



β -Mannosidosis caused by a novel homozygous intragenic inverted duplication in *MANBA*

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Ontology terms: abnormality of the vasculature of the conjunctiva; aggressive behavior; alacrima; angiokeratoma corporis diffusum; attention-deficit hyperactivity disorder; bilateral sensorineural hearing impairment; broad nasal tip; chronic constipation; hypohidrosis/hyperhidrosis; impulsivity; increased urinary disaccharide excretion; intellectual disability, mild; sleep disturbance; thin upper lip vermillion; upslanted palpebral fissure; wide nasal ridge

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Abstract β -Mannosidosis is a lysosomal storage disorder characterized by accumulation of disaccharides due to deficiency of the lysosomal enzyme β -mannosidase. The disease is caused by mutations in *MANBA* and is extremely rare in humans. Although the clinical presentation is heterogeneous, common symptoms include various degrees of developmental delay, behavioral disturbances, hearing loss, and frequent infections. We report a 15-yr-old girl presenting with mild intellectual disability, sensorineural hearing loss, severe behavioral disturbances, dysmorphic traits, and evolving angiokeratomas. Copy-number variation analysis of next-generation sequencing (NGS) data indicated increased coverage in exons 8–11 of *MANBA*. Low β -mannosidase activity (1 μ katal/kg protein, refv 25–40) established the diagnosis of β -mannosidosis. Whole-genome sequencing (WGS) and cDNA analysis revealed a novel homozygous intragenic inverted duplication in *MANBA*, where a 13.1-kb region between introns 7 and 11 was duplicated and inserted in an inverted orientation, creating a 67-base nonduplicated gap at the insertion point. Both junctions showed microhomology regions. The inverted duplication resulted in exon skipping of exons 8–9 or 8–10. Our report highlights the importance of copy-number variation analysis of data from NGS and in particular the power of WGS in the identification and characterization of copy-number variants.

[Supplemental material is available for this article.]

INTRODUCTION

β -Mannosidosis (OMIM #248510) is an autosomal recessive lysosomal storage disorder due to deficient activity of the enzyme β -mannosidase (E.C. 3.2.1.25). The disease is well known and relatively common in goats and other kind of cattle, but it is extremely rare in humans. β -Mannosidase is the final exoglycosidase involved in the degradation of N-linked oligosaccharides of glycoproteins, removing β -linked mannose residues (Winchester 2005; Samra and Athar 2008). Affected individuals have pronounced reduction of β -mannosidase activity that can be measured in white blood cells and fibroblasts. Enzyme deficiency in humans leads to lysosomal accumulation of disaccharides (primarily Man(β 1 \rightarrow 4)GlcNAc) (Cooper et al. 1988; van Pelt et al. 1990); therefore, the disease belongs to the group of glycoproteinoses. *MANBA* is located on Chromosome 4q24 and encodes an 879-amino acid

lysosomal protein (Alkhatat et al. 1998). So far, only 22 cases of β -mannosidose from 18 families have been reported in humans (Riise Stensland et al. 2008; Labauge et al. 2009; Broomfield et al. 2013). Disease-causing variants include both null mutations (splice, nonsense, small frameshift deletions/insertions) and missense mutations (Riise Stensland et al. 2008; Labauge et al. 2009; Broomfield et al. 2013).

The clinical presentation is heterogeneous, and a wide range of symptoms of different severity has been observed, with no clear genotype–phenotype correlation. Common symptoms include various degrees of developmental delay, behavioral disturbances, hearing loss, and frequent infections (Wenger et al. 1986). Angiokeratomas, facial and skeletal dysmorphic features, hypotonia, peripheral neuropathy, and seizures have also been reported in some of the patients (Cooper et al. 1991; Cherian 2004; Broomfield et al. 2013). Several of these symptoms are typical for glycoproteinoses.

We report a 15-yr-old girl presenting with mild intellectual disability, sensorineural hearing loss, severe behavioral disturbances, dysmorphic traits, and evolving angiokeratomas. A next-generation sequencing (NGS)-panel for lysosomal disorders suggested the diagnosis of β -mannosidosis, which was confirmed enzymatically. Subsequent whole-genome sequencing (WGS) analysis defined at the nucleotide level the presence of a homozygous intragenic inverted duplication in *MANBA*. This report highlights the importance of copy-number variation analysis of NGS data and in particular the power of WGS in the identification and characterization of copy-number variants.

RESULTS

Clinical Presentation and Family History

The patient is now a 15-yr-old girl born to consanguineous parents (second cousins once removed) of Norwegian ancestry. She has two older brothers. Pregnancy was uneventful until delivery by acute caesarean section in week 38 due to fetal distress; her Apgar scores were 9–10. Her birth weight was 2980 g, length 47 cm, and occipitofrontal head circumference 34.5 cm. Bottle feeding was initiated because of poor sucking. She showed globally delayed development from the newborn period, including muscular hypotonia, sitting at the age of 1 yr, crawling at the age of 17 mo, and walking at the age of 18 mo. At the age of 1 yr, a diagnosis of unilateral cerebral palsy (CP) was given because of hypertonia, hyperreflexia, and reduced function of her right arm. However, functions improved over the next years and the diagnosis was withdrawn. She still preferably uses her left arm. Cerebral MRI at the age of 3 showed delayed myelination but no structural abnormalities and no focal pathology that could explain unilateral CP. Because of her improvement in motor function, a repeat MRI was not performed.

Strabismus and hypermetropia was noted from the age of 1. At the age of 2 yr, sensorineural hearing loss was recognized and corrected with hearing aids. She suffered from recurrent infections in her childhood. Her parents commented early on that her facial features differed from parents and siblings. Dysmorphic features include upslanting palpebral fissures, blepharophimosis, broad nasal ridge and nasal tip, thin lips, and posteriorly rotated ears (Fig. 1B). A diagnosis of mild intellectual disability was given after cognitive testing in early school years. She displayed hyperactive behavior since early childhood and now has a formal diagnosis of attention-deficit hyperactivity disorder (ADHD). Her sleep pattern has always been disturbed. Severe behavioral disturbances in the form of self-biting, impulsiveness, improper language, aggression, and rubbing her glasses until they break have emerged over the years.

At 12 yr of age, autonomic features like no tear production, high pain threshold, constipation, and urinary incontinence as well as flushing of the skin on the scalp were identified



Figure 1. Clinical manifestations. (A) Flushing of the scalp/face. (B) Facial features with upslanting, narrow palpebral fissures, periorbital fullness, broad nasal root and tip, and thin lips. (C) Telangiectatic and tortuous conjunctival vessels. (D) Angiokeratomas on the tips of the fingers (evolving). (E) Close-up on angiokeratomas (thigh). (F) Multiple angiokeratomas on the dorsal side of both thighs.

(Fig. 1A). A new clinical examination at age 13 revealed numerous pinpoint red/dark spots—some macular, some papular—that did not blanch on pressure, on her thighs, lower torso, fingertips, and vermillion border of the lips (Fig. 1D–F). Varicose veins were found on both thighs, and eye examination showed dilated and tortuous conjunctival vessels (Fig. 1C) but normal appearance of the retina including the vasculature.

Biochemical and Molecular Studies

Initial genetic investigations undertaken at 1 yr of age showed normal G-band analysis, 46, XX, as well as normal results regarding Southern blot for fragile X syndrome (*FMR1*) and multiplex ligation-dependent probe amplification (MLPA) analysis for DiGeorge syndrome (P023B MRC Holland), for microdeletions/duplications causing mental retardation (MLPA P064 and P096, MRC Holland), and for subtelomere areas (MLPA P036B and P070, MRC

Holland). Screening for inborn errors of metabolism in urine showed normal results for amino acids and organic acids. At 5 yr of age, chromosomal microarray (Genome-Wide Human SNP array 6.0, Affymetrix) detected six large regions of homozygosity, including one in 4q24, where *MANBA* is located (data not shown).

At the age of 13 yr, genetic investigations with an NGS panel showed neither clinically significant SNVs nor small indels in any of the genes associated with lysosomal disorders. However, copy-number variation analysis showed increased coverage (average fold change +1.73) in exons 8–11 of *MANBA* (data not shown). This finding prompted the analysis of β -mannosidase in isolated lymphocytes which showed very low activity (1 μ katal/kg protein, reference value 25–40).

cDNA analysis of the patient revealed two abnormal *MANBA* transcripts lacking exons 8–9 and 8–10, respectively. No wild-type transcript was found, suggesting that the genetic variant was in homozygous form (Fig. 2). The patient's mother showed wild-type transcript in addition to the two truncated variants, as expected for a heterozygous carrier (Fig. 2).

WGS analysis showed that the increase in coverage found in the NGS panel analysis corresponded to an inverted duplication involving exons 8–11 (illustrated in Fig. 3A,B; see Table 1), which could also explain the exon skipping observed on cDNA analysis. Furthermore, WGS indicated the precise location of both junctions, which were confirmed by Sanger sequencing (Fig. 3C). The 13.1-kb duplicated fragment extends from position Chr 4:103,598,877 in intron 7 to position Chr 4:103,585,729 in intron 11 and was inserted in inverted orientation in positions Chr 4:103,590,920 and 103,590,854, creating a 67-base non-duplicated gap at the insertion point. Both junctions showed microhomology regions, as shown in Figure 3C. WGS did not detect any other pathogenic variants in the genes present in any of the six regions of homozygosity identified by SNP array.

Retrospectively, the duplication was visible on the SNP array analysis from 2009 as a gain in only seven probes (Genome-Wide Human SNP array 6.0, Affymetrix). However, with standard settings still in use, it falls below the threshold for reporting of the clinical laboratory.

DISCUSSION

The combination of intellectual disability, sensorineural hearing loss, behavioral difficulties, autonomic features, and angiokeratomas raised in our patient the suspicion of a lysosomal storage disorder. The homozygosity area in 4q24 where *MANBA* is located specifically pointed to β -mannosidosis and prompted genetic investigation with an NGS panel for lysosomal disorders, in which copy-number variation analysis showed increased coverage in exons 8–11 of *MANBA*. Analysis of β -mannosidase in isolated lymphocytes showed very low activity, thereby confirming the diagnosis of β -mannosidosis. Close analysis of NGS panel data suggested that the observed increase in coverage could correspond to an inverted duplication involving exons 8–11, matching the exon skipping observed on cDNA analysis. WGS further supported this hypothesis and indicated the precise location of both junctions.

β -Mannosidosis is a very rare lysosomal storage disorder with an estimated incidence of 0.1 per 100,000 (Poorthuis et al. 1999; Poupětová et al. 2010). So far, to our knowledge, only 22 cases of β -mannosidosis in 18 families from a range of ethnic backgrounds have been reported since the disease was first described in 1986 (Cooper et al. 1986; Wenger et al. 1986). The disease has a highly variable clinical presentation (Bedilu et al. 2002; Riise Stensland et al. 2008) including intellectual disability/developmental delay, behavioral disturbance, recurrent infections, hearing loss, and severe neurological phenotypes such as epileptic encephalopathy, hydrocephalus, and spinocerebellar ataxia (Sedel et al. 2006; Labauge et al. 2009; Broomfield et al. 2013). Dysmorphic traits have been reported (Poenu

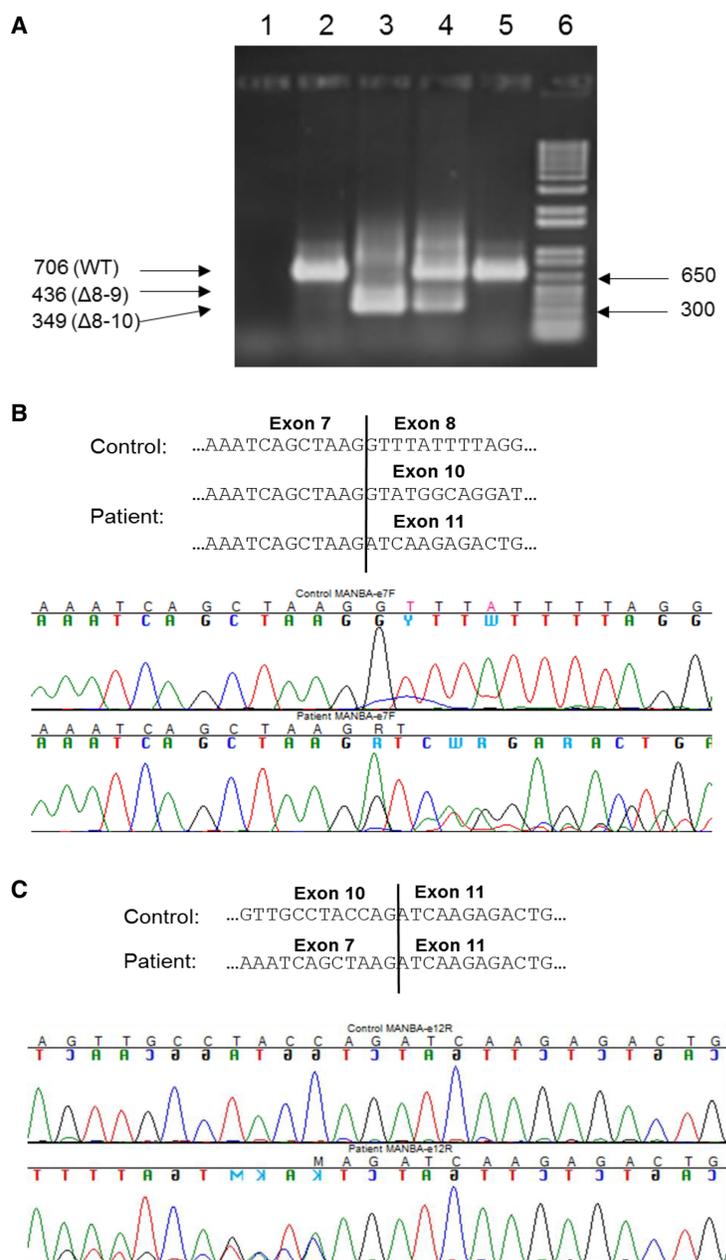


Figure 2. cDNA analysis reveals skipping of exons 8–9 and 8–10, respectively, in MANBA. (A) cDNA was prepared from RNA isolated from whole blood and PCR amplified using primers in exons 7 and 12. Agarose-gel electrophoresis of the PCR products. Lanes 1–6: PCR-mix (no cDNA); control 1; patient; mother; control 2; size standard (1 kb+ ladder, Sigma-Aldrich). (B) PCR products from control 1 and the patient were Sanger-sequenced in the forward direction with a primer in exon 7 and (C) reverse direction with a primer in exon 12.

et al. 1992; Labauge et al. 2009). Angiokeratomas are also described in several cases of β -mannosidosis (Rodríguez-Serna et al. 1996; Suzuki et al. 2004; Molho-Pessach et al. 2007). Thus several features of our patient are in line with β -mannosidosis patients previously described, including the presence of angiokeratomas.

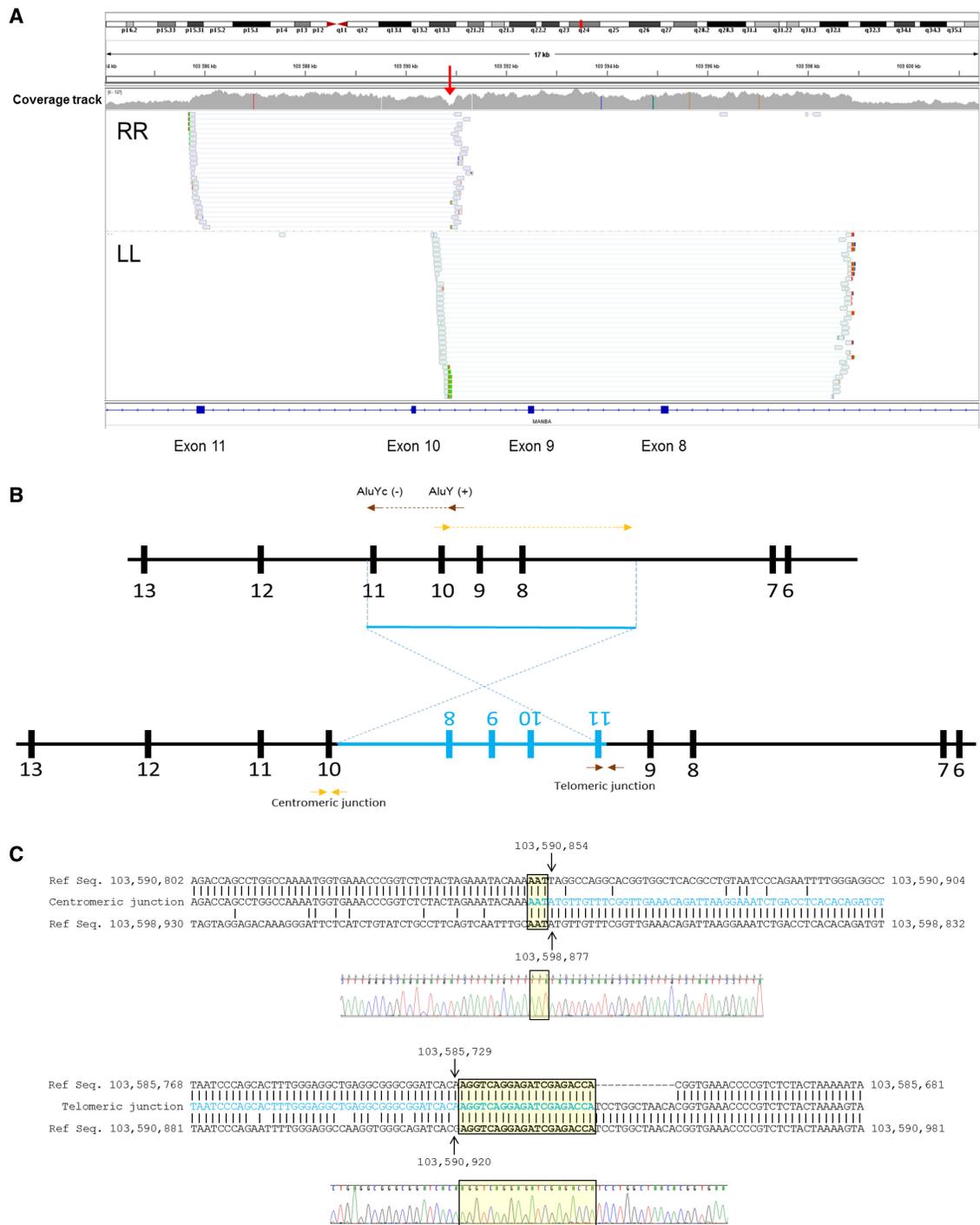


Figure 3. WGS analysis defines both junctions at the nucleotide level. (A) Overview of WGS data encompassing the duplicated region at *MANBA*. The coverage track clearly defines a duplicated region from introns 7–11. The drop in coverage observed in intron 9 (red arrow) corresponds to the 67-bp nonduplicated gap observed between the insertion points. (B) Paired reads with RR (right-right) and LL (left-left) orientation suggest the existence of an inversion, diagrammed in panel. (C) Definition of both junctions at the nucleotide level obtained from WGS data and confirmed by Sanger sequencing. The inserted sequence is marked in blue. Both junctions show areas of microhomology (yellow boxes).

Table 1. Variant table

Gene	Chromosome	HGVS DNA reference	HGVS protein reference	Variant type	Predicted effect (substitution, deletion, etc.)	dbSNP/dbVar ID	Genotype (heterozygous/homozygous)
MANBA	4q24	g.103590853_103590921ins103585729_103598877inv (hg19)	Not available	Inverted duplication	Exon skipping (8–9 or 8–10)	-	Homozygous

Angiokeratoma corporis diffusum (ACD) is thought to be the cutaneous hallmark of Fabry disease (Orteu 2010) but also occurs in other lysosomal storage disorders as well as a few other conditions, like hereditary hemorrhagic telangiectasia. Lesions typically appear in the first or second decade and increase in number with age. Lesions are red to black pinpoint—10-mm macules or papules, sometimes described as hyperkeratotic. The typical distribution is on the trunk, including the genital area and proximal limbs, but may also involve distal extremities and lips. Histologically, dilated capillaries with thin walls are found in the dermis, underlying an epidermis with hyperkeratosis (Molho-Pessach et al. 2007). Electron microscopy reveals lysosomal storage vacuoles in the cytoplasm in β -mannosidosis, whereas the findings in Fabry disease are distinct with electron-dense lysosomal granules (Molho-Pessach et al. 2007). The macroscopic appearance appears to be similar, but with different ultrastructural appearances due to unique substrate depositions in each of the lysosomal disorders. The pathogenesis of ACD in β -mannosidosis is not yet clear but probably could be explained by a similar mechanism as in Fabry disease. Pathophysiological studies in Fabry disease demonstrate accumulation of globotriaosylceramide in dermal endothelial cells, ensuing capillary wall defects, and vessel ectasias (Lidove et al. 2006).

Behavioral difficulties are frequently seen in the previously described cases of β -mannosidosis (Bedilu et al. 2002; Sabourdy et al. 2009). Hyperactivity, Tourette syndrome, obsessive-compulsive disorder, aggressiveness, and self-mutilation are reported (Sedel et al. 2006). In our patient, hyperactivity was present early with a later formal diagnosis of ADHD. Severe behavioral disturbances emerged later, including self-mutilation, disinhibition, and aggressiveness. Psychiatric symptoms in adolescence or adulthood together with systemic, cognitive, and neurological signs, as in our patient, can reveal inborn errors of metabolism. The coexistence of (mild) intellectual disability and emerging psychiatric symptoms or behavioral changes is found in several lysosomal storage disorders (Sedel et al. 2007). This is, however, difficult to bear in mind as each of these disorders are rare but should be considered especially in presence of other typical signs and symptoms and in cases in which recessive disease is suspected. Symptoms of autonomic dysfunction like hypohidrosis, facial flushing, absent tear production, and high pain threshold, as in our patient, have not been previously described in β -mannosidosis, but are well known in Mb Fabry (Biegstraaten et al. 2010). Biegstraaten et al. (2010) have suggested that the autonomic dysfunction is not due to autonomic neuropathy, as previously described, but rather due to end-organ damage. Formal testing for autonomic dysfunction (like tilt-test, special sweat tests) were not undertaken, but could give more information and quantification of this feature. Extensive WGS analysis did not reveal other disease-associated variants, supporting that these features are symptoms of β -mannosidosis in our patient.

β -Mannosidosis in humans is, with a few exceptions, generally less severe than the disease found in goats and other kind of cattle in which severe neonatal neurological onset together with dysmorphism are particularly common (Jones and Dawson 1981). It has been suggested that the phenotypic differences among species are caused by differences in the size and nature of the storage compounds (Winchester 2005). Furthermore, two

sequence variants found in ruminants are suggested to abolish the β -mannosidase enzyme activity, resulting in the severe neurodegenerative phenotype (Leipprandt et al. 1996, 1999). Comparing patients reported in the literature, no clear genotype–phenotype correlation could be shown in humans (Bedilu et al. 2002; Broomfield et al. 2013), and variability is suggested to be due to the involvement of other genetic and environmental factors (Gort et al. 2006; Riise Stensland et al. 2008; Lovell et al. 2014). However, comparative structural bioinformatics analyses of inherited mutations reported in *MANBA* until 2011 indicated the existence of genotype–phenotype correlation in β -mannosidosis (Huynh et al. 2011). In general, the authors suggest the proximity of mutations to the active site to be determinant of disease severity, and five mutational hotspots were identified. So far 18 disease-causing variants have been reported including both null mutations (splice, nonsense, small deletions/duplications) and missense mutations (The Human Gene Mutation Database Professional 2018.3; retrieved October 26, 2018 from <http://www.hgmd.cf.ac.uk/ac/>). Our patient shows a novel inverted duplication involving exons 8–11 in *MANBA*, resulting in exon skipping (exon 8–9 or 8–10), which in turn results in shortening of the protein by 90 or 119 amino acids, respectively. No western blot was performed to assess whether the abnormal transcripts gave stable protein products; however, despite the massive structural change involved, some residual β -mannosidase activity could be measured in lymphocytes, and thus we have to assume that some degree of translated protein is present.

WGS is a very efficient tool for identification and characterization of structural variants. Providing nucleotide-level resolution at the breakpoints, WGS gives us valuable information concerning the mechanism causing the observed inverted duplication. The insertion point of this duplication is located in an Alu element [AluY (+)] as well as the end of the duplication contributing to the telomeric junction [AluYc (–)]. Moreover, areas of microhomology can be observed in both junctions. Taken together, we propose that the duplication presented here is caused by a microhomology-mediated, replication-based template switching between Alu elements. The fact that both elements are in opposite orientations probably explains the inverted nature of the duplication (Fig. 4A). This is a common mechanism underlying nonrecurrent copy-number variations (Verdin et al. 2013; Carvalho and Lupski 2016). Analysis of cDNA revealed exon skipping as a result of the presence of the inverted duplication. Alternative splicing caused by insertion of inverted repeats is a well-known phenomenon and has been proposed to be caused by hairpin formation at the level of precursor mRNAs (Solnick 1985). Based on this, we propose that the presence of the inverted duplication causes a large hairpin at the pre-mRNA level that compromises correct splicing of exons 8–10 (Fig. 4B).

In conclusion, this study reports a new case of the ultra-rare disease β -mannosidosis with a novel pathogenic variant in *MANBA*—namely, a homozygous intragenic inverted duplication. Diagnostic clues to β -mannosidosis were tiny angiokeratomas, behavioral problems, intellectual disability, sensorineural hearing loss, and homozygosity of 4q24. Other symptoms such as hypohidrosis, facial flushing, no tears, and high pain threshold have not previously been described in β -mannosidosis but are well known in Fabry disease, another lysosomal storage disorder. Our case clearly shows the importance of reevaluation of undiagnosed patients, searching for tiny clues and special features and correct phenotyping. This report further highlights the importance of copy-number variation analysis of NGS data, and, in particular, the power of WGS in the identification and characterization of the inverted duplication in *MANBA*.

METHODS

NGS Panel for Lysosomal Storage Disorders

Mutation analysis was performed by using a custom-made NGS gene panel (SureSelect^{QXT}, Agilent Technologies). The panel targets coding exons of 61 genes described to be involved

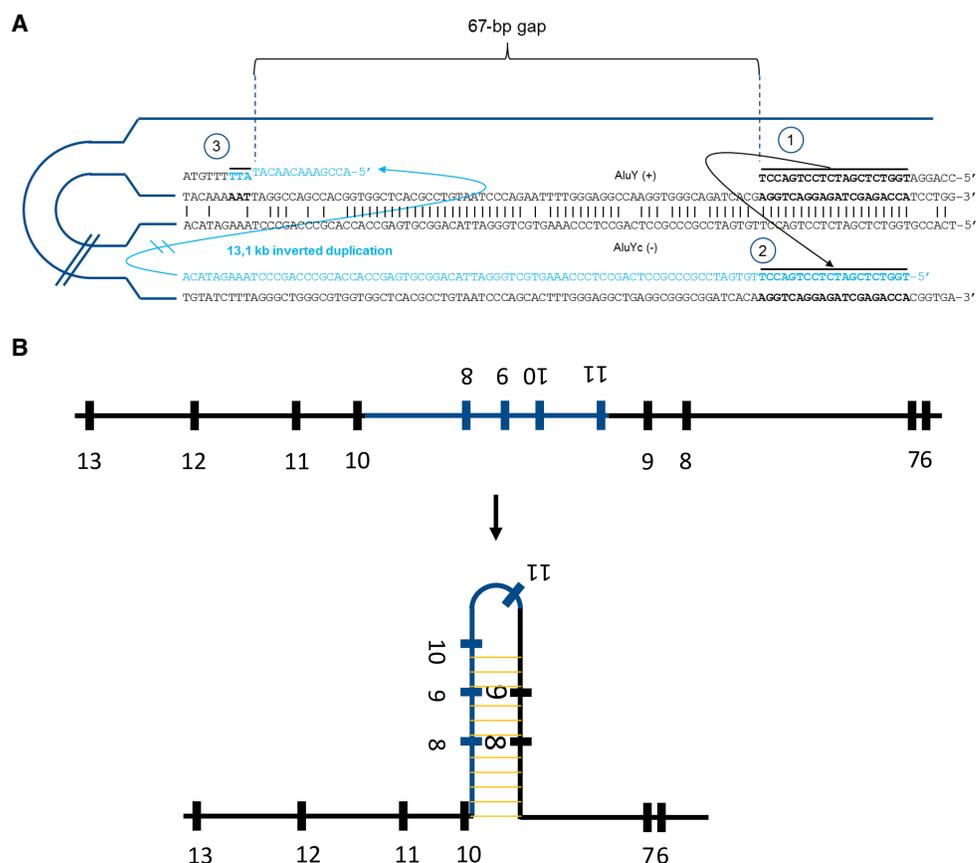


Figure 4. Proposed mechanisms underlying the observed inverted duplication and exon skipping. (A) We propose the following sequence of events causing the inverted duplication: (1) replication stalls at AluY (+), possibly facilitated by the interaction with a second Alu element, AluYc, in opposite orientation; (2) template switching between both Alu elements takes place, which is mediated by a 20-base-long microhomology region and causes the large inverted duplication; and (3) replication switches back to the original template directly downstream from the highly homologous regions between the Alu elements, leaving a 67-base-long nonreplicated gap. This template switching is mediated by a 3-base-long area of microhomology. (B) The inverted duplication creates a large area of full complementarity within the pre-mRNA that induces the formation of a hairpin, which in turn forces the spliceosome to skip exons 8 and 9. Even proper splicing of exon 10 is compromised.

in lysosomal disorders (± 25 bases, according to the RefSeq database and assembly February 2009 [GRCh37/hg19]). The list of genes is available upon request. Prepared libraries were sequenced in a paired-end run (2×150 bp) on the MiSeq instrument (Illumina). Demultiplexing, adaptor trimming, and mapping of sequencing reads to the human reference sequence hg19, as well as subsequent indel realignment and variant calling, was performed using the MiSeq Reporter Software (Illumina). Annotation and filtering of variants were addressed with Variant Studio Analysis Software (Illumina). Variants of interest were filtered according to allele frequency, exonic/splice site location, and autosomal recessive or X-linked pattern of inheritance. Copy-number variation analysis, executed with CLC Cancer Research Workbench 2.0 (CLC Bio, QIAGEN), was used to detect larger deletions (>1 kb). Quality assessment was performed by using Q30 as pass filter cutoff for a Phred score of called variants, together with a minimum sequencing read depth of $20\times$ for target regions. Statistics for the NGS panel are summarized in Supplemental Table 1.

RNA Isolation and cDNA Analysis

Blood was collected in Tempus Blood RNA Tubes (Thermo Fisher Scientific), and RNA was isolated using the Tempus Spin RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The RNA concentrations were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. Primers *APRT*-e3F (5'-GGGGAAGCTGCCAGGCCCACT-3') and *APRT*-e5R (5'-GCGAGGTCAGCTCCACCAGGCT-3'), positioned in exons 3 and 5, respectively, of the housekeeping gene *APRT* (NM_000485.2), were used to monitor the cDNA synthesis and to identify possible contamination of genomic DNA (presence of a 721-bp PCR product in addition to the expected 218-bp PCR cDNA product). Primers *MANBA*-e7F (5'-GAAACTTGGTGGCCTCATGG-3') and *MANBA*-e12R (5'-GGTTTTGAGAGACCCAGGCT-3'), positioned in exons 7 and 12, respectively, of the *MANBA* gene (NM_005908.3), were used for PCR amplification of *MANBA* exons 8–11 (PCR product 706 bp) using the JumpStart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich) (PCR conditions available on request). PCR products were visualized on agarose gels, purified using the A'SAP PCR Purification Kit (ArcticZymes), and Sanger-sequenced in both directions using PCR-primers and BigDye v.3.1 (Life Technology). Fragments were separated on a 3500xL Genetic Analyzer (Applied Biosystems), and the sequences were analyzed in Sequencher version 5.3 (Gene Codes Corporation). Primers were designed using the Primer3 software (Primer3 v0.4.0/).

Whole-Genome Sequencing

DNA extraction from blood, WGS, secondary analysis, and variant analysis was carried out essentially as described in Darin et al. (2018). Sequencing returned 900 million paired sequences, corresponding to a coverage of 40× across the human genome. Data was then trimmed and mapped, and variants were called using CLC Genomics Server (QIAGEN). Trimming was performed using an ambiguous limit of 2, minimum Phred score of 17, and minimal length of 30. Reads were mapped using default settings and three passes of local realignment. Variants were called with a 90% probability cutoff, minimum count of 2%, minimum coverage of 7%, and 15% minimum frequency. Long indels were called with Manta (Chen et al. 2016) and CNVs with Canvas (Roller et al. 2016). Variants were then uploaded into Ingenuity (QIAGEN) for analysis. Statistics for the WGS are summarized in Supplemental Table 2.

Sanger Verification of Duplication Junctions

DNA was extracted from EDTA blood using the DSP DNA Midi Kit 96 and the QIAasymphony extractor (both QIAGEN). DNA concentrations were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Primers *MANBA*-i10R (5'-AATCTGGGAGTCATCAGCCAA-3' in intron 10) and *MANBA*-i7R (5'-AGCAGTATTTGTGGCCAAGGA-3' in intron 7) were used for amplification of the centromeric junction, and primers *MANBA*-i10F (5'-GCAGAAATGCAAGGGCTTTA-3' in intron 10) and *MANBA*-i8F (5'-AACCATCATGATTTTAGAGTGTGTTT-3' in intron 8) were used for PCR amplification of the telomeric junction. PCR conditions are available on request. PCR products were visualized on agarose gels, purified using the A'SAP PCR Purification Kit (ArcticZymes), and Sanger-sequenced in both directions using PCR primers and BigDye v.3.1 (Life Technology). Fragments were separated on a 3500xL Genetic Analyzer (Applied Biosystems), and the sequences were analyzed in Sequencher version 5.3 (Gene Codes Corporation).

β-Mannosidase Activity in Lymphocytes

EDTA blood was collected and lymphocytes were isolated by Ficoll-Isopaque density centrifugation (Lymphoprep, Axis-Shield PoC AS). β-Mannosidase activity in isolated lymphocytes was measured using a fluorometric assay utilizing 4-metylumbelliferyl-β-D-mannopyranosid (Sigma-Aldrich) as substrate according to Panday et al. (1984). β-Galactosidase activity was measured as a control for sample quality, using the 4-metylumbelliferyl-β-D-galactopyranosid (TRC) as substrate. Protein concentration was assayed using the bicinchoninic protein assay (BCA, Pierce, Thermo Fisher Scientific).

ADDITIONAL INFORMATION

Data Deposition and Access

The inverted duplication reported in this manuscript has been submitted to the “Global Variome shared LOVD” (databases.lovd.nl/shared) as individual 00208206, variant 0000439394. Patient consent was not obtained to deposit raw sequencing data.

Ethics Statement

The patient was evaluated in a clinical diagnostic setting. Written informed consent for NGS in a diagnostic setting was obtained from the patient’s parents. Written informed consent for publication of results and any accompanying images was similarly obtained. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with Helsinki Declaration of 1975, as revised in 2000.

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Author Contributions

M.B. contributed to conception and design, data acquisition, interpretation of data, and drafting of the paper as well as final approval of the manuscript. H.M.F.R.S. performed the cDNA analysis and the Sanger verification of duplication junctions and contributed to the drafting and correction of the manuscript. J.L. coordinated the NGS-panel analysis, performed the data interpretation, and contributed to the drafting and correction of the manuscript. M.F.S. was physician in charge of the patient and contributed to manuscript drafting. J.A.-C. coordinated the WGS and performed the data interpretation, and contributed to the interpretation of NGS data and to the drafting and correction of the manuscript. P.S. performed the WGS data processing and analysis and contributed to the drafting and correction of the manuscript.

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Competing Interest Statement

The authors have declared no competing interest.

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