Identification of a *CTL4/Neu1* fusion transcript in a sialidosis patient

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Abstract The deficiency of the lysosomal neuraminidase (NEU1; sialidase) causes the storage disorder sialidosis with symptoms ranging from eye abnormalities and neurological disturbances to skeletal malformations, mental retardation and early death. Sialidosis patients encompassing a wide spectrum of clinical symptoms were screened for mutations in *neu1*. We identified the same homozygous interstitial deletion (11 kb) in two patients causing the fusion of exon 10 of *CTL4* (New Gene 22; NG22) with the 3′-UTR of *neu1*. In one patient we found the resulting *CTL4/Neu1* fusion transcript, in the other we detected an alternatively spliced *CTL4* transcript (retention of intron 9).

Key words: Lysosomal neuraminidase; Interstitial deletion; Fusion transcript; CTL4 (NG22); Sialidosis

1. Introduction

Sialidosis is characterised biochemically by the deficiency of lysosomal neuraminidase activity [1,2] and an abnormal tissue accumulation and urinary excretion of sialic acid-containing compounds. 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, progressive impaired vision and myoclonus syndrome. The type II or dysmorphic type with infantile onset is characterised by skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation and hepatosplenomegaly. The severe form often manifests itself prenatally and presents with ascites and hydrops fetalis (for review see [3]).

Sialidosis is an autosomal recessive disease due to lesions in the neu1 gene that maps to the MHC-III cluster on chromosome 6p21.3 [4–6]. Within this cluster *neu1* is closely neighboured (851 bp) to the choline transporter-like protein 4 gene (CTL4 also called NG22) [7]. Several genetic alterations have been reported in *neu1* of unrelated sialidosis patients. The majority of mutations consist of missense mutations (20 of 26 cases) [4,6,8–11].

In view of the fact that even an intronic mutation can cause a complete deficiency of the lysosomal neuraminidase [12], we performed a sequencing-based screening of genomic DNA from sialidosis patients. In two cases we identified the same 11 kb deletion that encompassed the entire coding region of *neu1*.

2. Material and methods

2.1. Patients

Available clinical data of the patients are summarised in Table 1.

2.2. Cell culture

Skin fibroblasts from the patients and healthy control donors were cultured in Eagle's modified Eagle's medium supplemented with 10% foetal calf serum (PAA Laboratories, Austria); non-essential amino acids were added as described previously [13].

2.3. Enzyme activity assay

Enzyme activity of lysosomal neuraminidase was determined using 2′-4-methylumbelliferyl-α-*N*-acetylneuramic acid as substrate according to [14]. The β-galactosidase activity was measured according to Gehler et al. [15]. Activities are expressed as specific activities based on the protein content of the samples that was determined using the Lowry procedure [16].

2.4. Isolation of genomic DNA and total RNA

Genomic DNA and total RNA were isolated from cultured skin fibroblast using the QIAamp DNA Mini Kit and the RNeasy Kit (Qiagen, Germany).

2.5. Polymerase chain reaction (PCR) amplification and direct sequencing

Genomic DNA was amplified with exon-specific oligonucleotides as described previously [8]. PCR for the intronic sequences was performed using combinations of the same oligonucleotides. Amplification of cDNA was carried out with three overlapping sets of oligonucleotides encompassing the entire coding region of the human lysosomal neuraminidase mRNA [6]. After purification the 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.

2.6. Reverse transcription (RT)-PCR and duplex-PCR

Reverse transcriptions were carried out with 1 μg DNase I treated total RNA in the presence of a RNAse inhibitor using the SuperScript II reverse transcriptase (Life Technologies, USA). Expression of the mature mRNAs of *neu1* and the protective protein cathepsin A (PPCA) were investigated by amplifications of oligo(dT) primed cDNA using exon-specific primers. For amplification of pre-mRNA of *neu1* we performed PCR with random hexamer primed cDNA and intron C-specific primers (Table 2). RT-PCR products were electrophoretically separated on an ethidium bromide stained 2% agarose gel and documented by digitalisation (Gel documentation system, Herolab, Germany).

To determine the heterozygosity of the 11 kb deletion (g.−8042–

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+3261 del) in the sister of patient 1, we performed a duplex-PCR with genomic DNA using one forward primer and two reverse primers (Table 2).

2.7. Transcriptional activity of the CTL4/Neu1 fusion gene

Based on the genomic sequencing data from both patients a hypothetical fusion transcript was assumed to consist of exons 1–10 of the CTL4 gene and the 3′-UTR of *neu1*. Transcription of the CTL4/Neu1 fusion gene was investigated by nested RT-PCR with two forward primers located within the CTL4 part and two reverse primers in the neu1 part of the fusion transcript (Table 2). Sequencing of the PCR product detected in patient 1 confirmed the occurrence of the *CTL4/Neu1* fusion transcript. As we obtained no amplification products in the sister of patient 1 and patient 2 a nested RT-PCR was performed using primers specific for the CTL4 part of the calculated fusion transcript. Here, the inner forward primer corresponded to the border of the exons 6 and 7 and both reverse primers were located within the remaining part of exon 10. In patient 2 a significantly longer PCR product was observed and sequenced.

3. Results

3.1. Activities of lysosomal neuraminidase and β-galactosidase in sialidosis patients

The lysosomal neuraminidase activities of fibroblasts derived from sialidosis patients were determined and compared to the activities of healthy controls (Table 1). The fibroblasts from both patients exhibited a complete lack of NEU1 activity. The activity of β-galactosidase was normal in both cases.

3.2. RNA expression of lysosomal neuraminidase and PPCA

The pre-mRNA and mRNA expression of *neu1* in sialidosis patients were investigated by RT-PCR. No pre-mRNA or mature mRNA was detectable in both patients, whereas the transcriptional activity of the PPCA gene was normal (Fig. 1).

Table 1
Clinical and biochemical data of the patients

	Patient 1	Patient 2
Clinical type	II	II
Age at diagnosis	prenatal	prenatal
Sex	male	male
Ethnicity	Turkish	Turkish
Clinical features		
Hydrops fetalis	+	+
Hepatomegaly	+	+
Ascites	+	+
Other symptoms	polydactyly	
Enzyme activities[a]		
NEU1	0	0
β-Galactosidase	21.2 mU/mg protein	16.3 mU/mg protein

[a]The normal range of healthy individuals for NEU1 ($n=6$) was 1.29–2.56 mU/mg protein and 9.16–27.28 mU/mg protein for β-galactosidase ($n=22$). 1 mU corresponds to 1 nmol substrate cleaved per min.

3.3. Localisation of the 11 kb deletion

Amplifications of the genomic and cDNA derived from the patients with primer combinations specific for *neu1* failed to generate any detectable PCR products. Screening of the entire neu1 gene and of the neighbouring upstream and downstream regions using primers located between g.−8992 and g.−8218, and between g.+5024 and g.+6056, relative to the start adenine of *neu1* led to the re-occurrence of a PCR product. Based on these findings, PCR was performed using a forward

Table 2
Oligonucleotides used for mutation analysis

Primer	Sequence	Position
RT-PCR		
f-neu1-a	5′-CGCTACGGAAGTGGGGTCAG-3′	+2261 to +2280[a]
r-neu1-a	5′-AGTCCTGAAGGCAGAATACC-3′	+3097 to +3078[a]
f-IN-C (neu1)	5′-GCTGCCTTGTGGAGAGTCTG-3′	+1619 to +1638[a]
r-IN-C (neu1)	5′-GGAACTGGGTGTCACAGAAGGAG-3′	+2144 to +2122[a]
f-PPCA-a	5′-CCTCAACATCTACAATCTCTATGCC-3′	+813 to +837[c]
r-PPCA-a	5′-CAGTATGGCTGCTTGTTCAGGA-3′	+1442 to +1421[c]
f-CTL4-a	5′-GATTGTGGCCTGGTTGTATGGAG-3′	+156 to +178[b]
f-CTL4-b	5′-GGGAGAACAAAGATAAGCCGTAT-3′	+233 to +255[b]
f-CTL4-c	5′-TAGGTTACATCGTGGTGGGGAT-3′	+94 to +113[b]
f-CTL4-d	5′-TGCTCCAGCTCTGGGACGC-3′	+522 to +540[b]
r-CTL4-a	5′-GCAGTAGTAGATGCCATATGCCAG-3′	+805 to +828[b]
r-CTL4-b	5′-GAAGCAAGATAAACAGTAGGCTCAAG-3′	+723 to +748[b]
r-CTL4-c	5′-CTCCTGCACGCTCTGGTAG-3′	+903 to +921[b]
f-GAPDH-a	5′-GATGCTGGCGCTGAGTACGTC-3′	+339 to +360[d]
r-GAPDH-a	5′-CGTTGTCATACCAGGAAATGAGC-3′	+1024 to +1002[d]
Fusion transcript CTL4/NEU1		
f-CTL4-a	see above	+156 to +178[b]
r-neu1-b	5′-CCCAAGACAGGACCAATCAATGTC-3′	+3330 to +3353[a]
f-CTL4-b	see above	+233 to +255[b]
r-neu1-c	5′-CCCAAGACAGGACCAATCAATGTC-3′	+3263 to +3287[a]
Duplex PCR		
f-CTL4/neu1	5′-CCATGGACCAACATTACTCCACC-3′	−8764 to −8787[a]
r-primer 1	5′-CCCTCTAGAAGGCCTATGTCATATTC-3′	−7574 to −7599[a]
r-primer 2	5′-CTACAGCTCACTAGACCTGCC-3′	+3352 to +3332[a]
Additional sequencing primer		
f-neu1-8242	5′-AGCCTACTGTTTATCTTGCTTCTGC-3′	−8242 to −8218[a]
r-neu1+3606	5′-GATGTGCATTGACAGAACCATAGAGA-3′	+3606 to +3581[a]

[a]Position relative to the start ATG of the genomic DNA sequence (accession number AF134726).
[b]Position relative to the start ATG of the mRNA sequence (accession number XM_016669).
[c]Position relative to the start ATG of the mRNA sequence (accession number NM_000308).
[d]Position relative to the start ATG of the mRNA sequence (accession number XM_006959).

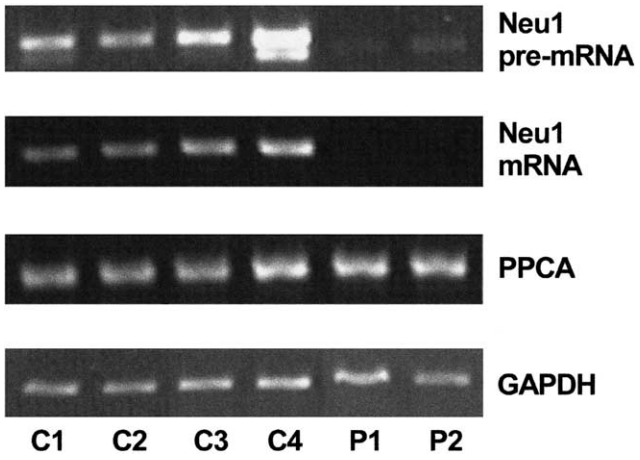

Fig. 1. RT-PCR analysis of *neu1* pre- and mature mRNA and *PPCA* mRNA in the sialidosis patients (P1, P2). *GAPDH* mRNA was used as control. C1–C4: control individuals.

primer (f-neu1−8242) upstream of the assumed deletion (~11 kb) and a reverse primer (r-neu1+3606) within the 3′-UTR of *neu1*. Details regarding this amplification procedure are shown in Fig. 2A. Direct sequencing of the obtained PCR products confirmed the expected 11 kb deletion. The breakpoints of the deletion were located between the nucleotides g.−8041 and g.−8042 (relative to the start adenine of *neu1*) in exon 10 of the CTL4 gene and g.+3261 and g.+3262 (Fig. 2B) in the 3′-UTR of *neu1*. Taken together, genomic sequencing and the complete lack of *neu1* transcripts suggested homozygosity of this gross deletion in both patients. In the 3′-UTR of *neu1* another small deletion was observed in both patients (c.*1566–1570delTGAAT, data not shown).

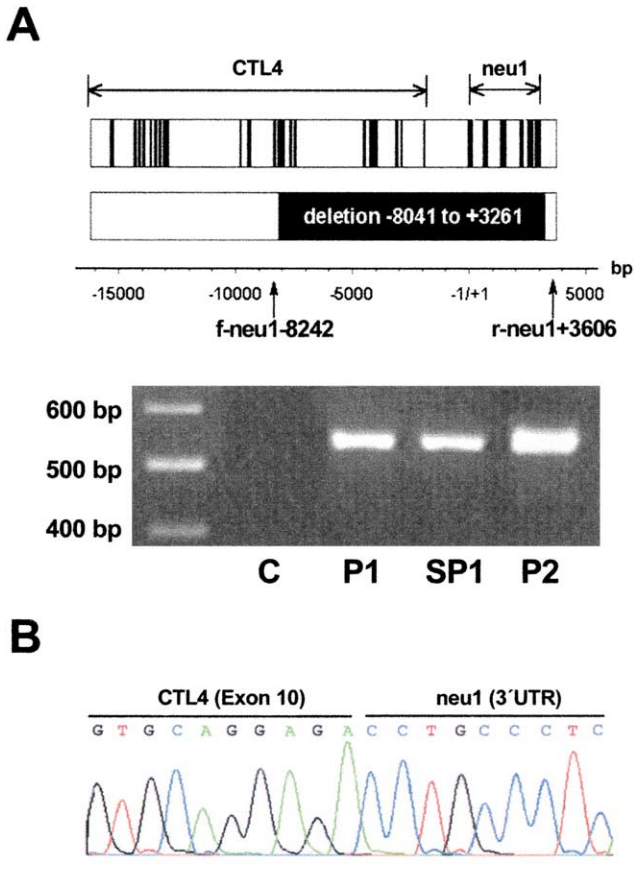

Fig. 2. Characterisation of the 11 kb deletion. A: Gel electrophoresis of a PCR using primers located on either side of the interstitial deletion of patient 1 (P1), his sister (SP1) and patient 2 (P2). The scheme above displays the closely neighboured genes *CTL4* and *neu1* (black boxes: exons, white boxes: introns), the exact range and localisation of the deletion and the positions of the used primers (arrows). B: Genomic sequence of the fusion region.

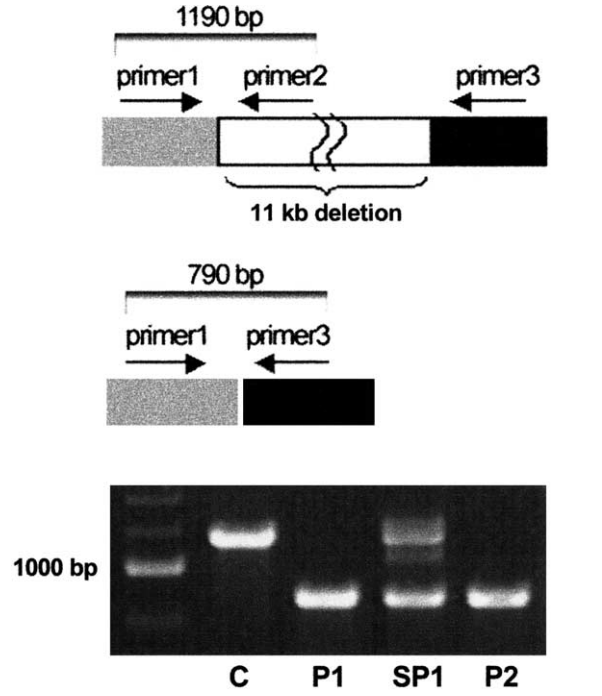

Fig. 3. Duplex PCR analysis of the allelic status of the affected patients. Top: Localisation and expected product length of the three primers used. Bottom: Gel electrophoresis showing the obtained PCR products. In the control (C) only the non-deletion-specific 1190 bp fragment was observed. The 790 bp fragment amplified from both patients (P1 and P2) indicates the homozygous deletion. The sister of patient 1 (SP1) is heterozygous.

3.4. Allelic status of patients affected by the gross deletion

We performed a duplex PCR using one forward and two reverse primers in the same reaction to confirm the allelic status for the 11 kb deletion in patients 1 and 2 and in the sister of patient 1 (Fig. 3). Subsequent gel electrophoresis revealed the occurrence of two different fragments in the sister of patient 1. The normal allele was represented by the 1190 bp fragment and the deletion-containing allele by the 790 bp PCR product indicating heterozygosity for this alteration. In contrast, the patients showed only the 790 bp fragment specific for the deletion, an unrelated control donor exhibited only the 1190 bp product.

3.5. Transcription of the CTL4/neu1 fusion gene

We performed an RT-PCR to verify the occurrence of the *CTL4/neu1* fusion transcript (GenBank accession number AF466766). Interestingly, only in patient 1 was the fusion transcript detected (Fig. 4A) and subsequently confirmed by direct sequencing. Nested RT-PCR was necessary to yield a detectable product for *CTL4* cDNA, indicating a low level transcription of *CTL4* mRNA in human fibroblasts. The nested amplification with outer primers specific for a *CTL4*

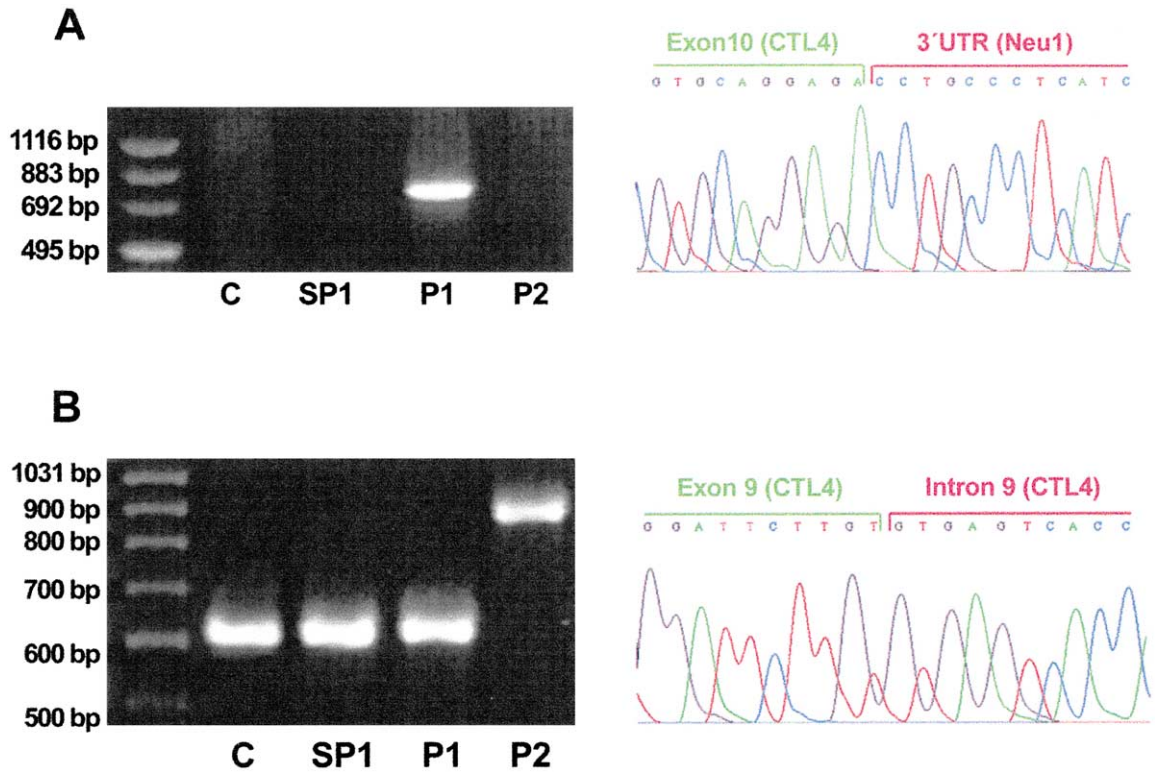

Fig. 4. Transcriptional activity of the CTL4/Neu1 fusion gene. A: RT-PCR with primers specific for the *CTL4/neu1* fusion transcript. The fusion transcript was only detected in patient 1 and verified by direct sequencing. B: With a reverse primer located in the beginning of exon 10 of *CTL4*, an enlarged fragment was amplified from patient 2. Sequencing revealed the retention of intron 9.

transcript ranging from exon 3 to the end of exon 10 and subsequent inner primers for a transcript from the end of exon 7 to the end of exon 10 resulted in a PCR product in both patient 1 and his sister, but not in patient 2 (data not shown). However, using an outer forward primer from the end of exon 3, and an inner forward primer from the end of exon 4 in combination with two different reverse primers corresponding to the beginning and the centre of exon 10 revealed a substantially longer PCR product in patient 2 compared to the control (Fig. 4B). Sequencing identified the retention of intron 9 in the *CTL4* transcript of patient 2.

4. Discussion

Since cloning of the cDNA of the lysosomal neuraminidase, several studies had characterised mutational alterations of *neu1* and attempts were made to correlate the impact of the mutations with the clinical phenotypes. The catalytic activity of the lysosomal neuraminidase relies on the formation of a quaternary complex with active β-galactosidase, *N*-acetylgalactosamine-6-sulphate sulfatase, and PPCA. The deficiency of lysosomal neuraminidase is associated with two different disorders of lysosomal metabolism: sialidosis and galactosialidosis [17]. Since both sialidosis and galactosialidosis are characterised by similar phenotypes, we excluded the possibility of galactosialidosis by determining the enzyme activity of β-galactosidase in the investigated patients. Both patients showed a total loss of neuraminidase activity that corresponded to their severe mutational alterations. Additionally, no pre-mRNA and mature mRNA of *neu1* were detectable in both patients. The finding of a total lack of *neu1* transcripts in our patients is in contrast to the cases so far published [4,6,9,10,12]. It was suggested that a complete absence of lysosomal neuraminidase is lethal during foetal development or at birth [9]. This hypothesis is confirmed by our findings in both patients who died shortly after birth. The complete absence of *neu1* transcripts in these patients is unambiguously caused by the observed homozygous gross deletion since it encompasses the entire coding and promotor regions of the *neu1* gene. The healthy sister of patient 1 was determined as a heterozygous carrier of the same deletion. The deletion also affects the CTL4 gene (NG22, GenBank accession number XM_016669), which is located 851 bp centromeric from *neu1* on chromosome 6p21.3. The CTL4 gene was sequenced as part of the gene-rich MHC-III cluster and consists of 21 exons coding for a hypothetical protein of 712 amino acids with as yet unknown function. The deduced polypeptide shares properties of a receptor-like protein such as several transmembrane domains and potential glycosylation sites [7]. The deletion leads to a genomic rearrangement between exon 10 of *CTL4* and the 3′-UTR of *neu1*. Analysis of the transcriptional activity of the CTL4/Neu1 fusion gene revealed different consequences of the same deletion in the three affected cases. In patient 1, the assumed *CTL4/Neu1* fusion transcript was observed, indicative for the existence of a fusion protein. In his heterozygous sister only the normal transcripts of *CTL4* and *neu1* were detectable concordant with the complete absence of clinical symptoms. In patient 2, we did not observe the fusion transcript, but found a retention of intron 9 in the *CTL4* mRNA. The reason for this alternatively spliced *CTL4* mRNA apparently terminating transcription before the fusion site remains unclear, since we could not

detect any alterations within the surrounding splice sites. Both patients were prenatally diagnosed by enzyme activity assays and exhibited a particularly severe clinical phenotype with hydrops fetalis and ascites. Additionally, only in patient 1 polydactyly was observed. Whether different clinical symptoms might be due to a different loss of CTL4 protein function cannot be addressed with the present data and requires more information about the physiological function of CTL4. In view of the fact that the parents of patient 1 and 2 and sister of patient 2 are heterozygous carriers of the deletion, yet are completely healthy, a partial loss of the CTL4 protein appears to have no clinical consequences.

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