Available online at www.sciencedirect.com
ScienceDirect
Neuromuscular Disorders 24 (2014) 642–647
www.elsevier.com/locate/nmd

Case report

Deep sequencing detects very-low-grade somatic mosaicism in the unaffected mother of siblings with nemaline myopathy

Satoko Miyatake[a,1], Eriko Koshimizu[a,1], Yukiko K. Hayashi[b,c], Kazushi Miya[d], Masaaki Shiina[e], Mitsuko Nakashima[a], Yoshinori Tsurusaki[a], Noriko Miyake[a], Hirotomo Saitsu[a], Kazuhiro Ogata[e], Ichizo Nishino[b], Naomichi Matsumoto[a,⇑]

[a] Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan
[b] Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan
[c] Department of Neurophysiology, Tokyo Medical University, Tokyo, Japan
[d] Department of Pediatrics, Faculty of Medicine, University of Toyama, Toyama, Japan
[e] Department of Biochemistry, Yokohama City University Graduate School of Medicine, Yokohama, Japan

Received 12 December 2013; received in revised form 1 April 2014; accepted 11 April 2014

Abstract

When an expected mutation in a particular disease-causing gene is not identified in a suspected carrier, it is usually assumed to be due to germline mosaicism. We report here very-low-grade somatic mosaicism in ACTA1 in an unaffected mother of two siblings affected with a neonatal form of nemaline myopathy. The mosaicism was detected by deep resequencing using a next-generation sequencer. We identified a novel heterozygous mutation in ACTA1, c.448A>G (p.Thr150Ala), in the affected siblings. Three-dimensional structural modeling suggested that this mutation may affect polymerization and/or actin's interactions with other proteins. In this family, we expected autosomal dominant inheritance with either parent demonstrating germline or somatic mosaicism. Sanger sequencing identified no mutation. However, further deep resequencing of this mutation on a next-generation sequencer identified very-low-grade somatic mosaicism in the mother: 0.4%, 1.1%, and 8.3% in the saliva, blood leukocytes, and nails, respectively. Our study demonstrates the possibility of very-low-grade somatic mosaicism in suspected carriers, rather than germline mosaicism.
© 2014 Elsevier B.V. All rights reserved.

Keywords: Nemaline myopathy; ACTA1; Deep resequencing; Next-generation sequencer; Low-grade somatic mosaicism

1. Introduction

Nemaline myopathy is a common form of congenital myopathy characterized clinically by general hypotonia and muscle weakness, and pathologically by the presence of nemaline bodies within the myofibers [1,2]. ACTA1 is one of the nine known genes associated with nemaline myopathy [3].

Sometimes in the clinic, an expected mutation is not identified in a suspected carrier. This is usually assumed to be because of germline mosaicism, in which mosaicism occurs in the carrier's germline only. Here we report, for the first time, very-low-grade somatic mosaicism detected by deep resequencing using a next-generation sequencer (NGS) in an unaffected mother of two affected siblings.

⇑ Corresponding author. Address: Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan, Tel.: +81 45 787 2604; fax: +81 45 786 5219.
E-mail address: naomat@yokohama-cu.ac.jp (N. Matsumoto).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.nmd.2014.04.002
0960-8966/© 2014 Elsevier B.V. All rights reserved.

2. Case report

2.1. The proband

The proband was a boy who was 6 years old at the time of the study. He was the first child of healthy nonconsanguineous Japanese parents. He had an affected sister (Fig. 1a). He was born by cesarean section at 40 weeks of gestation after an uneventful pregnancy. He was admitted to a neonatal intensive care unit immediately after birth because of asphyxia with loss of spontaneous respiration and general hypotonia. His birth weight was 2640 g, height 48 cm, and occipitofrontal head circumference 33 cm. His Apgar scores were 1 and 4 at 1 and 5 min, respectively. He quickly recovered with ventilatory support. Laboratory tests showed normal findings except mildly elevated creatine kinase (653 IU/L). When he was discharged at age 2 months, general hypotonia remained with absent deep tendon reflexes. Antigravity movements were not observed. Arthrogryposis and mild cardiomegaly were noted. Ultrasound cardiography revealed mild dilatation and dyskinesia of the left ventricle. At 7 months, he was given a tracheostomy, and home ventilation therapy was introduced because of aspiration pneumonia (Supplementary Fig. 1a). Tube-feeding was also started because of poor swallowing. At 9 months, a muscle biopsy was performed. On a modified Gomori trichrome stain, nearly all the muscle fibers contained nemaline rods. Intranuclear rods were also scattered (Fig. 1b). On staining for ATPases, type 1 fiber atrophy and predominance were seen. Immunolabeling of a muscle biopsy from the patient with a-actinin showed intensely stained rod bodies (Fig. 1c). Co-staining with a-actinin, lamin A (as a marker of the nuclear envelope), and DAPI clearly revealed intranuclear rods (Fig. 1c). His condition was diagnosed as nemaline myopathy. He could sit unassisted at age 2 years, move on his hip at age 3, and walk with assistance at age 5. He showed normal intellectual ability. At 5 years, his cardiac function was re-evaluated by ultrasound cardiography. Mild left ventricular dilatation and dyskinesia remained.

2.2. The affected sister

The proband's only sibling, a sister 3 years younger, was also affected (Fig. 1a). She was born by cesarean section at 38 weeks of gestation after an uneventful pregnancy. She was similarly admitted to a neonatal intensive care unit immediately after birth because of hypoventilation and hypotonia. Her birth weight was 2620 g, height 48.5 cm, and occipitofrontal head circumference 36.5 cm. Her Apgar scores were 4 and 6 at 1 and 5 min, respectively. Serum creatine kinase was 190 IU/L, which was within the normal range. She recovered with ventilatory support, but needed continuous oxygen therapy. She had difficulty thriving and tube-feeding was introduced. Her cardiac function was normal without cardiomegaly. General hypotonia remained. After she was discharged at age 2 months, frequent aspiration pneumonia occurred. She started to use nocturnal noninvasive positive pressure ventilation at the age of 22 months. At age 2 years, she was given a tracheostomy and was controlled under nocturnal ventilation after respiratory syncytial virus infection following respiratory failure (Supplementary Fig. 1a). Because of her clinical presentation, she was also suggested to have congenital (nemaline) myopathy. At present, she shows antigravity movement of the extremities although she has not acquired head control and cannot roll over.

2.3. Genetic and three-dimensional structural analysis

First we checked for ACTA1 (NM_001100.3) mutation in the proband's DNA, considering the presence of intranuclear rods. We identified a novel heterozygous missense mutation, c.448A>G (p.Thr150Ala), by Sanger sequencing. Because autosomal recessive inheritance was possible from the family tree, we performed whole-exome sequencing of the proband, affected sister, and their parents to identify a further genetic cause. Genomic DNA obtained from blood leukocytes was captured using a SureSelect^{XT} Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA) and sequenced on a HiSeq2000 (Illumina, San Diego, CA) with 101-bp paired-end reads, as previously described [4]. The mean depth of coverage was 123x to 143x. We selected rare protein-altering and splice-site variants after filtering against dbSNP135 and 408 in-house control exomes. Among the rare variant calls, we first screened for genes known to cause nemaline myopathies, namely ACTA1 (MIM 102610) [3], TPM3 (MIM 191030) [5], NEB (MIM 161650) [6], TPM2 (MIM 190990) [7], TNNT1 (MIM 191041) [8], CFL2 (MIM 601443) [9], KBTBD13 (MIM 613727) [10], KLHL40 (MIM 615340) [11], and KLHL41 (MIM 607701) [12]. We identified only the novel heterozygous missense mutation c.448A>G (p.Thr150Ala) in ACTA1 in both the affected brother and sister, which we confirmed by Sanger sequencing. Copy number analysis by eXome Hidden Markov Model (XHMM) [13] using next-generation sequencing (NGS) data revealed that there were no copy number changes within the ACTA1 locus (Supplementary Fig. 1b). This mutation was not identified in either of the parents, after testing DNA obtained from their saliva, hair, nails, and blood by Sanger sequencing (Fig. 2a). This mutation, which alters the evolutionarily well-conserved Thr150 to Ala (Fig. 2b), was not present in the NHLBI Exome Sequencing Project (ESP6500). Two of three web-based prediction programs suggested that this mutation is pathogenic (PolyPhen-2: benign; SIFT: deleterious; MutationTaster: disease-causing). We also searched for any rare variants that were compatible with an autosomal recessive inheritance model, such as a homozygous mutation or compound heterozygous

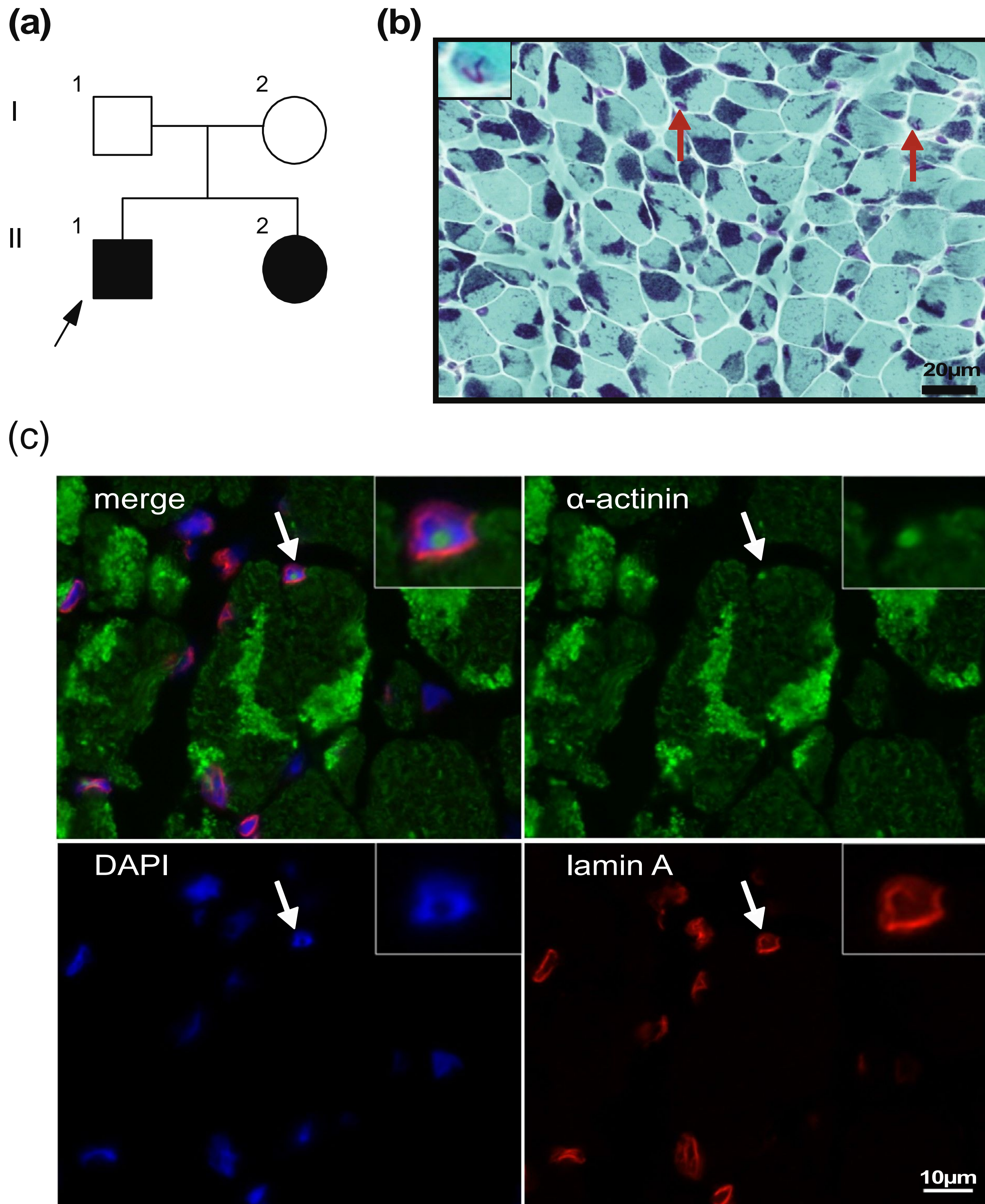

Fig. 1. (a) Family pedigree. (b) Light microscopic images of the muscle of the proband at age 9 months. With a modified Gomori trichrome stain, nearly all the muscle fibers can be seen to contain nemaline rods. Intranuclear rods (arrows, and upper-left window) are also scattered in some nuclei. Bar = 20 lm. (c) Immunohistochemical analysis of a muscle biopsy using anti-a-actinin (EA-53; Sigma, St. Louis, MO) (green) and anti-lamin A (red) antibodies [19]. Nuclei were stained with DAPI (blue). Nemaline rods were strongly stained by anti-a-actinin. An intranuclear rod was also seen (arrows, and higher-magnification inset boxes). Scale bar = 10 lm.

mutation, but no candidate mutations were identified (data not shown).

To explore the effect of the ACTA1 p.Thr150Ala mutation, we mapped the mutation onto reported crystal structures. Thr150 is located near the polymerization/interaction interfaces between actin monomers (Fig. 2c) and between actin and its interacting proteins (Fig. 2d, Supplementary Fig. 1c). Thus, p.Thr150Ala may

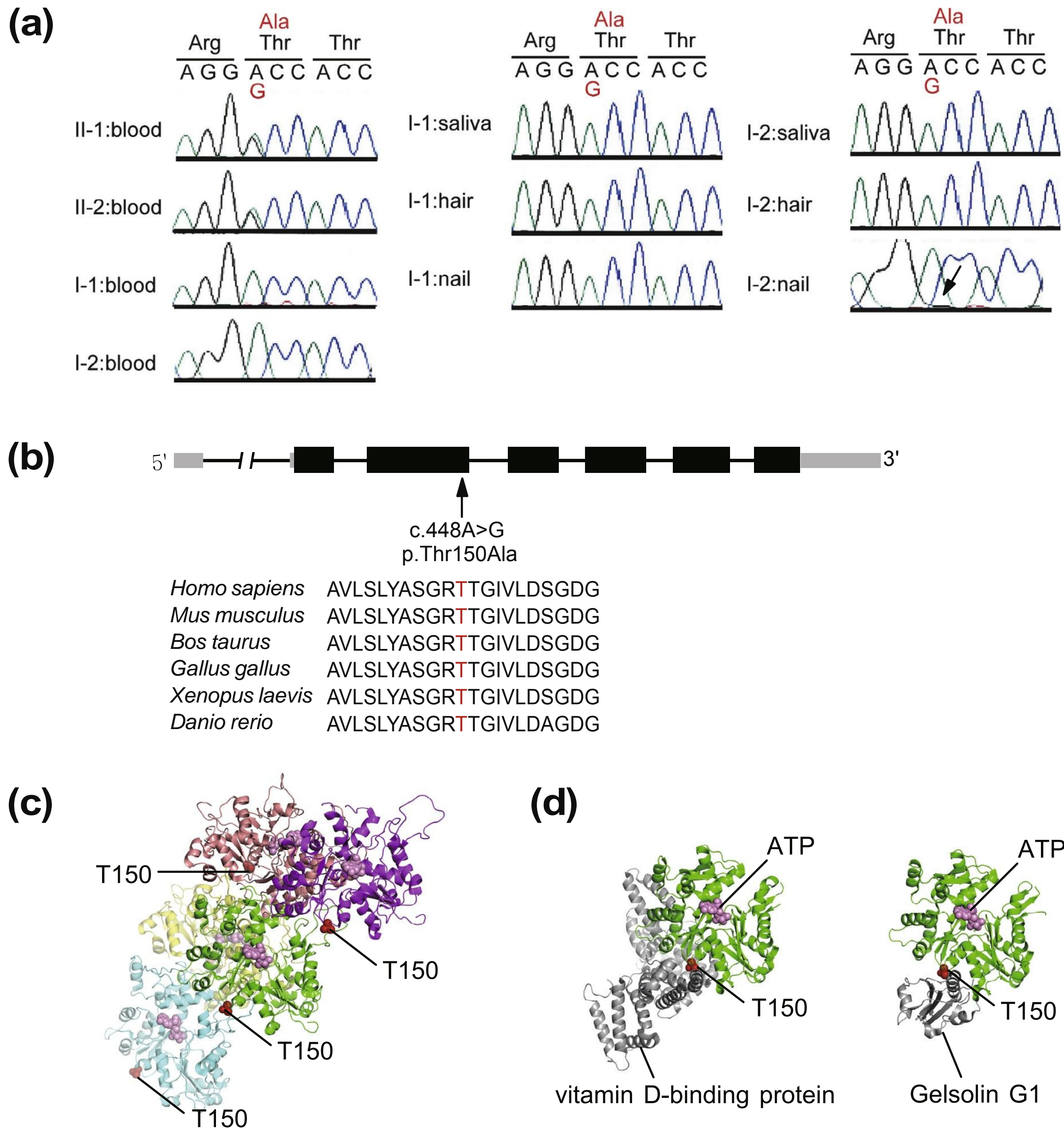

Fig. 2. (a) Sanger sequencing of the c.448A>G mutation using DNA from the affected siblings and the parents obtained from blood (left), and DNA from the father (middle) and mother (right) obtained from saliva, hair, and nails. The heterozygous mutation was identified in the affected siblings but not in any of the parental samples. No clear peak for the G allele was observed in the mother's nail DNA sample (arrow). (b) Schematic representation of ACTA1. The light gray bars represent untranslated regions and the black bars represent coding exons. Exon 1 is a non-coding exon. The c.448A>G mutation changes the well-conserved amino acid Thr150 (red) into Ala. (c, d) Structural implications of the p.Thr150Ala mutation in ACTA1. Structures of bare F-actin filaments determined by electron microscopy (Protein Data Bank (PDB) code 4A7N) (c) and G-actins in complex with vitamin D-binding protein (PDB code 1KXP) or gelsolin G1 (PDB code 1EQY) (d) are shown. The actin molecules are color-coded to discriminate each monomer in the F-actin filaments or are colored green in the G-actin complexes. Thr150 and ATP molecules are shown as red and pink space-filling spheres, respectively.

affect polymerization and/or the interactions of actin with other proteins.

We expected autosomal dominant inheritance from either parent. To test this, we performed deep resequencing for this mutation using an Illumina MiSeq platform. We used DNAs from the blood of the affected siblings, and from the saliva, nails, hair, and blood of their parents. The total read depth at c.448A in ACTA1 was 131495x to 425933x. Very-low-grade somatic mosaicism was confirmed in the mother: 0.4%, 1.1%, and 8.3% in saliva, blood, and nails, respectively (all beyond 0.1% of the background level) (Table 1).

We used allele-specific PCR to confirm the presence of the mutation in the mother. The primer sequences and

Table 1
Deep resequencing of c.448A>G in ACTA1 in various samples from each individual.

Individual	Tissue	Total depth (x)	Disease status	Wild-type allele (x)	Mutant allele (x)	% of wild-type allele	% of mutant allele
II-1 (Proband)	Blood	380016	Affected	192510	186808	50.7	49.2
II-2 (Sister)	Blood	425933	Affected	215496	209612	50.6	49.2
I-1 (Father)	Blood	261948	Unaffected	260897	355	99.6	0.1
I-2 (Mother)	Blood	364850	Unaffected	360030	4103	98.7	1.1
I-1 (Father)	Saliva	245895	Unaffected	245130	299	99.7	0.1
I-1 (Father)	Hair	190636	Unaffected	189929	253	99.6	0.1
I-1 (Father)	Nail	325282	Unaffected	324198	381	99.7	0.1
I-2 (Mother)	Saliva	239339	Unaffected	237872	999	99.4	0.4
I-2 (Mother)	Hair	324289	Unaffected	323120	400	99.6	0.1
I-2 (Mother)	Nail	131495	Unaffected	120210	10956	91.4	8.3

PCR conditions are available upon request. Both the wild-type and mutant alleles were amplified in the proband and the affected sister at a similar level. Both alleles could also be amplified in the mother, but the wild-type allele was amplified at a much greater level than the mutant allele. The wild-type allele only was amplified in the father (Supplementary Fig. 2a). Sanger sequencing of these amplicons confirmed the mutation in the proband, sister, and mother (Supplementary Fig. 2b).

There are various conventional methods to detect somatic mosaicism: Sanger sequencing to detect a small variant peak compared with the wild-type peak, high-resolution melting (HRM) analysis to detect an aberrant melting pattern, allele-specific PCR to amplify only the mutant allele, and pyrosequencing and SNaPshot analysis for quantitative variant detection [14]. We explored whether our very-low-grade somatic mosaicism could be detected by HRM, because this has been suggested to be one of the more sensitive methods [15]. We performed HRM analysis as previously described [16] using DNAs from normal controls, the affected siblings, the father (all DNA derived from blood), and the mother (DNA derived from the nails, which showed the highest rate of mosaicism (8.3%). The melting curves of both affected siblings were aberrant and were called mutant, but those of the father and mother were called normal (Supplementary Fig. 2c). In other words, this technique could not detect the 8.3% mosaicism.

3. Discussion

Here, we report very-low-grade somatic mosaicism in the unaffected mother of siblings with nemaline myopathy, identified by deep resequencing using NGS. Our study is significant in two ways. First, we demonstrate the possibility of very-low-grade somatic mosaicism in a suspected carrier, rather than germline mosaicism; this is likely to be a very rare event. Second, we present another example of the clinical application of NGS.

The novel heterozygous mutation c.448A>G (p.Thr150Ala) in ACTA1 is likely to be responsible for the nemaline myopathy in this pedigree based on four lines of evidence: the mutation is not registered in the ESP6500 database, the substituted amino acid is well conserved, two previously reported mutations also involve residue 150 (Thr150Asn, Thr150Ser) [17,18], and three-dimensional structural modeling suggests an impact on polymerization and/or the interactions of actin with other proteins. Interestingly, the mutation exists within a region where most of the mutations identified in patients with intranuclear rod myopathy, a variant of nemaline myopathy associated with ACTA1, are located [18].

The majority of reported ACTA1 mutations are de novo heterozygous mutations in sporadic cases. This is likely to be due to the severity of nemaline myopathy with ACTA1 mutation. However, autosomal dominant inheritance has been observed in a few situations: a pedigree with a relatively mild phenotype or incomplete penetrance, or parental somatic/germline mosaicism [2,18]. To date, there have been three reported cases of somatic mosaicism of ACTA1 in one parent of a severely affected patient [18].

In our study, allele-specific PCR, despite being non-quantitative, was sufficiently sensitive to detect mosaicism in blood leukocytes from the mother (mosaic rate 1.1%). Thus, this method is worth trying to confirm a suspected low-grade mosaicism. In contrast, as we were unable to detect 8.3% mosaicism using HRM, NGS should be the first choice for detecting very-low-grade somatic mosaicism that other methods might miss. A recent paper has described using NGS to detect somatic BRAF mutations down to 2% allele frequency, demonstrating the increased sensitivity of this method compared with HRM (limit 6.6% allele frequency), pyrosequencing (limit 5% allele frequency), and Sanger sequencing (limit 6.6% allele frequency) [15].

In our family, the mother does not seem to have any neurological problems in her daily activities, although she has not been clinically examined and no muscle imaging studies or biopsies have been undertaken.

The proband had mild left ventricular dilatation with dyskinesia without a hypertrophic phenotype. In the literature, cardiomegaly appears to be a rare complication. Patients with ACTA1 mutation usually have hypertrophic cardiomegaly [18,19].

In conclusion, we used NGS to confirm very-low-grade somatic mosaicism in the mother. Using conventional methods, the mother might have been judged to have germline mosaicism. Clinically, our data on the rate of

somatic mosaicism could be used to estimate the recurrence risk, although prenatal diagnosis would be required to provide certainty.

Acknowledgments

We thank all the participants for their cooperation in this research. We also thank Ms. K. Takabe and Mr. T. Miyama, from the Department of Human Genetics, Yokohama City University Graduate School of Medicine, for their technical assistance. This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan (N. Miyake, N. Matsumoto), a Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science (N. Matsumoto), a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (N. Miyake, H.S.), a Grant-in-Aid for Young Scientists (B) (E.K.), a research grant from the Yokohama Foundation for Advancement of Medical Science (S.M.), the Takeda Science Foundation (N. Miyake, H.S., N. Matsumoto), the Fund for the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program in the Project for Developing Innovation Systems (N. Matsumoto), the Strategic Research Program for Brain Sciences (E.K., N. Matsumoto), a Grant-in-Aid for Scientific Research on Innovative Areas (Transcription Cycle) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (N. Miyake, N. Matsumoto), a Grant-in-Aid for Research on Intractable Diseases, Comprehensive Research on Disability, Health and Welfare (Y.K.H., I.N.), a Grant-in-Aid for Applying Health Technology (Y.K.H., I.N.) from the Ministry of Health, Labour and Welfare, Japan, and an Intramural Research Grant 23-5 for Neurological and Psychiatric Disorders of NCNP (I.N.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2014.04.002.

References

[1] Nance JR, Dowling JJ, Gibbs EM, Bonnemann CG. Congenital myopathies: an update. Curr Neurol Neurosci Rep 2012;12:165–74.

[2] Romero NB, Sandaradura SA, Clarke NF. Recent advances in nemaline myopathy. Curr Opin Neurol 2013;26:519–26.

[3] Nowak KJ, Wattanasirichaigoon D, Goebel HH, et al. Mutations in the skeletal muscle alpha-actin gene in patients with actin myopathy and nemaline myopathy. Nat Genet 1999;23:208–12.

[4] Nakamura K, Kodera H, Akita T, et al. De novo mutations in GNAO1, encoding a galphao subunit of heterotrimeric g proteins, cause epileptic encephalopathy. Am J Hum Genet 2013;93: 496–505.

[5] Laing NG, Wilton SD, Akkari PA, et al. A mutation in the alpha tropomyosin gene TPM3 associated with autosomal dominant nemaline myopathy. Nat Genet 1995;9:75–9.

[6] Pelin K, Hilpela P, Donner K, et al. Mutations in the nebulin gene associated with autosomal recessive nemaline myopathy. Proc Natl Acad Sci USA 1999;96:2305–10.

[7] Donner K, Ollikainen M, Ridanpaa M, et al. Mutations in the beta-tropomyosin (TPM2) gene – a rare cause of nemaline myopathy. Neuromuscul Disord 2002;12:151–8.

[8] Johnston JJ, Kelley RI, Crawford TO, et al. A novel nemaline myopathy in the Amish caused by a mutation in troponin T1. Am J Hum Genet 2000;67:814–21.

[9] Agrawal PB, Greenleaf RS, Tomczak KK, et al. Nemaline myopathy with minicores caused by mutation of the CFL2 gene encoding the skeletal muscle actin-binding protein, cofilin-2. Am J Hum Genet 2007;80:162–7.

[10] Sambuughin N, Yau KS, Olive M, et al. Dominant mutations in KBTBD13, a member of the BTB/Kelch family, cause nemaline myopathy with cores. Am J Hum Genet 2010;87:842–7.

[11] Ravenscroft G, Miyatake S, Lehtokari VL, et al. Mutations in KLHL40 are a frequent cause of severe autosomal-recessive nemaline myopathy. Am J Hum Genet 2013;93:6–18.

[12] Gupta VA, Ravenscroft G, Shaheen R, et al. Identification of KLHL41 mutations implicates BTB-Kelch-mediated ubiquitination as an alternate pathway to myofibrillar disruption in nemaline myopathy. Am J Hum Genet 2013;93:1108–17.

[13] Fromer M, Moran JL, Chambert K, et al. Discovery and statistical genotyping of copy-number variation from whole-exome sequencing depth. Am J Hum Genet 2012;91:597–607.

[14] Tasca G, Fattori F, Ricci E, et al. Somatic mosaicism in TPM2-related myopathy with nemaline rods and cap structures. Acta Neuropathol 2013;125:169–71.

[15] Ihle MA, Fassunke J, Konig K, et al. Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p. V600E and non-p.V600E BRAF mutations. BMC Cancer 2014;14:13.

[16] Miyatake S, Miyake N, Touho H, et al. Homozygous c.14576G>A variant of RNF213 predicts early-onset and severe form of moyamoya disease. Neurology 2012;78:803–10.

[17] Sparrow JC, Nowak KJ, Durling HJ, et al. Muscle disease caused by mutations in the skeletal muscle alpha-actin gene (ACTA1). Neuromuscul Disord: NMD 2003;13:519–31.

[18] Laing NG, Dye DE, Wallgren-Pettersson C, et al. Mutations and polymorphisms of the skeletal muscle alpha-actin gene (ACTA1). Hum Mutat 2009;30:1267–77.

[19] Nowak KJ, Ravenscroft G, Laing NG. Skeletal muscle alpha-actin diseases (actinopathies): pathology and mechanisms. Acta Neuropathol 2013;125:19–32.