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Neuromuscular Disorders 17 (2007) 143–147

www.elsevier.com/locate/nmd

Quantitative analysis of CAPN3 transcripts in LGMD2A patients: Involvement of nonsense-mediated mRNA decay

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Received 10 July 2006; received in revised form 6 September 2006; accepted 11 October 2006

Abstract

Limb girdle muscular dystrophy type 2A (LGMD2A) is caused by single or small nucleotide changes widespread along the CAPN3 gene, which encodes the muscle-specific proteolytic enzyme calpain-3. About 356 unique allelic variants of CAPN3 have been identified to date. We performed analysis of the CAPN3 gene in LGMD2A patients at both the mRNA level using reverse transcription-PCR, and at the DNA level using PCR and denaturing high performance liquid chromatography. In four patients, we detected homozygous occurence of a missense mutation or an in-frame deletion at the mRNA level although the DNA was heterozygous for this mutation in conjuction with a frame-shift mutation. The relationship observed in 12 patients between the quantity of CAPN3 mRNA, determined using real-time PCR, and the genotype leads us to propose that CAPN3 mRNAs which contain frame-shift mutations are degraded by nonsense-mediated mRNA decay. Our results illustrate the importance of DNA analysis for reliable establishment of mutation status, and provide a new insight into the process of mRNA decay in cells of LGMD2A patients.

Keywords: LGMD2A; CAPN3; Calpain-3; Real-time RT-PCR; Nonsense-mediated mRNA decay

1. Introduction

Limb girdle muscular dystrophies (LGMDs) are a clinically and genetically heterogeneous group of disorders characterised by progressive involvement of proximal limb girdle muscles. Autosomal recessive LGMDs of type 2 include at least 10 different genetic entities. LGMD2A, the most prevalent form, is caused by mutations in the CAPN3 gene which is localized at 15q15.1-q21.1 and encodes the proteolytic enzyme calpain-3 [1]. The CAPN3 gene comprises 24 exons and covers a genomic region of 50 kb. Its transcript is spliced to a 3.5 kb muscle-specific mRNA, which is translated to a 94 kDa protein. Calpain-3 is a member of the calpain

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doi:10.1016/j.nmd.2006.10.001

superfamily, which processes enzymes involved in signalling pathways, transcription factors, and cytoskeletal proteins thereby modulating their activities [2].

In our previous studies [3,4], we described results of CAPN3 gene analysis and discussed the relation between the mutations detected and the patients' phenotype. In this study, we completed our analyses on DNA (using PCR, denaturing high performance liquid chromatography, and sequencing) and mRNA (using reverse transcription, PCR, and sequencing) to have both analyses in each patient. In four patients carrying the genotypes P82L/T184RfsX36, P82L/D772delK773NfsX3, F200_L204del/ T184RfsX36 and R490W/T184RfsX36, mRNA analysis detected the homozygous occurrence of the missense mutation or the in-frame deletion, but DNA analysis detected heterozygous occurrence of these together with the frame-shift mutation (T184RfsX36 or D772delK773NfsX3) which was not detected by mRNA analysis. It is probable that the process termed nonsense mediated mRNA decay (NMD) induced degradation of mRNA carrying these frame-shift mutations [5–8]. Further, we performed quantitative analysis of CAPN3 mRNA in 12 LGMD2A patients: 2 patients had a genotype missense/missense mutation; 1 in-frame deletion/missense mutation; 3 missense/frame-shift mutation, 1 in-frame deletion/frame shift mutation, 1 in-frame deletion/nonsense mutation, 3 frame-shift/frame-shift mutation and 1 patient had the genotype in-frame deletion/intron mutation. We evaluated the relationship between the amount of CAPN3 mRNA and the genotype.

2. Patients and methods

2.1. Patients

Clinical findings for patients 2, 3, 4, 6–12 were presented in our previous works [3,4]. Patients 1 and 5 are newly described here. Age of disease onset was 8 and 5 years, respectively. At present, patient 1 is 33 years old and is wheel-chair bound since the age of 30. Patient 5 is 7 years old, and suffers from mild proximal muscle weakness in the upper and lower extremities. In both patients, immunohistochemical analysis of calpain-3 using Western blot showed 94 kDa band of a low intensity.

2.2. Immunohistochemical analysis, Western blot, RNA analysis, and DNA analysis

Immunohistochemical analysis of muscle proteins in tissue sections, Western blot of calpain-3 as well as RNA isolation from muscle tissue, reverse transcription, and amplification of calpain-3 mRNA, DNA isolation, amplification of all CAPN3 exons and DHPLC analysis have been described in our previous studies [3,4].

2.3. Real-time PCR

100 ng of RNA were used for reverse transcription (RT) using the SuperScript™ First-Strand Synthesis System (Invitrogen). The cDNA synthesis reactions were primed by random hexamers. RT products were amplified using SybrGreen PCR Core Reagents (Applied Biosystems) under the following cycling conditions: initial denaturation: 95°C/10 min; followed by 40 cycles: 95 °C/10 s, 55 °C/15 s, 72 °C/30 s. Fluorescence was monitored at 72 °C and specificity of amplification was evaluated by melting curve analysis. The analysis of each RNA sample was performed in two parallel PCRs in two or three independent experiments. Primer sequences are shown in Table 1. Amplification of the CAPN3 mRNA was performed using the set of three couples of primers that amplified three various regions of calpain-3 mRNA. Relative amount of CAPN3 mRNA was taken as the average of the results of relative quantifications of three different CAPN3 mRNA regions. The data obtained were analysed by the $2^{-\Delta\Delta Ct}$ method that evaluates the target gene expression with respect to the reference gene expression and normalizes this value relative to expression in a calibrator sample [9]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxantine guanine phosphoribosyltransferase 1 (HPRT), and ABL1 protein tyrosine kinase (ABL) were used as the reference genes and the RNA of patient 1 was used as the calibrator.

3. Results

3.1. Analysis of calpain3 mRNA and DNA

In 12 LGMD2A patients, mRNA analysis of the CAPN3 gene was subsequently confirmed by DNA

Table 1
Primers used for real-time PCR

Primer	Sequence of forward primer (5′ → 3′direction)	Primer	Sequence of reverse primer (5′ → 3′direction)	Length of PCR product
GAPDH-F	cctgcaccaccaactgctta	GAPDH-R	gaggcagggatgatgttctg	178
HPRT-F	gcagactttgctttccttgg	HPRT-R	tcaagggcatatcctacaacaa	153
ABL-F	tggagataacactctaagcataactaaaggt	ABL-R	gatgtagttgcttgggaccca	123
CAPN-1F	tcctacgaagctctgaaagg	CAPN-1R	ttccataggtcatgttcgtg	183
CAPN-2F	gacgatgaccctgatgactc	CAPN-2R	aggcgttgtacaggaagaag	186
CAPN-3F	ttcatctgctgcttcgttag	CAPN-3R	gctttggaaatagagggtga	193

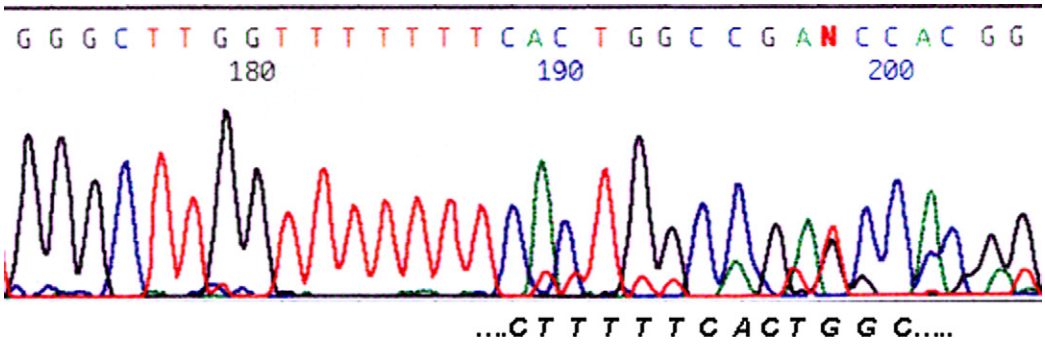

Fig. 1. Example of sequencing of the mRNA region with the in-frame mutation K595_K596. The sequence of the mRNA bearing standard sequence in this region, and mutation R748X further to the 3′ end, is represented by lower peaks and is given below in bold italics.

analysis. However, in patients 3, 4, 5, and 6 the results of mRNA and DNA analysis were not concordant. We detected homozygous occurence of the mutations P82L (patients 3 and 4), F200_L204del (patient 5) and R490W (patient 6) by mRNA analysis, but analysis at the DNA level did not confirm the homozygous status of these mutations. DHPLC analysis of all CAPN3 exons and subsequent sequencing detected the mutation T184RfsX36 in addition to the mutation P82L (patient 3), D772delK773NfsX3 in addition to P82L (patient 4), T184RfsX36 in addition to F200_L204del (patient 5) and T184RfsX36 in addition to R490W (patient 6). In the case of patient 12 (genotype K595_K596del/R748X), DNA as well as mRNA analysis gave concordant results, but it is evident (Fig. 1) that the amounts of mRNA carrying K595_K596del and R748X are not similar. In the case of patient 11, we detected heterozygous occurence of the in-frame deletion F200_L204del by mRNA analysis but no second mutation. DNA analysis detected the mutation in intron 13 (1746-20C > G) but this CAPN3 variant probably has no noticeable phenotypic effect (Leiden muscular dystrophy pages, www.dmd.nl). The mutation Y75C detected in case of patient 1 was not described so far. The tyrosine in the position 75 is the second amino acid of the calpain-3 catalytic domain. Sequence alignment of this region of several species (human, macaca, pig, mouse, rat, and chicken) confirmed the conservation of tyrosine in this position. Some species like sheep and bovine have another aromatic amino acid, phenylalanine, in this position. The phenotype of patient 1 is rather similar to phenotype of patients carrying mutations affecting translational reading frame. The results of mRNA and DNA analyses are shown in Table 2.

3.2. Real-time PCR

Quantitative analyses of CAPN3 mRNA using real-time PCR and the $2^{-\Delta\Delta Ct}$ method were evaluated in relation to the mRNAs of three housekeeping genes GAPDH, HPRT, and ABL. Results of these analyses are shown in Table 3. Relative to GAPDH, the patients with genotypes missense/missense or in-frame deletion/missense had an average amout of CAPN3 mRNA 1.07; the patients with genotype missense/frame-shift, in-frame deletion/frame-shift or in-frame deletion/nonsense had an average amount 0.39; and the patients with genotypes frame-shift/frame-shift had an average amount 0.03. Relative to HPRT, these values were 0.69; 0.41; and 0.01, respectively, and relative to ABL,

Table 2
Mutations detected in the set of LGMD2A patients

Patient	Mutation detected in allele 1	Position of mutation	Mutation detected in allele 2	Position of mutation	Age of disease onset
1	c.224A>G; p.Y75C	Exon 1	c.224A > G; p.Y75C	Exon 1	8
2	c.133G>A; p.A45T	Exon 1	c.133G > A; p.A45T	Exon 1	15
3	c.245C>T; p.P82L	Exon 1	c.550delA; p.T184RfsX36	Exon 4	12
4	c.245C>T; p.P82L	Exon 1	c.2314-2317del; p.D772delK773NfsX3	Exon 22	13
5	c.550delA; p.T184RfsX36	Exon 4	c.598-612del; p.F200_L204del	Exon 4	5
6	c.550delA; p.T184RfsX36	Exon 4	c.1468C > T; p.R490W	Exon 11	12
7	c.550delA; p.T184RfsX36	Exon 4	c.550delA; p.T184RfsX36	Exon 4	10
8	c.550delA; p.T184RfsX36	Exon 4	c.1981delA; p.I661X	Exon 17	7
9	c.550delA; p.T184RfsX36	Exon 4	c.1722delC; p.F574FfsX21	Exon 13	10
10	c.598-612del; p.F200_L204del	Exon 4	c.2245A > C; p.N749H	Exon 21	17
11	c.598-612del; p.F200_L204del	Exon 4	c.1746-20C > G	Intron 13	25
12	c.1783-1788del; p.K595_K596del	Exon 15	c.2242C > T; p.R748X	Exon 21	20

Table 3
Results of real-time PCR: Relative amount of CAPN3 transcripts relative to three housekeeping genes using CAPN3 mRNA of patient 1 as calibrator

Patient	Relative amount of CAPN3 mRNA with respect to			Average value
	GAPDH	HPRT	ABL	
1	1.00	1.00	1.00	1.00
2	1.2047 ± 0.0084	0.6462 ± 0.0045	0.5804 ± 0.0040	0.810 ± 0.010
3	0.285 ± 0.075	0.354 ± 0.082	0.257 ± 0.059	0.30 ± 0.13
4	0.313 ± 0.044	Not evaluated	0.238 ± 0.035	0.276 ± 0.056
5	0.435 ± 0.014	0.405 ± 0.012	0.374 ± 0.011	0.405 ± 0.022
6	0.604 ± 0.053	0.415 ± 0.037	0.732 ± 0.071	0.584 ± 0.087
7	0.0139 ± 0.0093	0.0066 ± 0.0044	0.0187 ± 0.0058	0.013 ± 0.012
8	0.0231 ± 0.0039	0.0097 ± 0.0038	0.0287 ± 0.0035	0.0205 ± 0.0067
9	0.0412 ± 0.0072	0.0118 ± 0.0021	0.0176 ± 0.0031	0.0235 ± 0.0081
10	0.995 ± 0.037	0.417 ± 0.015	0.569 ± 0.021	0.660 ± 0.045
11	0.795 ± 0.044	0.876 ± 0.049	1.092 ± 0.061	0.921 ± 0.089
12	0.295 ± 0.018	0.483 ± 0.035	0.638 ± 0.046	0.472 ± 0.061

0.72; 0.45; 0.02, respectively. When the results of all reference genes were taken into account, these values were 0.82; 0.41; and 0.02, respectively.

4. Discussion

The relative abundance and lifetime of any RNA molecule is governed by the balance between the rates of its synthesis and degradation. Increasing the destruction rate is perhaps the fastest means of modulating RNA level. Therefore, although the field of RNA decay is relatively young [10], its importance for regulation of RNA metabolism has become swiftly recognized as an apparent equivalent to proteasome-mediated degradation of proteins. NMD is a post-transcriptional process that rapidly degrades mRNA with premature translation termination codons. This limits the production of potentially toxic truncated proteins. The NMD pathway is initiated during translation when the responsible proteins discriminate premature from normal translation termination and mark the transcript for rapid decay. The distinction between a normal and a premature translation stop codon is made on the basis of its location with respect to the last exon–exon junction: If the termination codon is positioned >50–55 nucleotides upstream it is considered premature, and the mRNA is targeted for rapid decay [11]. NMD is an evolutionarily selected response because it reduces the severity of some genetic diseases caused by truncating dominant-negative mutations [12,13]. However NMD can also increase the severity of genetic diseases when truncating mutation does not destroy completely normal function. Thus, evidence suggests that NMD worsens the phenotype of many genetic diseases including e.g. Duchenne muscular dystrophy [14] and Ullrich's congenital muscular dystrophy [15,16]. Here we examined for the first time potential involvement of NMD in pathology of LGMD using a set of patients carrying various combinations of mutated alleles. To do this, we complemented our previous work [3,4] by performing DNA and mRNA analyses in all the patients presented and, especially, by quantitative analysis of their CAPN3 transcripts.

Positions of all frame-shift and nonsense mutations detected in our patients match the above mentioned exon–exon-junction rule [11] and thus constitute potential targets of NMD. In patients 3, 4, 5, and 6, mRNA analysis detected homozygous occurence of a mutation with no effect on translation reading frame, but DNA analysis detected a further frame-shift mutation. Degradation of mRNA carrying this frame-shift mutation was probably caused by NMD. It is interesting that in patient 12 mRNA analysis detected both the in-frame deletion and the nonsense mutation, but it is clear (Fig. 1) that the level of the mRNA carrying R748X is lower than that of the mRNA carrying K595_K596del. Further, we performed quantitative analysis of CAPN3 transcripts using real-time PCR using the $2^{-\Delta\Delta Ct}$ method for data evaluation; the amount of CAPN3 mRNA was determined with respect to GAPDH, HPRT, and ABL mRNAs, and then relative quantification was performed by reference to patient 1 chosen as the calibrator (quantity of CAPN3 mRNA = 1). Relative to GAPDH, a common housekeeping gene, the patients with genotypes missense/missense or in-frame/missense had an average amout of CAPN3 mRNA 1.07; the patients with a missense mutation or an in-frame deletion on one allele and a frame-shift mutation or a nonsense mutation on the second allele had an average amount 0.39; and the patients with genotypes frame-shift/frame-shift mutation had an average amount 0.03. Quantitative analysis of CAPN3 mRNA in 5 control samples (mRNA from patients without suspicion of inherited neuromuscular disease) showed relative transcript levels from 1.00 to 1.35.

While in the presence of a single frame-shift or a nonsense mutation, the level of CAPN3 transcripts

decreases to about 40%, its presence on both alleles results in just 2% of the reference value. Accordingly, in the case of a single frame-shift or nonsense mutated allele, the transcript of the other allele (maintaining the reading frame) is strongly overrepresented. These quantitative differences in CAPN3 transcript levels, which reflect precisely the type of mutations involved, provide the first evidence for a role of NMD in destruction of aberrant transcripts in LGMD patients. Our results thus illustrate the practical importance of DNA analysis for a reliable establishment of mutation status, and provide an interesting insight into the processes of mRNA decay in cells of LGMD2A patients.

Acknowledgements

This work was supported by IGA MHCR, Project No. NR/8087-3, and the institutional support MSM0021622415. The authors thank Professor Ronald Hancock, Hôtel-Dieu Hospital, Quebec, Canada, for the manuscript revision.

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