

# Pilocytic Astrocytoma Tissue from a 25-year-old Patient with Duchenne Muscular Dystrophy Expresses the *DMD* Transcript but Not Protein

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## ABSTRACT

Duchenne muscular dystrophy (DMD) is a lethal progressive skeletal muscle wasting disease caused by mutations of the *dystrophin* (*DMD*) gene. *DMD* has also been reported to function as a tumor suppressor gene. We report a case of recurrent glioma in a 25-year-old male with a nonsense mutation (c.8460G>A) in exon 57 of the *DMD* gene. The glioma was diagnosed as pilocytic astrocytoma (PA) through histological analysis. Evaluation of cDNA samples prepared from the tumor tissue-derived RNA showed the presence of *DMD* transcripts which might produce dystrophin isoforms. However, anti-dystrophin antibodies could not detect any dystrophin isoform by immunostaining of the tumor tissue. This is the first case of PA in a DMD patient to the best of our knowledge. Although the occurrence of PA in this patient could be by chance, the discordant expression of transcript and protein might provide clues to support the new role of dystrophin as a tumor suppressor. Nevertheless, this is a single case and further analysis with many more patients is required to provide a definitive conclusion.

**Key words:** Pilocytic astrocytoma, *DMD*, Duchenne muscular dystrophy, splicing, intron retention, exon skipping, tumor suppressor

## INTRODUCTION

**D**uchenne muscular dystrophy (DMD), a lethal progressive skeletal muscle wasting disease, is caused by mutations of the *dystrophin* (*DMD*) gene, the largest known human gene.<sup>[1]</sup> The full-length 427 kDa dystrophin isoform, Dp427, is expressed in the brain (B), muscle (M), and Purkinje (P) cells. The shortest

dystrophin, Dp71, is ubiquitously expressed in non-muscle tissues.<sup>[2]</sup>

DMD patients have been reported to exhibit non-muscle symptoms such as heart complications,<sup>[3]</sup> mental retardation or cognitive dysfunction,<sup>[4,5]</sup> retinal abnormalities and hearing defects,<sup>[6]</sup> and gastrointestinal problems.<sup>[7]</sup> These, non-muscle symptoms suggest that the *DMD* gene has several other functions apart from its role in the muscles.<sup>[2]</sup>

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Recently, a new function of the *DMD* gene is arising. This is the function as a tumor suppressor or an anti-metastatic agent because *DMD* aberrations have been identified in a variety of tumors.<sup>[8-14]</sup> On the other hand, some neoplasms in DMD patients may also support the hypothesis of *DMD* as a tumor suppressor gene.<sup>[15,16]</sup>

Here, we studied a pilocytic astrocytoma (PA) in a DMD patient. PAs are slow-growing benign tumors that grow in the optic nerve pathways.<sup>[17-19]</sup> They are low-grade gliomas and occur most frequently in children and teenagers with a percentage survival of 98%.<sup>[18]</sup> The diagnosis is based on histological findings as well as the presence of the *KIAA1549/BRAF* fusion genes.<sup>[20,21]</sup> About 5% of gliomas have been associated with a variety of very rare inherited diseases<sup>[22]</sup> that arise due to mutation in tumor suppressor genes. In addition, two cases of medulloblastoma have been reported in DMD patients.<sup>[23,24]</sup> However, there have been no reports so far of PA in a DMD patient.

In this study, a DMD patient diagnosed with a recurrent PA is presented. After diagnosis of the tumor type, we carried out an evaluation of the dystrophin expression pattern in the resected tumor sample at both the transcript and protein levels.

## CASE PRESENTATION

### History and examination

The patient was a 25-year-old male (height 160 cm, weight 27 kg, BMI 10.5) with DMD. At the age of 1 year and 6 months, motor developmental delay was pointed out at the health checkup because he could only stand with support. He was referred to the local hospital and high level of serum creatinine kinase (CK) was found there. At the age of 2 years, he was admitted to our university hospital to undergo thorough examination for persistent high level of serum CK (1314–3754 U/L).

Immunohistological examination of a muscle biopsy sample revealed a complete absence of dystrophin, and genetic testing with white blood cells identified a transition mutation of G>A at position c.8460 in the exon 57 of the *DMD* gene. He was thereby diagnosed as having DMD. As muscle wasting progressed, he lost ambulation at the age of 11. His respiratory and cardiac functions were normal at the time. However, at the age of 19, due to a high pCO<sub>2</sub> (56.4 mmHg), he started non-invasive positive pressure ventilation (NPPV) use. He was discomforted by it so presently he continues only with nocturnal NPPV use. Assessment of cardiac involvement by electrocardiography and electrocardiogram indicated a left ventricular ejection fraction of 60.4%. Oral prednisolone (dosage: 0.75 mg/kg/2 days) was administered for a period of 4 years but there was no favorable result.

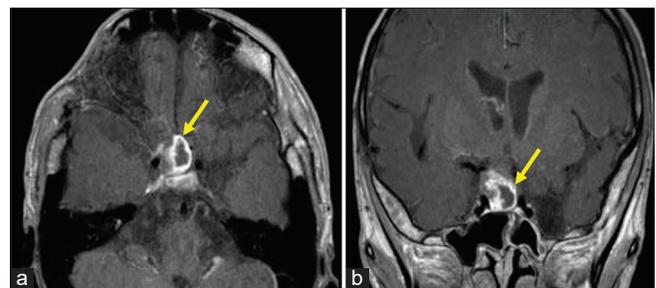
Brain computerized tomography, which was routinely performed for neurological evaluation of the muscle disease patients, indicated the presence of a hypothalamic mass at the first admission to our university hospital at the age of 2 years. The mass was diagnosed as a right optic glioma with no subjective symptoms. He underwent tumorectomy and lost his right eyesight. After tumorectomy, routine follow-up by ophthalmologist for visual acuity testing, tonometry, and eye field examination was normal. However, tumor regrowth was observed with a magnetic resonance imaging (MRI) examination at the age of 25 years [Figure 1], and retumorectomy was performed.

After obtaining informed consent from the patient and his family, we did molecular analysis and immunofluorescence examination of the tumor specimen. This study was approved by the Ethical Review Board of Kobe University Graduate School of Medicine (Approval No. 1210).

### Post-tumorectomy Analysis of tumor sample

A hematoxylin and eosin staining of the tumor tissue showed consisting bipolar neoplastic cells with long pilocytic or hair-like processes and round to oval nuclei, and Rosenthal fibers. There were varying degrees of mucoid material in the background, but no malignant findings such as hypercellularity, mitotic figures, or necrosis were observed. These findings were consistent with PA [Figure 2].

Subsequently, we examined the cDNA derived from the tumor sample for the common *KIAA1549/BRAF* fusion genes. We performed reverse transcription (RT)-PCR for fusion genes of *KIAA1549* exon 16 with *BRAF* exon 9 or exon 11 and *KIAA1549* exon 15 with *BRAF* exon 9 or exon 11.<sup>[20]</sup> However, none of the above fusion genes were identified (data not shown).



**Figure 1:** Magnetic resonance images showing a pilocytic astrocytoma in a 25-year old DMD boy. (a) An axial CT image showed a well circumscribed hypointense mass in the hypothalamic region of the brain (arrow), (b) a coronal CT image showed a well circumscribed hypointense mass in the hypothalamic region of the brain (yellow arrow)

### DMD Transcript analysis

#### Transcripts from DMD promoters

To characterize the expression of *DMD* transcripts in the PA-derived cDNA from the DMD patient, we perform a RT-PCR amplification of the *DMD* promoters. RT-PCR amplification demonstrated the presence of transcripts from Dp427m, Dp412e, Dp116, and Dp71 promoters [Figure 3].

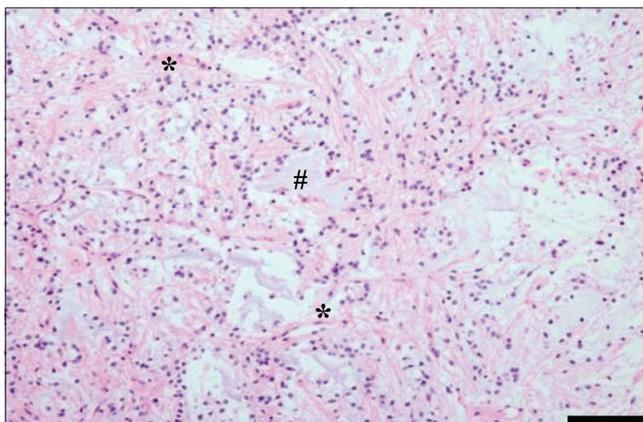
#### Alternative splicing of DMD transcripts

We next analyzed the full-length *DMD* transcript in the tumor-derived cDNA amplified as 20 separate overlapping fragments by RT-PCR.<sup>[9]</sup> The primers used were designed, as shown in Figure 4a. These findings were compared with the results from normal whole human brain-derived cDNA. All fragments that gave more than 1 band in the tumor tissue-derived cDNA were picked up for confirmation even if the bands were present in the whole human brain-derived cDNA control. The alternatively spliced products were confirmed by comparison with the sequencing of normal transcripts as reported before.<sup>[9]</sup> The analysis of tumor-derived cDNA showed that 16 out of 20

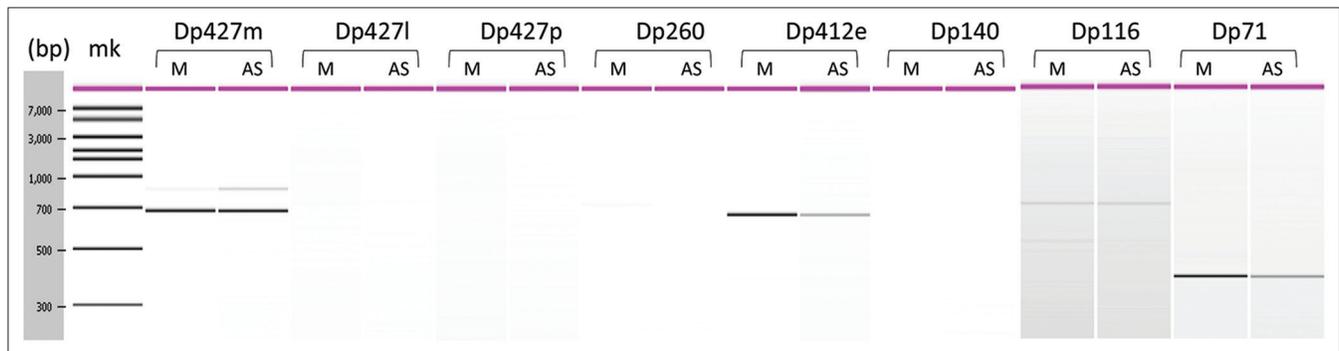
fragments gave single bands similar in both the control brain-derived cDNA (B) and tumor-derived cDNA (AS) preparations [Figure 4b]. We confirmed the cDNA integrity by amplifying the housekeeping gene, *GAPDH* in whole human brain- and tumor-derived cDNAs, respectively [Figure 4c].

Subsequently, the alternatively spliced products presented by the four remaining transcripts were investigated by direct sequencing. From the sequencing results, we observed that, in the exons 34–38 region, two bands were observed in both whole brain- and tumor-derived cDNAs. The larger band was the normally spliced product, whereas the smaller band indicated deletion of exon 37 [Figure 5a]. Next, in the exons 36–41 region, four amplified products were disclosed in tumor-derived cDNA [Figure 5b]. The normal transcript was the only one seen in the control brain-derived cDNA. For the abnormal bands, the first one indicated the presence of whole intron 40 sequence between exons 40 and 41 [Figure 5b-1]. The second one was exon 38-skipped product [Figure 5b-2] while the third one was the exon 37-skipped product [Figure 5b-3], respectively. Furthermore, in the exon 67–72 region, two products were revealed in both brain- and tumor-derived cDNAs with the larger band being the normally spliced product and the smaller band being exon 71-skipped product [Figure 5c]. Finally, in the exon 70–79 region, a set of bands were seen in both brain- and tumor-derived cDNA [Figure 5d]. Remarkably, cloning and sequencing of the product from the tumor-derived cDNA produced a normal transcript as well as five different splicing patterns which were mostly skipping of exon 71, 78, 71, and 72, 71 and 78, and 71–74, respectively [Figure 5d]. The partial sequences of these products are shown in Figure S1.

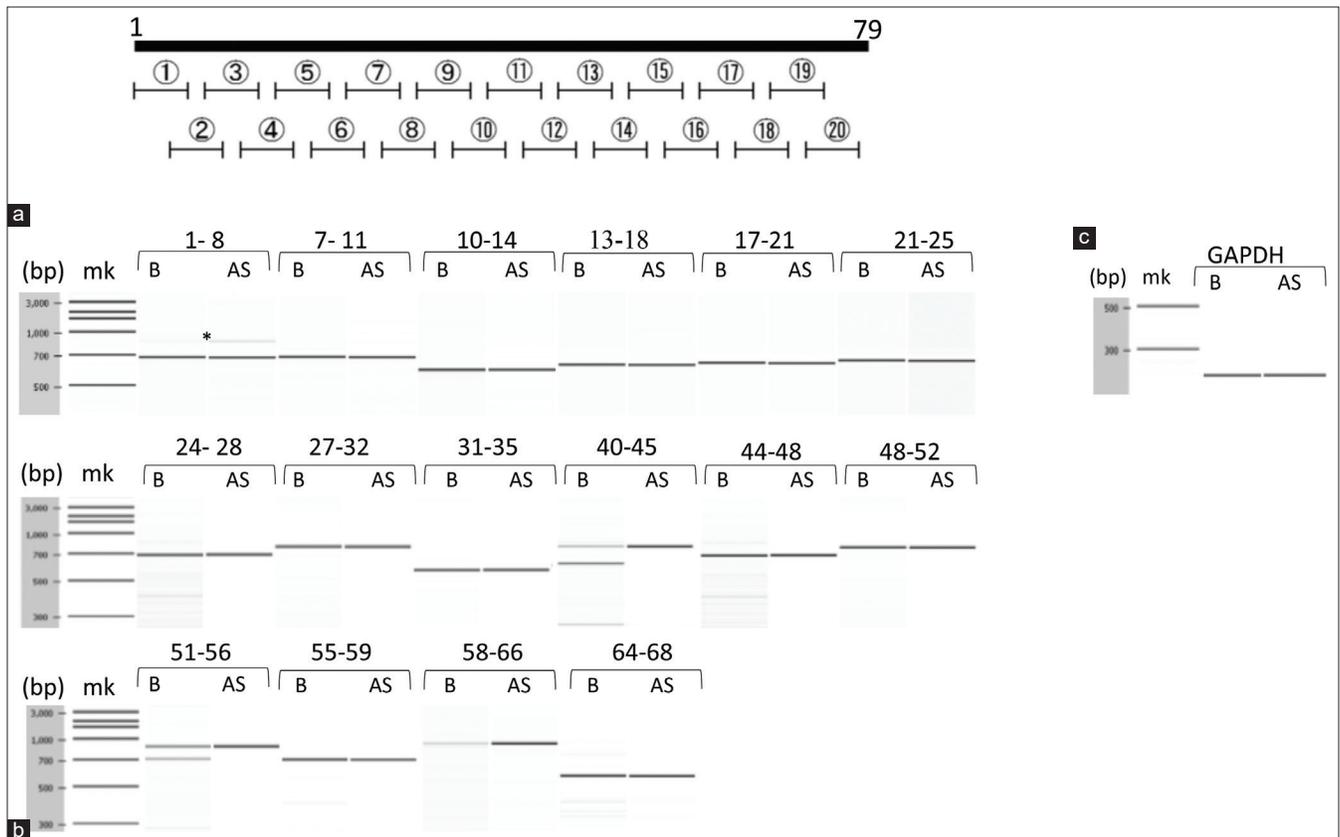
For intron retention (IR) analysis, RT-PCR of *DMD* short introns was examined as reported before.<sup>[9]</sup> Introns 40, 58, 70, and 75 showed retention [Figure S2]. Intron 40 was the most retained intron among all the other retained introns.



**Figure 2:** Histological analysis. An H & E staining is shown. There is characteristic bipolar cells with hair-like processes, Rosenthal fibers (\*) and mucoid material (#). Black bar: 100  $\mu$ m



**Figure 3:** Reverse transcription PCR of the *DMD* isoform transcripts of the pilocytic astrocytoma cDNA. Electrophoregram showing results of RT-PCR amplification in *DMD* isoforms from Skeletal Muscle cDNA (M) and pilocytic astrocytoma cDNA (AS). Dp427m, Muscle dystrophin; Dp427l, Lymphocyte dystrophin; Dp427p, Purkinje dystrophin; Dp260, Retinal dystrophin; Dp412e, Human embryonic dystrophin; Dp140, Brain distal dystrophin; Dp116, Schwann cell-specific dystrophin and Dp71, General dystrophin. bp, basepair; mk, molecular marker



**Figure 4:** RT-PCR of the conventional DMD transcript of the pilocytic astrocytoma cDNA. (a) Schematic representation of the primer design strategy to amplify the 14-kb DMD transcript in 20 overlapping fragments covering the 79 exons. All fragment sizes were limited to approximately 1 kb for efficient PCR amplification. (b) Electrophoregram showing results of PCR amplification. All fragments were amplified from normal whole brain cDNA (B) and pilocytic astrocytoma cDNA (AS). The results of 16 fragments are shown. The numbers above the electrophoregram signifies the 5' and 3' exons of each amplified fragment. (c) As a control, GAPDH was also amplified (GAPDH). bp, basepair; mk, molecular marker. \*has been described before<sup>[9]</sup>

#### Exon skipping in C-terminal region of DMD transcripts

To characterize the splicing pattern in the C-terminal region of *DMD* transcripts, we evaluated the percentage of exon skipping in this region [Figure S3]. Exon 71-skipped pattern was most abundant and made up 65% of the amount of the spliced products followed by exon 78 skipped patterns with 19%. Skipping of exon 72 made up 7% and exons 73 and 74 skipping were the least observed with 4% abundance each.

#### Dystrophin protein analysis

To investigate for dystrophin protein expression in the PA, immunostaining was performed. The following antibodies were employed to analyze the N-terminal, rod domain, and C-terminal of dystrophin: DYS3 (recognizes an epitope within exons 9–12), DYS1 (recognizes an epitope within exons 26–30), and 12715-1-AP, DYS2, and ab15277 (all three recognize the C-terminal region). The result of immunostaining was compared to a control human brain tissue. From the findings, none of the antibodies detected any signals of dystrophin isoforms, including Dp71, in the tumor tissue [Figure 6a] when compared to the control human brain [Figure 6b].

## DISCUSSION

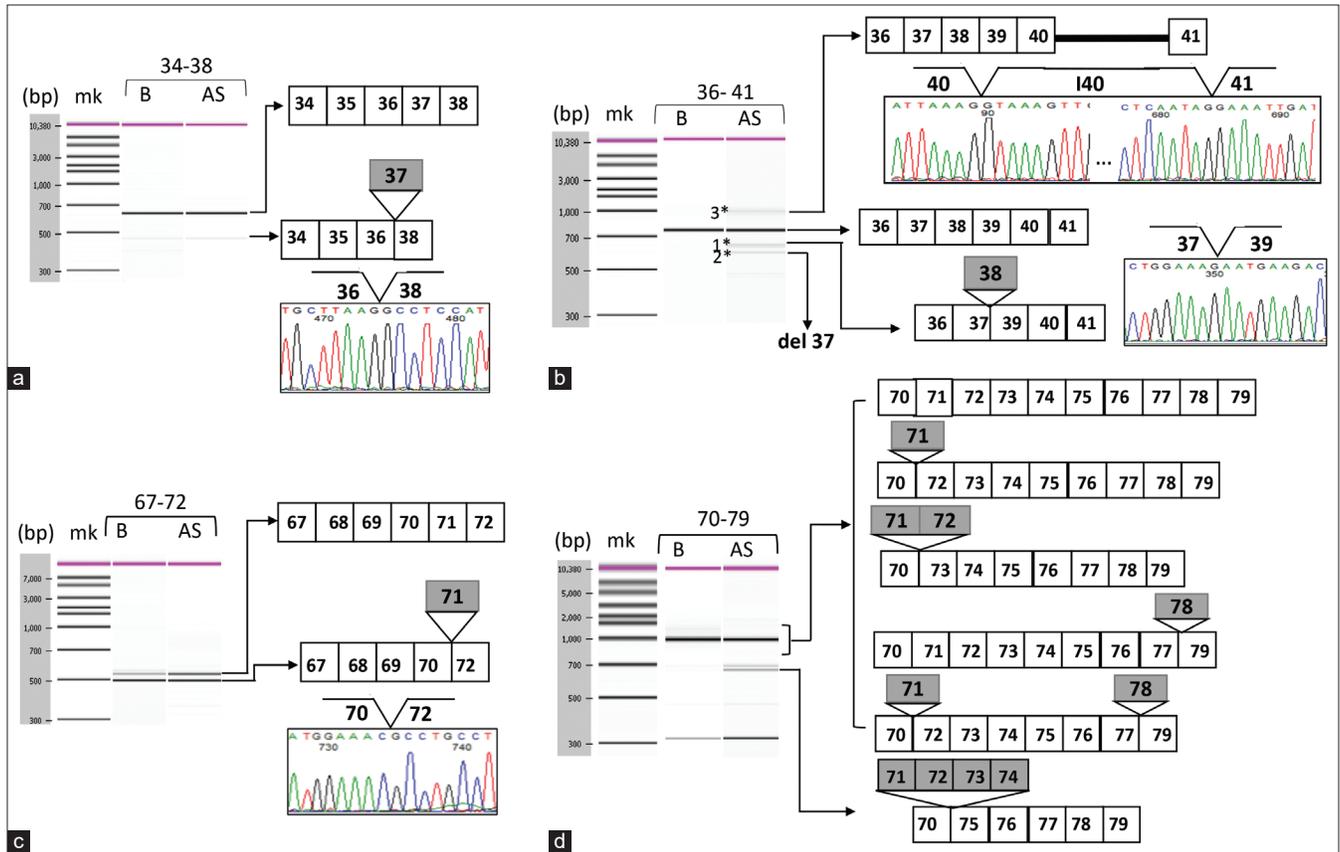
#### Diagnosis of PA in DMD patient

PA in a DMD patient is rare. Our case was diagnosed solely by histological findings because we did not observe any of the common *KIAA1549-BRAF* fusion genes. Reports have indicated that only 72% of PA show presence of the *KIAA1549-BRAF* fusion genes.<sup>[20]</sup> Our DMD patient developed a recurrent PA, nevertheless, it is very unclear whether the observation here is by chance or there is a causal connection between the two conditions.

#### A variety of DMD splicing patterns in the tumor sample

Following the assessment of *DMD* splicing in the tumor, three major observations were found: Multiple exon skipping, IR, and high abundance of exon 71-skipped *DMD* transcripts.

In the case of multiple exon skipping, all the skipped transcripts did not lead to distortion of the *DMD* reading frame. Skipping of exon 37 (171 bp), exon 38 (123 bp), and exon 71 (39 bp) all presented with in-frame mRNA. Besides,



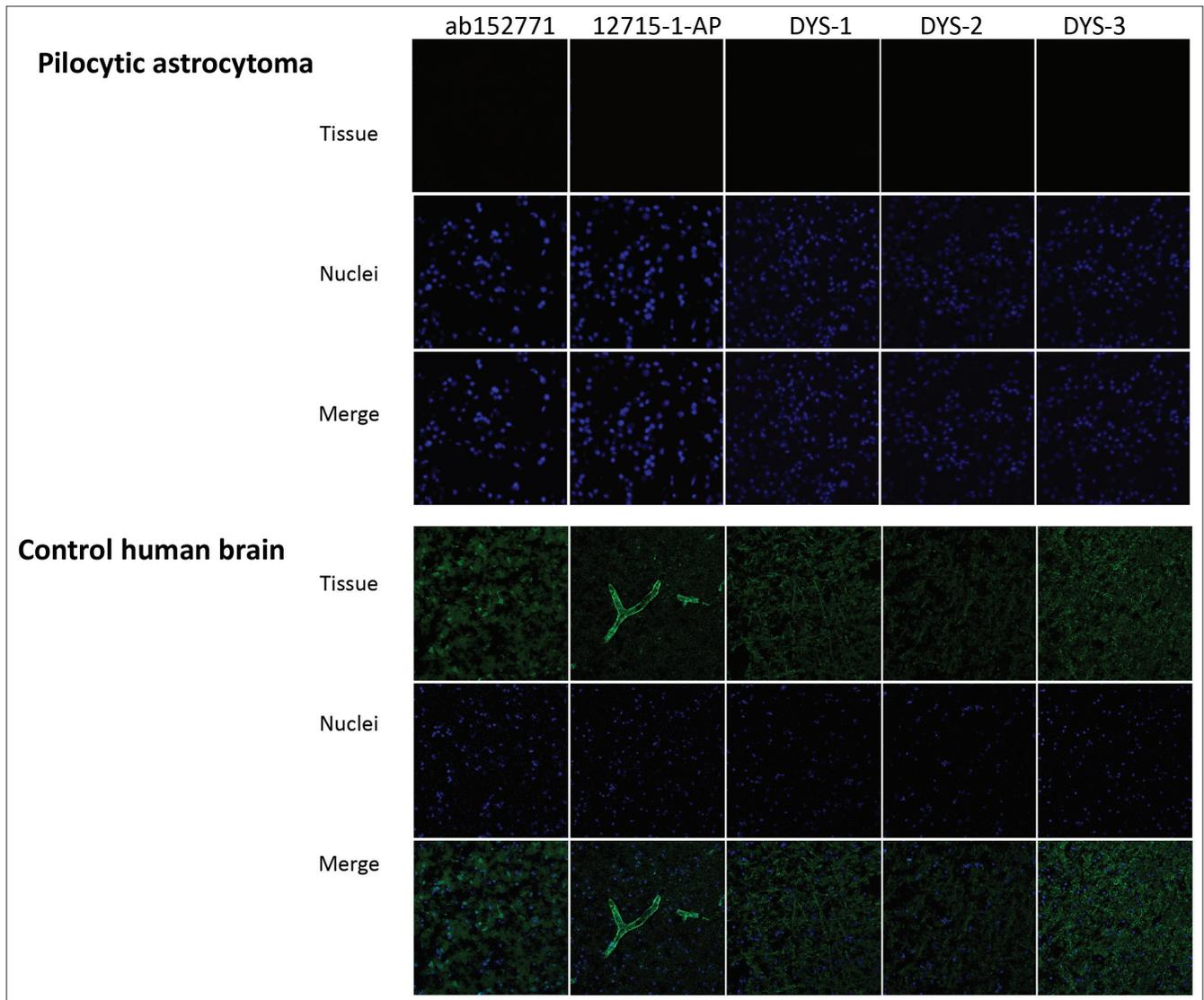
**Figure 5:** Direct sequencing analysis of alternatively spliced products. (a) Exon 34-38; a faint lower band was observed in both the normal whole brain (B) and the pilocytic astrocytoma (AS) in addition to the normal band. The exon structure of the two products is shown schematically on the right. Boxes and numbers in the boxes represent exons and exon numbers, respectively. Partial nucleotide sequences at the junctions between exons 36 and 38 are shown under the boxes. (b) Exon 36-41; a single normal band was amplified in the normal whole brain (B) whereas three less dense bands were observed in the pilocytic astrocytoma (AS) in addition to the normal band. The exon structure of the three products is shown schematically on the right. Boxes and numbers in the boxes represent exons and exon numbers, respectively. Bar indicates intron 40. Partial nucleotide sequences under the boxes show the junctions between exons 38 and 39, exon 40 and intron 40, and intron 40 and exon 41, respectively. (c) Exon 67-72; Two products were obtained from the normal whole brain (B) and the pilocytic astrocytoma (AS). The exon structure of the two products is shown schematically on the right. Boxes and numbers in the boxes represent exons and exon numbers, respectively. Partial nucleotide sequences at the junctions between exons 70 and 72 are shown under the boxes. (d) Exons 70–79; A coarse band together with some faint lower band were identified in both the normal whole brain (B) and the pilocytic astrocytoma (AS). The exon structures of these products are shown schematically on the right. Boxes and numbers in the boxes represent exons and exon numbers, respectively. The shaded exon boxes indicate the exon that was skipped within that transcript

transcripts with skipping of C-terminal located exons have been shown to express some kind of shorter isoforms.<sup>[25,26]</sup>

In the case of IR, only intron 40 was found retained in the conventional transcript [Figure 5b]. IR is sometimes caused by non-sequential splicing as has been shown in some genes,<sup>[27-29]</sup> and such abnormally spliced products may be associated with carcinogenesis.<sup>[30]</sup> Examples of IR as a possible cause of cancer have already been reported in breast cancer *BRCA1* intron 21<sup>[31]</sup> and in pancreatic cancer gastrin receptor intron 4.<sup>[32]</sup> Thus, it is anticipated that the presence of intron-retained *DMD* transcript could be relevant in translation. That is, intron 40 retention might generate a

premature termination codon that could render it a target for nonsense mediated decay pathway.<sup>[28,29]</sup> However, this does not seem to justify the absence of the protein because the amount of intron 40 retained transcript was deemed insignificant compared to the normal transcript in the exon 36–41 region.

Finally, we observed a high abundance of exon 71-skipped *DMD* transcripts. The high abundance of exon 71 skipping in the brain tumor may be one of the characteristics inherited from the neuronal cancer stem cells. Moreover, transcripts with exon 71 skipping have been reported to produce functional isoforms.<sup>[5]</sup>



**Figure 6:** Immunostaining of pilocytic astrocytoma tissue from DMD patient and human brain control tissue. (a) Immunostaining with ab15277, 12715-1-AP, DYS1, DYS2, and DYS3, antibodies, respectively in pilocytic astrocytoma tissues from DMD patient, the images showed negative expression of dystrophin in all cases. (b) Immunostaining with ab15277, 12715-1-AP, DYS1, DYS2, and DYS3 antibodies, respectively in control human brain tissue, the images showed positive expression of dystrophin in all cases

### Absence of dystrophin isoforms in spite of the presence of DMD transcripts

In the PA tissue, immunostaining analysis of tumor samples showed no staining with any of the antibodies indicated above. Furthermore, contrary to our expectations, the results showed absence of ubiquitous Dp71 [Figure 6a]. One of the antibodies targeting the C-terminal used here, ab15277 has its epitope on exons 77 and 78. The exon 78-skipped transcript was calculated as only 19%, implying that some amount of translated protein could still be detected by the ab15277, but this was not the case.

The patient carried a nonsense mutation, c.8460G>A, in *DMD* exon 57. The *DMD* gene has several alternative promoters leading to different translation start sites that translate to

protein isoforms of various sizes.<sup>[2]</sup> The exon 57 mutation resides on the muscle isoform, so no muscle dystrophin was detected in the muscle biopsy sample at the time of diagnosis (data not shown). As for other dystrophin isoforms, we had expected at least the presence of Dp71 in the brain tumor, because (1) *Dp71* promoter is located in the downstream of the mutation site in exon 57, and no aberration was found in the downstream exon, and (2) Dp71 is the most abundant *dystrophin* gene product in the brain.<sup>[33,34]</sup> In addition, RT-PCR demonstrated the presence of *Dp71* transcript expression in the tumor [Figure 3]. Nonetheless, no dystrophin isoforms were detected in the tumor. There remains a possibility that our method could not detect dystrophin simply because dystrophin is present at very low levels in the brain as reported before.<sup>[35]</sup>

## CONCLUSION

Here, we report a 25-year-old DMD boy with a recurrent right optic glioma at ages 2 and 25, diagnosed as PA with no subjective symptoms. Subtotal resection was performed as the only treatment procedure. Consequently, he lost his right eye sight from the first operation but his ophthalmologic conditions have been stable for years. He is still hospitalized in our hospital for his DMD condition and follow-up MRIs are constantly performed.

This is the first report for PA in a DMD patient to the best of our knowledge and it is possible that this could have occurred by chance. The discordant expression of dystrophin transcripts and protein in the tumor tissue is somehow intriguing and might suggest a connection between the two conditions, however, this is just a single case. Hence, it is disproportionate to make a declarative statement at this point although many reports support the role of dystrophin as a tumor suppressor. Further analysis of more cases will be required to provide a definitive conclusion.

## ACKNOWLEDGMENTS

We thank the patient and their family for their participation in this study. This work was supported by a Grant-in-Aid for Scientific Research(C) (ETENiba: 16k07216). The funding body played no role in the design, acquisition, analysis, and interpretation of data and in writing the manuscript.

## AUTHORS' CONTRIBUTIONS

ETEN, HA, and MM designed the work; HA and MN reviewed the clinical data; ETEN, HA, MT, and KI<sup>5</sup> contributed to data acquisition; KI<sup>2</sup>, MM, and HN analyzed and interpreted the data; ETEN, HA, and NN drafted and revised the manuscript.

### Availability for data and material

The datasets used and/or analyzed during the current study are only those presented in the main text and supplemental material, respectively.

### Declaration

#### **Ethics approval and consent to participate**

This study was approved by the Ethical Review Board of Kobe University (Approval No. 1210) and informed consent was obtained from the patient and his family.

#### **Consent for publication**

Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

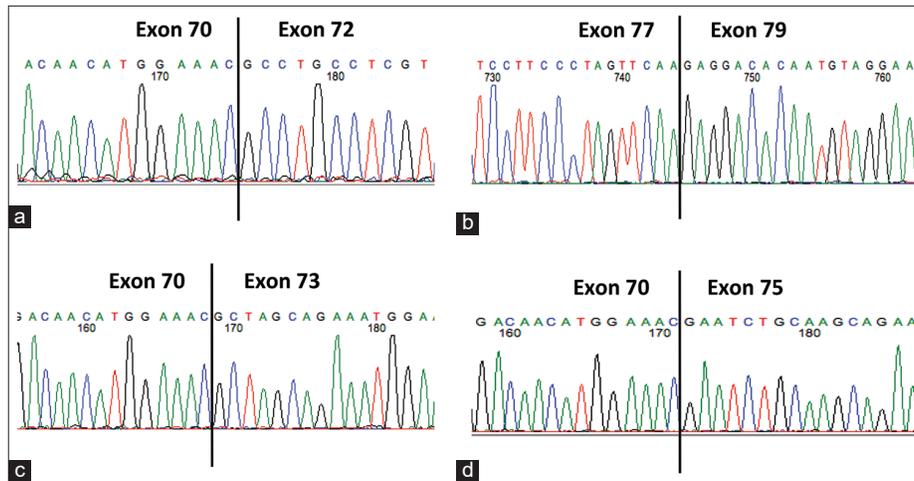
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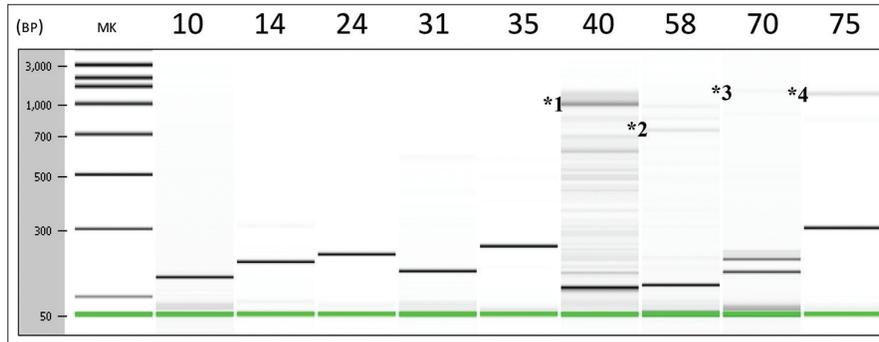
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**How to cite this article:** Niba ETE, Awano H, Nagai M, Taniguchi M, Itoh K, Nishimura N, Iijima K, Nishio H, Matsuo M. Pilocytic Astrocytoma Tissue from a 25-year-old Patient with Duchenne Muscular Dystrophy Expresses the DMD Transcript but Not Protein. *Clin Res Pediatr* 2020;3(1):1-9.

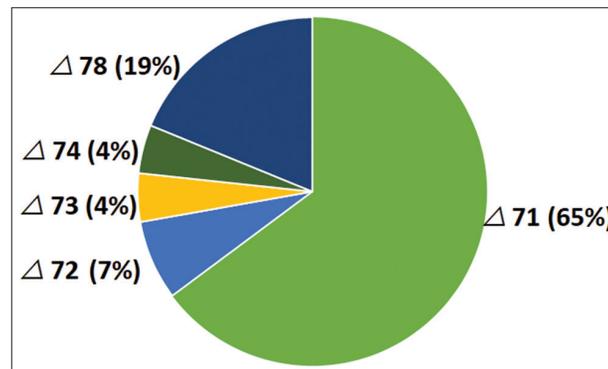
## SUPPLEMENTARY FIGURES



**Figure S1:** Results of Sanger sequencing. Results of Sanger sequencing. (a-d) Partial nucleotide sequences at the junctions between the exons are shown (a) exon 71 is skipped, (b) exon 71 + 72 is skipped, (c) exon 78 is skipped, and (d) exon 71–74 is skipped



**Figure S2:** RT-PCR results of Intron retention analysis of DMD short introns. Electrophoregram showing the intron-specific PCR results: Nine *DMD* short introns of not more than 1 kb were amplified with primers that bind to the neighboring exons. Introns 40, 58, 70, and 75 showed retention. Intron 40 (\*1) was the most retained amongst all other retained introns. Numbers at the top of the electrophoregram indicate the intron investigated. Asterisks (\*) indicate the specific intron retained band. bp: Basepair, mk: Molecular marker



**Figure S3:** Pie chart display of the amount of exon-skipped transcript observed in the *DMD* exon 70–79 region of PA tissue-derived cDNA. The number of clones carrying each skipped pattern was expressed as a percentage of the total number of clones with exon skipped patterns. The numbers in the chart represents the respective exon and ‘Δ’ indicates deletion