Splice donor site mutation in the lysosomal neuraminidase gene causing exon skipping and complete loss of enzyme activity in a sialidosis patient

Roland Penzel[a,1,*], Johannes Uhl[b,1], Jürgen Kopitz[b], Michael Beck[c], Herwart F. Otto[a], Michael Cantz[b]

[a] *Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 220, D-69120 Heidelberg, Germany*
[b] *Department of Pathochemistry and Neurochemistry, University of Heidelberg, Im Neuenheimer Feld 220, D-69120 Heidelberg, Germany*
[c] *Children's Hospital of the Johannes-Gutenberg University, Langenbeckstrasse 1, D-55131 Mainz, Germany*

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Abstract Sialidosis is a lysosomal storage disease caused by the deficiency of α-*N*-acetylneuraminidase (NEU1; sialidase), the key enzyme for the intralysosomal catabolism of sialylated glycoconjugates. We have identified a homozygous transversion in the last intron (IVSE +1 G > C) in *neu1* of a sialidosis patient. Sequencing of the truncated cDNA revealed an alternatively spliced *neu1* transcript which lacks the complete sequence of exon 5. Skipping of exon 5 leads to a frameshift and results in a premature termination codon. This is the first description of an intronic point mutation causing a complete deficiency of the lysosomal neuraminidase activity.

Key words: Lysosomal neuraminidase; Mutation; Donor splice site; Exon skipping; Sialidosis

1. Introduction

The catabolism of sialylated glycoconjugates is strictly dependent on the function of specific hydrolases, which occur in viruses, bacteria, protozoa, birds and mammals [1–3]. Among these exoglycosidases are members of a family of neuraminidases or sialidases that catalyze the cleavage of α-glycosidically linked terminal *N*-acetylneuraminic acid (sialic acid) from glycoconjugates [4]. The intralysosomal *N*-acetyl-α-neuraminidase (NEU1) in mammals, unlike the membrane-bound and cytosolic neuraminidases, is catalytically active only when it is bound to a carboxypeptidase called protective protein/cathepsin A (PPCA) in a multi-protein complex that also contains β-galactosidase (GAL) and *N*-acetylgalactosamine-6-sulfate sulfatase [5–10]. Genetically based alterations of constituents of this complex result in lysosomal storage diseases such as sialidosis and galactosialidosis. Sialidosis is due to lesions in the *neu1* gene located on chromosome 6p21.3 [11] and is characterized biochemically by the deficiency of lysosomal neuraminidase activity and an abnormal tissue accumulation and urinary excretion of sialic acid-containing compounds (for review see [12]). According to the clinical symptoms, sialidosis has been divided into two subtypes with different ages of onset and severity. Sialidosis type I, also called non-dysmorphic type, is the mild form of the disease with late onset, bilateral macular cherry-red spots, progressively impaired vision and myoclonus syndrome. The type II or dysmorphic type with infantile onset is characterized by skeletal dysplasia, Hurler-like phenotype, dystosis multiplex, mental retardation and hepatosplenomegaly. The severe form often occurs prenatally and manifests itself by ascites and hydrops fetalis [13]. Galactosialidosis is associated with primary genetic defects of PPCA causing a disruption of the complex resulting in a combined deficiency of GAL and neuraminidase activities (for review see [14]).

Since the cloning of the *neu1* mRNA [11,15], several studies have reported the identification of genetic alterations in *neu1* of unrelated sialidosis patients. The majority of reported mutations consist of missense mutation (15 cases), followed by nonsense mutations (four cases), two insertions and two deletions [11,15–19]. All mutations are exonic and distributed over the entire coding region of *neu1*. Recent work using 3D modeling of mutated protein sequences indicates that even amino acid changes which do not interfere with structurally important domains have a strong influence on enzyme activity as residues located in specific regions on the surface of the enzyme may be involved in functionally important protein interactions [20].

In order to identify novel mutations within the *neu1* gene, we performed a sequencing-based screening of cultured skin fibroblast derived from sialidosis patients. In the present patient with the severe type II, we found a homozygous $_{g2736}$G > C transversion (IVSE +1 G > C) located in the splice donor site of intron E causing skipping of exon 5 and complete loss of lysosomal neuraminidase activity.

2. Materials and methods

2.1. Clinical data

The patient was a full-term small-for-date female newborn delivered by Cesarean section at week 41 of pregnancy (weight: 2740 g, length: 43 cm, circumference of the head: 31 cm). She was the first child of healthy parents without any known consanguinity. At week 27 of pregnancy, hydrops fetalis was detected by sonography. After a pathological cardiotocogram, birth was initiated by Cesarean section. Pre- and postnatally, the patient suffered from ascites and edema. In sonographic examinations of the brain fine calcifications of the thalamic striatum blood vessels were seen. Besides this, symmetrical and synchronic myoclonus of the extremities lasting several minutes was

*Corresponding author. Fax: (49)-6221-565251.
E-mail address: roland_penzel@med.uni-heidelberg.de (Roland Penzel).

[1] These authors contributed equally to this work.

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observed. The hemogram and further parameters were inconspicuous. The analysis of chromosomes was normal (46XX).

2.2. Tissue culture

Skin fibroblasts derived from the patient and healthy donors were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Life Technologies, USA) and non-essential amino acids as described previously [21].

2.3. Enzyme activity assays

Enzyme activity of the lysosomal neuraminidase was determined using 2′,4-methylumbelliferyl-α-*N*-acetylneuraminic acid as substrate according to Harzer et al. [22]. The GAL activity was measured according to Gehler et al. [23].

2.4. Isolation of genomic DNA and total RNA

Genomic DNA and total RNA were isolated from cultured skin fibroblasts derived from the patient and healthy donors using the QIAamp DNA Mini kit and the RNeasy kit (Qiagen, Germany). 1 μg of DNase I (Roche Diagnostics GmbH, Germany)-treated total RNA was reverse transcribed by oligo(dT) and random hexamer priming using the SuperScript II reverse transcriptase (Life Technologies, USA).

2.5. neu1 mRNA expression

Semi-quantitative RT-PCR following oligo(dT)-primed reverse transcription was used to assess the *neu1* transcript level in the sialidosis patient. PCR was carried out for 25 cycles using 5′-CCC AAG CTT AGA TCT TGG AGT CTA GCT GCC AGG GT-3′ and 5′-CCA GGG GCA AAC ACT TCA GT-3′ for *neu1*-specific amplification and for 19 cycles using 5′-GAT GCT GGC GCT GAG TAC GTC-3′ and 5′-CGT TGT CAT ACC AGG AAA TGA GC-3′ for GAPDH-specific amplification. PCR was performed with 2 μl of the reverse transcription samples in a total volume of 50 μl using the hot start method for both target sequences. Denaturation was done at 94.5°C for 45 s, annealing at 60°C for 90 s, primer extension at 72°C for 150 s and final extension for 10 min. Amplification products were visualized on an ethidium bromide-stained 2% agarose gel and documented by digitalization (Gel documentation system, Herolab GmbH, Germany). Signal intensities were quantitated by densitometry using the LabImage 2.51 program (Labsoft Diagnostics AG, Germany). The resulting values of the *neu1*-specific fragments were normalized with GAPDH as internal standard and the result of the sialidosis patient was displayed as a relative change compared to the mean of three control donors.

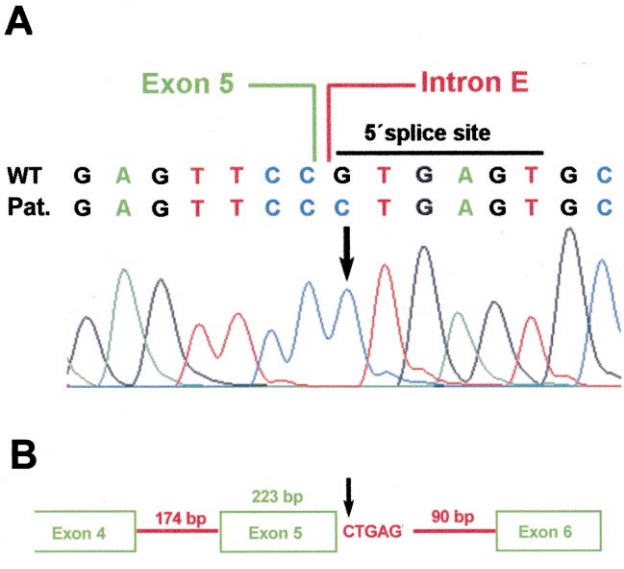

Fig. 1. Splice donor site mutation in the sialidosis patient. A: The wild-type (WT) genomic DNA sequence is shown above the patient's sequence. The homozygous IVSE +1 G>C transversion is marked by an arrow in the chromatogram. B: The scheme displays the localization of the detected mutation in the *neu1* genomic sequence.

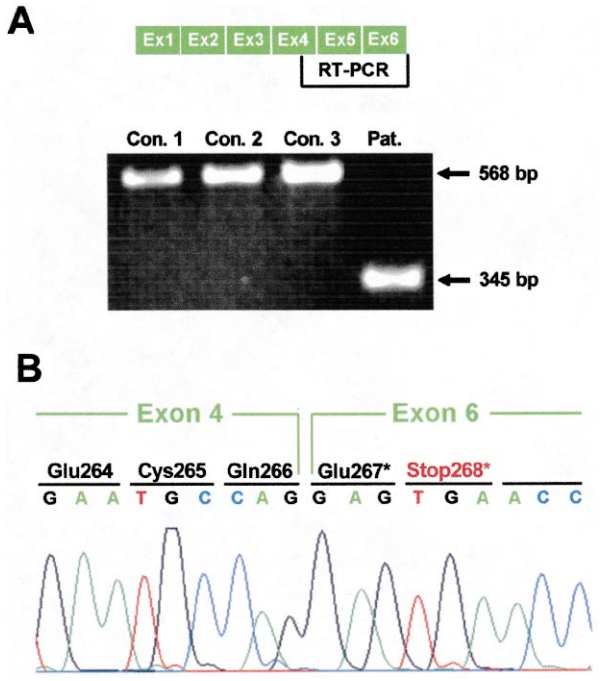

Fig. 2. RT-PCR and cDNA sequencing of the truncated amplification product of the sialidosis patient. A: Total RNAs were isolated from three control donors (Con.) and the sialidosis patient (Pat.), reverse-transcribed, and PCR performed to generate the indicated fragment. B: Sequencing of the truncated RT-PCR product (345 bp) detected in the sialidosis patient revealed the complete loss of exon 5. Amino acid positions altered consecutive to the reading frame shift are indicated by asterisks.

2.6. PCR amplification and direct sequencing

Genomic DNA was amplified with exon-specific sets of oligonucleotides as described previously [16]. To amplify intron sequences, PCR of genomic DNA was carried out with combinations of these exon-specific oligonucleotides. Amplification of cDNA was performed with three overlapping sets of oligonucleotides, which encompassed the entire coding region of the human lysosomal neuraminidase mRNA [15]. Amplification products were separated on 1% agarose gels to confirm the expected length. After purification, PCR fragments were sequenced directly in the forward and reverse directions on an Applied Biosystems 377-18 DNA sequencer using the Big Dye Termination kit (Applied Biosystems, USA) and the corresponding oligonucleotides.

3. Results

The enzymatic activities of lysosomal neuraminidase and GAL of fibroblasts derived from the sialidosis patient were compared to the activities of healthy donors. The patient's fibroblasts exhibited no detectable neuraminidase activity, whereas that of GAL was normal, establishing the biochemical diagnosis of sialidosis.

The transcriptional activity of *neu1* was investigated by semi-quantitative RT-PCR. The *neu1*-specific PCR fragments representing nucleotide positions ca. −55 to +741 of the patient and healthy control donors were normalized to GAPDH amplicons. Compared to control donors the sialidosis patient showed a normal amount of *neu1* mRNA.

In order to identify mutations which could form the basis

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NEU1 MTGERPSTALPDRRWGPRILGFWGGCRVWVFAAIFLLLSLAASWSKAENDFGLVQPLVTM  60
Pat. MTGERPSTALPDRRWGPRILGFWGGCRVWVFAAIFLLLSLAASWSKAENDFGLVQPLVTM

NEU1 EQLLWVSGRQIGSVDTFRIPLITATPRGTLLAFAEARKMSSSDEGAKFIALRRSMDQGST 120
Pat. EQLLWVSGRQIGSVDTFRIPLITATPRGTLLAFAEARKMSSSDEGAKFIALRRSMDQGST

NEU1 WSPTAFIVNDGDVPDGLNLGAVVSDVETGVVFLFYSLCAHKAGCQVASTMLVWSKDDGVS 180
Pat. WSPTAFIVNDGDVPDGLNLGAVVSDVETGVVFLFYSLCAHKAGCQVASTMLVWSKDDGVS

NEU1 WSTPRNLSLDIGTEVFAPGPGSGIQKQREPRKGRLIVCGHGTLERDGVFCLLSDDHGASW 240
Pat. WSTPRNLSLDIGTEVFAPGPGSGIQKQREPRKGRLIVCGHGTLERDGVFCLLSDDHGASW

NEU1 RYGSGVSGIPYGQPKQENDFNPDECQPYELPDGSVVINARNQNNYHCHCRIVLRSYDACD 300
Pat. RYGSGVSGIPYGQPKQENDFNPDECQE*

NEU1 TLRPRDVTFDPELVDPVVAAGAVVTSSGIVFFSNPAHPEFRVNLTLRWSFSNGTSWRKET 360

NEU1 VQLWPGPSGYSSLATLEGSMDGEEQAPQLYVLYEKGRNHYTESISVAKISVYGTL 415
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Fig. 3. Comparison of the amino acid sequences predicted from the complete *neu1* mRNA and the truncated *neu1* transcript of the sialidosis patient. The normal *neu1* transcript encodes a polypeptide with 415 amino acids (Neu1). The deduced polypeptide sequence of the sialidosis patient (Pat.) lacks 148 amino acids of the C-terminal region. The five presumed catalytically active residues (bold), the five Asp boxes (underlined) and the premature termination codon (asterisk) are indicated.

for the complete deficiency of the NEU1 activity we isolated genomic DNA from patient's skin fibroblasts. Subsequent to amplification of the six exons and the five intervening introns (A–E) the PCR products were directly sequenced using the corresponding oligonucleotides. As shown in Fig. 1, there was a homozygous $_{g2736}G>C$ transversion in the PCR fragment encompassing the 3′ region of exon 5, the intervening intron E and exon 6. The mutation affected the +1 position within the donor splice site of intron E (IVSE +1 G > C).

RT-PCR amplification of total RNA isolated from skin fibroblasts of three unrelated healthy donors generated a 568-bp fragment corresponding to cDNA positions 721–1288 (Fig. 2A). As confirmed by direct DNA sequencing, the products contained part of exon 4 and the complete sequences of exon 5 and exon 6. RT-PCR performed under the same conditions with total RNA from skin fibroblasts of the sialidosis patient produced exclusively a 345-bp fragment. Direct sequencing of this truncated cDNA fragment revealed the complete deletion of exon 5 (Fig. 2B). Skipping of exon 5 will result in a frame shift and a premature termination at amino acid position 268.

4. Discussion

To date, 23 genetic alterations were detected in the lysosomal neuraminidase gene of sialidosis patients. They all were observed throughout the entire coding region without any indications for a hot spot region [11,15–19]. Recent studies using 3D modeling of the mutated sequences were helpful in understanding the potential influence of amino acid substitutions on tertiary protein structure. The majority of mutations consisted of missense mutations affecting amino acid positions that were not located within important functional domains such as the active site or the central core of the molecule. It was therefore suggested that the position of some of the affected residues on the surface of the enzyme would be critical for the correct association of NEU1 with the multi-protein complex that is necessary for catalytic function [20]. In other cases, the deficiency of the enzyme activity could be explained by nonsense mutations, small insertions or deletions that cause a shift in the reading frame resulting in a premature termination codon.

In this study, we have identified the first intronic mutations in the *neu1* gene of a patient diagnosed as having sialidosis type II. The mutated position +1 in intron E (IVSE +1 G > C) is part of the highly conserved 5′ splice donor site. Three different consequences could be expected for a splice site mutation, viz. skipping of the entire preceding exon, read-through of the retained intron, or use of a cryptic splice donor site. Exon skipping is the most frequently observed result of a splice site mutation in humans [24] and mammals [25], because intron inclusion or the use of a cryptic splice site instead of the destroyed consensus splice site depends on specific sequence properties. Retention of an intron may be favored if the size of the intron does not yield a redefined exon of more than 300 bp [26]. This is unlikely for the case reported here because the redefined exon would exceed the limiting size of 300 bp. Recognition of the donor splice site by the U1 small nuclear ribonucleoprotein particle is the initial step in formation of the spliceosome and essential for further processing of the pre-mRNA transcript during the splicing procedure [27]. If no acceptable cryptic splice donor site is recognized in the vicinity of the authentic splice site, exon skipping seems to occur [25]. Obviously, no suitable cryptic site could be found for the mutated splice donor site of intron E, since exon 5 was completely absent from the mature *neu1* transcript of the sialidosis patient.

As the junction between exon 5 and exon 6 of *neu1* is not in frame (the last base of exon 5 and the first two bases of exon 6 make up codon 341), this frame shift led to a premature termination codon at position 268 in the aberrantly spliced mRNA. As shown in Fig. 3, the deduced polypeptide exhibits a truncation of 148 amino acids at the C-terminus. The complete enzyme deficiency can thus be explained by the fact that the lost C-terminal region of NEU1 contains two of five structurally important Asp boxes and four of five strictly conserved active site residues.

References

[1] Saito, M. and Yu, R.K. (1995) in: Biology of Sialic Acids (Rosenberg, A., Ed.), pp. 261–313, Plenum Press, New York.
[2] Miyagi, T., Konno, K., Emori, Y., Kawasaki, H., Suzuki, K., Yasui, A. and Tsuiki, S. (1993) J. Biol. Chem. 268, 26435–26440.
[3] Ferrari, J., Harris, R. and Warner, T.G. (1994) Glycobiology 4, 367–373.
[4] Traving, C. and Schauer, R. (1998) Cell. Mol. Life Sci. 54, 1330–1349.
[5] van der Spoel, A., Bonten, E. and d'Azzo, A. (1998) EMBO J. 17, 1588–1597.
[6] Verheijen, F. and Brossmer, R. (1982) Biochem. Biophys. Res. Commun. 108, 868–875.
[7] Verheijen, F.W., Palmeri, S. and Hoogeveen, A.T. (1985) Eur. J. Biochem. 149, 315–321.
[8] Yamamoto, Y. and Nishimura, K. (1987) J. Biol. Chem. 19, 435–442.
[9] Pshezhetsky, A.V. and Potier, M. (1994) Arch. Biochem. Biophys. 313, 64–70.
[10] Pshezhetsky, A.V. and Potier, M. (1996) J. Biol. Chem. 271, 28359–28365.
[11] Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G. and d'Azzo, A. (1996) Genes Dev. 10, 3156–3169.
[12] Cantz, M. and Ulrich-Bott, B. (1990) J. Inher. Metab. Dis. 13, 523–537.
[13] Thomas, G.H. and Beaudet, A.L. (1995) in: The Metabolic and Molecular Bases of Inherited Disease, 7th edn. (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.), pp. 2529–2561, McGraw-Hill, New York.
[14] d'Azzo, A., Andria, G., Strisciuglio, P. and Galjaard, H. (1995)

in: The Metabolic and Molecular Bases of Inherited Disease, 7th edn. (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.), pp. 2825–2837, McGraw-Hill, New York.
[15] Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M.-A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L. and Potier, M. (1997) Nature Genet. 15, 316–320.
[16] Lukong, K.E., Elsliger, M.-A., Chang, Y., Richard, C., Thomas, G., Carey, W., Tylki-Szymanska, A., Czartoryska, B., Buchholz, T., Rodríguez Criado, G., Palmeri, S. and Pshezhetsky, A.V. (2000) Hum. Mol. Genet. 9, 1075–1085.
[17] Bonten, E.J., Arts, W.F., Beck, M., Covanis, A., Donati, M.A., Parini, R., Zammachi, E. and d'Azzo, A. (2000) Hum. Mol. Genet. 9, 2715–2725.
[18] Naganawa, Y., Itoh, K., Shimmoto, M., Takiguchi, K., Doi, H., Nishizawa, Y., Kobayashi, T., Kamei, S., Lukong, K.E., Pshezhetsky, A.V. and Sakuraba, H. (2000) J. Hum. Genet. 45, 241–249.
[19] Buchholz, T., Molitor, G., Lukong, K.E., Praun, M., Genzel-Boroviczeny, O., Freund, M., Pshezhetsky, A.V. and Schulze, A. (2001) Eur. J. Pediatr. 160, 26–30.
[20] Lukong, K.E., Landry, K., Elsliger, M.-A., Chang, Y., Lefrancois, S., Morales, C.R. and Pshezhetsky, A.V. (2001) J. Biol. Chem. 276, 17286–17290.
[21] Cantz, M., Chrambach, A., Bach, G. and Neufeld, E.F. (1972) J. Biol. Chem. 10, 5456–5462.
[22] Harzer, K., Cantz, M., Sewell, A.C., Dhareshwar, S.S., Roggendorf, W., Heckl, R.W., Schofer, O., Thumler, R., Peiffer, J. and Schlote, W. (1986) Hum. Genet. 74, 209–214.
[23] Gehler, J., Cantz, M., Stoeckenius, M. and Spranger, J. (1976) Eur. J. Pediatr. 122, 201.
[24] Krawczak, M., Reiss, J. and Cooper, D.N. (1992) Hum. Genet. 90, 41–54.
[25] Nakai, K. and Sakamoto, H. (1994) Gene 141, 171–177.
[26] Robberson, B.L., Cote, G.J. and Berget, S.M. (1990) Mol. Cell. Biol. 10, 84–94.
[27] Newman, A. (1998) Curr. Biol. 8, 903–905.