J Hum Genet (2008) 53:565–572
DOI 10.1007/s10038-008-0263-5

SHORT COMMUNICATION

Novel synonymous substitution in *POMGNT1* promotes exon skipping in a patient with congenital muscular dystrophy

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Received: 21 December 2007 / Accepted: 21 January 2008 / Published online: 11 March 2008

Abstract Walker-Warburg syndrome, muscle-eye-brain disease, Fukuyama congenital muscular dystrophy, congenital muscular dystrophy type 1C, and congenital muscular dystrophy type 1D are overlapping clinical entities belonging to a subgroup of the congenital muscular dystrophies (CMD), collectively designated dystroglycanopathies, in which the common underlying defect is hypoglycosylation of alfa-dystroglycan. Currently, six different genes are known to be implicated in these diseases: *POMT1*, *POMT2*, *POMGNT1*, *FCMD*, *FKRP*, and *LARGE*. We report the molecular characterization of a patient presenting clinical features of CMD and reduced immunostaining for alfa-dystroglycan in muscle. Three candidate genes (*FCMD*, *POMT1* and *POMGNT1*) were analyzed, and a total of 18 sequence variants were detected: 15 polymorphisms in *POMT1* [including three unreported single nucleotide polymorphisms (SNPs)], two polymorphisms in *FCMD*, and the exonic silent mutation c.636C > T in *POMGNT1*. Expression analysis revealed that this apparently silent mutation compromises correct premessenger RNA (mRNA) splicing, promoting skipping of the entire exon 7, with a consequent frameshift. In silico analysis of this mutation did not predict alterations in the canonical splice sequences, but rather the creation of a new exonic splice silencer. The recognition of such disease-causing elements may contribute to the further understanding of RNA processing and assist mutation screening in routine diagnosis, where such changes may be underestimated. To aid clinical diagnosis, we generated publicly available LOVD-powered Locus Specific Databases for these three genes and recorded all known sequence variants (http://www.dmd.nl).

Keywords Congenital muscular dystrophy · POMGNT1 · Silent mutation · Exon skipping · Exonic splice silencer

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Introduction

The dystroglycanopathies constitute a subgroup of the congenital muscular dystrophies (CMD), in which abnormal glycosylation of the alfa-dystroglycan subunit is the common underlying defect. These include Walker–Warburg syndrome (WWS; MIM 236670), muscle-eye-brain disease (MEB, MIM 253280), Fukuyama congenital muscular dystrophy (FCMD, MIM 253800), congenital muscular dystrophy type 1C (MDC1C, MIM 606612), and congenital muscular dystrophy type 1D (MDC1D, MIM 608840). These disorders represent a continuum in severity, sharing similar clinical features that include congenital or neonatal hypotonia, ocular abnormalities, and mental retardation associated with defects in neuronal migration (reviewed by Martin 2006).

Several genes have been identified as implicated in these diseases: *FCMD* (Kobayashi et al. 1998), *FKRP* (Brockington et al. 2001a), *POMGNT1* (Yoshida et al. 2001), *POMT1* (Beltran-Valero de Bernabe et al. 2002), *LARGE* (Longman et al. 2003), and *POMT2* (van Reeuwijk et al. 2005). Mutations in the *POMGNT1* gene, encoding protein O-mannose β-1,2-*N*-acetylglucosaminyltransferase, were initially described as the underlying cause of MEB (Yoshida et al. 2001). Since then, patients with FCMD and WWS phenotypes have been reported as having mutations in this gene as well, suggesting a broader clinical heterogeneity than initially recognized (Taniguchi et al. 2003).

Previous biochemical studies demonstrated that *POMGNT1* catalyzes the transfer of *N*-acetylglucosamine to *O*-mannose of glycoproteins. Although this type II membrane protein is similar to other Golgi glycosyltransferases, *O*-mannosylation is an uncommon type of glycosylation in mammals, occurring in a limited number of glycoproteins from brain, nerve, and skeletal muscle (Liu et al. 2006).

To date, 29 different mutations have been reported in the *POMGNT1* gene, the majority of which are single-base changes resulting in missense mutations (34.5%) or in the disruption of consensus splice-site motifs (31.0%). Other mutation types include deletions (17.2%), duplications (10.3%), and nonsense mutations (6.9%). These are seen to be distributed throughout the *POMGNT1* gene, with no obvious mutational hotspots. A single frequent recurrent mutation, c.1539 + 1G > A, is found in the Finnish population, in which it accounts for around 99% of the MEB chromosomes (Diesen et al. 2004).

This report describes a new pathogenic variant in exon 7 of the *POMGNT1* gene detected in homozygosity in a patient presenting clinical features of severe CMD and reduced alfa-dystroglycan expression in the muscle. This single silent base change, located 16 nucleotides upstream of intron 7, was predicted to be silent at the translational level and not expected to interfere with RNA splicing. However, when analyzed at the RNA level, it was seen to cause exon skipping, resulting in a frameshifted transcript with a premature stop codon. We discuss the possible mechanism(s) by which correct pre-messenger RNA (pre-mRNA) splicing may be compromised in this case and emphasize the importance of investigating silent base changes that at first glance appear to be benign.

Materials and methods

Patient

The patient was an 8-year-old girl presenting CMD with clinical features compatible with MEB or severe FCMD. She presented multifocal epilepsy, mental retardation, visual impairment, axial hypotonia with distal hypertonia, hyporeflexia, and elevated serum creatine phosphokinase (CPK) levels (>1,500 U/l). Brain magnetic resonance imaging (MRI) revealed bilateral deep frontal and temporal leukodystrophy, involving cerebellar white matter, associated with brainstem atrophy. The common metabolic and mitochondrial disorders were excluded. The parents where not available for study. Informed consent was obtained from the child's legal tutor.

Muscle biopsy

Muscle sections from a left deltoide specimen were subjected to routine histochemical evaluation and immunohistochemical analysis. No necrosis or regeneration was observed. The only alteration was a slight increment in the normal variability of the size of muscle fibers, internal nuclei, and some fibrosis. Immunostaining was normal for dystrophin, alfa-, beta-, and gama-sarcoglycans, as well as merosin. Analysis using the monoclonal antibody VIA4-1 (Upstate Biotechnology, Lake Placid, NY, USA), which binds specifically to glycosylated alfa-dystroglycan residues, revealed markedly reduced staining.

Molecular analysis

Based on onset, clinical signs, and muscle biopsy data, three candidate genes were selected for analysis: *FCMD*, *POMT1,* and *POMGNT1*. Genomic DNA was extracted from peripheral blood by the salting-out method (Miller et al. 1988). M13-tailed primers for genomic DNA (gDNA) sequencing were designed to amplify all the coding exons and directly flanking intronic sequences, as well as the entire sequence of introns 6 and 7 of the *POMGNT1* gene (primer sequences available upon request). Sequencing was carried out with M13 universal primers using the BigDye^{TM} Terminator Cycle Sequencing Kit V1.1 and the products resolved on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Mutation analysis was aided by SeqScape V2.5 software (Applied Biosystems). Population screening for all new variants was also carried out by sequencing in 110 anonymized control samples.

RNA analysis

The effect of the c.636C > T variant on splicing of *POMGNT1* pre-mRNA was analyzed by conventional reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR. Total RNA was isolated from cryopreserved muscle specimens from the patient and three

controls (with no related disease) using TRIzol isolation reagent (Invitrogen, CA, USA), and complementary DNA (cDNA) obtained using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems). For conventional RT-PCR, the following primers were designed to encompass exons 4–9: (Pomgnt1-4F) 5′-GTAGAGGTGTAT TCAAGTCGCAGC-3′, and (Pomgnt1-9R) 5′-ACA-CTTCCATAGCCCTCAACTTTG-3′. The same pair was used for subsequent cDNA sequencing. *POMGNT1* gene expression levels were quantified by real-time RT-PCR using the comparative CT method (ΔΔ CT; Livak and Schmittgen 2001). Three sets of TaqMan probes and primers were used: a beta-actin gene-specific set as an endogenous control and two different target regions for the *POMGNT1* transcripts, spanning the junctions of exons 2–3 and 6–7 (assay ID Hs01086332_m1 and Hs01086328_m1; Applied Biosystems). Singleplex reactions were prepared with TaqMan Universal PCR master mix with primers, probe, and 100 ng of cDNA, according to the manufacturer's instructions. For each sample, three replicate reactions were prepared and amplified on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Data acquisition and analysis was performed using the Relative Quantification software version 1.1.

Bioinformatic tools

The Genscan program (http://genes.mit.edu/GENSCAN.html[t1]) was used to evaluate the influence of the mutation on gene splicing (Burge and Karlin 1997). Normal and mutated sequences were also scanned for exonic splice enhancers (ESE) using the program ESEfinder version 2.0 (http://rulai.cshl.edu/tools/ESE/[t2]) (Cartegni et al. 2003) to check for a possible influence on elements responsive to human splicing regulatory proteins SF2/ASF, SC35, SRp40, and SRp55. Scanning for exonic splice silencers (ESS) was carried out with the aid of the application Sequence Manipulation Suite—DNA pattern find tool (http://www.bioinformatics.org/sms2/dna_pattern.html[t3]) using the consensus sequences CUAGAGGU, [U/G]

Table 1 Sequence changes detected in *FCMD*, *POMT1*, and *POMGNT1* genes

Gene/location	Sequence variation	Protein effect	Interpretation[a]
FCMD—NM_139309.2			
Exon 5	c.[608A > G] + [=]	p.Arg203Gln	Polymorphism—reference sequence ambiguity
Exon 8	c.[1026A > C] + [=]	–	NCBI—SNP rs17309806
POMT1—NM_007171.2			
Intron 2	c.[123-5dupT] + [=]	–	New polymorphism—frequency: 14.3%
Intron 4	c.[281-82C > T] + [281-82C > T]	–	NCBI—SNP rs6597501
Intron 5	c.[428-21T > C] + [428-21T > C]	–	NCBI—SNP rs11243404
Exon 8	c.[751C > T] + [=]	p.Arg251Trp	Polymorphism (Beltran-Valero de Bernabe et al. 2002)
Intron 8	c.[766-48A > G] + [766-48A > G]	–	NCBI—SNP rs2018621
Intron 9	c.[922-65G > A] + [=]	–	New polymorphism—frequency: 7.1%
Intron 9	c.[922-49T > G] + [922-49T > G]	–	NCBI—SNP rs4740163
Intron 10	c.[1052 + 49G > A] + [1052 + 49G > A]	–	NCBI—SNP rs10901066
Intron 11	c.[1148 + 16G > A] + [=]	–	Polymorphism (Beltran-Valero de Bernabe et al. 2002)
Intron 11	c.[1149-64C > T] + [=]	–	New polymorphism—frequency: 14.3%
Intron 14	c.[1432-61A > G] + [1432-61A > G]	–	NCBI—SNP rs1547768
Intron 17	c.[1764 + 48C > G] + [1764 + 48C > G]	–	NCBI—SNP rs2277152
Intron 17	c.[1764 + 107C > A] + [1764 + 107C > A]	–	NCBI—SNP rs2277153
Intron 19	c.[2069 + 13C > T] + [2069 + 13C > T]	–	NCBI—SNP rs4740165
Intron 19	c.[2070-70C > T] + [=]	–	NCBI—SNP rs10122068
POMGNT1—NM_017739.2			
Exon 7	c.[636C > T] + [636C > T]	p.Asp179ValfsX23	Splicing mutation—not detected in 220 control alleles

NCBI National Center for Biotechnology Information, *SNP* single nucleotide polymorphism

[a] Frequency of new polymorphisms as determined in 110 normal controls

G[U/A]GGGG and UCUCCCAA, as described by Sironi et al. 2004. Alterations in mRNA secondary structure were tentatively inferred with the aid of the mFold program (Mathews et al. 1999; Zuker 2003) (http://www.bioinfo.rpi.edu/applications/mfold[t4]) by comparing stretches of the normal and mutated pre-mRNA sequence.

Mutation nomenclature and databases

Sequence variations were described according to the Human Gene Variation Society (HGVS) mutation nomenclature recommendations (den Dunnen and Antonarakis 2001). The following cDNA reference sequences from RefSeq project [National Center for Biotechnology Information (NCBI)] were used: NM_139309.2 (*FCMD*), NM_007171.2 (*POMT1*), and NM_017739.2 (*POMGNT1*). The locus-specific databases (LSDB) for the *POMT1*, *POMGNT1*, and *FCMD* genes were created using the LOVD software (Fokkema et al. 2005) available through the Leiden Muscular Dystrophy pages (http://www.dmd.nl[t5]; see respectively http://www.LOVD.nl/FCMD[t6], http://www.LOVD.nl/POMT1[t7], and http://www.LOVD.nl/POMGNT1[t8]). All the variants detected in this patient were submitted to the respective LSDBs.

Results

The three genes chosen for direct sequencing, *FCMD*, *POMT1*, and *POMGNT1*, seemed the best candidates based on the patient's clinical presentation and muscle histology. Table 1 summarizes all the sequence variants that were detected and their significance. The involvement of *FCMD* and *POMT1* was excluded because all the variants were polymorphisms. *POMT1* was found to be particularly polymorphic, with a total of 15 noncausative variants in this patient, three of which had not been reported previously (see http://www.LOVD.nl/POMT1).

Sequencing results for the *POMGNT1* gene revealed the presence of an apparently silent nucleotide change in exon 7, c.636C > T, in homozygosity (Fig. 1a). This variation was not described in the literature or in the main publicly available SNP databases. None of the 110 controls harbored the c.636C > T variation, suggesting a frequency lower than 0.5%. In the patient, no sequence variants were detected in introns 6 or 7.

Although the base change was located 16 nucleotides upstream of intron 7 and thus unlikely to interfere with the canonical splice-site sequences, cDNA analysis was carried out to exclude the presence of altered *POMGNT1* transcripts. The results revealed the presence of a single cDNA fragment, smaller than that of the control sample (Fig. 1b). Upon sequencing, the observed size difference was seen to correspond to the absence of exon 7 (r.535_652del). No normal transcripts were detected in the patient's sample.

To rule out a possible amplification bias toward the smaller fragment, and therefore to further characterize the *POMGNT1* gene expression changes in the patient, relative quantification of this transcript was performed using two different target regions in real-time RT-PCR. Results with the probe for the exon 6–7 boundary showed that the amount of transcript carrying exon 7 was residual, as revealed by the high cycle-threshold (CT > 34, Fig. 2a). Additionally, results for the probe that binds to the exon 2–3 boundary suggested a sixfold reduction in the overall expression of this gene when compared with normal controls (Log 10 0.79 RQ, Fig. 2b).

Several bioinformatics tools were used with the aim of understanding the mutation's effect on splicing. The Genscan program indicated a low donor splice-site score for exon 7 (5′ splice-site score = 0.40) but did not predict any splicing alteration when comparing the reference and the mutated sequences (data not shown). In the screening for other splice control elements, rather than the suspected disruption of an ESE, a striking change was predicted with the creation of a new consensus ESE motif for SRp40 as

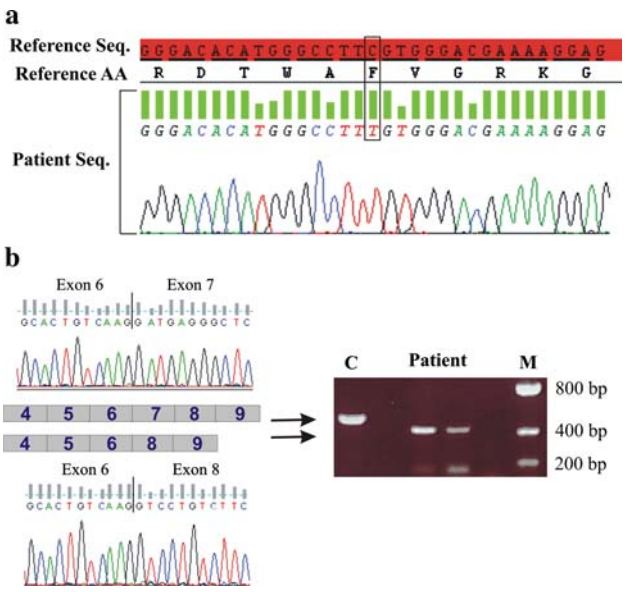

Fig. 1 **a** Electropherogram of patient's exon 7 disclosing the homozygous mutation c.636C > T. **b** *POMGNT1* expression analysis at the messenger RNA (mRNA) level by reverse transcriptase polymerase chain reaction (RT-PCR). Amplification of *POMGNT1* transcripts encompassing exons 4–9 revealed the presence of a shorter fragment in the patient (118 bp smaller) compared with the control. Direct sequencing from complementary DNA (cDNA) confirmed skipping of exon 7 in the patient (the *faint lower band* is a product of nonspecific amplification). *Vertical bars* above the sequence indicate PHRED quality values. *Seq* sequence, *AA* aminoacids, *C* control, *M* molecular weight marker

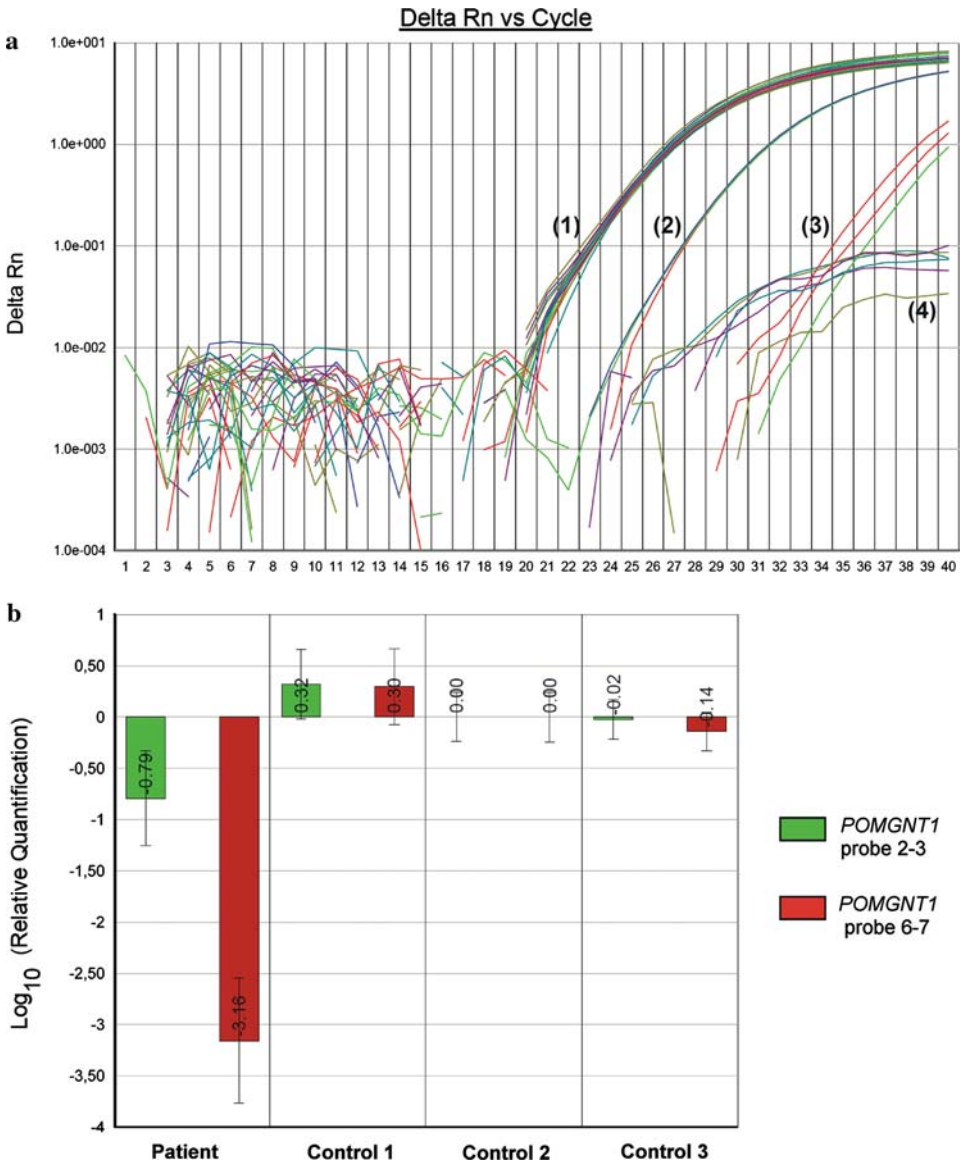

Fig. 2 Quantification of *POMGNT1* gene expression levels by real-time reverse transcriptase polymerase chain reaction (RT-PCR). **a** Amplification plot ΔRn vs. cycle number in experiments performed in triplicate: *1* three control samples with probe 2–3 and probe 6–7 (18 plots), *2* the patient's sample with probe 2–3 (three plots), *3* the patient's sample with probe 6–7 (three plots), and *4* no template reaction for each probe set (six plots). **b** Relative quantification results for probes 2–3 (*first bar*) and 6–7 (*second bar*) using control 2 as a calibrator sample

well as for the ESS motif TGTGGG (Fig. 3a). The mFold program, with the pre-mRNA sequence arbitrarily chosen from nucleotides 51705–50385 (genomic reference sequence AL672043.15), did not predict a significant alteration in the secondary structure, although there was an increase in the number of consecutive nucleotide connections and hence a possible increase in the stability of the stem-loop structure (Fig. 3b).

Discussion

The characterization of genes and enzymes involved in the glycosylation pathway of alfa-dystroglycan has been a subject of intensive investigation in the last few years. However, the posttranslational processing of this dystroglycan subunit is yet to be fully understood, and some important steps of this pathway remain to be elucidated (Barresi and Campbell 2006). In terms of the molecular diagnosis of CMD patients, the main difficulty is the identification of target genes, because very few specific biochemical markers are available, linkage analysis is restricted to a few informative families, and some clinical phenotypes overlap and are not easily distinguished. Often, the only solution is to extend the molecular study to consecutive candidate genes in search for a causative variant.

In our case, the severe clinical presentation of the disease, with cerebral and ocular involvement and the marked reduction of alfa-dystroglycan in the muscle, prompted the search for mutations in the *FCMD*, *POMT1*, and *POMGNT1* genes. Since LSDBs were missing, part of our study involved collecting and reviewing all known DNA variants

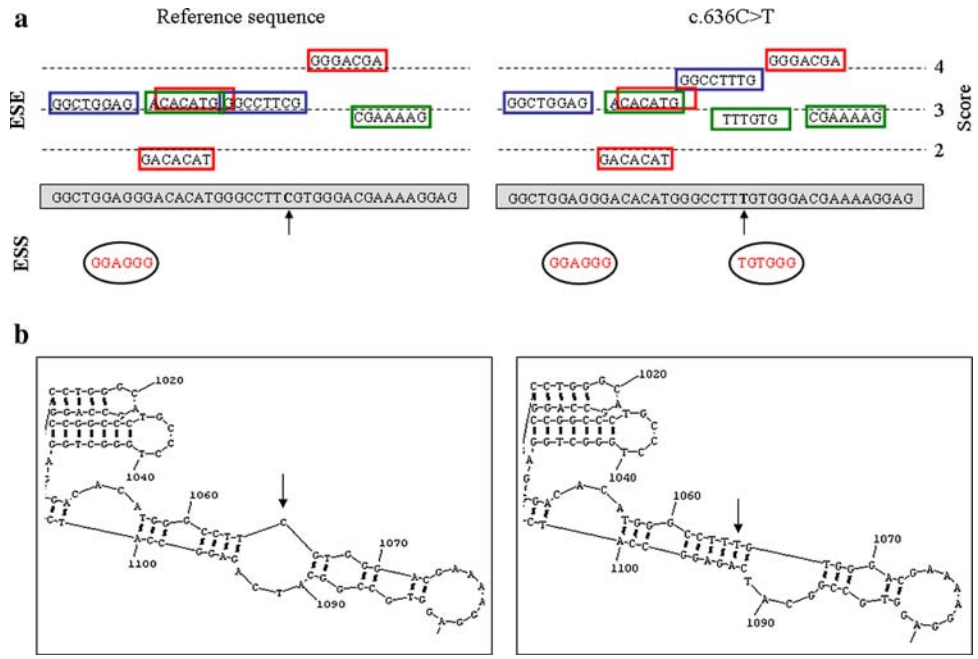

Fig. 3 In silico analysis of the c.636C > T mutation detected in exon 7 of the *POMGNT1* gene. **a** Distribution of splicing control motifs in the normal and mutated sequence. Exon splice enhancer (ESE) motifs as predicted by ESEfinder are *boxed*: SF2/ASF (GACACAT; CACATGG; GGGACGA), SC35 (GGCTGGAG; GGCCTTTG; GGCCTTCG), and SRp40 (ACACATG; TTTGTG; CGAAAAG). Exon splice silencer (ESS) motifs detected for the consensus sequences described by Sironi et al. 2004 are *circled*. **b** RNA secondary structure prediction by mFold software, with thermodynamically most stable pre-messenger RNA (mRNA) structure for part of exon 7 and intron 7. *Arrows* indicate the affected nucleotide

for these three genes from sources such as HGMD, OMIM, and scientific literature. Based on this work, and to aid clinical diagnosis in general, as advocated by Cotton et al. (2007), we decided to initiate Web-accessible databases (LSDBs) for *FCMD*, *POMT1*, and *POMGNT1*. These LSDBs can be accessed freely through the Leiden Muscular Dystrophy pages (urls http://www.LOVD.nl/FCMD[t10], http://www.LOVD.nl/POMT1[t11], and http://www.LOVD.nl/POMGNT1[t12] resp.). To keep the knowledge on these genes as up to date as possible, we suggest that researchers and clinicians worldwide submit their new findings there.

Among a total of 18 new and known sequence variants that were detected, only the transcriptionally silent c.636C > T change in the *POMGNT1* gene appeared not to be a polymorphism, since it was not detected in 220 control alleles.

RNA analysis showed that this synonymous base change has a severe effect on *POMGNT1* gene expression, compromising correct pre-mRNA splicing by removal of exon 7. This aberrant transcript results in a frameshift leading to a premature stop codon that is predicted to produce a truncated polypeptide (p.Asp179ValfsX23). No changes were detected in introns 6 or 7, which makes c.636C > T the only obvious change accountable for the aberrant splicing.

Silent mutations and predicted missense mutations have often been reported to interfere with pre-mRNA splicing (reviewed by Cartegni et al. 2002; Faustino and Cooper 2003; Wang and Cooper 2007). This results from either (a) direct disruption of canonical splice sequences and/or activation of cryptic splice sequences, (b) abolition or creation of cis-acting exonic signals such as enhancers, silencers, or composite exonic regulatory elements (CERES) (Pagani et al. 2003a), or (c) alteration of the pre-mRNA secondary structure (Buratti et al. 2004; Baralle and Baralle 2005).

Exon 7 of the *POMGNT1* gene has a suboptimal donor splice site, so its exonization is likely to rely heavily on cis-acting elements. In this case, in silico analysis of the base change surprisingly revealed the creation of an SRp40-specific ESE adjacent to a preexisting similar sequence and overlapping with an Sc35-specific ESE. However, the mutation also appeared to create a new ESS, overlapping with the same Sc35-binding sequence. This particular ESS motif (TGTGGG), previously shown to exert its negative effect by binding heterogeneous nuclear ribonucleoprotein (hnRNP) H (Chen et al. 1999), appears to be a powerful splicing silencer (Chen et al. 1999; Pagani et al. 2003b; Sironi et al. 2004). Additionally, the mFold program revealed that pre-mRNA folding may suffer a change toward a slightly more stable stem-loop structure in the

vicinity of the mutation. The available algorithms are currently unable to integrate all these factors as well as other restraints such as sterical hindrance and RNA processivity to provide a reliable prediction of the mutation's effect.

It is conceivable that the almost complete absence of normal transcript in this case reflects the cumulative effect of the different inhibitory influences or the upset of a fine balance between positive and negative regulatory elements. The elucidation of how all such factors intervene in the splicing process will contribute toward the development of upcoming personalized therapy. Indeed, depending on the outlay of the neighboring authentic regulatory elements in each case, such mutation-derived ESS motifs should represent further candidates amenable to correction with antisense oligonucleotide therapeutics. The feasibility of this approach has recently been demonstrated in examples of mutation-driven involvement of cryptic splice sites (Uchikawa et al. 2007).

Direct experimental evidence of the mechanism(s) involved in this case is beyond the scope of our study; however, this report widens the spectrum of disorders in which synonymous base changes have a drastic effect on pre-mRNA processing. The knowledge of such sequence effects, which are thought to be underrepresented in the literature, may contribute toward the refinement of predictive algorithms used in the diagnostic setting and ultimately aid in the development of targeted therapeutic approaches.

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