop codon could affect protein synthesis and/or stability. It should also be noted that skipping of serine 117, although not altering IDS maturation, results in an inactive enzyme, as reported for a mutation at the contiguous amino acid, T118I [10]. However, in this last case the involved missense alteration was found in a mildly affected patient, probably due to the marked amount (78%) of IDS polypeptides achieving a normal lysosomal localization; therefore, it is possible that polypeptides lacking serine 117 are not able to reach lysosomes in significant amounts, accounting for the severe phenotype of the involved patient.

In conclusion, we have confirmed as deleterious the K347T, N265I, 473delTCC and 533delTT mutations in the IDS gene demonstrating that at least the first three molecular defects do not affect IDS processing but have possible effects on enzyme conformation and function. This type of information is potentially useful for genotype–phenotype correlation, prognosis and possible therapeutic intervention.

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References

[1] E.F. Neufeld, J. Muenzer, The mucopolysaccharidoses, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 7th edn., McGraw-Hill, New York, 1995, pp. 2465–2494.

[2] R.H. Flomen, P.M. Green, D.R. Bentley, F. Giannelli, Determination of the organization of coding sequences within the iduronate sulfatase (IDS) gene, Hum. Mol. Genet. 2 (1993) 5–10.

[3] P.J. Wilson, C.A. Meaney, J.J. Hopwood, C.P. Morris, Sequence of the human iduronate-2-sulfatase (IDS) gene, Genomics 17 (1993) 773–775.

[4] P.J. Wilson, C.P. Morris, D.S. Anson, T. Occhiodoro, J. Bielicki, P.R. Clements, J.J. Hopwood, Hunter syndrome: isolation of an iduronate-2-sulfatase cDNA clone and analysis of patient DNA, Proc. Natl. Acad. Sci. USA 87 (1990) 8531–8535.

[5] R. Froissart, G. Millat, M. Mathieu, D. Bozon, I. Maire, Processing of iduronate 2-sulfatase in human fibroblasts, Biochem. J. 309 (1995) 425–430.

[6] G. Millat, I. Froissart, I. Maire, D. Bozon, IDS transfer from overexpressing cells to IDS-deficient cells, Exp. Cell Res. 230 (1997) 362–367.

[7] K. Sukegawa, S. Tomatsu, T. Fukao, H. Iwata, X.Q. Song, Y. Yamada, S. Fukuda, K. Isogai, T. Orii, Mucopolysaccharidosis type II (Hunter disease): identification and characterization of eight point mutations in the iduronate-2-sulfatase gene in Japanese patients, Hum. Mutat. 6 (1995) 136–143.

[8] G. Millat, R. Froissart, I. Maire, D. Bozon, Characterization of iduronate sulfatase mutants affecting N-glycosylation sites and the cysteine-84 residue, Biochem. J. 326 (1997) 243–247.

[9] G. Millat, R. Froissart, S. Cudry, V. Bonnet, I. Maire, D. Bozon, COS cell expression studies of P86L, P86R, P480L and P480Q Hunter's disease-causing mutations, Biochim. Biophys. Acta 1406 (1998) 214–218.

[10] G.R.D. Villani, A. Daniele, N. Balzano, P. Di Natale, Expression of five iduronate-2-sulfatase site-directed mutations, Biochim. Biophys. Acta 1501 (2000) 71–80.

[11] M. Filocamo, G. Bonuccelli, F. Corsolini, R. Mazzotti, R. Cusano, R. Gatti, Molecular analysis of 40 Italian patients with mucopolysaccharidosis type II: new mutations in the iduronate-2-sulfatase gene, Hum. Mutat. (2001) in press.

[12] G. Bonuccelli, S. Regis, M. Filocamo, F. Corsolini, F. Caroli, R. Gatti, A deletion involving exons 2–4 in the iduronate-2-sulfatase gene of a patient with intermediate Hunter syndrome, Clin. Genet. 53 (1998) 474–477.

[13] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.

[14] W.P. Deng, J.A. Nickoloff, Site-directed mutagenesis of virtually any plasmid by eliminating a unique site, Anal. Biochem. 200 (1992) 81–88.

[15] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, K.J. Randall,

Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[16] P. Di Natale, Mutations in sulfatase-deficient mucopolysaccharidoses, Curr. Top. Biochem. Res. 2 (2000) 207–217.
[17] S. Tomatsu, S. Fukuda, M. Masue, K. Sukegawa, T. Fukao, A. Yamagishi, T. Hori, H. Iwata, T. Ogawa, Y. Nakashima, Y. Hanyu, T. Hashimoto, K. Titani, R. Oyama, M. Suzuki, K. Yagi, Y. Hayashi, T. Orii, Morquio disease: isolation, characterization and expression of full-length cDNA for human N-acetylgalactosamine-6-sulfate sulfatase, Biochem. Biophys. Res. Commun. 181 (1991) 677–683.