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

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A R T I C L E   I N F O
Keywords:
GALNS
GALNS transcript isoform
Aberrant splicing
Morquio syndrome A
Mucopolysaccharidosis IVA

A B S T R A C T
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 catalyzes 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 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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https://doi.org/10.1016/j.ymgmr.2022.100875
Received 22 April 2022; Accepted 22 April 2022
Available online 3 May 2022
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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
ezyme 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 nucleo-
tides [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 tran-
scripts 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%). How-
ever, sequence and copy number analysis of the GALNS gene 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%). How-
ever, sequence and copy number analysis of the GALNS gene 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 fea-
tures 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 aber-
rant 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 pa-
tients 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 labora-
tory 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 anal-
ysis 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 (QiAxxel, Qiagen) and then
separated by agarose gel electrophoresis, gel excised, and purified
(Qiagen) prior to Sanger sequencing. Sequences were aligned to tran-
script NM_000512.4 (ENST00000268695.9). Exon spanning oligosac-
charides 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 evalu-
ation 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 car-
Inatum, lumbar kyphosis, dysplastic vertebral bodies, forearm de-
formities, 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, 0–6.2) and chondroitin-
sulfate, (19.18 g/mol creatinine, normal range, 0–8.54). 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 pre-
sent, 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, 0–6.5)
and keratan sulfate, (19.18 μg/mg Cr, normal range, 0–6.2), and chondroitin-
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.

| Phenotype      | Patient 1–1 | Patient 2–1 | Patient 2–2 | Patient 2–3 |
|----------------|-------------|-------------|-------------|-------------|
| 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 (mg/mg creatinine)* | 6.55 (normal range, 0.2–1.4) | 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.Gly-67Arg Heterozygous | p.Gly-67Arg Heterozygous | No data |
| 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 [−3.22] | 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 |
| Osseous hypertrophy | No | Yes | Yes | No |
| Spine | Kyphosis, Platypondyly and beaking of the vertebral bodies | Scoliosis Lordosis | Mild Lordosis | Mild Lordosis |
| Hip | Cox vara | Flattened femoral heads | Dysplastic femoral heads | Cox vara | Dysplastic femoral heads |
| Knee | Genu valgum | Irregular ossification of patella | Dysplastic acetabuli | Genu valgum | Genu valgum |
| Upper and lower extremities | Irregularity of multiple epiphysis 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 hemiepiphiodesis and medial malleolar screws (9 years) | Bilateral femoral and tibial hemiepiphiodesis and medial malleolar screws (9 years) | Tonsillotomy and adenoidectomy (11 years) |

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) 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
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 oligonucleotides 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.
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 quantification 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, 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.

Acknowledgements

We would like to thank the biochemical and molecular diagnostic laboratories at the Greenwood Genetic Center for their help in these analyses.
Appendix A. Supplementary data

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

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