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RNA analysis of the GALNS transcript reveals novel pathogenic mechanisms associated with Morquio syndrome A

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ABSTRACT

Morquio syndrome A (Mucopolysaccharidosis IVA, MPS IVA) is an autosomal recessive lysosomal storage disorder caused by deficiency of *N*-acetyl-galactosamine-6-sulfatase (GALNS) which catabolizes the glycosaminoglycans (GAG), keratan sulfate and chondroitin-6-sulfate. Homozygous or compound heterozygous pathogenic variants in the *GALNS* result in the deficiency of the enzyme and consequent GAG accumulations. DNA sequence and copy number analysis of the *GALNS* coding region fails to identify biallelic causative pathogenic variants in up to 15% of patients with Morquio syndrome A. RNA transcript analysis was performed to identify pathogenic alterations in two unrelated families with Morquio syndrome A in whom a single heterozygous or no pathogenic alteration was detected by standard analysis of the *GALNS* gene. RNA sequencing and quantitative expression analysis identified the overabundance of an aberrant GALNS transcript isoform and a reduction of the clinically relevant isoform (NM_000512.4) in the Morquio syndrome A patients from both families. The aberrant isoform (ENST00000568613.1) was produced by alternative splicing and contained intronic sequence that was likely a cryptic exon predicted to result in a reading frame shift and generation of a premature termination codon. These findings indicated that the aberrant splicing is likely the novel molecular defect in our patients. RNA transcript analysis could be useful to identify pathogenic alterations and increase the yield of molecular diagnosis in patients with Morquio syndrome A whose genetic variants are not found by standard sequencing or gene dosage analysis.

1. Introduction

Morquio syndrome A (Mucopolysaccharidosis IVA, MPS IVA) is an autosomal recessive lysosomal storage disease caused by deficiency of *N*-acetyl-galactosamine-6-sulfatase (GALNS), which catabolizes the glycosaminoglycans (GAG) keratan sulfate (KS) and chondroitin-6-sulfate (C6S) [1,2]. Homozygous or compound heterozygous pathogenic variants in the *GALNS* gene, located at 16q24, result in the deficiency of the enzyme and consequent GAG accumulations. Patients with Morquio syndrome A have characteristic musculoskeletal manifestations including short stature, short neck and trunk, spine abnormalities, pectus carinatum, hip dysplasia, genu valgum, and hypermobility of joints [3–5]. The radiographic features show dysostosis multiplex presented with odontoid hypoplasia, atlantoaxial subluxation, anterior beaking of the vertebral bodies, dysplastic femoral heads, coxa valga deformity, genu valum, and shortening and cortical thinning of long bones [2,6]. They may have non-skeletal manifestations including cardiorespiratory complications, corneal clouding, and hearing loss, which are shared with other types of mucopolysaccharidoses. The presentation can be variable with respect to age of onset, severity, and rate of progression of both skeletal and non-skeletal features. Most patients with the classical (severe) phenotype have initial recognizable skeletal symptoms between 1 and 3 years of age and are diagnosed before 5 years old, while individuals with the non-classical (attenuated) phenotype

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have fewer or milder late-onset symptoms, and longer life span [2,5,7]. The confirmatory diagnosis of Morquio syndrome A can be made by enzyme assay and molecular analysis of the *GALNS* gene.

In the Ensembl database 13 different transcripts have been described for the *GALNS* gene, which range from 456 nucleotides to 2363 nucleotides [8]. Of these, four isoforms are classified as protein coding, and the remaining transcripts are degraded by nonsense mediated decay or do not encode protein. The transcript NM_000512.4 (ENST00000268695.9) is considered the clinically relevant transcript given that it is the only isoform that contains the correct coding sequence for the enzymatically active protein, whereas the other transcripts contain cryptic exons or exon-skipping which are predicted to produce aberrant products that lack the necessary protein domains needed for enzyme activity. Postmortem tissue analyses reveal high levels of the full length clinically relevant transcript (NM_000512.4) which is the most ubiquitously expressed, however the other isoforms are also detected at varying levels in all tissue types analyzed [9].

Molecular analysis is typically restricted to the clinically relevant transcript, and to date there are over 330 reported single nucleotide or structural variants published in the Human Gene Mutation Database (http://www.hgmd.org) in association with Morquio syndrome A. Zanetti et al. [10] reported the mutational spectrum of 1190 patients with Morquio syndrome A demonstrating the distribution of *GALNS* variants was missense (65.0%), followed by nonsense (8.1%), splicing (7.2%), small frameshift deletions(del)/insertions(ins) (7.0%), intronic (4.0%), and large del/ins and complex rearrangements (3.8%). However, sequence and copy number analysis of the *GALNS* coding region fails to identify two causative pathogenic alterations in up to 15% of patients with a biochemical and clinical diagnosis of Morquio syndrome A [1].

In this report we described two families with individuals affected with Morquio syndrome A presenting with characteristic clinical features and null GALNS enzyme activities in whom Sanger sequencing of the *GALNS* gene failed to identify two pathogenic alterations. RNA sequencing and quantitation identified the overabundance of an aberrant *GALNS* transcript isoform and a reduction of the clinically relevant isoform. These findings, combined with a recent report of a different deep intronic splicing defect in the *GALNS* gene [11], support the use of RNA transcript analysis in clinically affected Morquio syndrome A patients in whom a single heterozygous or no pathogenic alterations are detected by Sanger sequencing.

2. Patients and methods

2.1. Patient and control samples

Four patients with Morquio syndrome A from two unrelated families were enrolled in this study (Patient 1–1 from family 1 and Patient 2–1, 2–2, and 2–3 from family 2). Clinical data of the all four patients were collected by retrospective review of medical chart. In family 1, blood samples for RNA analysis were available from proband (Patient 1–1), parents, and four unaffected siblings. In family 2, blood samples for RNA analysis were available only from two affected patients (Patient 2–1 and 2–2) and mother. Additionally, a specimen from an unaffected male was used as a negative control. Informed consent from patients and their families were obtained in accordance with policies in place at the Greenwood Genetic Center and University of California San Francisco Benioff Children's Hospital Oakland.

2.2. Enzyme activity and urinary GAG analysis

GALNS enzyme analysis was performed using the standard laboratory procedures as previously described [12]. Total urinary GAG was measured using 1,9-dimethyl-methylene blue (DMB) incorporation and spectrophotometry. Urinary KS was measured using LC-MS/MS analysis as previously described [13].

2.3. DNA sequencing and copy number variation analysis

Standard laboratory procedures were used for DNA nucleotide extraction, PCR amplification, Sanger sequencing, and copy number analysis from peripheral blood. Specifically, the coding exons plus flanking intronic regions of *GALNS* gene were PCR amplified and each amplified product was analyzed by a standard fluorescent sequencing protocol in both the forward and reverse direction. Copy number analysis was performed using the Agilent 4x180K custom microarray system.

2.4. RNA analysis

RNA was isolated from peripheral blood (Qiagen) and converted to cDNA by reverse transcription (Invitrogen). GALNS cDNA was sequenced in overlapping amplicons using standard PCR and Sanger sequencing conditions with primers listed in Supplemental table 1. For the sequence analysis of exon 1 to exon 4, PCR fragments were first visualized using capillary electrophoresis (QIAxcel, Qiagen) and then separated by agarose gel electrophoresis, gel excised, and purified (Qiagen) prior to Sanger sequencing. Sequences were aligned to transcript NM_000512.4 (ENST00000268695.9). Exon spanning oligosaccharides were used for transcript-specific quantitation utilizing SYBR green (ABI) detected on an ABI One touch PCR machine (Supplemental table 2). Samples were run in triplicate and normalized against a control transcript, TBP, as previously described [14].

3. Results

3.1. Clinical manifestations

The Patient 1–1 from family 1 was a 14 year-old male from a non-consanguineous family of Mexican descent. He was referred for evaluation of mucopolysaccharidosis due to hip pain and abnormal x-ray findings. He was followed for unclassified skeletal dysplasia since 11 years old. He had no history of previous surgery. He was the firstborn child and had four unaffected younger siblings (one sister and three brothers). At diagnosis, his height was 162 cm (Z score − 0.23) and weight was 50 kg (Z score − 0.1). He had slightly coarse facial features, corneal clouding, multiple skeletal deformities including pectus carinatum, lumbar kyphosis, dysplastic vertebral bodies, forearm deformities, hip joint deformities, genu valgum and hypermobile wrist joints. His intelligence was normal. He had increased urinary total GAG and keratan sulfate, 7.57 mg/mmol Cr (normal range, 0–6.5) and 6.55 μg/mg Cr (normal range, 0–2.14), respectively. GALNS enzyme activity in leukocytes was 0 nmol/17 h/mg protein (normal range, 49–255) while other sulfatase activities were within normal ranges. GALNS enzyme activities of the parents and the other siblings were within the normal range. Beta-galactosidase activity was 42.5 nmol/h/mg protein (normal range, 13.5–176) which excluded the diagnosis of Morquio syndrome B. However, no sequence-level mutations were identified in the GALNS gene. To determine if a large insertion or deletion was present, copy number analysis was performed using a custom microarray system which indicated normal *GALNS* dosage. Clinical and biochemical findings are summarized in Table 1.

Family 2 includes three siblings with Morquio syndrome A from a non-consanguineous family of Mexican descent. The proband (Patient 2–1) was a 12 year-old female diagnosed at 6 years of age due to short stature and multiple skeletal deformities. At diagnosis, her height was 104 cm (Z score − 2.21) and weight was 20 kg (Z score − 0.08). She had increased urinary total GAG (21.62 mg/mmol Cr, normal range, 0–12), keratan sulfate, (19.18 μg/mg Cr,normal range, 0–6.2), and chondroitin-6-sulfate (16.7 g/mol creatinine, normal range, 0–8.54). GALNS enzyme activity in leukocytes was 0.99 nmol/17 h/mg protein (normal range, 49–255) (Table 1). The other two younger siblings (Patient 2–2 and 2–3) were diagnosed by the family history and skeletal symptoms (Table 1). Sequence analysis of the *GALNS* gene identified a heterozygous c.139G

Table 1
Clinical, biochemical and genetic findings of the patients.

	Patient 1–1	Patient 2–1	Patient 2–2	Patient 2–3
Phenotype	Non-classic	Classic	Classic	Classic
Sex	Male	Female	Male	Male
Age (years)	14	12	11	9
Age at Diagnosis (years)	14	6	5	6.6
Age of ERT initiation (years)	14	9.3	8	7.3
Reason for Diagnosis	Hip pain with abnormal skeletal x-ray	Short stature, multiple skeletal deformities	Family history, short stature, multiple skeletal deformities	Family history, short stature, multiple skeletal deformities
Total urine GAG (mg/mmol creatinine)*	7.57 (normal range, 0–6.5)	21.62 (normal range, 0–12)	19.8 (normal range, 0–12)	30.21 (normal range, 0–12)
Keratan Sulfate (mcg/mg creatinine)*	6.55 (normal range, 0–2.14)	19.18 (normal range, 0–6.2)	28.6 (normal range, 0–6.2)	35.97 (normal range, 0–6.2)
Chondroitin-6-Sulfate (g/mol creatinine)*	Not available	16.7 (normal range, 0–8.54)	23.19 (normal range, 0–8.54)	33.75 (normal range, 0–8.54)
GALNS activity in leukocytes (normal range, 49–255 nmol/17 h/mg prot)*	0	0.99	1.12	0
DNA alteration	None	c.139G > A Heterozygous (NM_000512.4)	c.139G > A Heterozygous (NM_000512.4)	c.139G > A Heterozygous (NM_000512.4)
Protein alteration	None	p.Gly47Arg Heterozygous	p.Gly47Arg Heterozygous	p.Gly47Arg Heterozygous
RNA alteration	<1% expression of GALNS normal transcript	~50% expression of GALNS normal transcript	~50% expression of GALNS normal transcript	No data
Height at diagnosis (cm) [Z score]	162 [−0.23]	104 [−2.21]	98 [−2.32]	102.7 [−3.23]
Weight at diagnosis (kg) [Z score]	50 [−0.10]	20.0 [−0.08]	15.9 [−1.21]	17.6 [−1.87]
Facial features	Slightly coarse	Broad forehead, depressed nasal bridge	Mild midfacial hypoplasia, Mild depression of nasal bridge	Midfacial hypoplasia
Pectus carinatum	Yes	Yes	Yes	Yes
Odontoid hypoplasia	No	Yes	Yes	No
Spine	Kyphosis, Platyspondyly and beaking of the vertebral bodies	Scoliosis Lordosis Beaking vertebral bodies	Mild Lordosis Beaking vertebral bodies	Mild Lordosis
Hip	Coxa vara Flattened femoral heads Dysplastic acetabuli	Coxa vara	Dysplastic femoral heads	Coxa vara Dysplastic femoral heads
Knee	Genu valgum Irregular ossification of patella	Genu valgum	Genu valgum	Genu valgum
Upper and lower extremities	Irregularity of multiple epiphyses and metaphyses	Shortened in all segments	Shortened in all segments	Mild meosmelic shortening, Dysplastic epiphyses with metaphyseal flaring
Joint hypermobility	Yes	Yes	Yes	Yes
Bone or joint pain	Yes	No	No	No
Spinal cord compression	No	No	No	No
Corneal clouding	Yes	None at initial visit, later mild	None at initial visit, later mild	Mild
Hearing loss	No	No	No	No
Obstructive sleep apnea (age at diagnosis)	No	Yes (12.2 years)	Yes (10.9 years)	Yes (8.4 years)
Cardiac complications	No	No	No	No
Surgery (age at done)	None	Bilateral femoral and tibial hemiepiphysiodesis and medial malleolar screws (9 years) Ulnar and radial osteotomy with external fixator and pinning (10 years)	Tonsillectomy and adenoidectomy (11 years) Bilateral medial malleolar screws and right proximal tibial hemiepiphysiodesus (11.2 years)	None

ERT, enzyme replacement therapy; GAG, glycosaminoglycan; GALNS, *N*-acetyl-galactosamine-6-sulfatase; *, pre-ERT values.

> A (p.Gly47Arg) change in all three siblings that has been previously reported and was classified as pathogenic [15–18]. A second sequence-level change was not identified, and parental studies indicated that this change was non-maternally inherited (The paternal sample was not available). *GALNS* gene dosage determined using a custom microarray system was normal.

3.2. RNA analysis reveals increased levels of an aberrant GALNS transcript

To investigate if the GALNS transcript contained an intragenic inversion or splicing defect which would be undetectable by DNA analysis, total RNA from the Patient 1–1 was converted to cDNA, PCR amplified, and sequenced in overlapping sections. The clinically relevant GALNS transcript (NM_000512.4) contains 14 exons which encodes a full-length functional protein. Sequencing analysis of the GALNS transcript was normal from exon 3 to exon 14. PCR amplification of the 5′ end of the transcript spanning exons 1–4 should have yielded a product of approximately 480 base pairs; however, in Patient 1–1, this product was absent and instead, one larger (approximately 650 base pairs) and one smaller (approximately 350 base pairs) product was observed (Fig. 1A). The control sample produced both the expected 480-base pairs product and the two additional products (350 and 650-base pairs), but the unexpected bands had lower intensities than those observed in Patient 1–1. To rule out that these unexpected products were a PCR artifact the PCR conditions were altered to increase stringency; however, the same bands were observed, and were never present in the no template control. To elucidate the origin of the aberrant products, the

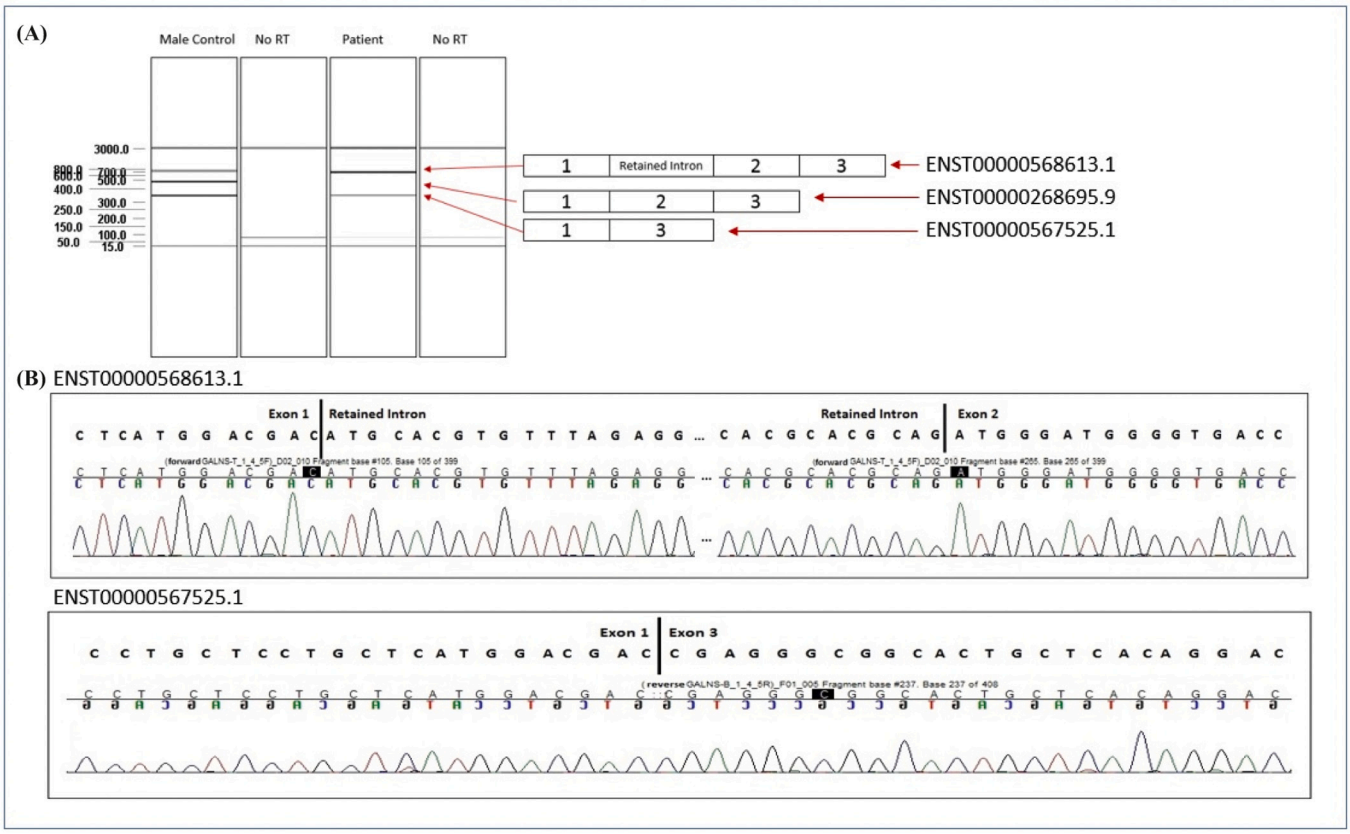

Fig. 1. mRNA sequence analysis of Patient 1–1 identifies imbalanced expression of a GALNS transcript containing a retained intron. mRNA was reverse transcribed to cDNA, PCR amplified with exon-spanning primers, and separated by capillary electrophoresis. Three different PCR products are present in the control sample, whereas only two products are present in the patient (A). To ensure that genomic DNA was not contaminating the assay, samples that lacked reverse transcriptase (No RT) were analyzed as a negative control. The same PCR products were then separated by agarose gel electrophoresis (data note shown) and Sanger sequencing of gel excised bands indicates that the larger product contains a retained exon, whereas the smaller product is a result of skipping of exon 2 (B).

PCR products were separated by agarose gel electrophoresis and the bands were gel extracted and sequenced. Sequence analysis confirmed that the products were alternatively spliced isoforms of GALNS. The larger predominant fragment present in the proband contains intronic sequence that is likely a cryptic exon that is predicted to result in a reading frame shift and generation of a premature termination codon. This isoform is present in the Ensembl database as ENST00000568613.1 (Fig. 1B). Additionally, the lower molecular weight fragment is also an alternatively spliced isoform of GALNS, but originates from skipping exon 2, resulting in a reading frame shift and generation of a premature termination codon. This smaller isoform is also present in the Ensembl database as ENST00000567525.1 (Fig. 1B). The expected 480 base pair product was not detected in the Patient 1–1, and was therefore analyzed in the control sample which confirmed it to be the properly spliced isoform containing exons 1 through 3, which encodes the clinically relevant full length transcript (NM_000512.4, ENST00000268695.9). These findings indicate that splicing of exons 1, 2, and 3 is dynamic and that aberrant splicing is likely the molecular defect resulting in a diagnosis of Morquio syndrome A in Patient 1–1. Visual inspection of the relative intensity of the bands corresponding to each of the three GALNS isoforms indicates that the abundance of the larger isoform is increased in Patient 1–1 compared to the control. To quantify the relative abundance of the three different isoforms, exon-spanning oligonucelotides were used to perform allele specific qPCR. Compared to an unaffected control, the Patient 1–1 only expressed approximately 1% of the properly spliced transcript, whereas parents (obligate carriers) and unaffected siblings expressed 20%–60% of the properly spliced transcript (Fig. 2A). Conversely, expression of the longer transcript containing the cryptic exon (ENST00000568613.1) was 16-fold higher in Patient 1–1, and six to eight fold higher in parents and unaffected siblings, compared to expression in the control (Fig. 2B). Finally, qPCR analysis of the shorter transcript with skipping of exon 2 (ENST00000567525.1) indicated that the Patient 1–1's expression was not significantly reduced compared to the unaffected parents and siblings (data not shown). These findings suggest that the molecular cause for the patient's clinical and biochemical diagnosis of Morquio syndrome A is a splicing defect of the GALNS gene that causes mal-incorporation of intronic sequence between exons 1–2, resulting in significantly decreased amounts of the full length clinically relevant transcript.

Several key sequences influence proper transcript processing including donor splice sites, acceptor splice sites, and branch points [19]. To identify the DNA alteration responsible for the misincorporation of the cryptic exon between exons 1 and 2 of the GALNS gene, intron 1 was sequenced in the Patient 1–1 and family members. Segregation analysis revealed only a single variant that was homozygous in the patient and heterozygous in the other family members: a C > T alteration located at position c.121-210C (NM_000512.4: c.121-210C > T) which has been reported in the public SNP databases (rs75552025). Specifically, in the African/African American subpopulation in the Genome Aggregation Database version v3.1.2 the c.121-210C > T has an allele frequency of 3% with 20 homozygous individuals and in the Latino/Admixed American subpopulation has an allele frequency of 0.4% with zero homozygotes. Given this information, this change is classified as likely benign. Therefore, the causative mutation responsible for improper splicing was not identified within the intron 1.

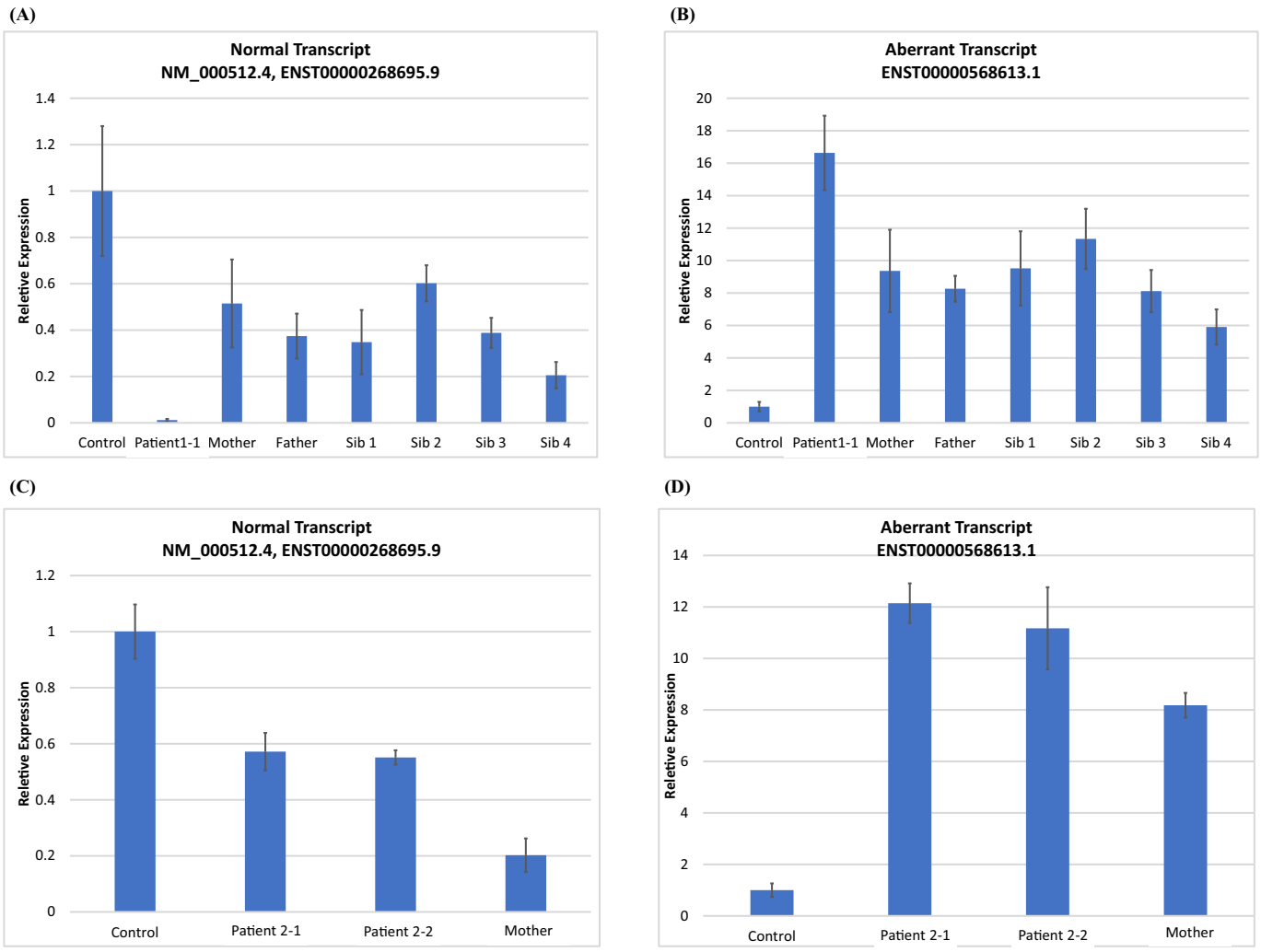

Fig. 2. Transcript specific oligos and quantitative PCR of cDNA confirms reduced expression of the normal GALNS transcript and increased expression of the larger, aberrant transcript. (A) Patient 1–1 in family 1 expresses approximately 1% of the normal transcript compared to a normal control and first degree relatives. (B) The aberrant transcript is approximately 16 fold higher in patient 1–1 compared to a normal control and two-fold higher than in first degree relatives. (C) Patient 2–1, Patient 2–2, and mother (obligate carrier) in family 2 expresses approximately half of the normal transcript compared to a normal control. (D) The aberrant transcript is approximately 10 fold higher in Patient 2–1, Patient 2–2, and mother (obligate carrier) compared to a normal control.

3.3. Increased expression of the larger GALNS isoform also identified in family 2

Although the DNA lesion responsible for the altered splicing observed in the Patient 1–1 above is not known, it is presumed to be homozygous, given the transcript expression profiles observed in the obligate carrier parents. To elucidate if this splicing error could be a recurrent disease-causing alteration, qPCR was performed for the patients from family 2, for whom only one heterozygous pathogenic alteration was detected in the GALNS gene (c.139G > A, p.Gly47Arg). The father of the affected siblings was not available for testing, but is presumed to be a carrier for this missense change, as the patients' mother was negative for this sequence change. This also suggests that the mother is a carrier for the second undetected pathogenic change in *GALNS*.

Samples from two of the three affected siblings (Patient 2–1 and 2–2) and mother in Family 2 were available for analysis. Quantitation of the GALNS isoforms in the affected siblings and their obligate carrier mother reveals reduced levels of the properly spliced isoform (Fig. 2C), and inversely, increased levels of the isoform containing the cryptic exon (ENST00000568613.1) (Fig. 2D). This expression pattern is similar with that observed for the obligate carrier parental samples in Family 1. This indicates that the second, previously undetected, disease-causing mutation in the affected siblings in Family 2 is the same alteration present in the homozygous state in the proband in Family 1.

4. Discussion

Pathogenic alterations in the *GALNS* gene are associated with autosomal recessive Morquio syndrome A, a lysosomal storage disease characterized by multiple progressive skeletal manifestations including short trunk dwarfism, pectus carinatum, kyphoscoliosis, hip dysplasia, genu valgum, corneal clouding, and joint hypermobility [3]. However, there is wide clinical variability thus some patients have non-classical phenotype, presenting with mild skeletal dysplasia and bone or joint pain only [4]. The clinical diagnosis of Morquio syndrome A is challenging and often delayed given the rarity of the disease and the overlapping phenotype with other various skeletal dysplasia [20]. When patients have suggestive symptoms of Morquio syndrome A, urine GAG testing could be performed first; however, total urine GAG levels alone do not clearly distinguish the Morquio syndrome A patients from normal individuals, especially in patients with a non-classical phenotype. Patients from family 2 with classic phenotype had marked elevation (2–3 times of reference value) of total urine GAG and KS levels in contrast the

patient 1–1 presented with non-classical phenotype had mild elevation suggesting possible correlation between phenotype and urinary GAG or KS. Approximately 20% of the patients with Morquio syndrome A have total urine GAG levels within the normal range [2]. The quantitation of urine KS is a more sensitive assay to distinguish patients from normal controls [2]. Once the urinary GAG levels especially KS is elevated, the confirmatory diagnosis of Morquio syndrome A is typically made by demonstrating deficient GALNS enzyme activity in peripheral leukocytes or cultured fibroblasts [4]. Together with reduced enzyme activity, identification of pathogenic variants in *GALNS* contributes to clarify the diagnosis [4]. Establishing accurate diagnostic strategies, including the requisite genetic analyses, is essential to distinguish between Morquio syndrome A patients and individuals with other skeletal disorders sharing the clinical and radiological findings [20–22]. Moreover, timely diagnosis is crucial for the prompt initiation of enzyme replacement therapy (ERT), which can lead to better outcomes when applied earlier before the irreversible damages occur. Indeed, the diagnosis of Patient 1–1 was challenging due to his non-classical phenotype, subtle elevation of total urine GAGs, and normal sequence and gene dosage analysis resulting in delay of diagnosis and ERT initiation.

Molecular analysis of the *GALNS* gene is currently restricted to sequence and copy number analysis of the clinically relevant transcript (NM_000512.4, ENST00000268695.9). The standard molecular testing covering all exons and exon-intron boundaries by sequence analysis fails to identify two pathogenic alterations in up to 15% of individuals with Morquio syndrome A [1]. Additional copy number analysis to verify the presence of large deletion/insertion or genetic rearrangements could improve the diagnostic yield [10,23]; however, some genetic alterations remain unidentified. Zanetti et al. [10] reported that 10.7% of a large multinational cohort of Morquio syndrome A patients had only one variant detected in the *GALNS* gene. This can be particularly problematic for prenatal testing or familial carrier testing. Moreover, accurate identification of pathogenic genetic alterations has become more crucial in recent years, as phenotype-specific targeted next-generation sequencing (NGS) panels and whole exome sequencing are being used as first-tier tests in place of traditional biochemical testing such as enzyme analysis and/or urine GAG analysis. The diagnosis of patient 1–1 would be missed by these NGS-based approaches.

In this report, we used RNA sequencing and quantitative expression analysis to identify a novel defect that decreases the steady state levels of the normal GALNS isoform and increases the levels of an aberrant longer transcript (ENST00000568613.1). This method could be useful to identify pathogenic alterations in patients whose genetic variants are not found by standard sequencing or gene dosage analysis. In general, pathogenic alterations in the *GALNS* gene are familial, and it's unclear if this specific splicing defect could account for a large portion of the missing molecular diagnoses. However, we identified a second unrelated family who also shares this aberrant isoform expression pattern, which suggests that this aberrant splicing within intron 1 of the *GALNS* gene, resulting in production of a longer, non-coding transcript, appears to be a recurrent mutation, and likely accounts for at least a portion of the currently undetected mutations in 10–15% of Morquio syndrome A patients. In combination with a recent study by Caciotti et al. [11] that identified different aberrant splice defects in the *GALNS* gene by mRNA analysis, our results indicate that transcript analysis of all known isoforms may increase the diagnostic yield of *GALNS* molecular analyses. If RNA splicing defects represent a significant proportion of pathogenic changes in GALNS, additional therapeutic options may be available to these patients.

As some newborn screening (NBS) programs begin screening for Morquio syndrome A, neonates can be diagnosed before the presence of apparent clinical symptoms, due to low GALNS enzymatic activity in dried blood spots [24,25]. The diagnosis is then confirmed by the analysis of GALNS enzyme activity in leukocytes or fibroblasts [20,26] followed by molecular analysis. However, prediction of the newborn's phenotype in terms of disease severity and rate of progression would be difficult. Although genotype-phenotype studies of Morquio A patients indicate that predicted loss of function alterations are more common in patients with a severe phenotype, there can be significant clinical variability even within the same family [1,21]. The inconsistency of genotype-phenotype correlation may reflect the heterogeneity of the Morquio syndrome A. For example, c.901G > T (p.Gly301Cys) was reported as the most common variant of classical phenotype but also associated with the non-classical phenotype in a large Morquio syndrome A cohort [10]. The patients from family 2 had heterozygous missense variant (c.139G > A, p.Gly47Arg) in GALNS, which was previously reported to be identified in patients with classic phenotype [15,16,27] including a 4-year-old patient with classic phenotype carrying homozygous p.Gly47Arg variants [15].p.Gly47 is located in the hydrophobic core of GALNS tertiary structure which is known as a critical protein region for enzyme activity [17,28] and completely conserved position in 20 species [16]. The *in silico* analysis demonstrated deteriorating effect of p.Gly47Arg variant [18]. The classic phenotype of the patients from family 2 could be related with this missense variant. It is uncertain that the aberrant splicing found in this study is related to classical or non-classical phenotype. Given that the Patient 1–1 who was homozygote for this novel variant had an attenuated phenotype, it could be associated with a non-classical phenotype. However, more data is necessary to establish the firm genotype-phenotype correlation. Knowledge of genotype-phenotype correlations is essential for correct diagnosis, prenatal diagnosis and appropriate genetic counseling and will be an important component if Morquio syndrome A is added to newborn screening panels [24,25].

Although we could not identify the causative gDNA level variant by sequencing intron 1 of *GALNS* in this study, our identification of a novel, yet recurrent, aberrant splicing variant in *GALNS* using RNA analysis suggests that it may be the elusive pathogenic variant in at least a portion of the Morquio syndrome A patients for whom standard sequencing or gene dosage analysis failed to identify two pathogenic variants. This additional RNA analysis could be a useful tool for the timely and accurate diagnosis of Morquio syndrome A, which would allow for more rapid therapeutic intervention and therefore better clinical outcomes. Also, it could expand the knowledge of disease pathogenicity and expand genotype-phenotype correlations. Whole genome sequencing is currently being performed with the hopes to identify cis- or trans-acting regulatory factors that are outside of intron 1 that could be responsible for the splice defect identified in this study. Additionally, future studies may include optical genomic mapping to identify complex structural rearrangements that could be disrupting proper transcript processing and *in vitro* functional studies by making mini-gene expression vector of this novel splicing variant to prove the gene product, transcripts and enzyme activities.

5. Conclusion

Molecular analysis reveals that transcripts that are considered non-clinically relevant can play a role in pathogenesis, and RNA analysis should be considered in third tier *GALNS* gene analysis. Specifically, RNA studies should be designed to detect both splicing errors and quantify the relative abundance of different isoforms.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2022.100875.

References

[1] S. Tomatsu, A.M. Montaño, T. Nishioka, M.A. Gutierrez, O.M. Peña, G.G. Tranda Firescu, P. Lopez, S. Yamaguchi, A. Noguchi, T. Orii, Mutation and polymorphism spectrum of the GALNS gene in mucopolysaccharidosis IVA (Morquio A), Hum. Mutat. 26 (2005) 500–512.

[2] K. Sawamoto, J.V. Álvarez González, M. Piechnik, F.J. Otero, M.L. Couce, Y. Suzuki, S. Tomatsu, Mucopolysaccharidosis IVA: diagnosis, treatment, and management, Int. J. Mol. Sci. 21 (2020).

[3] P. Harmatz, K.E. Mengel, R. Giugliani, V. Valayannopoulos, S.P. Lin, R. Parini, N. Guffon, B.K. Burton, C.J. Hendriksz, J. Mitchell, A. Martins, S. Jones, N. Guelbert, A. Vellodi, C. Hollak, P. Slasor, C. Decker, The Morquio A clinical assessment program: baseline results illustrating progressive, multisystemic clinical impairments in Morquio A subjects, Mol. Genet. Metab. 109 (2013) 54–61.

[4] S. Tomatsu, A.M. Montaño, H. Oikawa, M. Smith, L. Barrera, Y. Chinen, M. M. Thacker, W.G. Mackenzie, Y. Suzuki, T. Orii, Mucopolysaccharidosis type IVA (Morquio A disease): clinical review and current treatment, Curr. Pharm. Biotechnol. 12 (2011) 931–945.

[5] A.M. Montaño, S. Tomatsu, G.S. Gottesman, M. Smith, T. Orii, International Morquio A Registry: clinical manifestation and natural course of Morquio a disease, J. Inherit. Metab. Dis. 30 (2007) 165–174.

[6] K.K. White, A. Jester, C.E. Bache, P.R. Harmatz, R. Shediac, M.M. Thacker, W. G. Mackenzie, Orthopedic management of the extremities in patients with Morquio A syndrome, J. Child. Orthop. 8 (2014) 295–304.

[7] S. Khan, C.J. Alméciga-Díaz, K. Sawamoto, W.G. Mackenzie, M.C. Theroux, C. Pizarro, R.W. Mason, T. Orii, S. Tomatsu, Mucopolysaccharidosis IVA and glycosaminoglycans, Mol. Genet. Metab. 120 (2017) 78–95.

[8] D.R. Zerbino, P. Achuthan, W. Akanni, M.R. Amode, D. Barrell, J. Bhai, K. Billis, C. Cummins, A. Gall, C.G. Girón, L. Gil, L. Gordon, L. Haggerty, E. Haskell, T. Hourlier, O.G. Izuogu, S.H. Janacek, T. Juettemann, J.K. To, M.R. Laird, I. Lavidas, Z. Liu, J.E. Loveland, T. Maurel, W. McLaren, B. Moore, J. Mudge, D. N. Murphy, V. Newman, M. Nuhn, D. Ogeh, C.K. Ong, A. Parker, M. Patricio, H. S. Riat, H. Schuilenburg, D. Sheppard, H. Sparrow, K. Taylor, A. Thormann, A. Vullo, B. Walts, A. Zadissa, A. Frankish, S.E. Hunt, M. Kostadima, N. Langridge, F.J. Martin, M. Muffato, E. Perry, M. Ruffier, D.M. Staines, S.J. Trevanion, B. L. Aken, F. Cunningham, A. Yates, P. Flicek, Ensembl 2018, Nucleic Acids Res. 46 (2018) D754–d761.

[9] L.J. Carithers, K. Ardlie, M. Barcus, P.A. Branton, A. Britton, S.A. Buia, C. C. Compton, D.S. DeLuca, J. Peter-Demchok, E.T. Gelfand, P. Guan, G. E. Korzeniewski, N.C. Lockhart, C.A. Rabiner, A.K. Rao, K.L. Robinson, N.V. Roche, S.J. Sawyer, A.V. Segrè, C.E. Shive, A.M. Smith, L.H. Sobin, A.H. Undale, K. M. Valentino, J. Vaught, T.R. Young, H.M. Moore, A novel approach to high-quality postmortem tissue procurement: the GTEx project, Biopreserv. Biobank. 13 (2015) 311–319.

[10] A. Zanetti, F. D'Avanzo, M. AlSayed, A.C. Brusius-Facchin, Y.H. Chien, R. Giugliani, E. Izzo, D.C. Kasper, H.Y. Lin, S.P. Lin, L. Pollard, A. Singh, R. Tonin, T. Wood, A. Morrone, R. Tomanin, Molecular basis of mucopolysaccharidosis IVA (Morquio a syndrome): a review and classification of GALNS gene variants and reporting of 68 novel variants, Hum. Mutat. 42 (2021) 1384–1398.

[11] A. Caciotti, R. Tonin, M. Mort, D.N. Cooper, S. Gasperini, M. Rigoldi, R. Parini, F. Deodato, R. Taurisano, M. Sibilio, G. Parenti, R. Guerrini, A. Morrone, Mis-splicing of the GALNS gene resulting from deep intronic mutations as a cause of Morquio a disease, BMC Med. Genet. 19 (2018) 183.

[12] O.P. van Diggelen, H. Zhao, W.J. Kleijer, H.C. Janse, B.J. Poorthuis, J. van Pelt, J. P. Kamerling, H. Galjaard, A fluorimetric enzyme assay for the diagnosis of Morquio disease type A (MPS IV A), Clin. Chim. Acta 187 (1990) 131–139.

[13] L.A. Martell, R.L. Cunico, J. Ohh, W. Fulkerson, R. Furneaux, E.D. Foehr, Validation of an LC-MS/MS assay for detecting relevant disaccharides from keratan sulfate as a biomarker for Morquio A syndrome, Bioanalysis 3 (2011) 1855–1866.

[14] M. Padró, R.J. Louie, B.V. Lananna, A.J. Krieg, L.A. Timmerman, D.A. Chan, Genome-independent hypoxic repression of estrogen receptor alpha in breast cancer cells, BMC Cancer 17 (2017) 203.

[15] S. Bunge, W.J. Kleijer, A. Tylki-Szymanska, C. Steglich, M. Beck, S. Tomatsu, S. Fukuda, B.J. Poorthuis, B. Czartoryska, T. Orii, A. Gal, Identification of 31 novel mutations in the N-acetylgalactosamine-6-sulfatase gene reveals excessive allelic heterogeneity among patients with Morquio A syndrome, Hum. Mutat. 10 (1997) 223–232.

[16] S. Olarte-Avellaneda, A. Rodríguez-López, C.J. Alméciga-Díaz, L.A. Barrera, Computational analysis of human N-acetylgalactosamine-6-sulfate sulfatase enzyme: an update in genotype-phenotype correlation for Morquio A, Mol. Biol. Rep. 41 (2014) 7073–7088.

[17] Y. Rivera-Colón, E.K. Schutsky, A.Z. Kita, S.C. Garman, The structure of human GALNS reveals the molecular basis for mucopolysaccharidosis IV A, J. Mol. Biol. 423 (2012) 736–751.

[18] E.R. Tamarozzi, E. Torrieri, E.P. Semighini, S. Giuliatti, In silico analysis of mutations occurring in the protein N-acetylgalactosamine-6-sulfatase (GALNS) and causing mucopolysaccharidosis IVA, Genet. Mol. Res. 13 (2014) 10025–10034.

[19] K. Pan, J.T. Lee, Z. Huang, C.M. Wong, Coupling and coordination in gene expression processes with pre-mRNA splicing, Int. J. Mol. Sci. 16 (2015) 5682–5696.

[20] C.J. Hendriksz, P. Harmatz, M. Beck, S. Jones, T. Wood, R. Lachman, C. G. Gravance, T. Orii, S. Tomatsu, Review of clinical presentation and diagnosis of mucopolysaccharidosis IVA, Mol. Genet. Metab. 110 (2013) 54–64.

[21] A. Morrone, A. Caciotti, R. Atwood, K. Davidson, C. Du, P. Francis-Lyon, P. Harmatz, M. Mealiffe, S. Mooney, T.R. Oron, A. Ryles, K.A. Zawadzki, N. Miller, Morquio A syndrome-associated mutations: a review of alterations in the GALNS gene and a new locus-specific database, Hum. Mutat. 35 (2014) 1271–1279.

[22] S. Tomatsu, K. Sawamoto, C.J. Alméciga-Díaz, T. Shimada, M.B. Bober, Y. Chinen, H. Yabe, A.M. Montaño, R. Giugliani, F. Kubaski, E. Yasuda, A. Rodríguez-López, A. J. Espejo-Mojica, O.F. Sánchez, R.W. Mason, L.A. Barrera, W.G. Mackenzie, T. Orii, Impact of enzyme replacement therapy and hematopoietic stem cell transplantation in patients with Morquio A syndrome, Drug. Des. Devel. Ther. 9 (2015) 1937–1953.

[23] A. Caciotti, R. Tonin, M. Rigoldi, L. Ferri, S. Catarzi, C. Cavicchi, E. Procopio, M. A. Donati, A. Ficcadenti, A. Fiumara, R. Barone, L. Garavelli, M.D. Rocco, M. Filocamo, D. Antuzzi, M. Scarpa, S.D. Mooney, B. Li, A. Skouma, S. Bianca, D. Concolino, R. Casalone, E. Monti, M. Pantaleo, S. Giglio, R. Guerrini, R. Parini, A. Morrone, Optimizing the molecular diagnosis of GALNS: novel methods to define and characterize Morquio-a syndrome-associated mutations, Hum. Mutat. 36 (2015) 357–368.

[24] Y.H. Chien, N.C. Lee, P.W. Chen, H.Y. Yeh, M.H. Gelb, P.C. Chiu, S.Y. Chu, C. H. Lee, A.R. Lee, W.L. Hwu, Newborn screening for Morquio disease and other lysosomal storage diseases: results from the 8-plex assay for 70,000 newborns, Orphanet. J. Rare Dis. 15 (2020) 38.

[25] H.Y. Lin, C.L. Lee, C.Y. Chang, P.C. Chiu, Y.H. Chien, D.M. Niu, F.J. Tsai, W. L. Hwu, S.J. Lin, J.L. Lin, M.C. Chao, T.M. Chang, W.H. Tsai, T.J. Wang, C. K. Chuang, S.P. Lin, Survival and diagnostic age of 175 Taiwanese patients with mucopolysaccharidoses (1985–2019), Orphanet. J. Rare Dis. 15 (2020) 314.

[26] H. Peracha, K. Sawamoto, L. Averill, H. Kecskemethy, M. Theroux, M. Thacker, K. Nagao, C. Pizarro, W. Mackenzie, H. Kobayashi, S. Yamaguchi, Y. Suzuki, K. Orii, T. Orii, T. Fukao, S. Tomatsu, Molecular genetics and metabolism, special edition: diagnosis, diagnosis and prognosis of Mucopolysaccharidosis IVA, Mol. Genet. Metab. 125 (2018) 18–37.

[27] T. Ogawa, S. Tomatsu, S. Fukuda, A. Yamagishi, G.M. Rezvi, K. Sukegawa, N. Kondo, Y. Suzuki, N. Shimozawa, T. Orü, Mucopolysaccharidosis IVA: screening and identification of mutations of the N-acetylgalactosamine-6-sulfate sulfatase gene, Hum. Mol. Genet. 4 (1995) 341–349.

[28] S. Tomatsu, T. Nishioka, A.M. Montaño, M.A. Gutierrez, O.S. Pena, K.O. Orii, W. S. Sly, S. Yamaguchi, T. Orii, E. Paschke, S.G. Kircher, A. Noguchi, Mucopolysaccharidosis IVA: identification of mutations and methylation study in GALNS gene, J. Med. Genet. 41 (2004), e98.