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## GALNS gene expression profiling in Morquio A patients' fibroblasts

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Mucopolysaccharidosis IVA (MPS IVA, Morquio type A) OMIM disorder/gene accession number, 253000

### ABSTRACT

**Background:** Quantification studies of mutated mRNAs have not been carried out on Morquio A patients. Such studies are very important for the determination of stability of premature termination codons (PTC) bearing transcripts in order to assess the appropriateness of introducing the newly developed therapeutic strategies such as “stop codon read-through therapy”.

**Methods:** This paper focuses on the study of the GALNS gene and mRNAs in two severe forms of Morquio A patients' fibroblasts with development of a new and rapid real-time RT-PCR for detection and quantification of absolute mRNA copy number.

**Results:** We identified two new mutations c.385A>T (p.K129X) and c.899–1G>C in Pt1 and a known splicing defect c.120+1G>A in Pt2. Using RT-PCR and real-time RT-PCR in Pt2 we detected low levels of mRNAs, suggesting its instability; in Pt1, we detected three aberrant mRNAs introducing premature stop codons, suggesting that both the c.385A>T and c.899–1G>C mutations produce mRNAs capable of escaping the nonsense-mediated decay (NMD) pathway.

**Conclusions:** The development of a real-time RT-PCR assay allows to absolutely quantify the GALNS mRNAs carrying mutations that lead to PTCs bearing transcripts, which escape the NMD process and are potentially suitable for the new therapeutic approach.

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### 1. Introduction

Deficiency of the lysosomal enzyme N-acetylgalactosamine-6-sulfate sulfatase (GALNS; E.C. 3.1.6.4; OMIM 253000) leads to mucopolysaccharidosis IVA (MPS IVA; Morquio type A). GALNS hydrolyses the sulfate ester groups of galactose-6-sulfate at the nonreducing end of keratan sulphate (KS) and of N-acetylgalactosamine-6-sulfate (GalNAc6S) at the nonreducing end of chondroitin-6-sulfate (C6S) [1,2].

MPS IVA patients show a broad spectrum of clinical severity, ranging from classical (severe) forms to milder phenotypes. Classical forms are characterised by a lifespan of 20–30 years, short trunk dwarfism, spondyloepiphyseal dysplasia, coxa valga, odontoid hypo-

plasia, corneal clouding, preservation of intelligence, hepatosplenomegaly and an excessive urinary excretion of KS and C6S [3,4]. Mild forms imply little bone and visceral organ involvement and a relatively good quality of life until 50–60 years of age [5,6].

The GALNS enzyme is encoded by the GALNS gene, the latter, mapped on chromosome 16q24.3 [7–9], encompassing about 50 kb and organized in 14 exons and 13 introns [10].

About 150 mutations have so far been identified, revealing a high degree of genetic heterogeneity that is probably responsible for the clinical variability in MPS IVA patients.

Only a few studies have been carried out on human GALNS mRNA [10,11] none of which including mRNA quantification by real-time PCR. However mRNAs quantification can be helpful in studying mutations causing disease such as splicing and nonsense alterations. These mutations can cause very unstable mRNA transcripts or transcripts capable of escaping the nonsense-mediated decay (NMD) pathway, a molecular mechanism that reduces the amount of transcripts carrying premature termination codons (PTCs). Consequently toxic or gain-of-function proteins would be generated [12], resulting in different phenotypes. Here we report characterisation of GALNS gene mutations

**Abbreviations:** GALNS, N-acetylgalactosamine-6-sulfate sulfatase; MPS IVA, mucopolysaccharidosis IVA; GalNAc6S, N-acetylgalactosamine-6-sulfate; KS, keratan sulphate; C6S, chondroitin-6-sulfate; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; PTC, premature termination codon; NMD, nonsense-mediated decay; MRD, minimal residual disease.

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and mRNA stability verifying a genotype/phenotype correlation in two MPS IVA patients with a severe form.

## 2. Materials and methods

### 2.1. Patients

The clinical and biochemical findings of the two patients (Pt1 and Pt2) are shown in Table 1. Deficiency of GALNS enzyme activity was confirmed on fibroblasts by the fluorogenic method previously reported by Van Diggelen et al. [13].

The molecular study was performed after informed consent, for genetic testing from patients' parents, was obtained.

### 2.2. Analysis of genomic DNA

To identify genetic lesions in the GALNS gene, genomic DNA was isolated from peripheral blood lymphocytes and fibroblasts. The GALNS exons were amplified with the primers reported in Table 2.

Amplification of genomic fragments was performed on 400 ng of genomic DNA and PCR conditions for all the exons were: denaturation at 94 °C for 5 min, 28 cycles at 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, and a final extension cycle at 72 °C for 10 min.

PCR products were visualized on a 2% agarose gel, excised and purified using Nucleospin Extract II extraction kit (MACHEREY-NAGEL, Duren, Germany). About 100 ng of purified DNA was analyzed for mutation detection by nucleotide sequencing on ABI PRISM 310 Genetic Analyzer using BigDye terminator chemicals (Applied Biosystems, Foster City, CA).

### 2.3. RNA isolation and retrotranscription

Isolation of total RNA from cultured skin fibroblasts was performed with the TRIzol reagent (Life Technologies, Rockville, MD). RNA integrity and concentrations were both checked by 1% agarose gel and Nanodrop® ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, USA).

RNA reverse transcription was carried out as follows:

- 1–7 µg of total RNAs were reverse transcribed with Display THERMO-RT (Eppendorf, Hamburg, Germany) using the specific 3' UTR primer 5' GGAGGGTCTGAAATCTGAGG 3', according to the manufacturer.
- 400 ng total RNAs were reverse transcribed in 80 µl of final volume in a reaction mixture containing 10 µl TaqMan RT buffer 1×, 5.5 mM MgCl<sub>2</sub>, 500 µM each dNTP, 2.5 µM random hexamers, 0.4 U/µl RNase Inhibitor and 1.25 U/µl Multi-Scribe Reverse transcriptase provided by Applied Biosystems. The profile of the one-step reverse transcriptase was: 10 min at 25 °C, 30 min at 48 °C and 2 min at 95 °C.

The reverse transcripts obtained from the second method were used for quantitative real-time analysis.

**Table 1**

Clinical, biochemical and molecular data of MPS IV A patients

	Patient 1	Patient 2
Sex	F	F
Age at diagnosis (y)	1	2
Qualitative urinary MPS	Keratan excretion	Keratan excretion
GALNS activity (fibroblasts) (105–380 nmol/mg/17h)	0.2	1.1
Genotype <sup>a</sup> Allele 1	c.385A>T (p.K129X) [This study]	c.120+1G>A [36]
Allele 2	c.899–1G>C [This study]	c.120+1G>A [36]
Parents' consanguinity	No	Yes
At present		
Age (y)	9	7
Height(cm)	91	89
Weight (kg)	22.8	14
Spondylo-Epiphyseal-dysplasia	Yes	Yes
Chest deformity	Yes	Yes
Hearing loss	Yes	Yes
Corneal clouding	Yes	Yes
Coxa valga	Yes	Yes
Odontoid hypoplasia	Yes	Yes
Visceromegaly	Yes	Yes
Cardiac involvement (left ventricular hypertrophy/valva disease)	Mitral, aortic and tricuspidal regurgitation	Mitral regurgitation

y=year; na=not available.

<sup>a</sup> In square brackets, citation refers to the first report.

**Table 2**

Primers for PCR amplification of GALNS gene

Primer name	Segment <sup>a</sup>	Oligonucleotide	Fragment length
Exon 1			
galns 1F	Sense –168/–148 nt <sup>b</sup>	5' CTGGTCACGAGGCAGTCCAG3'	351
galns 1R	Antisense +46/+64 nt	5' CTCATCCGCCCTCCCT3'	
Exon 2			
galns 2F	Sense –54/–34 nt	5' CCAGAAGCACCTGCAGAAGGC3'	231
galns 2R	Antisense +34/+53 nt	5' CCTCGCCTGTGGGCTCAC3'	
Exon 3			
galns 3F	Sense –65/–46 nt	5' GTTGACCCCGGGTCTGAG3'	208
galns 3R	Antisense +49/+68 nt	5' CACCACCCGTAGCCACCTG3'	
Exon 4			
galns 4F	Sense –66/–46 nt	5' GTTAGGATGGGGTGTGGCA3'	231
galns 4R	Antisense +42/+62 nt	5' GGAACCAAGCCAGGAAGTGG3'	
Exon 5			
galns 5F	Sense –51/–33 nt	5' CCGAGTGTCCACGTGGG3'	245
galns 5R	Antisense +31/+50 nt	5' CGGGCACAGCAGTTCAGGAC3'	
Exon 6			
galns 6F	Sense –71/–51 nt	5' GGTGAAATCAGGGAGAACGGG3'	205
galns 6R	Antisense +48/+67 nt	5' GGATGAGGTTGGTCCGTCC3'	
Exon 7			
galns 7F	Sense –67/–49 nt	5' GGGGACCGTGGGAGGCATG3'	279
galns 7R	Antisense +48/+68 nt	5' CCCATCTCTGAGTCAAGCAC3'	
Exon 8			
galns 8F	Sense –70/–51 nt	5' GCCGTTGGCTGCCTGATCCA3'	277
galns 8R	Antisense +48/+67 nt	5' GGTCCAGGCACCTCTCCGCTG3'	
Exon 9			
galns 9F	Sense –107/–88 nt	5' CCGGGCCCTTTTCCTATG3'	284
galns 9R	Antisense +55/+73 nt	5' GAGGGGCCACACACCTTG3'	
Exon 10			
galns 10F	Sense –72/–54 nt	5' CCCAGGATTGGCCCCAG3'	292
galns 10R	Antisense +65/+83 nt	5' GGCCTGGGGTTGCACTG3'	
Exon 11			
galns 11F	Sense –69/–51 nt	5' CCCAGACCTCCAGGTGGC3'	239
galns 11R	Antisense +49/+67 nt	5' GCTCAGGGCCACGTCTGG3'	
Exon 12			
galns 12F	Sense –120/–101 nt	5' CTCTGTCCCTGTGGAGCCTG3'	321
galns 12R	Antisense +60/+79 nt	5' CTCTGTCCCTGTGGAGCCTG3'	
Exon 13			
galns 13F	Sense –69/–51 nt	5' GTCAGGCCAGCCCTCTC3'	261
galns 13R	Antisense +56/+74 nt	5' CGGTCATCTGGGCCCCG3'	
Exon 14			
galns 14F	Sense –67/–49 nt	5' CCGTGTCTGTTGGTCCAG3'	533
galns 14R-int	Antisense +361/+379 nt <sup>c</sup>	5' CCCAGCCACTCGGCACC3'	

<sup>a</sup> Numbered from each intron/exon junction according to den Dunnen et al. [14,15].

<sup>b</sup> 1 is the adenine of the initiator Met.

<sup>c</sup> 1 is the guanine of the stop codon.

### 2.4. RT-PCR analysis

A first full length reverse transcriptase PCR (RT-PCR) was performed with the following amplification primers:

I° PCR	Primer forward GALNS c1F 5' CAGCCCAGCCCGAAGGGCC 3' or GALNS OMF139 5' CGGGGCTCCGCGCTCCCGTGGTTG 3' or Primer reverse GALNS c6R 5' GGCAAAGCTGAGGGGGAACC 3'
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1–3 µl of first RT-PCR amplification was used as a template in a nested PCR using the following primers:

II° PCR (nested)	Primer forward GALNS c1F 5' CAGCCCAGCCCGAAGGGCC 3' or GALNS c2F 5' GGACCGGATGCTGCAGAA 3' or GALNS c3F 5' CAAGGCCAGGCCAACATCC 3' or Primer reverse GALNS c3R 5' CGCCGTTGCCGACGTGAAG 3' or GALNS c5R 5' ACACGTTGAGCTGGGCTGC 3'
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Cycle conditions were the same as those used for the analysis of genomic DNA.

### 2.5. Mutation nomenclature

The reported mutations are described according to mutation nomenclature (<http://www.hgvs.org/mutnomen/recs.html>) considering nucleotide +1 the A of the first ATG

translation initiation codon [14,15]. Nucleotide numbers are derived from cDNA *GALNS* sequence (EMBL/Gen Bank/ DDBJ; accession number NM\_000512).

## 2.6. Quantification of *GALNS* mRNA

The measurement of *GALNS* gene mRNA was performed using a quantitative real-time RT-PCR method, based on TaqMan™ technology. Probe and primers were selected by the computer program "Primer Express" (Applied Biosystems, Foster City, USA). For the detection of *GALNS* mRNA the following probe and primers were chosen on *GALNS* cDNA (1569 bp): probe 635/649 nt: 5' AAGCCCTGGACTTCA 3' labeled with FAM, located on exon 7, forward primer 608/629 nt: 5' CCAACCTCACCCAGATCTACT 3' which hybridizes on exon 6, reverse primer 664/651 nt: 5' GGTGCCGTGCTGTCTCTTA 3' located on exon 7. PCR analysis was performed using 25 ng of cDNA in a reaction mixture containing 300 nM of forward and reverse primers and 200 nM of the fluorescent probe, and 12.5 µl Universal master Mix. Plates were treated 2 min at 50 °C, 10 min at 95 °C and then submitted to 40 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min in the ABI Prism 7000 Sequence Detector PE Applied Biosystems (Foster City, USA). Plasmid vector, carrying *GALNS* gene transcript (pCXN-*GALNS*), was tenfold serially diluted from a starting quantity of  $11 \times 10^6$  plasmid copies to 11 plasmid copies and used as standard curve. The absolute values of *GALNS* gene mRNA products were expressed as copy/µg total RNA (mean ± SD).

## 2.7. Splice site score calculation

A numerical score expressing adherence to the consensus sequences was calculated by the <http://www.fruitfly.org/>.

## 2.8. Statistical analysis

Statistical analysis of different real-time assay measurement was carried out using the SPSS software package (SPSS INC, Chicago, IL). Statistical differences between

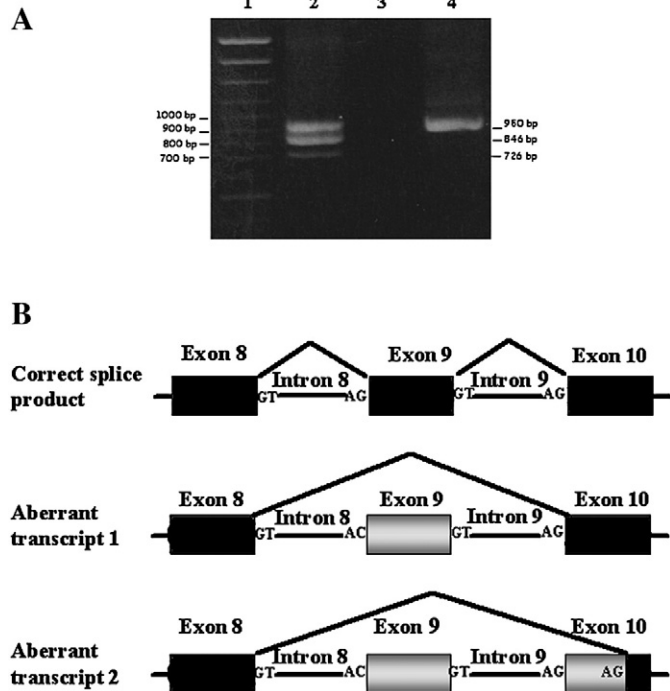


Fig. 1. A. RT-PCR analysis of MPS IVA patients. The PCR products, run on 2% agarose gel, were obtained after total RNA retrotranscription with random hexamers and two subsequent amplifications with the following primers:

I° PCR	GALNSc1F 5' CAGCCAGCCGGAAGGGCC 3'
	GALNSc6R 5' GGCAAAGCTGAGGGGGAACC 3'
II° PCR (nested)	GALNSc3F 5' CAAGCCAGGCCAACATCC 3'
	GALNSc5R 5' ACACGTTGAGCTGGGGCTGC 3'

1. Molecular weight marker, 2. Pt 1, 3. Pt 2, 4. Normal control. B. Schematic representation of the aberrant transcripts detected in MPS IVA Pt1. Black boxes mark the exons, white boxes indicate exonic sequence loss.

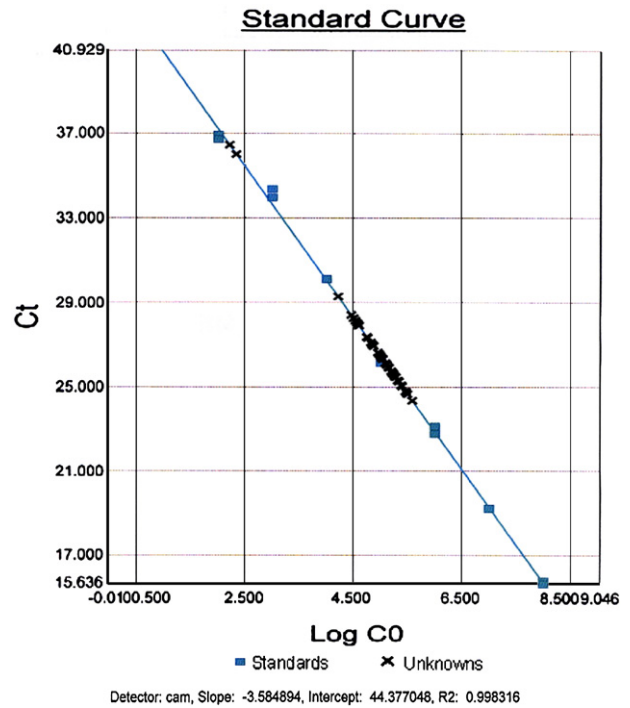


Fig. 2. Real-time standard curve. Amplification plot showing the standard curve of plasmid vector pCXN-*GALNS*, generated by seven tenfold serial dilution from a starting quantity of  $11 \times 10^6$  plasmid copies, and controls, Pt1 and Pt2 samples.

groups were assessed by *t*-test. Differences with  $p < 0.05$  were considered statistically significant.

## 3. Results

The fibroblasts from patients with enzymatic diagnosis of MPS IVA disease underwent molecular characterisation (Table 1). Exons and exon–intron boundaries of the *GALNS* gene were amplified and directly sequenced on both strands.

Sequence analyses of the *GALNS* gene of the MPS IVA patients led to the identification of two new alterations and a known splicing mutation. The new c.385A>T leading to the premature stop codon p. K129X and the c.899–1G>C splicing mutations were identified in Pt1 and the reported c.120+1G>A mutation was identified in a homozygous state in Pt2.

The splice acceptor site mutation c.899–1G>C, located in intron 8, led to the detection, by RT-PCR, of two aberrantly spliced transcripts (Fig. 1A): transcript 1, devoid of exon 9, and transcript 2, lacking the entire exon 9 and the first 120 bp of exon 10 (Fig. 1B). Sequence analysis showed that both transcripts led to a frameshift, introducing a premature stop codon.

The new c.385A>T nucleotide change, in exon 4, leading to the p. K129X nonsense mutation, was also detected in the mRNA by conventional RT-PCR and sequence analyses.

The known c.120+1G>A splicing mutation was identified in a homozygous state in Pt 2 with North African background. In this patient conventional RT-PCR analysis, performed with different amplification primers (by both specific 3' UTR primer and random hexamers), on agarose gel, failed to detect, any aberrant transcript (Fig. 1A).

Quantitative analysis of *GALNS* gene mRNA was performed by absolute real-time PCR, using probe and primers encompassing exon 6–7 junction, present in all Pt1's *GALNS* transcripts. To determine the quantity of mRNA in patients' specimens, pCXN-*GALNS* vector was used to generate the standard curve. Results, reported in Fig. 2, indicated that this assay effectively distinguished 10-fold differences

in concentration from about 11 to  $11 \times 10^6$  vectors' molecules per reaction mixture.

We analyzed total RNA from the fibroblasts of two MPS IVA patients and 15 normal controls. With respect to the standard deviation, the mean value of *GALNS* control mRNAs was not significantly different from that of Pt 1 sample (normal mean value:  $6.76 \times 10^5 \pm 3.17 \times 10^5$ , patient's mean value  $5.59 \times 10^5 \pm 2.8 \times 10^5$  molecules). Pt 2, homozygous for the c.120+1G>A mutation, showed an almost complete absence of the *GALNS* mRNA (only  $8.46 \times 10^2 \pm 2.61 \times 10^2$  molecules of *GALNS* mRNA were detected), confirming conventional RT-PCR data (Fig. 2 and Table 3).

In Table 4 we report the splice site score calculated for physiological and cryptic sites for the splicing of exon 1 and exon 9.

#### 4. Discussion

About 150 genetic lesions have been reported in the *GALNS* gene of mucopolysaccharidosis IVA (MPS IVA) patients, indicating remarkable genetic heterogeneity.

Here we report two Mediterranean patients affected by a severe form of the disease. We identified one known and two new mutations causing severe genetic lesions and contributing to the wide spectrum of allelic heterogeneity of this disease.

Computational analysis showed that these two splicing mutations dramatically decrease the numerical score of the invariant affected motifs (Table 4). Conventional *GALNS* RT-PCR analysis showed the almost complete absence of transcripts in Pt2 while in Pt1 the presence of three different transcripts was identified. One of such transcripts takes advantage of a cryptic splice site inside exon 10, whose computational score resulted to be very high (0.97).

Anyhow such end-point analysis was not precise enough to quantify the mRNAs. We therefore developed a real-time RT-PCR assay to quantify absolute mRNA copy numbers and to determine whether the *GALNS* transcripts of Pt1 were subject to NMD degradation.

NMD is a mechanism that, with some exceptions [16–20], down-regulates spliced mRNAs presenting premature termination codons about 50 to 55 nucleotides upstream of the subsequent exon–exon junction. Consequently, it prevents the production of the potentially toxic truncated proteins they encode [12,21].

Quantitative real-time PCR has been used in hematology and oncology to monitor response to treatment and detect minimal residual disease (MRD) in leukemia and lymphoma [22–24]. This accurate quantification of mRNAs expression levels can also be utilised for genotype–phenotype correlation and for investigating the pathogenetic mechanism of diseases. The real-time technology is now highly sensitive, accurate and simple enough to be adopted as a routine method for measuring gene levels. However, although a number of studies on real-time mRNA quantification of lysosomal genes have been performed [25–32], only one did reported on absolute mRNA copy number detection [33].

The presence in Pt1 of normal *GALNS* transcripts levels, identified by the real-time assay technique we describe, suggests that such mRNAs are not sensitive to NMD, according to what conventional RT-PCR analysis has indicated.

These results increase the number of known cases in which the nonsense-mediated mRNA decay surveillance system is bypassed

**Table 4**

Score calculation of canonical and cryptic splice sites by the <http://www.fruitfly.org/>

Patient	Transcript	Sequence	Splice site score
Pt1	Normal	INT8 acaagggccctctctcccaggtggcagcaacggcccttt EX9	0.94
	Mutant	INT8 acaagggccctctctcccaggtggcagcaacggcccttt EX9	<0.40
	Aberrant transcript 1	INT tgttgctgccatgttttcaggtgagccaccagctggcag EX	0.87
	Aberrant transcript 2	INT ctctccccacctctctcagggtggcctgatggacagtt EX	0.97
Pt2	Normal	EX1 ggacgacgtgagtg EX INT1	0.99
	Mutant	EX1 ggacgacatgagtg EX INT1	<0.40

[16,20,31,34,35]. Only the c.385A>T (p.K129X) mutation, which is 38 nucleotides upstream of the donor splice site of intron 4, fulfilled the condition that PTCs located within a distance of 50 to 55 nucleotides from the subsequent exon–exon junction do not trigger NMD. The reason why mRNAs, such as aberrant transcripts 1 and 2, caused by Pt 1's c.899–1G>C mutation, escape mRNA surveillance is unknown, but a modulation of NMD sensitivity mediated by additional, unidentified factors cannot be excluded [36].

Conventional RT-PCR analysis performed on Pt 2, who carried the c.120+1G>A at a homozygous level, failed to detect any aberrant transcript and the more sensitive and accurate real-time RT-PCR analysis showed very low *GALNS* transcript levels: only  $8.46 \times 10^2 \pm 2.61 \times 10^2$  copies compared to the  $6.76 \times 10^5 \pm 3.17 \times 10^5$  copies of normal controls.

In Pt2 a constitutive 5' splice donor site is mutated with a significant reduction of splice site score (0.99 vs <0.40). It is known that mutations affecting constitutive 5' splice site lead to the complete exon skipping or to the use of a nearby cryptic site. This could lead, in Pt2, to aberrant transcripts resulting in frame shift products containing PTCs. Such transcripts can be highly unstable and subject to NMD [12,21]. This could explain the almost complete absence of *GALNS* mRNA in this patient.

Pt 2, who was homozygous for the c.120+1G>A mutation, had consanguineous North African parents who were heterozygous for the mutation. The c.120+1G>A mutation has also been described in five Tunisian patients, with the classical form of MPS IVA, segregating in three families [37]. These results suggest a common ancestor in North African populations, as suggested by Laradi et al. [36].

Detection of severe mutations in these patients, leading to truncated proteins, with potentially deleterious activities, indicates a correlation between the genotype and severe phenotype severity.

We believe that it is important to determine the stability of PTC bearing transcripts in MPS IVA patients in order to assess the appropriateness of new therapeutic strategies. Recent studies indicate that it may be possible to correct the negative effect of mutations causing in-frame nonsense codons (stop codon read-through) using aminoglycoside antibiotics which, by virtue of their low molecular weight, are able to enter, by diffusion, cells, tissues and organs that are otherwise not easily accessible [38,39]. High levels of nonsense containing mRNAs, able to escape NMD, will result in an enhanced level of drug-induced read-through, making transcripts, from the allele with the c.385A>T mutation good candidates for this kind of therapy.

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**Table 3**

Number of *GALNS* copies detected in MPS IVA patients and control samples

Patient	Number of copies <i>GALNS</i> ±SD
1	$5.59 \times 10^5 \pm 2.8 \times 10^5$
2	$8.46 \times 10^2 \pm 2.61 \times 10^2$
Normal mean value	$6.76 \times 10^5 \pm 3.17 \times 10^5$

## References

- [1] Matalon R, Arbogast B, Justice P, Brandt IK, Dorfman A. Morquio's syndrome: deficiency of a chondroitin sulfate *N*-acetylhexosamine sulfate sulfatase. *Biochem Biophys Res Commun* 1974;61:759–65.
- [2] Singh J, Di Ferrante N, Niebes P, Tavella D. *N*-acetylglactosamine-6-sulfate sulfatase in man. Absence of the enzyme in Morquio disease. *J Clin Invest* 1976;57:1036–40.
- [3] Morquio L. Sur une forme de dystrophie osseuse familiale. *Bull Soc Pediatr (Paris)* 1929;27:145–52.
- [4] Maroteaux P, Lamy M. Studying the mucopolysaccharidoses. *Lancet* 1967;II:510.
- [5] Nelson J, Thomas PS. Clinical findings in 12 patients with MPS IVA (Morquio's disease). *Clin Genet* 1988;33:126–30.
- [6] Montaña AM, Tomatsu S, Gottesman GS, Smith M, Orii T. International Morquio A Registry: clinical manifestation and natural course of Morquio A disease. *J Inher Metab Dis* 2007;30:165–74.
- [7] Tomatsu S, Fukuda S, Masue M, et al. Mucopolysaccharidosis IVA: characterization and chromosomal localization of *N*-acetylglactosamine-6-sulfate sulfatase gene and genetic heterogeneity. *Am J Hum Genet (Suppl)* 1992;51:A178.
- [8] Baker E, Guo XH, Orsborn AM, et al. The Morquio A syndrome (mucopolysaccharidosis IVA) gene maps to 16q24.3. *Am J Hum Genet* 1993;52:96–8.
- [9] Masuno M, Tomatsu S, Nakashima Y, et al. Mucopolysaccharidosis IVA: assignment of the human *N*-acetylglactosamine-6-sulfate sulfatase (GALNS) gene to the chromosome 16q24. *Genomics* 1993;16:777–8.
- [10] Nakashima Y, Tomatsu S, Hori T, et al. Mucopolysaccharidosis IVA: molecular cloning of the human *N*-acetylglactosamine-6-sulfate sulfatase gene (GALNS) and analysis of the 5'-flanking region. *Genomics* 1994;20:99–104.
- [11] Tomatsu S, Filocamo M, Orii KO, et al. Mucopolysaccharidosis IVA (Morquio A): identification of novel common mutations in the *N*-acetylglactosamine-6-sulfate sulfatase (GALNS) gene in Italian patients. *Hum Mutat* 2004;24:187–8.
- [12] Maquat LE. Nonsense-mediated mRNA decay in mammals. *J Cell Sci* 2005;118(Pt 9):1773–6.
- [13] van Diggelen OP, Zhao H, Kleijer WJ, et al. A fluorimetric enzyme assay for the diagnosis of Morquio disease type A (MPS IVA). *Clin Chim Acta* 1990;187:131–9.
- [14] den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 2000;15:7–12.
- [15] den Dunnen JT, Paalman MH. Standardizing mutation nomenclature: why bother? *Hum Mutat* 2003;22:181–2.
- [16] Asselta R, Duga S, Spena S, et al. Congenital afibrinogenemia: mutations leading to premature termination codons in fibrinogen A alpha-chain gene are not associated with the decay of the mutant mRNAs. *Blood* 2001;98:3685–92.
- [17] Buzina A, Shulman MJ. Infrequent translation of a nonsense codon is sufficient to decrease mRNA level. *Mol Biol Cell* 1999;10:515–24.
- [18] Denecke J, Kranz C, Kemming D, Koch HG, Marquardt T. An activated 5' cryptic splice site in the human *ALG3* gene generates a premature termination codon insensitive to nonsense-mediated mRNA decay in a new case of congenital disorder of glycosylation type Id (CDG-Id). *Hum Mutat* 2004;23:477–86.
- [19] Perrin-Vidoz L, Sinilnikova OM, Stoppa-Lyonnet D, Lenoir GM, Mazoyer S. The nonsense-mediated mRNA decay pathway triggers degradation of most *BRCA1* mRNAs bearing premature termination codons. *Hum Mol Genet* 2002;11:2805–14.
- [20] Zhang J, Maquat LE. Evidence that translation reinitiation abrogates nonsense-mediated mRNA decay in mammalian cells. *EMBO J* 1997;16:826–33.
- [21] Solis AS, Shariat N, Patton. Splicing fidelity, enhancers, and disease. *JG Front Biosci* 2008;13:1926–42.
- [22] Dölken G. Detection of minimal residual disease. *Adv Cancer Res* 2001;82:133–85.
- [23] Jaeger U, Kainz B. Monitoring minimal residual disease in AML: the right time for real time. *Ann Hematol* 2003;82:139–47.
- [24] van der Velden V, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003;17:1013–34.
- [25] Potratz A, Huttler S, Bierfreund U, Proia RL, Suzuki K, Sandhoff K. Quantification of m-RNAs encoding proteins of the glycosphingolipid catabolism in mouse models of GM2 gangliosidosis and sphingolipid activator protein precursor (prosaposin) deficiency. *Biochim Biophys Acta* 2000;502:391–7.
- [26] Rice JE, Sanchez JA, Pierce KE, Wangh LJ. Real-time PCR with molecular beacons provides a highly accurate assay for detection of Tay-Sachs alleles in single cells. *Prenat Diagn* 2002;22:1130–4.
- [27] Hermans MM, van Leenen D, Kroos MA, et al. Twenty-two novel mutations in the lysosomal alpha-glucosidase gene (*GAA*) underscore the genotype-phenotype correlation in glycogen storage disease type II. *Hum Mutat* 2004;23:47–56.
- [28] Dhami R, Passini MA, Schuchman EH. Identification of novel biomarkers for Niemann-Pick disease using gene expression analysis of acid sphingomyelinase knockout mice. *Mol Ther* 2006;13:556–64.
- [29] Lualdi S, Pittis MG, Regis S, et al. Multiple cryptic splice sites can be activated by IDS point mutations generating misspliced transcripts. *J Mol Med* 2006;84:692–700.
- [30] Lualdi S, Di Rocco M, Corsolini F, et al. Identification of nine new IDS alleles in mucopolysaccharidosis II. Quantitative evaluation by real-time RT-PCR of mRNAs sensitive to nonsense-mediated and nonstop decay mechanisms. *Biochim Biophys Acta* 2006;1762:478–84.
- [31] Montfort M, Chabás A, Vilagelju L, Grinberg D. Analysis of nonsense-mediated mRNA decay in mutant alleles identified in Spanish Gaucher disease patients. *Blood Cells Mol Dis* 2006;36:46–52.
- [32] Raja M, Azzoni A, Giona F, et al. Movement and mood disorder in two brothers with Gaucher disease. *Clin Genet* 2007;72:357–61.
- [33] Caciotti A, Donati MA, Procopio E, et al. GM1 gangliosidosis: molecular analysis of 9 patients and development of an RT-PCR assay for *GLB1* gene expression profiling. *Hum Mut* 2007;28:204–14.
- [34] Romão L, Inácio A, Santos S, et al. Nonsense mutations in the human beta-globin gene lead to unexpected levels of cytoplasmic mRNA accumulation. *Blood* 2000;96:2895–901.
- [35] Wang J, Gudikote JP, Olivas OR, Wilkinson MF. Boundary-independent polar nonsense-mediated decay. *EMBO Rep* 2002;3:274–9.
- [36] Laradi S, Tukul T, Khediri S, et al. Mucopolysaccharidosis type IV: *N*-acetylglactosamine-6-sulfatase mutations in Tunisian patients. *Mol Genet Metab* 2006;87:213–8.
- [37] Danckwardt S, Neu-Yilik G, Thermann R, Frede U, Hentze MW, Kulozik AE. Abnormally spliced beta-globin mRNAs: a single point mutation generates transcripts sensitive and insensitive to nonsense-mediated mRNA decay. *Blood* 2002;99:1811–6.
- [38] Brooks DA, Muller VJ, Hopwood JJ. Stop-codon read-through for patients affected by a lysosomal storage disorder. *Trends Mol Med* 2006;12:367–73.
- [39] Kuzmiak HA, Maquat LE. Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. *Trends Mol Med* 2006;12:306–16.