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ORIGINAL ARTICLE

Next-generation sequencing discloses a nonsense mutation in the *dystrophin* gene from long preserved dried umbilical cord and low-level somatic mosaicism in the proband mother

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Duchene muscular dystrophy (DMD) is a progressive muscle wasting disease, caused by mutations in the dystrophin (*DMD*) on the X chromosome. One-third of patients are estimated to have *de novo* mutations. To provide in-depth genetic counseling, the comprehensive identification of mutations is mandatory. However, many DMD patients did not undergo genetic diagnosis because detailed genetic diagnosis was not available or their mutational types were difficult to identify. Here we report the genetic testing of a sporadic DMD boy, who died > 20 years previously. Dried umbilical cord preserved for 38 years was the only available source of genomic DNA. Although the genomic DNA was severely degraded, multiplex ligation-dependent probe amplification analysis was performed but no gross mutations found. Sanger sequencing was attempted but not conclusive. Next-generation sequencing (NGS) was performed by controlling the tagmentation during library preparation. A nonsense mutation in DMD (p.Arg2095*) was clearly identified in the proband. Consequently, the identical mutation was detected as an 11% mosaic mutation from his healthy mother. Finally, the proband's sister was diagnosed as a non-carrier of the mutation. Thus using NGS we have identified a pathogenic *DMD* mutation from degraded DNA and low-level somatic mosaicism, which would have been overlooked using Sanger sequencing.
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INTRODUCTION

Duchene muscular dystrophy (DMD; OMIM 310200) is an X-linked recessive disorder that affects approximately 1 in 3500 live male newborns. DMD patients present severe muscle weakness and they lose their ability to walk before the age of 12 years, coupled with deterioration in respiratory and cardiac functions.[1] DMD is caused by mutations in the *dystrophin* gene (*DMD*), located at Xp21.2, which is 2.4 Mb in size, and consists of 79 exons that form a 14-kb mRNA transcript.[2] Introduction of the quantitative PCR-based technique of multiplex ligation-dependent probe amplification (MLPA), which can amplify all 79 exons of the *DMD* and is commercially available, has significantly improved mutation detection of exon deletions or duplications.[3–7] Using MLPA, approximately 70% of DMD patients have been shown to have partial deletions or duplications.[8] However, Sanger sequencing identification of small mutations within *DMD*, including small insertions or deletions within an exon (~7%),

single-nucleotide point mutations (~20%) and splice site intronic mutations (<1%), requires great effort with associated time and cost issues owing to the size and number of exons of *DMD*.[7,8] Recently, targeted next-generation sequencing (NGS) approaches have identified previously unknown causative genes for Mendelian disorders.[9] Disease panels for NGS are now widely used in the clinical field.[10] It is likely that genetic analysis of small *DMD* mutations will soon be replaced by NGS sequencing. Moreover, because of progress in NGS, more and more diseases with mosaic mutations have been identified, including *AKT1* in Proteus syndrome,[11] *IDH1* in Maffucci syndrome[12] and *GNAQ* in Sturge Weber syndrome.[13] Although somatic mosaicism is caused by many factors such as DNA mutations, epigenetic factors and chromosomal abnormalities, its influence on the clinical phenotype and transmission to the next generation is not negligible.

Here we report a sporadic case with a deceased, phenotypically diagnosed but genetically undiagnosed DMD patient. Using NGS, we

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identified a pathogenic nonsense mutation in *DMD* from dried umbilical cord. Here we report a sporadic case of a deceased, phenotypically diagnosed but genetically undiagnosed DMD patient by using dried umbilical cord for NGS. We also detected low-level somatic mosaic mutation in the proband's mother.

MATERIALS AND METHODS

Consultee

A 36-year-old woman, the sister of the proband, visited our genetic counseling unit to have a carrier genetic test for DMD. The consultee wanted to know whether she had inherited the *DMD* mutation from her mother as she knew that her elder brother had DMD (Figure 1a). Because mutation analysis was not performed on the proband, examining the mutation in her genome was difficult. First, MLPA was performed, but no gross mutations were identified. This suggested that if she was a carrier, there were only small mutations in her genomic DNA. Identifying single-nucleotide changes in females is difficult and indicated the need for mutational analysis in the proband.

Proband

The proband was a Japanese boy, born 38 years previously. He was diagnosed with DMD because of clinical manifestations at 5 years of age. The case had no familial background (Figure 1a), and his mother had no symptoms, such as muscle cramps or high serum creatine kinase. None of the maternal relatives presented with muscle weakness. The proband had a healthy younger sister (the consultee). He passed away from cardiomyopathy in 1991, aged 14 years. Genetic diagnosis was not performed.

Genetic analysis of the family

The diseased boy, his sister and their parents were analyzed for genetic mutations. Informed consent was obtained from the proband's family, and the experimental protocols and ethical approval for the study were obtained from the Review Boards of Kobe University. Peripheral blood samples were obtained from the proband's sister and parents. DNA was extracted from these blood samples using the QuickGene Kit (FujiFilm, Tokyo, Japan), following the manufacturer's instructions. The proband's DNA was extracted from dried umbilical cord using DNeasy (Qiagen, Venlo, The Netherlands). Initially, deletion or amplification mutations were examined by MLPA (SALSA MLPA; MRC-Holland, Amsterdam, The Netherlands). Following this, point mutations in *DMD* were detected using the Trusight One Sequencing Panel on the MiSeq platform (Illumina, San Diego, CA, USA). This panel can detect mutations in approximately 4321 genes (http://www.illumina.com/products/trusight-one-sequencing-panel.ilmn), including *DMD*. For dried umbilical cord, the genomic DNA tagmentation step was shortened to 3 min (instead of 10 min), as recommended in the standard protocol. All procedures were performed according to the manufacturer's instructions. For quantification and validation of genomic library samples, the Qubit 2.0 Fluorometer system

(Life Technologies, Carlsbad, CA, USA) and 2100 Bioanalyzer Instrument (Agilent Technologies, Palo Alto, CA USA) were used. Sequenced data was analyzed using the Variant Studio Software (Illumina), with *Homo sapiens* data (hg19, build 37.2) for the workflow. The reference genome provides variant annotations and fixes chromosome size in the BAM file output. Each single variant reported in the VCF output file was examined for coverage and Qscore and visualized via the Integrative Genome Viewer (IGV). For variant calling and reporting, the Variant Studio Software was used. Mutations identified as pathological were confirmed by Sanger sequencing following a standard protocol (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems by Life Technologies). Sequencing primers for point mutations in the proband were: DMD_exon9_F, 5′-ttactcagcaggcagtctgg-3′ and 5′-tggcacaaatgtcaaaatga-3′. Confirmation of umbilical cord identity was performed using microsatellite markers (D9S306, D9S2105, D9S2170, D9S2171 and D9S21072) on chromosome 9 from the parents' genome, as reported previously.[14]

Deep sequencing of genomic DNA from the mothers of sporadic DMD cases

For six sporadic DMD cases with *de novo* point mutations, peripheral blood samples were also obtained from the patients and their mothers. For deep sequencing of maternal somatic mosaic mutations, 500-bp PCR products harboring suspected mutation sites were purified by gel extraction using the QIAquick Gel Extraction Kit (Qiagen). Each mutation was then analyzed using the Truseq LT PCR-Free Kit (Illumina). All mutations tested in the mothers of sporadic cases and PCR primers are listed in Table 1. In Table 1, depth and frequency of the mutant to consensus allele obtained from ultra-deep sequencing are shown. All procedures and data analysis were performed according to the manufacturer's instructions.

RESULTS

Genetic test for *DMD* in the proband and his sister

We were unable to obtain genomic DNA from the proband as he had been dead for 23 years. However, the parents offered dried umbilical cord from the deceased patient, as such preservation is a cultural norm for parents in Japan. As the sample had been preserved at room temperature for >38 years, we carefully extracted genomic DNA from the dried umbilical cord. The extracted DNA was degraded to small fragments, ranging from 200 to 3000 bp in size (Figure 2a). Sample identity of the umbilical cord was confirmed using five microsatellite markers on chromosome 9 (Figure 1b). All five markers were found to be inherited from both parents. Next we used MLPA to examine *DMD* in the proband but detected no exonic deletions or duplications (data not shown). We then attempted to examine all 79 exons by Sanger sequencing but failed to obtain PCR products, especially of longer base lengths (data not shown), which was attributed to genomic DNA degradation. Subsequently, NGS was chosen to examine *DMD* in the proband. We usually enzymatically digest genomic DNA to obtain fragmented DNA ranging in size from 200 to 1000 bp. However, the degraded genomic DNA had already been naturally fragmented (Figure 2a). Therefore, by shortening the enzyme step for the degraded genomic sample, we were able to obtain a DNA sample of sufficient quantity and quality from the proband for NGS analysis. The proband's DNA sample had a similar smear pattern (DNA sizes between 200 and 1000 bp) as a control (Figure 2b). NGS identified many nucleotide changes within *DMD*, but c.6292>T was considered to be the strongest candidate (Figure 3a). Indeed, this change has already been reported as a pathogenic mutation for DMD in the Leiden database (http://www.dmd.nl/). Consequently, we concluded that a hemizygous point mutation (c.6292C>T, p.Arg2095*) in *DMD* caused DMD in the proband. This nucleotide change was confirmed by Sanger sequencing (Figure 3b). Moreover, also using NGS, we found the same heterozygous somatic mosaic mutation in the mother, at an 11% mutation rate within the total depth (29 out of the
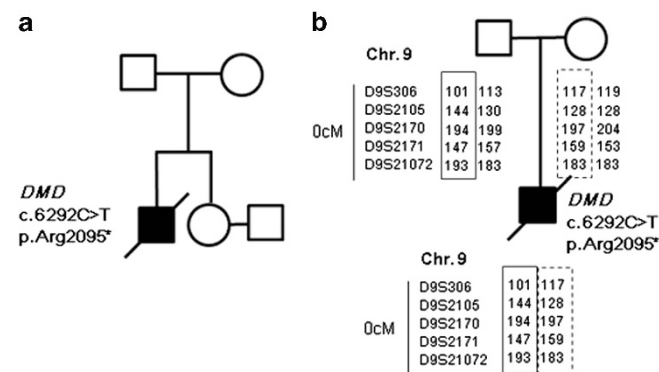

Figure 1 Familial pedigree. (**a**) Familial pedigree. Squares, males; circles, females; open shape, unaffected; filled shape, DMD patient. (**b**) Haplotype analysis between the proband (using umbilical cord DNA) and his parents. All five markers on chromosome 9 were inherited from both parents.

Table 1 Deep sequencing of the mothers of *DMD* in sporadic DMD cases

Sample ID	Disease	Mutation type	Exon/intron	Mutation	Mutation	Depth	Frequency %	Result	Forward (5′ → 3′)/reverse (5′ → 3′) primer
1	DMD	Nonsense	ex41	c.5899C>T	p.Arg1967*	0/2512	0.0	Non-carrier	GGGTTATTGAGCGAGGATGA/ AAGCCCAAAGTGAGGGAAAC
2	DMD	Microdeletion	ex18	c2230-2231delAG	p.Phe745*	0/71 945	0.0	Non-carrier	ATTCAAGCAGAGGCTGGGTA/ CACAGATAACAAAGCACGGAGT
3	DMD	Splice site mutation	58i	c.8669-1G>C	p.Glu2890Alafs*	0/81 672	0.0	Non-carrier	CCATTTAAGAGCCTGCCTGA/ TCCCTTGATCACCTCAGCTT
4	DMD	Nonsense	ex32	c.4414C>T	p.Gln1472*	0/59 617	0.0	Non-carrier	TCCTGTGTTGGATGAATGGA/ GCCACAATACATGTGCCAAT
5	DMD	Splice site mutation	ex32	c.4518+5G>A	p.[Ser1474Lysfs*4, Asn1507Valfs*12]	0/983	0.0	Non-carrier	AGGACCAGTTATTGTTTGAAAGG/ AATCATAAAAATTAAACACAAGCAACA
6	DMD	Nonsense	ex44	c.6292C>T	p.Arg2098*	1017/9437	11.0	Somatic mosaic carrier	TTTGTTACTTGAAACTAAACTCTGCAA/ CCAGGCAAACTCTCTCATCC

Abbreviation: DMD, Duchene muscular dystrophy.
Sample no. 6: present case.
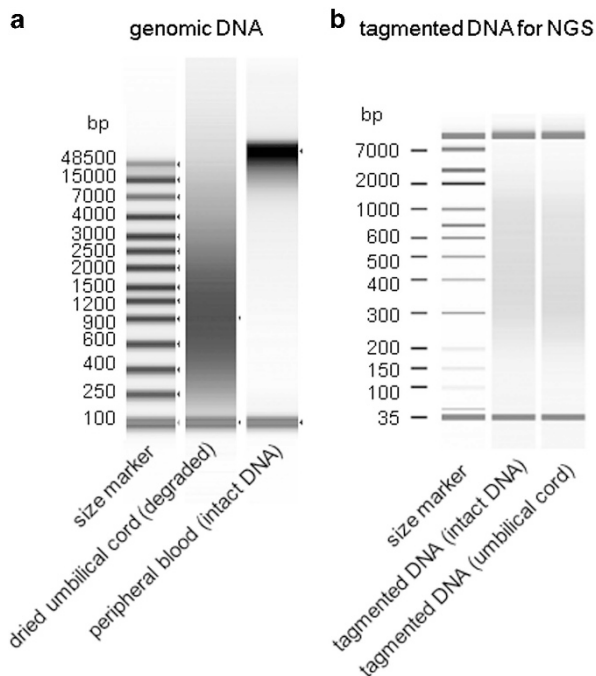*(asterisk) denotes translation termination codon.

Figure 2 Genomic DNA and library preparation from dried umbilical cord. (**a**) Degraded genomic DNA from dried umbilical cord. Electrophoresed genomic DNA. Note smeared degraded DNA of 200– 3000 bp. Lane 1, DNA size marker; Lane 2, genomic DNA from umbilical cord; Lane 3, genomic DNA from peripheral blood. (**b**) Electrophoresed tagmented DNA. Both degraded genomic DNA and fresh genomic DNA are enzymatically digested to the expected fragment sizes. Smeared bands are located within 200–1000 bp. Lane 1, DNA size marker; Lane 2, tagmented DNA from genomic DNA from peripheral blood; Lane 3, tagmented genomic DNA from dried umbilical cord. bp, base pairs.

265 depths) detected by the Trusight One Sequencing Panel (Figure 3a). Sanger sequencing showed slight elevation of the mutation allele, although this may not represent a mosaic mutation and instead be considered a nonspecific wave (Figure 3b). Using these results, the patient's sister was tested for carrier analysis and found to be free of the mutation (Figure 3b).

DMD ultra-deep sequencing detects mosaicism in the mothers of six sporadic DMD cases

Our maternal results indicate that non-carrier mothers of DMD cases may actually be mosaic carriers. Therefore, we additionally tested the mothers of sporadic DMD cases carrying point mutations. Genomic DNA from six mothers (including the present case) was analyzed by NGS using targeted ultra-deep *DMD* sequencing of the *DMD* mutation site (500 bp around the mutation site; Table 1). In the present case, we detected 11% of mosaic mutations (1017/9437 depth) in *DMD*, which is exactly the same mosaic rate as our previous result (29/265 depth, Figure 3a) obtained with the Trusight One disease panel. Thus this suggests that the mosaic rate obtained by the Trusight One disease panel is not coincidentally biased. No mutations were detected in the other five mothers of sporadic cases (Table 1).

DISCUSSSION

Although mutational analysis of point or small *DMD* mutations has already been vigorously performed, there are many undiagnosed patients with DMD but negative MLPA results. Recently, more and more mutational analyses are reported using NGS. Moreover, NGS disease panels such as Trusight One enable identification of pathogenic mutations when the genetic analysis is not performed in clinically diagnosed or even undiagnosed patients by relatively simple methods. Especially in large genes such as *DMD*, *myosin* and *titin*, reports on pathogenic mutation analysis using NGS have increased.[15] Regarding the clinical use of NGS analysis, we informed the patient on certain issues. First, as there are thousands of *DMD* variants that are not likely pathogenic or with unknown significance, we discussed the limitation of accuracy and validation of the test result. For this study, we were able to confirm a validated pathogenic variant in the proband to the patient's family because the variant p.Arg2095* is a definite pathogenic mutation. Second, because of the limitation and nature of this study, we did not attempt to detect other gene variants not related to muscular dystrophy. Third, if we find incidental findings, we will discuss the ethics and benefit for the patient with an appropriate board of Kobe University.

It is estimated that more unaffected, female, DMD carriers will consult genetic counseling units for genetic carrier testing because of their family plans. Prenatal genetic testing for DMD is also performed for children of DMD patients or carrier mothers. The *a priori* carrier
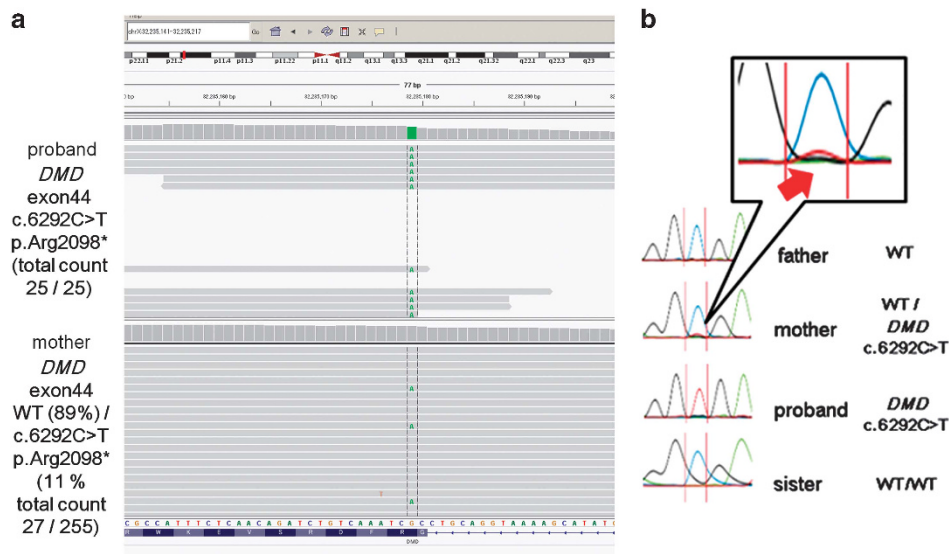
Figure 3 Mutation analysis of the proband and his mother. (**a**) IGV picture of the proband (upper lane) and mother (lower lane). Pathogenic *DMD* mutation (c.6292C>T, p.Arg2095*) in the proband. Eleven percent mutation rates (29 out of the 265 depths) of the same somatic mosaic mutation was also detected in blood-derived maternal genomic DNA. (**b**) Sanger sequencing of the pathogenic *DMD* mutation. Note the slightly elevated mutant allele wave (T) (closed arrow) in the mother.

risk for mothers of sporadic DMD patients is 66%.[16] The presence of unaffected sons and brothers, in combination with low maternal blood creatine kinase levels, reduces this risk. Carrier detection and first trimester prenatal diagnosis has attained >99% reliability in every case.[16] Therefore, accurate diagnosis for the proband and probands' mothers is crucial.

In this report, we used NGS in a sporadic DMD case to genetically diagnose a proband with DMD. Using this information, we could perform carrier genetic testing for a potential female carrier in a family. As a tissue sample (for example, peripheral blood or skeletal muscle) from the proband was unavailable, we extracted genomic DNA from dried umbilical cord, which the majority of Japanese families keep as a memento of their child's birth. Although the DNA quality was not good enough to amplify long PCR products for Sanger sequencing, shorter (around 300 bp in size) DNA fragments were at least suitable for preparing NGS libraries for subsequent sequencing. Indeed, we failed to amplify PCR products of some exons within *DMD*. Given that the degraded DNA quality is below the 50% range required for customized PCR sequencing primers, re-arranging the primers for *DMD* sequencing may cost at least $500. Sequencing 79 exons by Sanger sequencing costs at least $1500. Moreover, validating and re-examining PCRs for failed samples would also be time-consuming. In contrast, a commercially available genetic disease gene panel for NGS analysis encompasses all *DMD* exonic regions and costs no more than $1000 to detect pathogenic variants in only 4 days of lab experiments and with detailed data analysis using updated software. Therefore, it is more straightforward, faster and cost-effective to use NGS tools instead of conventional methods. To our knowledge, there have been no reports that have tested dried umbilical cord for identifying pathogenic mutations by NGS. As genetic testing has been widely accepted worldwide, the need for carrier genetic testing of small *DMD* mutations will likely increase. This practice may be beneficial in Japan for genetic diagnosis of deceased patients.

Deep sequencing by NGS has recently detected very-low-grade somatic mosaicism in unaffected mothers of siblings with nemaline myopathy.[17] Thus NGS enables deep resequencing of previously undetected low-grade mosaic mutations by Sanger sequencing. The background baseline signal noise of bases in Sanger sequencing can sometimes be confusing or misleading for identifying true variants. Especially in cases with low-grade somatic mosaicism, Sanger methods have limitations for accurately diagnosing mosaic rates. If the mosaic rate is low and/or the background baseline signal is high, mosaic mutations are likely to have been overlooked by Sanger methods. Germline mosaicism was previously suggested if a recurrent DMD patient is born to a non-carrier mother. Therefore, even if a *de novo* mutation is identified in the proband, prenatal genetic testing for the next child has sometimes been indicated for the pregnant mother. Germline *DMD* mutations can be problematic in determining recurrent from sporadic DMD cases in families.[18] Somatic mosaicism may not have the same rate as germline mosaicism, but NGS may be a useful tool for diagnosing maternal low-grade mosaic mutations, especially in sporadic DMD cases. If low-grade mosaicism were found in such cases, sisters of the proband could also be carriers for the mutations. As *DMD* is the largest gene, it is not surprising to find somatic mosaic variants over the course of a lifetime. Although we did not detect somatic pathogenic mutations in an additional five mothers, genetic testing by NGS will allow a more correct diagnosis in terms of recurrent risk evaluation in genetic counseling for genetic diseases.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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