A cDNA analysis disclosed the discordance of genotype-phenotype correlation in a patient with attenuated MPS II and a 76-base deletion in the gene for iduronate-2-sulfatase

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ARTICLE INFO

Keywords:
- Mucopolysaccharidosis type II
- cDNA analysis
- Splice variants
- Signal peptide

ABSTRACT

We previously showed that the genotype-phenotype correlation in MPS II is well-conserved in Japan (Kosuga et al., 2016). Almost all of our patients with attenuated MPS II have missense variants, which is expected to result in residual activity of iduronate-2-sulfatase. In contrast, our patients with severe MPS II have so-called null-type disease-associated variants, such as nonsense variants, frame-shifts, gene insertions, gene deletions and rearrangement with pseudogene (IDS2), none of which are expected to result in residual activity. However, we recently encountered a patient with attenuated MPS II who had a presumable null-type disease-associated variant and 76-base deletion located in exon 1 that extended into intron 1. To investigate this discordance, we extracted RNA from the leukocytes of the patient and performed reverse transcription polymerase chain reaction. One of the bands of the cDNA analysis was found to include a nucleotide sequence whose transcript was expected to generate an almost full-length IDS mature peptide lacking only part of its signal peptide as well as only one amino acid at the end of the N-terminus. This suggests that an alternative splicing donor site is generated in exon 1 upstream of the deleted region. Based on these observations, we concluded that the phenotype-genotype discordance in this patient with MPS II was due to the decreased amount of IDS protein induced by the low level of the alternatively spliced mRNA, lacking part of the region coding for the signal peptide but including the region coding almost the full mature IDS protein. The first 25 amino acids at the N-terminus of IDS protein are a signal peptide. The alternative splice transcript has only 13 (1 M-13 L) of those 25 amino acids; 14G-25G are missing, suggesting that the exclusively hydrophobic 1 M-13 L of the signal peptide of IDS might have a crucial role in the signal peptide.

1. Introduction

Mucopolysaccharidosis type II (MPS II, MIM #309900) is an X-linked recessive lysosomal storage disorder caused by deficiency of the enzyme iduronate-2-sulfatase (IDS, EC 3.1.6.13) [1,2]. The lack of the IDS enzyme results in the accumulation of glycosaminoglycans (GAGs), including dermatan sulfate and heparan sulfate, in various tissues and organs, leading to prominent skeletal and cardiac abnormalities, hearing difficulty, inguinal hernia, hepatosplenomegaly and occasionally neurological involvement [2,3]. In Asia including Japan, MPS II has been reported to be the most frequent type of MPS [4–7].

IDS is located on chromosome Xq28, spanning about 24 kb and

Abbreviations: DS, dermatan sulfate; ER, endoplasmic reticulum; ERT, enzyme replacement therapy; GAG, glycosaminoglycan; HSCT, hematopoietic stem cell therapy; HS, heparan sulfate; IDS, iduronate 2-sulfatase; LSD, lysosomal storage disorder; MPS II, mucopolysaccharidosis II.

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https://doi.org/10.1016/j.ymgmr.2020.100692

Received 27 November 2020; Accepted 27 November 2020
containing 9 exons. The Human Gene Mutation Database (HGMD) on the May 2019 edition (http://www.hgmd.cf.ac.uk/ac/all.php) shows a list of the 664 variants of IDS. Only about 9% of the disease-associated variants listed in the HGMD are described as affecting splice-site signals. However, the effect of splicing disease-associated variants in IDS is probably underestimated, since specific splicing effects were experimentally examined in only a few cases [8-14]. IDS is alternatively spliced, with seven different transcripts possible [15], suggesting that the mechanisms that controls splicing in the IDS gene are complex. Previous reports have shown that the occurrence of missense, nonsense or silent variants, whether in coding or noncoding regions, might also be related to the downregulation of splicing mechanisms, especially in genes with complicated splice control mechanisms, like IDS [12-14].

MPS II is classified into two disease subtypes; (1) the severe type of MPS II involves a decline in the cognitive ability of a patient, and (2) the attenuated type does not have any such intellectual impairment [3,4]. In Japanese patients with MPS II, most of the attenuated phenotype is derived from the missense variants of IDS, whereas disease-associated variants associated with large structural alterations, including recombinations, splice site errors and frame-shift and nonsense variants, are linked to the severe phenotype [16].

We herein report a patient with the attenuated phenotype of MPS II who had a 76-base deletion located in exon 1 that extended into intron 1. The phenotype-genotype discordance in this patient with MPS II may be attributed to a decreased amount of IDS protein induced by a low level of alternatively spliced mRNA, lacking part of the region coding for the signal peptide but having the region coding almost the full mature IDS protein. The first 25 amino acids at the N-terminus of IDS protein are a signal peptide. The alternative splice transcript has only 13 (1 M-13 L) of those 25 amino acids; 14G-25G are missing, suggesting that the exclusively hydrophobic 1 M-13 L of the signal peptide of IDS might have a crucial role in the signal peptide.

2. Material and methods

2.1. The patient

The primary patient was a 28-year-old male who was born and grew up in Japan as the second child of non-consanguineous Japanese parents. When he was five years old, he was suspected of having MPS because he had specific dysmorphic features, recurrent otitis media and mild hearing difficulty. As the IDS activity in his leukocytes was less than measurement sensitivity (reference value is 6.5 ± 1.7 nmol/mg/h), he was diagnosed with MPS II. He had no intellectual disability or other severe physical difficulties, and given that enzyme replacement therapy was not yet allowed to be used in Japan at the time, he was simply observed at a local hospital without any special treatment. His family photos showed that his mother’s brother seemed to have MPS II, although a genetic test had not been performed. His grandmother and mother were thought to be obligate carriers, so his sister had a 50% risk of being a carrier. He and his sister were referred to our hospital because his sister had just gotten married and wanted to find out if she was a carrier or not.

At the time of our visit, he was 168.5 cm, the height of an average Japanese male. He had typical dysmorphic facial features. He had mild hearing difficulty and non-severe heart valve disease. His liver and spleen were enlarged, and his fingers showed slight joint contractures. However, he had never had any problems in his daily life and was...
working at a large company, living independently. He had ‘multiplex dysostosis’ with typical findings on X-ray, including ‘oar-shaped’ widening of the ribs (Fig. 1). However, there was no evidence of gibbus deformity or dorsolumbar kyphosis. His pelvic bone shape was well-preserved. In addition, no point-shaped metacarpal bones were observed, although this may have been because we encountered him once he had reached adulthood. These data suggested that his skeletal symptoms were quite mild compared with a typical MPS II patient. We performed direct sequencing of IDS at first, as requested. Informed consent from the patient and his sister was obtained, as approved by the Institutional Review Board of the National Center for Child Health and Development. All of the samples were prepared and analyzed in accordance with the protocols approved by the ethics committee of the National Center for Child Health and Development.

2.2. Molecular diagnoses

2.2.1. Direct sequencing of IDS

We performed direct sequencing of IDS, as previously reported [16]. In brief, genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). The concentration of the isolated DNA was quantified spectrophotometrically using a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). All exons and intron/exon boundaries of the IDS were amplified using specific primers (Table 1) and assessed by polymerase chain reaction (PCR) and direct sequencing. The obtained sequences were compared with the reference sequences NM_000202.8 to identify disease-associated variants.

2.2.2. The IDS cDNA analysis

Total cellular RNA was isolated from the blood using a QIAamp RNA Blood Mini Kit (Qiagen) followed by DNase treatment using an Ambion TURBO DNA-free Kit (animal-free) (Thermo Fisher Scientific). The amount of RNA isolated was quantified and reverse-transcribed using the SuperScriptIII First-strand Synthesis System for RT-PCR kit (Invitrogen, CA, USA). The cDNA was amplified with specific primers (Table 1) and assessed by polymerase chain reaction (PCR) and direct sequencing. The obtained sequences were gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced.

2.2.3. Modelling splicing patterns

Prediction of the effect of the 76-base deletion on the splicing motif was performed with the Human Splicing Finder software program (http://www.umd.be/HSF/). The consensus value ranges from 0 to 100. The threshold was set at 65.

3. Results (Molecular diagnosis)

3.1. Direct sequencing of IDS

IDS direct sequencing detected a probably disease-associated hemizygous 76-base deletion of IDS, c. 93_103 + 65del76, in the primary patient first. His sister was then found to be a heterozygous carrier of the variant (Fig. 2). This 76-base deletion was located in exon 1, extending into intron 1, and was thought to cause a frame-shift, leading to nonsense mediated mRNA decay induced by a premature stop codon within intron 1. The variant, c. 93_103 + 65del76, was novel and not registered in any databases, such as HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), 1000Genomes data and ExAC.

**Fig. 2.** Partial sequence chromatograms of IDS in the primary patient. The sequence around the boundary between exon 1 and intron 1 is shown. IDS direct sequencing detected a probably disease-associated hemizygous 76-base deletion of IDS, c. 93_103 + 65del76, in the primary patient.

| Primer no | Primer name | From | To | Length | Sequence (5’ to 3’) | Product size (bp) |
|-----------|-------------|------|----|--------|---------------------|-----------------|
| TO-pri2732 | IDS-ex1-F | 29,026 | 20 | 3663 | TGGTTGTTAGGGCATGCC | 533 |
| TO-pri2731 | IDS-ex1-R | 29,671 | 20 | 3663 | TAGTGATTGATCTCTCC | 533 |
| TO-pri2734 | IDS-ex2-F | 30,041 | 20 | 3663 | CTGCCGTTAGCTCTCC | 533 |
| TO-pri2736 | IDS-ex3-F | 30,915 | 20 | 3663 | GTCTCTGTTAGCTCTCC | 533 |
| TO-pri2737 | IDS-ex3-R | 31,391 | 20 | 3663 | CTGCCGTTAGCTCTCC | 533 |
| TO-pri2738 | IDS-ex4-F | 33,357 | 20 | 3663 | TAGGTAGGATCTCTCC | 533 |
| TO-pri2739 | IDS-ex4-R | 33,786 | 20 | 3663 | TGGTTGTTAGGGCATGCC | 533 |
| TO-pri2740 | IDS-ex5-F | 36,033 | 20 | 3663 | TAGTGATTGATCTCTCC | 533 |
| TO-pri2741 | IDS-ex5-R | 36,565 | 19 | 3663 | GTCTCTGTTAGCTCTCC | 533 |
| TO-pri2742 | IDS-ex6-F | 37,895 | 20 | 3663 | CTGCCGTTAGCTCTCC | 533 |
| TO-pri2743 | IDS-ex6-R | 38,336 | 20 | 3663 | GTCTCTGTTAGCTCTCC | 533 |
| TO-pri2744 | IDS-ex7-F | 43,931 | 20 | 3663 | TAGTGATTGATCTCTCC | 533 |
| TO-pri2745 | IDS-ex7-R | 44,365 | 20 | 3663 | GTCTCTGTTAGCTCTCC | 533 |
| TO-pri2746 | IDS-ex8-F | 47,285 | 23 | 3663 | TAGTGATTGATCTCTCC | 533 |
| TO-pri2747 | IDS-ex8-R | 47,784 | 20 | 3663 | TAGTGATTGATCTCTCC | 533 |
| TO-pri2748 | IDS-ex9-F | 51,169 | 21 | 3663 | TAGTGATTGATCTCTCC | 533 |
| TO-pri2749 | IDS-ex9-R | 51,914 | 22 | 3663 | TAGTGATTGATCTCTCC | 533 |

Sequences of primers used to amplify the several DNA fragments analyzed

| Primer no | Primer name | From | To | Length | Sequence (5’ to 3’) | Product size (bp) |
|-----------|-------------|------|----|--------|---------------------|-----------------|
| TO-pri2830 | IDS-ex1-F | 264 | 283 | 20 | GTGTGTAAGGGCATGCC | 533 |
| TO-pri2840 | IDS-ex2-R | 456 | 437 | 20 | TGGTTGTTAGGGATGCTGCC | 533 |
| TO-pri2841 | IDS-ex3-R | 622 | 603 | 20 | TGGTTGTTAGGGATGCTGCC | 533 |

Sequences of primers used to amplify the several cDNA fragments analyzed

**Table 1**

Primer sequences used for PCR amplification.
3.2. IDS cDNA analysis results

We conducted a cDNA analysis (Fig. 3). The wild-type transcript produced using the primers TO-pri2828 located in exon 1 and TO-pri2840 located in exon 2 was 364 bases. The primary patient lacked this 364-base transcript but had a longer 1029-base transcript, the MS_transcript1 (MS1), and another shorter 301-base transcript, the MS_transcript2 (MS2). MS1 was a full-length transcript except for a 76-base deletion. When translated, it would lead to a frame-shift and then a premature stop codon. MS2 was considered an alternative splice transcript produced by the disruption of c.103, which was the original splicing donor site, and alternative activation of c.40, located 63 bases upstream of the original splicing donor site and considered a cryptic splicing donor site. MS2 was found to be in-frame and did not cause a frame-shift.

3.3. Modelling splicing patterns

Using the Bioinformatics tool Human Splicing Finder (HSF) (http://www.umd.be/HSF/), we tried to confirm the validity of our data (Fig. 4). The HSF system combines 12 different algorithms to identify and predict disease-associated variant’s effect on splicing motifs, including the acceptor and donor splice sites, the branch point and auxiliary sequences known to either enhance or repress splicing: Exonic Splicing Enhancers (ESE) and Exonic Splicing Silencers (ESS). The consensus value ranges from 0 to 100. The threshold was set at 65.

The top diagram in Fig. 4 shows the correct splice product. The bottom diagram shows MS2. The score of c.103 was 76.09, and that of c.40 was 77.4. Given that the score of c.40 was nearly as high as that of c.103, the original splicing donor site, it was entirely possible that should c.103 be lost, c.40 would become the alternative splicing donor site.

4. Discussion

MPS II is the most frequent type of MPS in Asian countries, including Japan [4-7], while the incidence of MPS III is higher in Western Australia, Germany and the Netherlands [17-19], and the incidence of MPS VI is high in Brazil [20]. MPS II is classified into two disease subtypes: (1) the severe type, involving a decline in the cognitive ability of a
patient, and (2) the attenuated type, which is associated with no such intellectual disabilities [3,4]. To determine the relationship between the phenotypes and genotypes, we analyzed disease-associated variants in the IDS gene of 65 patients with MPS II in Japan registered between 2004 and 2014 [16]. The 65 alleles from our patients consisted of 33 missense variants (33/65, 50.8%), 8 nonsense variants (8/65, 12.4%), 6 small deletions or small insertions (6/65, 9.2%), 4 splice site errors (4/65, 6.2%), 7 recombinations involving IDS-IDS2 (7/65, 10.8%) and others (7/65, 10.8%). Forty-three patients (43/65, 66.2%) had severe type, and 22 (22/65, 33.8%) had the attenuated type. Among the 33 missense variants, the severe type of MPS II was found in 42.4% (14/33) and the attenuated type in 57.6% (19/33). Among the 8 nonsense variants, the severe type of MPS II was found in 75% (6/8) and the attenuated type in 25% (2/8). The four small deletions and two small insertions were all linked to the severe phenotype.

Premature termination codons (PTCs), such as a nonsense variant, frame-shift (deletion or insertion) or splice site error, are generally associated with the severe phenotype. First, PTCs prematurely terminate the translation of mRNA, leading to the production of a truncated polypeptide that often lacks a normal function and/or is unstable. PTCs then trigger the degradation of the mRNA by activating nonsense-mediated mRNA decay (NMD), a cellular pathway that recognizes and degrades mRNAs containing a PTC [23]. Transcripts from genes with PTCs are subject to extreme downregulation, often by 10- to 100-fold, and sometimes mutant mRNA can be degraded completely. As these two mechanisms cause a severe reduction in gene expression, PTC-generating disease-associated variants are generally expected to have a more severe disease phenotype than missense variants, which may allow the retention of at least part of a protein’s function [23]. Indeed, all six patients with frame-shifts in our study had the severe phenotype.

4.1. IDS enzyme activity and urinary uronic acid

The primary patient was diagnosed with MPS II because his IDS enzyme activity was reported to be extremely low. We confirmed this in our laboratory based on the urinary accumulation of uronic acid and the IDS enzyme activity in the leukocytes and dried blood spots by a fluorometric procedure, as previously described [21,22]. The urinary uronic acid was 37 mg/L (reference value is less than 20 mg/L), the dermatan sulfate (DS1) was 42% (reference value is 0%–10%), DS2 was 19%, heparan sulfate was 10% (reference value is 1%–30%), and chondroitin sulfate was 29% (reference value is 60%–98%). Urinary uronic acid and dermatan sulfate levels were high, while those of chondroitin sulfate were low. These data were consistent with those of other MPS II patients. The IDS enzyme activity in his lymphocytes as well as dried blood spots was also less than the measurement sensitivity.

4.2. X-ray findings

As mentioned above, the primary patient had a surprisingly mild phenotype. His height of 168.5 cm was average for a Japanese male, making him 40 cm taller than the average Japanese patient with MPS II [24,25]. His X-ray findings showed ‘multiplex dysostosis’, a typical finding of MPS, including ‘oar-shaped’ widening of the ribs. As a whole, his skeletal symptoms were quite mild compared with those of a typical MPS II patient.

4.3. Signal sequence

IDS direct sequencing detected a probably disease-associated hemizygous 76-base deletion of IDS, c. 93,103 + 65del76, in the primary patient. This 76-base deletion was located in exon 1, extending into intron 1, and was thought to cause a frame-shift leading to nonsense-mediated mRNA decay by a premature stop codon in intron 1. A cDNA analysis showed that the primary patient did not have the wild-type transcript, instead showing a shorter transcript: MS2. MS2 is thought to be an alternative splice transcript. It is produced by the alternative activation of c.40, which is located 63 bases upstream of the original splicing donor site and considered a cryptic splicing donor site. MS2 is found in-frame and does not cause a frame-shift.

The amino acid sequences for human IDS proteins contain 550 amino acids [26]. Previous studies have reported several key regions and residues for human and mouse IDS proteins [27], including the N-terminal signal peptide (25 residues including the N-terminal methionine) followed by a propeptide, which is an 8-residue segment (residues 25–33) [28]. The eight amino acids of the propeptide are removed from the IDS precursor shortly after the signal peptide is cleaved by the signal peptidase [28]. A comparison of 10 mammalian IDS sequences for the N-terminus, including the signal peptide, propeptide and exon 1, revealed species-specific variability in these sequences and that the signal peptide contains multiple proline and hydrophobic residues [26].

Although the precise number of proteins remains to be determined, it is generally recognized that the majority of proteins are delivered by the signal recognition particle (SRP), a universally conserved protein-targeting machine [29–32]. The N-terminal signal peptide mediates the targeting of nascent secretory and membrane proteins to the endoplasmic reticulum (ER) in an SRP-dependent manner. The signal peptide consists of three parts: a hydrophobic core region (h-region), c-region and c-region. Usually, the signal peptide is cleaved off co-translationally so that the signal peptide and mature proteins are generated [33–36,37]. The N-terminal signal peptide on the nascent polypeptide works as the ‘signal’ and allows the ribosome nascent chain complex (RNC) to engage the SRP and be delivered to the target membrane [37]. The SRP pathway is evolutionarily conserved, although the size and composition of the SRP vary widely across species [37]. The bacterial SRP contains the universally conserved SRP54 protein (called Ffh in bacteria) bound to the 4.5S SRP RNA. Ffh has a methionine-rich M-domain that recognizes the signal peptide and binds to the SRP RNA [38–40]. The signal peptide must be recognized by the SRY in a timely manner in order to start cotranslational protein targeting. The signal peptide that engages the SRP is characterized, in general, by a core of 8–12 hydrophobic amino acids [41,42]. In support of this model, crystallographic analyses of Ffh [43] and SRP54-signal peptide fusions [44,45] have shown that the signal peptide binds to a groove in the Ffh/SRP54 M-domain comprised almost exclusively of hydrophobic residues. The first 25 amino acids at the N-terminus of IDS protein form a signal peptide, MPPPRTGRGLLWLGLVLSVSCVALG. There are 18 hydrophobic amino acids: 2P, 3P, 4P, 7G, 9G, 10 L, 11 L, 12 W, 13 L, 14G, 15 L, 16 V, 17 L, 20 V, 22 V, 23A, 24 L, 25G. The GLVLSVSCVALG portion of the signal peptide and SETQANST of the propeptide are missing in MS2, which retains the hydrophobic amino acids 2P, 3P, 4P, 7G, 9G, 10, L, 11, 12, 13, 14G, 15, 16, 17, 19, 20, 22, 23A, 24 L, 25G. The GLVLSVSCVALG portion of the signal peptide and SETQANST of the propeptide are missing in MS2, which retains the hydrophobic amino acids 2P, 3P, 4P, 7G, 9G, 10, L, 11, 12, 13 L and 13 L. These retained hydrophobic amino acids might contribute to the retention of the protein-targeting ability of MS2 (Fig. 5). However, there have been reports that RNCs containing no signal sequences or even empty ribosomes bind the SRP with kd values of 80–100 mM
In addition, an increasing number of post-translational protein-targeting pathways have been identified [29,30,49–52]. It is therefore also possible that there are other unknown post-translational targeting pathways aside from SRP that may have caused the primary patient’s unbelievably mild phenotype.

Once the NRC reaches the ER, MS2 is expected to include a certain degree of IDS activity, as it has an almost full-length mature IDS protein missing only one amino acid (T34) at the N-terminus. T34 is not an active site of IDS and is not conserved in the other 16 human sulfatases [33], suggesting that T34 is not essential for the catalytic function of IDS. LDSs are known to have a relatively low threshold for correction compared to other genetic diseases. For example, the amount of protein function that needs to be restored in order to alleviate cystic fibrosis [54] compared to other genetic diseases. For example, the amount of protein

Additionally, the phenotype of MPS can be alleviated by an extremely small quantity of the enzyme activity. We believe that MS2 must be related to the phenotype-genotype
discordance in the primary patient.

5. Conclusion

We herein report a patient with the attenuated phenotype of MPS II who had a 76-base deletion located in exon 1 that extended into intron 1. The phenotype-genotype discordance in this patient with MPS II may be attributed to a decreased amount of IDS protein induced by a low level of alternatively spliced mRNA, lacking part of the region coding for the signal peptide but having the region coding almost the full mature IDS protein. The first 25 amino acids at the N-terminus of IDS protein are a signal peptide. The alternative splice transcript has only 13 (1 M-13 L) of those 25 amino acids; 14G-25G are missing, suggesting that the exclusively hydrophilic 1 M-13 L of the signal peptide of IDS might be an essential region allowing the protein to target the ER.

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