Research Article • DOI: 10.2478/s13380-011-0028-z • Translational Neuroscience • 2(3) • 2011 • 241-245
Translational Neuroscience
VERSITA
Springer

NON-INVASIVE PROTEIN ANALYSIS IN THE FIRST DYSFERLINOPATHY CROATIAN FAMILIES

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Abstract

Mutations in human dysferlin (*DYSF*) gene cause both limb girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy (MM), also named dysferlinopathy. They are autosomal recessive muscular dystrophies characterized by degeneration and weakness of proximal and/or distal limb girdle muscles caused by partial or complete absence of a sarcolemmal protein dysferlin. The size and large mutational spectrum of *DYSF* impose a multistep diagnosis strategy before gene analysis. Here we report the first three patients from two unrelated Croatian families in which diagnosis of dysferlinopathy was suggested on the basis of clinical picture, family history and linkage analysis. In order to confirm the presumed diagnosis, we performed a blood-based assay in which dysferlin expression is screened in blood monocytes. All three tested patients showed complete absence of dysferlin expression, giving strong evidence of dysferlinopathy that was recently confirmed by mutation analysis. In conclusion, we would suggest the presented diagnostic strategy as a reliable and non-invasive method to be used as an alternative to muscle tissue protein analysis in routine diagnostics of dysferlinopathies, prior to the more complex and demanding search for causative *DYSF* mutations. This non-aggressive approach seems especially useful in situation in which multiplex Western blot (WB) analysis of different muscular dystrophy proteins on muscle sample is not available.

Keywords

• LGMD2B • MM • *DYSF* • Dysferlin • Monocytes • Western blotting • Linkage analysis

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Received 22 August 2011
accepted 11 September 2011

1. Introduction

The term "dysferlinopathy" [1] is related to autosomal recessive muscular disorders involved in at least two clinically distinct muscular dystrophies due to mutations in the same gene (phenotypic heterogeneity): proximal disease designated LGMD2B (OMIM #253601) [2], affecting limb girdle muscles and the distal Miyoshi myopathy (MM; OMIM #254130) [3]. They are both caused by mutations in the *DYSF* gene (OMIM #603009), located on chromosome 2p13, spanning region of more than 150kb and containing 55 exons [4,5]. *DYSF* mutations are distributed along the entire length of the gene, with no obvious hot spots and where the majority of more than 300, so far reported, sequence variants (including disease-causing mutations and non-pathogenic polymorphisms) represent single nucleotide changes and small insertion and/or deletions [Leiden Muscular Dystrophy pages database: www.dmd.nl].

DYSF gene encodes dysferlin, a 237 kDa transmembrane protein belonging to a ferlin family of proteins, named after homology to *C. elegans* spermatogenesis factor Fer-1. The main characteristic of this protein family is the presence of multiple C2 domains (ranging from four to seven, latter being the number present in dysferlin), which are implicated in various biological processes [6]. Recent data suggest a role of dysferlin in the muscle fiber repair process [7-9], myoblast differentiation [10], T-tubulogenesis [11], monocyte phagocytosis [12] and muscle regeneration [13]. Dysferlin is predominantly expressed in skeletal and heart muscle, where it localizes to the plasma membrane of muscle fibers [14,15]. In addition, dysferlin is also found in blood monocytes, brain, lung and placenta [2,3,15-17].

Although accurate and definitive diagnosis of dysferlinopathies relies on direct gene mutation analysis, screening of the whole gene for mutations is laborious, expensive, time-consuming and an inappropriate approach for routine laboratories. Protein analysis, on the other hand, proved to be reliable for differential diagnostics of muscular dystrophies [1,18]. Moreover, above mentioned dysferlin expression in blood monocytes enabled the development of a blood-based assay that analyses dysferlin expression from a peripheral blood sample [16]. This assay is a relatively simple and quick method of dysferlin analysis, resembling the usual protein analysis from muscle. However, it is a significantly less invasive and painless approach that overcomes the problems associated with handling and storage

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of muscle samples. Therefore it represents a valuable alternative to muscle tissue protein analysis, as well as an important and decisive step prior to the start of demanding gene mutational analysis.

Herein, we present the application of the specific diagnostic strategy [19] adapted to our population of 4,29 milion people, followed by linkage anaylsis and monocyte protein analysis of dysferlin that has permitted us to confirm the first diagnosis of dysferlinopathy in two families from Croatia.

2. Materials and Methods

2.1. Patients

A total of three patients from two unrelated families were included in the study (Figure 1). In Family No.1, two siblings (brother and younger sister) were born to non-affected, apparently non-related parents from close villages. Both patients, aged 39 and 28 years, respectively, showed characteristic clinical features (both were good in sports in childhood and adolescence; they had early adult onset with massive elevation of serum CK levels, asymmetry of legs, "dysferlin gait" and showed relatively slow progression) which, together with genealogical data, directed us to the diagnosis of LGMD2B. On the other hand, a 34-year old male patient from Family No.2 with two year older, healthy brother was born to unrelated parents originating from the same, small geographic region. In this family, clinical data together with disease course during 10-year follow-up (active in sport in childhood and early adolescence; was accidentally found to have high transaminases at the age of 19 years; seven years later his slender right leg was noticed and CK was 21x normal; slow progression of symptoms with bilateral asymmetric muscle wasting and weakness in the lower legs) suggested a diagnosis of Miyoshi myopathy (MM).

2.2. Methods

Following informed consent, peripheral blood samples were used for DNA extraction of patients and family members (presented in Figure 1) and monocyte isolation of the three patients. DNA was extracted using standard salting-outand procedure [20], while monocyte isolation is described later in the text.

Protein analysis of dysferlin was done in monocytes for all three patients and one healthy individual who was used as a positive control of dysferlin expression. A muscle biopsy sample obtained from an individual not affected by any muscle disorder served as another positive control.

Haplotype analysis. Haplotype analysis was performed using two intragene markers (Cy172-H32 and 104-sat), as well as four polymorphic microsatellite markers located in the close vicinity of *DYSF* locus: D2S292, D2S2113, D2S291 and D2S2111. The marker genotyping consisted of PCR reaction for each marker and DNA-PAGE followed by polyacrylamide gel silver staining. PCR amplification of DNA (200 ng) was performed in a 25 µl reaction mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM of each dNTP (Eppendorf, Germany), 1 U Taq polymerase (Eppendorf, Germany) and 3 pmol of each primer. Initial denaturation (5 min at 94°C) was followed by 30 cycles consisting of denaturation (30 sec at 92˚C), annealing (30 sec), and elongation (30 sec at 72˚C). Annealing temperatures were as follows: 52˚C (D2S292), 56˚C (Cy172-H32 and 104-sat), 60˚C (D2S291 and D2S2113) and 64˚C (D2S2111). The primer sequences and marker allele sizes were obtained from literature [2,3] and from www.gdb.org and www.cephb.fr, respectively.

Muscle biopsy. The muscle biopsy sample, frozen in liquid nitrogen, was homogenized in 20 volumes of Treatment buffer (75mM Tris, 15% SDS, 20% glycerol, 5% p-mercaptoethanol and bromophenol blue solution). After denaturation at 95°C for 5 minutes, homogenate was centrifuged for 5 minutes at 14000 rpm.

Monocyte isolation. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood sample of patients by Ficoll-Hypaque gradient centrifugation. In continuation, PBMCs were separated into monocytes (CD14$^+$) and lymphocytes (CD14$^-$) using CD14-coated microbeads (Milteny Biotech, Germany) and MACS separator according to manufacturer's instructions and as previously described [16,21]. Isolated monocytes were then lysed with a lysis buffer consisting of 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na$_2$EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium-pyrophosphate, 1mM β-glycerolphosphate, 1mM Na$_3$VO$_4$, 1µg/ml leupeptin and 1mM PMSF.

Protein analysis. Protein concentration was determined using the Bradford protein assay. Approximately 20 µg of proteins (from muscle homogenate and cell lysate, respectively) were separated on a 4-7% gradient SDS-PAGE. Immunoblotting was performed as previously described [22], using anti-dysferlin primary

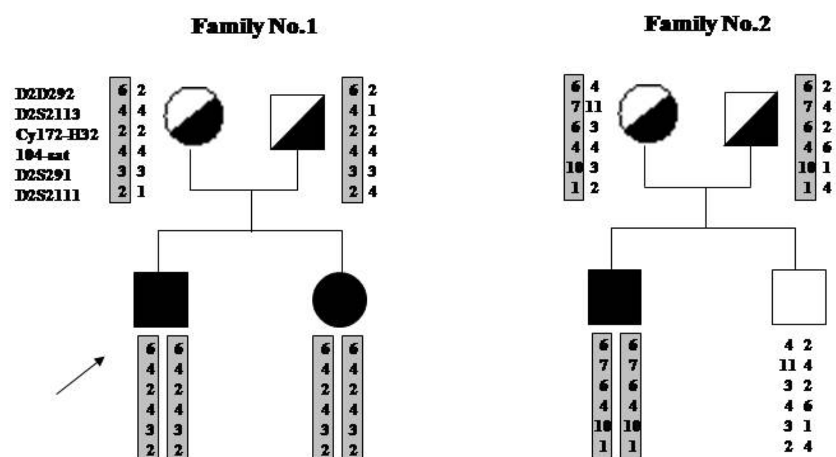

Figure 1. Pedigrees of two Croatian dysferlinopathy families and results of haplotype analysis. Black squares and circles represent affected males and females, respectively. Healthy individual is represented by white square, while half black squares and circles represent healthy heterozygotes. Marker loci are listed from telomere to centromere and are presented in front of Family No 1. Haplotypes carrying dysferlin mutations are shown within the grey box, while unboxed haplotypes represent wild type chromosomes.

monoclonal antibodies (Hamlet; Novocastra, Newcastle upon Tyne, UK). Protein bands were detected with chemiluminescence system Super Signal®West Pico Chemiluminescent Substrate (Pierce, Rockford, USA).

3. Results

Diagnosis suggested by clinical features was followed by linkage analysis and then by protein analysis on isolated monocytes from peripheral blood.

Haplotype analysis. The results of haplotype analysis are presented in Figure 1. In both families affected members are homozygous for two intragene and four polymorphic markers flanking the *DYSF* gene. However, haplotypes are different in each family: 6/4/2/4/3/2 in Family No.1 and 6/7/6/4/10/1 in Family No.2, respectively.

Protein analysis. Western blot analysis of monocytes from all three patients demonstrated the absence of dysferlin expression, when compared with the two controls: skeletal muscle sample and monocyte's cell lysate from two healthy individuals, respectively (Figure 2).

4. Discussion

Direct gene analysis, with identification of causative mutation(s), represents definitive confirmation of a clinically suspected diagnosis of dysferlinopathy. Such an accurate diagnosis of a genetic defect is nowdays prerequisite for participation in gene [23] and/or pharmacological [24-26] therapy treatments. However, in some disorders including dysferlinopathy, the size of the gene, as well as the large mutational spectrum impose the need for a different, usually multistep diagnostic approach prior to gene screening.

In our first family, clinical and genealogical data (two of three affected children of different gender born to apparently non-related parents originating from the same village), together with characteristic clinical features and disease course in the second family, directed us to the diagnoses of LGMD2B and MM, respectively. The availability of the patients' parents and a healthy sibling in family No. 2 permitted the first step in confirming the suspected diagnosis i.e. linkage analysis using two intragene (Cy172-H32 and 104-sat) and four polymorphic markers located in a close vicinity of DYSF gene (D2S292, D2S2113, D2S291 and D2S2111). Intragene markers are located in intron 2 (Cy172-H32) and intron 29 (104-sat) of *DYSF*, respectively. While the first is a CCAT repeat, the latter is characterised by a CA dinucleotide repeating unit [2,3]. The same repeats are also found in four polymorphic microsatellite markers used in this study. They are positioned on chromosome 2p as listed in the text, D2S2113 and D2S291 being the closest to the *DYSF* gene [27]. Results of linkage analysis (Figure 1), suggested the existence of linkage for dysferlinopathy in both families. Different haplotypes were obtained in each family, but interestingly in both families haplotypes of affected persons were in a homozygous state, both for intragene and extragene markers. This might indicate the existence of the same mutation on both alleles in each of the two studied families. These findings could be useful in future testings of new dysferlinopathy families from the same region of Croatia.

As linkage analysis was in accordance with dysferlinopathy, our next diagnostic step was to check dysferlin expression, as previously published studies showed the reliability of this approach in confirming LGMD2B diagnosis [18,28]. In order to avoid the more invasive method of muscle biopsy we focused on a recently developed blood based assay, that analyses dysferlin expression in non-muscle tissue, i.e. a blood sample. Western blotting (WB) on monocytes was done according to the protocol described first by Ho *et al*. [16] and later improved by de Luna *et al*. [21]. This improved protocol emphasizes the importance of monocyte separation from other peripheral mononuclear cells. As Western blot of total peripheral mononuclear cells can raise false negative results, monocyte separation makes the assay more reliable and effective [21]. In our experience the main advantage of this improvement was a significant decrease in the expression of an additional protein band (mostly present in non-monocyte cells), which reacted very strongly with used antibodies and therefore made difficult the interpretation of obtained results.

Analysis of blood samples of all 3 patients revealed the total absence of dysferlin expression (Figure 2) providing strong evidence of the clinically suggested diagnosis. Nevertheless, confirmatory mutational analysis (done, but not shown here) is nowadays mandatory, in order to ensure accurate genetic counseling and future therapy.

There are several publications containing data that suggest that dysferlin analysis from a blood sample is a major improvement in

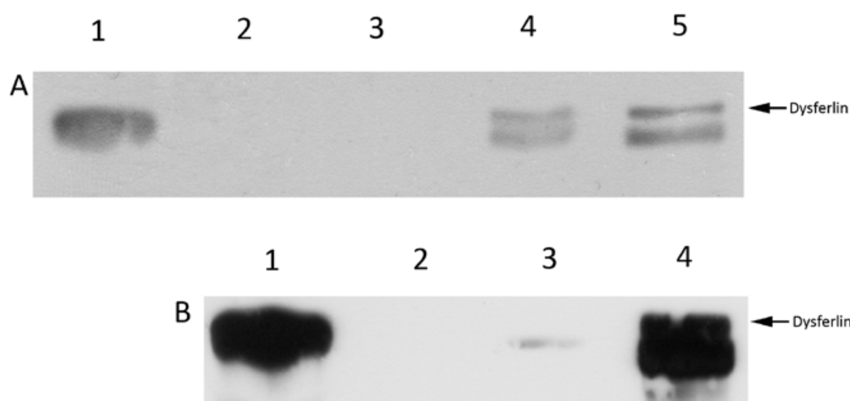

Figure 2. Western blot analysis of dysferlin expression. a. Column 1 represents control muscle, while columns 4 and 5 correspond to monocyte cell lysate from a healthy individual. Columns 2 and 3 represent two LGMD2B patients (Family No.1) with complete absence of dysferlin expression. b. Column 1 corresponds to the control muscle sample, while no sample was loaded to column 2 (empty); column 3 represents monocyte cell lysate of patient from Family No.2 in which complete absence of dysferlin expression was obtained; column 4 corresponds to monocyte cell lysate from the control healthy individual.

the diagnostics of dysferlinopathy, with the possibility of routine use. In almost all of them, lack and reduction of dysferlin expression was followed by molecular confirmation of dysferlinopathy [16,21,29,30]. In the first published article based on 12 patients with dysferlinopathy (LGMD2B, MM, distal anterior compartment myopathy) [16] blood-based assay proved to be as reliable as previously performed protein analysis from muscle sample, showing dysferlin absence in all 12 studied patients. Moreover, in 3 of them the causative *DYSF* mutation was identified. In another article [29], concerning two dysferlin gene mutation carriers, significant reduction of dysferlin expression both in monocytes and muscle was also confirmed by *DYSF* mutation found on one allele by genetic analysis. De Luna *et al*. [21] performed WB on monocytes in 25 patients, and all of them displayed absence of dysferlin. These results were confirmed several times and were compared either by dysferlin analysis in skeletal muscle and/or by genetic analysis, giving the 100% reproducibility of the used blood based assay. Just recently, Meznaric *et al.* [30] described the first two dysferlinopathy patients in Slovenia, in which a severe reduction of dysferlin was found both in muscle samples and in monocytes and finally confirmed by *DYSF* causative mutations.

However, it should be emphasized that the use of blood-based assay is still limited, i.e. the majority of routine laboratories are still giving priority to common dysferlin analysis from muscle. This can be explained by routine work in which only muscle samples permit multiplex western blot analyses of different muscular dystrophy proteins [22], which is a very useful initial step in the analysis of patients with limb-girdle muscular dystrophy in centers equipped with numerous antibodies available for direct testing of proteins encoded by LGMD2 genes. On the other hand, in cases where economic or other circumstances do not permit such study, dysferlin blood-based assay seems to be more suitable as it has proven to be a reliable initial orientation prior to the final diagnosis confirmation by gene mutational analysis.

Acknowledgements

We gratefully acknowledge the participation of patients and their family members in this study. This study was supported by grants No. 0108052/2002 and 10800000003435/2007 from the Ministry of Science, Education and Sports of the Republic of Croatia.

References

[1] Bushby K. M., Making sense of limb girdle muscular dystrophies, Brain, 1999, 122, 1403-1420

[2] Bashir R., Britton S., Strachan T., Keers S., Vafiadaki E., Lako M. et al., A gene related to Caenorhabditis elegans spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B, Nat. Genet., 1998, 20, 37-42

[3] Liu J., Aoki M., Illa I., Wu C., Fardeau M., Angelini C. et al., Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb-girdle muscular dystrophy, Nat. Genet., 1998, 20, 31-36

[4] Bashir R., Strachan T., Keers S., Stephenson A., Mahjneh I., Marconi G. et al., A gene for autosomal recessive limb-girdle muscular dystrophy maps to chromosome 2p, Hum. Mol. Genet., 1994, 3, 455-457

[5] Bejaoui K., Hirabayashi K., Hentati F., Haines J. L., Ben Hamida C., Belal S. et al., Linkage of Miyoshi myopathy (distal autosomal recessive muscular dystrophy) locus to chromosome 2p12-14, Neurology, 1995, 45, 768-772

[6] Lek A., Lek M., North K.N., Cooper S. T., Phylogenetic analysis of ferlin genes reveals ancient eukaryotic origins, BMC Evolutionary Biology, 2010, 10, 231-247

[7] Bansal D., Miyake K., Vogel S. S., Groh S., Chen C. C., Williamson R. et al., Defective membrane repair in dysferlin-deficient muscular dystrophy, Nature, 2003, 423, 168-172

[8] Bansal D., Campbell K. P., Dysferlin and the plasma membrane repair in muscular dystrophy, Trends. Cell Biol., 2004, 14, 206-213

[9] Han R., Campbell K. P., Dysferlin and muscle membrane repair, Curr. Opin. Cell Biol., 2007, 19, 409-416

[10] De Luna N., Gallardo E., Soriano M., Dominguez-Perles R., de la Torre C., Rojas-García R. et al., Absence of dysferlin alters myogenin expression and delays human muscle differentiation «in vitro», J. Biol. Chem., 2006, 281, 17092-17098

[11] Klinge L., Laval S., Keers S., Haldane F., Straub V., Barresi R. et al., From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis, FASEB J., 2007, 21, 1768-1776

[12] Nagaraju K., Rawat R., Veszelovszky E., Thapliyal R., Kesari A., Sparks S. et al., Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B, Am. J. Pathol., 2008, 172, 774-785

[13] Chiu Y. H., Hornsey M. A., Klinge L., Jørgensen L. H., Laval S. H., Charlton R. et al., Attenuated muscle regeneration is a key factor in dysferlin-deficient muscular dystrophy, Hum. Mol. Genet., 2009, 18, 1976-1989

[14] Matsuda C., Aoki M., Hayashi Y. K., Ho M. F., Arahata K., Brown R. H. Jr., Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy, Neurology, 1999, 53, 1119-11122

[15] Anderson L. V., Davison K., Moss J. A., Young C., Cullen M. J., Walsh J. et al., Dysferlin is a plasma membrane protein and is expressed early in human development, Hum. Mol. Genet., 1999, 8, 855-861

[16] Ho M., Gallardo E., McKenna-Yasek D., De Luna N., Illa I., Brown R. H., A novel blood-based diagnostic assay for limb girdle muscular dystrophy 2B and Miyoshi myopathy, Ann. Neurol., 2002, 51, 129-133

[17] Vandre D. D., Ackerman W. E., Kniss D. A., Tewari A. K., Mori M., Takizawa T., Dysferlin in expressed in human placenta but does not associate with caveolin, Biol. Reprod., 2007, 77, 533-542

[18] Vainzof M., Anderson L. V., McNally E. M., Davis D. B., Faulkner G., Valle G. et al., Dysferlin protein analysis in limb-girdle muscular dystrophies, J. Mol. Neurosci., 2001, 17, 71-80

[19] Canki-Klain N., Milić A., Kovač B., Trlaja A., Grgičević D., Zurak N. et al., Prevalence of the 550delA mutation in calpainopathy (LGMD 2A) in Croatia, Am. J. Med. Genet., 2004, 125A, 152-156

[20] Miller S. A., Dykes D. D., Polesky H. F., A simple salting-out procedure for extracting DNA from human nucleated cells, Nucl. Acids. Res., 1998, 16, 1215

[21] De Luna N., Freixas A., Gallano P., Caselles L., Rojas-Garcia R., Paradas C. et al., Dysferlin expression in monocytes: A source of mRNA for mutation analysis, Neuromuscul. Disord., 2007, 17, 69-76

[22] Anderson L.V., Davison K., Multiplex Western blotting system for the analysis of muscular dystrophy proteins, Am. J. Pathol., 1999, 154, 1017-1022

[23] Lostal W., Bartoli M., Bourg N., Roudaut C., Bentaib A., Miyake K. et al., Efficient recovery of dysferlin deficiency by dual adeno-associated vector-mediated gene transfer, Hum Mol Genet, 2010, 19, 1897-1907

[24] Hattori H., Nagata E., Oya Y., Takahashi T., Aoki M., Ito D. et al., A novel compound heterozygous dysferlin mutation in Miyoshi myopathy siblings responding to dantrolene, Eur J Neurol, 2007, 14, 1288-1291

[25] Lerario A., Cogiamaninan F., Marchesi C., Belicchi M., Bresolin N., Porretti L. et al., Effects of rituximab in two patients with dysferlin-deficient muscular dystrophy, BMC Musculoskelet. Disord., 2010, 11, 157

[26] Wang B., Yang Z., Brisson B. K., Feng H., Zhang Z., Welch E. M. et al., Membrane blebbing as an assessment of functional rescue of dysferlin-deficient human myotubes via nonsense suppression, J. Appl. Physiol., 2010, 109, 901-905

[27] Illarioshkin S. N., Ivanova-Smolenskaya I. A., Tanaka H., Poleshchuk V. V., Markova E. D., Tsuji S., Refined genetic location of the chromosome 2p-Linked preogressive muscular dystrophy gene, Genomics, 1997, 42, 345-348

[28] Anderson L.V., Harrison R. M., Pogue R., Vafiadaki E., Pollitt C., Davison K. et al., Secondary reduction in calpain 3 expression in patients with limb girdle muscular dystrophy type 2B and Miyoshi myopathy (primary dysferlinopathies), Neuromuscul. Disord., 2000, 10, 553-559

[29] Illa I., De Luna N., Domínguez-Perles R., Rojas-García R., Paradas C., Palmer J. et al., Symptomatic dysferlin gene mutation carriers: characterization of two cases, Neurology, 2007, 68, 1284-1289

[30] Meznaric M., Gonzales-Quereda L., Gallardo E., De Luna N., Gallano P., Fanin M. et al., Abnormal expression of dysferlin in skeletal muscle and monocytes supports primary dysferlinopathy in patients with one mutated allele, Eur. J. Neurol., 2011, 18, 1021-1023