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GALNS gene expression profiling in Morquio A patients' fibroblasts

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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 condroitin-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 hypoplasia, 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. 1–7 μg of total RNAs were reverse transcribed with Display THERMO-RT (Eppendorf, Hamburg, Germany) using the specific 3′ UTR primer 5′ GGAGGGTCCTGAAA-TCTGAGG 3′, according to the manufacturer.
2. 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 $MgCl_2$, 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[a]	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.
[a] In square brackets, citation refers to the first report.

Table 2
Primers for PCR amplification of *GALNS* gene

Primer name	Segment[a]	Oligonucleotide	Fragment length
Exon 1			
galns 1F	Sense −168/−148 nt[b]	5′ CTGGTCACGAGGCAGTCCAG3 ′	351
galns 1R	Antisense +46/+64 nt	5′ CTCCATCCGCCCCTCCCCT 3′	
Exon 2			
galns 2F	Sense −54/−34 nt	5′ CCAGAAGCACCTGCAGAAGGC 3′	231
galns 2R	Antisense +34/+53 nt	5′ CCTCGGCCTGTTGGGCTCAC 3′	
Exon 3			
galns 3F	Sense −65/−46 nt	5′ GTTGACGCCCGGGGTCTGAG 3′	208
galns 3R	Antisense +49/+68 nt	5′ CACCACCCGTAGCCCACCTG 3′	
Exon 4			
galns 4F	Sense −66/−46 nt	5′ GTTAGGATGGGGTTGGTGGCA 3′	231
galns 4R	Antisense +42/+62 nt	5′ GGAACCAAGGCCAGGAAGTGG 3′	
Exon 5			
galns 5F	Sense −51/−33 nt	5′ CCGAGTGTCCCCACGTGGG 3′	245
galns 5R	Antisense +31/+50 nt	5′ CGGGCACAGCAGTTCAGGAC 3′	
Exon 6			
galns 6F	Sense −71/−51 nt	5′ GGTGAAATCAGGGAGAACGGG 3′	205
galns 6R	Antisense +48/+67 nt	5′ GGATGAGGTTGGTGCGGTCC 3′	
Exon 7			
galns 7F	Sense −67/−49 nt	5′ GGGGACCGTGGGAGGCATG 3′	279
galns 7R	Antisense +48/+68 nt	5′ CCCATCTCTGGAGTCAAGCAC 3′	
Exon 8			
galns 8F	Sense −70/−51 nt	5′ GCCGTTGGCTGCCTGATCCA 3′	277
galns 8R	Antisense +48/+67 nt	5′ GGTCCAGGCACTCTTCGCTG 3′	
Exon 9			
galns 9F	Sense −107/−88 nt	5′ CCGGGCCCTTTGTCCCTATG 3′	284
galns 9R	Antisense +55/+73 nt	5′ GAGGGGCGCACACACCCTG 3′	
Exon 10			
galns 10F	Sense −72/−54 nt	5′ CCCAGGGATTGGCCCCCAG 3′	292
galns 10R	Antisense +65/+83 nt	5′ GGCCTGGGGGTTGCACCTG 3′	
Exon 11			
galns 11F	Sense −69/−51 nt	5′ CCCAGAGCCTCCAGGTGGC 3′	239
galns 11R	Antisense +49/+67 nt	5′ GCTCAGGGGCCACGTCTGG 3′	
Exon 12			
galns 12F	Sense −120/−101 nt	5′ CTCTGTCCCTGTGGAGCCTG 3′	321
galns 12R	Antisense +60/+79 nt	5′ CTCTGTCCCTGTGGAGCCTG 3′	
Exon 13			
galns 13F	Sense −69/−51 nt	5′ GTCCAGGCCAGCCCCTCTC 3′	261
galns 13R	Antisense +56/+74 nt	5′ CGGTTCATCCTGGGCCCCG 3′	
Exon 14			
galns 14F	Sense −67/−49 nt	5′ CCGCTGCTGCTGGGTCCAG 3′	533
galns 14R-int	Antisense +361/ +379 nt[c]	5′ CCCAGCCACCTCGGCACC 3′	

[a] Numbered from each intron/exon junction according to den Dunnen et al. [14,15].
[b] 1 is the adenine of the initiator Met.
[c] 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′ CAGCCCAGCCGGAAGGGCC 3′ or GALNS OMF139 5′ CGGGGCTCCGCGGCTCCCGTGGTTG 3′ Primer reverse GALNS c6R 5′ GGCAAAGCTGAGGGGGAACC 3′

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′ CAGCCCAGCCGGAAGGGCC 3′ or GALNS c2F 5′ GGACCGGATGGCTGCAGAA 3′ or GALNS c3F 5′ CAAGGCCAGGCCCAACATCC 3′ Primer reverse GALNS c3R 5′ CGCCGTTGTCCGACGTGAAG 3′ or GALNS c5R 5′ ACACGTTGAGCTGGGGCTGC 3′

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′ CCAACCTCACCCAGATCTACCT 3′ which hybridizes on exon 6, reverse primer 664/651 nt: 5′ GGTGCCGTGCTTGTCTCTTA 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 11×10^6 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 groups were assessed by *t*-test. Differences with $p<0.05$ were considered statistically significant.

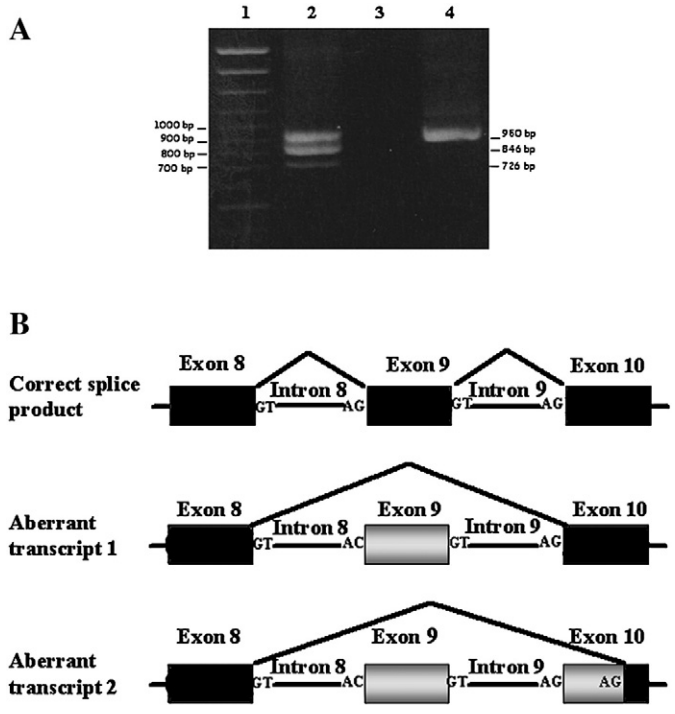

Fig. 1. A. RT-PCR analysis of MPS IVA patients. The PCR products, run on 2% agarose gel, were obtained after total RNA retrotranscription with random hexamers and two subsequent amplifications with the following primers:

I° PCR	GALNSc1F 5′ CAGCCCAGCCGGAAGGGCC 3′
	GALNSc6R 5′ GGCAAAGCTGAGGGGGAACC 3′
II° PCR (nested)	GALNSc3F 5′ CAAGGCCAGGCCCAACATCC 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.

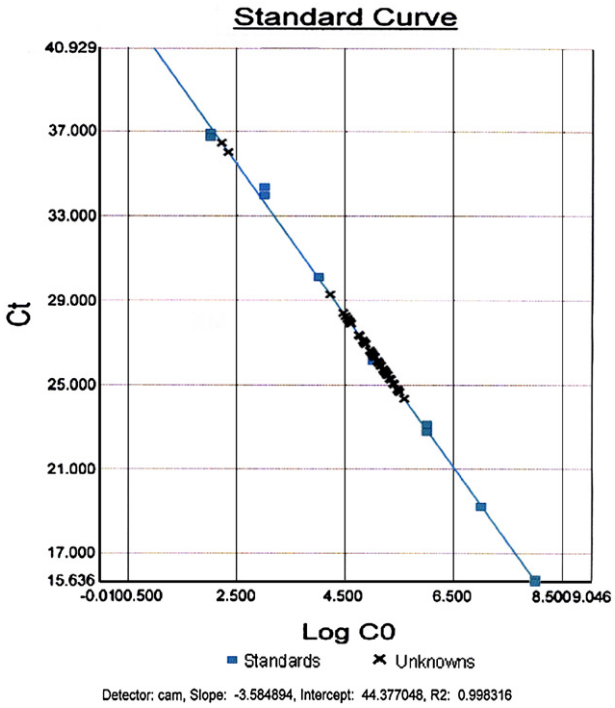

Fig. 2. Real-time standard curve. Amplification plot showing the standard curve of plasmid vector pCXN-*GALNS*, generated by seven tenfold serial dilution from a starting quantity of 11×10^6 plasmid copies, and controls, Pt1 and Pt2 samples.

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 at 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 11×10^6 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: $6.76\times10^5\pm3.17\times10^5$, patient's mean value $5.59\times10^5\pm2.8\times10^5$ molecules). Pt 2, homozygous for the c.120+1G>A mutation, showed an almost complete absence of the *GALNS* mRNA (only $8.46\times10^2\pm2.61\times10^2$ 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.

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 acaagggcccctctctccc**ag**gtggcagcaacggccccttt EX9	0.94
	Mutant	INT8 acaagggcccctctctccc**ac**gtggcagcaacggccccttt EX9	<0.40
	Aberrant transcript 1	INT tgttgctgccatgtgtttc**ag**gtgagccaccagctgggcag EX	0.87
	Aberrant transcript 2	INT ctcctccccaccctcctgc**ag**ggccggctgatggacaggtt EX	0.97
Pt2	Normal	EX1 ggacgac**gt**gagtgc INT1	0.99
	Mutant	EX1 ggacgac**at**gagtgc INT1	<0.40

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 [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 $8.46\times10^2\pm2.61\times10^2$ copies compared to the $6.76\times10^5\pm3.17\times10^5$ 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.

Table 3
Number of *GALNS* copies detected in MPS IVA patients and control samples

Patient	Number of copies *GALNS*±SD
1	$5.59\times10^5\pm2.8\times10^5$
2	$8.46\times10^2\pm2.61\times10^2$
Normal mean value	$6.76\times10^5\pm3.17\times10^5$

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