Journal of Human Genetics (2017) 62, 459–463

www.nature.com/jhg

ORIGINAL ARTICLE

Comparison of the phenotypes of patients harboring in-frame deletions starting at exon 45 in the *Duchenne muscular dystrophy* gene indicates potential for the development of exon skipping therapy

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Exon skipping therapy has recently received attention for its ability to convert the phenotype of lethal Duchenne muscular dystrophy (DMD) to a more benign form, Becker muscular dystrophy (BMD), by correcting the open reading frame. This therapy has mainly focused on a hot-spot (exons 45–55) mutation in the *DMD* gene. Exon skipping of an entire stretch of exons 45–55 is an approach applicable to 46.9% of DMD patients. However, the resulting phenotype is not yet fully understood. Here we examined the clinical profiles of 24 patients with BMD resulting from deletions starting at exon 45. The Δ45–55 group ranged in age from 2 to 87 years; no mortality was observed, and one patient was ambulatory at 79 years of age. The age at which patients became wheelchair-bound in the Δ45–48 group (18–88 years old) was approximately 50 years. Cardiomyopathy was well controlled by pharmaceuticals in both deletion groups. In contrast, the Δ45–47 and Δ45–49 groups exhibited more severe phenotypes than those with other mutations: the age at which patients in the Δ45–49 group became wheelchair-bound was around 30–40 years. Our study shows that clinical severity differs between each hot-spot deletion.
Journal of Human Genetics (2017) **62,** 459–463; doi:10.1038/jhg.2016.152; published online 15 December 2016

INTRODUCTION

Exon skipping therapy using antisense oligonucleotides has recently been proposed as a treatment for Duchenne muscular dystrophy (DMD), a lethal X-linked muscle disorder.[1] DMD is caused by a mutation in the *DMD* gene that encodes the sarcolemmal protein, dystrophin. The mutation causes a disruption in the open reading frame (ORF), known as an out-of-frame mutation, and prevents dystrophin from being expressed.[2] Conversely, sometimes the ORF is preserved despite the presence of a mutation (that is, in-frame mutation), resulting in a truncated but still functional dystrophin and that leads to the comparatively benign disorder, Becker muscular dystrophy (BMD).[2] Accordingly, antisense oligonucleotides have been used to restore the *DMD* ORF by causing skipping of the mutated exon(s) during translation and converting the severe DMD phenotype into a milder form, similar to BMD.[3]

Dystrophin is a rod-shaped structure consisting of four domains: an N-terminal actin-binding domain, a central rod domain, a cysteine-rich domain, and a C-terminal domain.[1] The central rod domain is composed of 24 spectrin-like repeats with four hinges. It includes over 76% of the structure's protein and contains the great majority of the deletions that cause BMD.[4,5] In particular, a significant number of DMD-causative mutations occur between exons 45 and 55, making this a mutation hot spot.[6] We previously described the cases of three unrelated patients with a Δ45–55 who showed very mild skeletal muscle involvement and could ambulate late in their lives.[7] Similarly, two reports independently described patients with the same deletion mutation who exhibited a very mild or asymptomatic phenotype.[8,9] These observations suggest that skipping the entire 45–55 stretch of exons, using multiexon skipping, is a feasible treatment for DMD and severe BMD cases that are caused by mutations in this region.[8,10] Indeed, based on the Universal Mutation Database (UMD-DMD; http://www.umd.be/DMD/), this multiexon skipping could be applicable to 69.2% of DMD patients with large deletions (⩾1 exon) and 46.9% of all DMD patients with various mutations (Table 1). Furthermore, this approach has been reported to restore the dystrophin expression at the sarcolemma and lead to recovery of muscle pathology and function in a DMD mouse model.[11]

Whether or not restoring the ORF will also ameliorate symptoms in more benign BMD cases still needs to be clarified. Very recently, molecular homology modeling revealed that the proteins resulting

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Received 29 August 2016; revised 10 November 2016; accepted 10 November 2016; published online 15 December 2016

Table 1 Exon 45–55 skipping applicability for patients with DMD based on UMD-DMD database[a]

	Total mutations	*Exon 45–55 skipping applicability*	*% applicability*
Total	1716	805	46.9
Large lesions	1259	754	59.9
Large deletions (⩾1 exon)	1028	711	69.2
Large duplications (⩾1 exon)	231	43	18.6
Small lesions	449	51	11.4
Small deletions (<1 exon)	117	17	14.5
Small insertions (<1 exon)	44	8	18.2
Splice-site mutations (<10 bp from exon)	78	7	9.0
Point mutations	210	19	9.0
Nonsense mutations	198	19	9.0
Missense mutations	12	0	0
Mid-intronic lesions	8	0	0

Abbreviations: DMD, Duchenne muscular dystrophy; UMD-DMD, Universal Mutation Database—Duchenne muscular dystrophy.
[a]http://www.umd.be/DMD/ (the data were obtained on 4th November 2016).

from an in-frame deletion consist of a structure similar to normal dystrophin (hybrid-repeat) and an unrelated structure (fractional-repeat), and disease progression in BMD patients appears to be dependent on the structure and associated with a specific structure of dystrophin by deletion pattern.[12] This report focused on deletion mutations Δ45–57, Δ45–48, Δ45–49 and Δ45–51;[12] however, Δ45–55, which have attracted much attention, were not considered. We have already reported that truncated dystrophin with Δ45–55 is associated with a hybrid phenotype.[11]

Therefore, we also suggest that it is important to clarify the correlation between phenotype and genotype in BMD patients with in-frame mutations. In this study, we focused on the phenotype of patients with BMD, who had in-frame deletion starting at exon 45, to investigate the appropriate restoration of the reading frame by exon skipping therapy.

MATERIALS AND METHODS

A total of 43 patients with BMD were included in the study, who were receiving outpatient care from our hospital in Nagano Prefecture, Japan. The mutation analyses were performed by multiplex-ligation prove amplification, a combination of multiplex PCR and Southern blotting, or a combination of multiplex PCR and reverse transcription PCR (RT-PCR) using muscle biopsy specimens.[5,11,12] Among them, the *DMD* mutation was identified in 35 patients, and we selected 24 patients harboring an in-frame deletion within the mutation hot spot, starting at exon 45. We examined their clinical profiles based on dystrophin conformation: hybrid type or fractional type, and performed a statistical analysis of the age at which patients became wheelchair-bound for each deletion mutation using the Kaplan–Meier method and log-rank test. Statistical significance was set at $P<0.05$. This study was approved by the institutional ethical committee of the Shinshu University School of Medicine, Japan (approval number 2270).

RESULTS

Twenty-four patients with in-frame, hot-spot deletions were divided into groups based on the location of their deletions as follows: Δ45–57 ($n=3$), Δ45–48 ($n=5$), Δ45–49 ($n=3$), Δ45–51 ($n=6$), and Δ45–55 ($n=7$). We further divided the five deletion mutations into two groups, namely, hybrid type (Δ45–55, Δ45–48, and Δ45–51) and fractional type (Δ45–57 and Δ45–49) of dystrophin conformation.

The clinical profiles of hybrid-type mutations are shown in Table 2. In patients having Δ45–55, the age range was 2–87 years and no mortality occurred. We have previously reported three patients (Nos. 5–7) with this deletion who exhibited a very mild phenotype,[5] and we have now added four younger patients (Nos. 1–4) as well. In the younger patients, the cause for diagnosis was hyperCKemia, and calf hypertrophy without muscle atrophy and weakness was observed.

The ages of the five patients with Δ45–48 ranged widely from 18 to 88 years. The 88-year-old patient died as a result of gall-bladder cancer. Three of these patients became wheelchair-bound around the age of 50 years. Cardiomyopathy was well controlled using angiotensin-converting enzyme inhibitor and/or a β-blocker. Our six patients with Δ45–51 are all young (<18 years) and have not yet shown any phenotype aside from hyperCKemia; therefore, we are continuing to care for their conditions, including cardiomyopathy.

The clinical profiles of fractional-type mutations (Δ45–47 and Δ45–49) are shown in Table 3. We had a small number of cases of these mutations ($n=3$, each); however, the age at which patients became wheelchair-bound in the Δ45–49 group was comparatively younger (30–40) and their cases were more severe than those of the hybrid-type form of dystrophin. Additionally, no mortality occurred and cardiomyopathy was controllable by pharmaceutical agencies, such as angiotensin-converting enzyme inhibitor or β-blockers.

We next examined the age at which patients became wheelchair-bound in each deletion group using Kaplan–Meier methods (Figure 1). With the exception of the Δ45–51 and Δ45–47 groups, in which patients were young and had not become wheelchair-bound, we performed log-rank tests between each group as follows: Δ45–55 vs Δ45–58, Δ45–55 vs Δ45–49, and Δ45–48 vs Δ45–49. We found a statistically significant difference between the Δ45–55 and Δ45–49 groups ($P<0.05$) and the Δ45–48 and Δ45–49 groups ($P<0.05$).

DISCUSSION

The clinical phenotype of our cases having Δ45–55 was consistently very mild and this might be associated with the fact that Δ45–55 forms a hybrid-type dystrophin.[11] Similar to Δ45–55 cases, patients with Δ45–58 also presented a relatively mild phenotype. This observation is consistent with a previous report,[10] and this deletion also created a functionally stable hybrid-type dystrophin.[10] The Δ45–51 created a hybrid type of dystrophin, indicating that this deletion might also result in a mild phenotype.

Table 2 Clinical profiles of patients with BMD who have hybrid-type mutations: Δ45–55, Δ45–48 or Δ45–51

No	Age (years)	Age at diagnosis (years)	Cause of diagnosis	Muscle involvement	Serum CK ($IU\ l^{-1}$)	Cardiac involvement	Medication	Diagnostic analysis	DMD mutation	Ref.
1	2	1 year 3 months	High CK	(−)	600–3500	(−)	(−)	MLPA	Δ45–55	(−)
2	14	10	High CK	(−)	5300	(−)	(−)	MLPA		(−)
3	23	14	High CK	Calf hypertrophy	2800–10 000	(−)	(−)	MLPA		(−)
4	26	14	High CK	Calf hypertrophy, exertional myalgia	1000–4000	(−), EF 63%	(−)	MLPA		(−)
5	39	26	High CK	(−)	650	(+), EF 63%	ACE-I	mPCR/RT-PCR		5
6	49	36	High CK	(−)	1300	(+)	ACE-I	mPCR/Southern		5,15
7	87	69	Walking disability	Wheelchair-bound at 79 years	670	(+), EF 50%	ACE-I, β-blocker	mPCR/Southern		5,16
8	19	12	Family history	(−)	1000–5000	(−)	(−)	MLPA	Δ45–48	(−)
9	21	5	High CK	(−)	2200–11 500	(−)	(−)	MLPA		(−)
10	69	53	Walking disability	Wheelchair-bound at 54 years	217	(+)	ACE-I	mPCR/Southern		16
11	74	39	Walking disability	Wheelchair-bound at 48 years	756	(+)	ACE-I	mPCR/Southern		16
12	88[a]	70	Walking disability	Wheelchair-bound at 55 years	681	(+)	ACE-I, β-blocker	mPCR/Southern		16
13	6	5 months	High CK	(−)	800–3800	(−)	(−)	MLPA	Δ45–51	(−)
14	9	4	High CK	(−)	5900	(−)	(−)	MLPA		(−)
15	14	8	High CK	(−)	1000–4000	(−)	(−)	MLPA		(−)
16	15	9	High CK	(−)	300–2000	(−)	(−)	MLPA		(−)
17	17	12	High CK	(−)	1000–6000	(−)	(−)	MLPA		(−)
18	18	18	High CK	(−)	750	(−)	(−)	MLPA		(−)

Abbreviations: ACE-I, angiotensin-converting enzyme inhibitor; BMD, Becker muscular dystrophy; CK, creatine kinase; EF, ejection fraction; MLPA, multiplex ligation-dependent probe amplification; mPCR, multiplex PCR; Ref., reference number; RT-PCR, reverse transcription PCR; Southern, Southern blotting.
[a]Death.

Table 3 Clinical profiles of patients with BMD who have fractional-type mutations: Δ45–47 or Δ45–49

No	Age (years)	Age at diagnosis (years)	Cause of diagnosis	Muscle involvement	Serum CK ($IU\ l^{-1}$)	Cardiac involvement	Medication	Diagnostic analysis	DMD mutation	Ref.
19	14	3	High CK	(−)	3000	(−)	(−)	MLPA	Δ45–47	(−)
20	17	4	Myalgia, High CK	(−)	5000–10 000	(−)	(−)	MLPA		(−)
21	31	3	High CK	Walking disability	Unknown	(+)	β-Blocker	mPCR/Southern		(−)
22	31	7	Limb muscle weakness	Walking disability	Unknown	(−)	(−)	MLPA	Δ45–49	(−)
23	33	7	High CK	Wheelchair-bound at 31 years	1400	(−)	(−)	MLPA		(−)
24	49	4	Walking retardation	Wheelchair-bound at 42 years	Unknown	(−)	(−)	MLPA		(−)

Abbrviations: BMD, Becker muscular dystrophy; CK, creatine kinase; MLPA, multiplex ligation-dependent probe amplification; mPCR, multiplex PCR; Ref., reference number; Southern, Southern blotting.

In contrast, the clinical phenotypes having fractional-type mutations Δ45–47 and Δ45–49 were more severe than the hybrid types. Despite our small number of patients, our study is the first to detect a significant difference in the degree of walking disturbance between the hybrid-type mutation (Δ45–55 or Δ45–58) and fractional-type mutation (Δ45–49) in Japanese BMD patients. It is important to note that the patient's phenotypes could also depend on their environmental backgrounds and degree of exercise.

Regarding the cause of diagnosis, 16 of the 24 patients were diagnosed after hyperCKemia was detected in the asymptomatic stage. In patients with DMD, clinical severity and course are roughly homogenous because of a lack of functional dystrophin, and genetic counseling at the time of diagnosis has little effect. In contrast, the clinical severity and course of patients with BMD are heterogeneous and can depend on the type of mutation as well as other genetic or environmental factors. However, the specific details of these interactions are currently unknown. In the future, this information will be important for choosing the appropriate care and treatment courses for patients with BMD who are diagnosed at an early or asymptomatic stage. Furthermore,

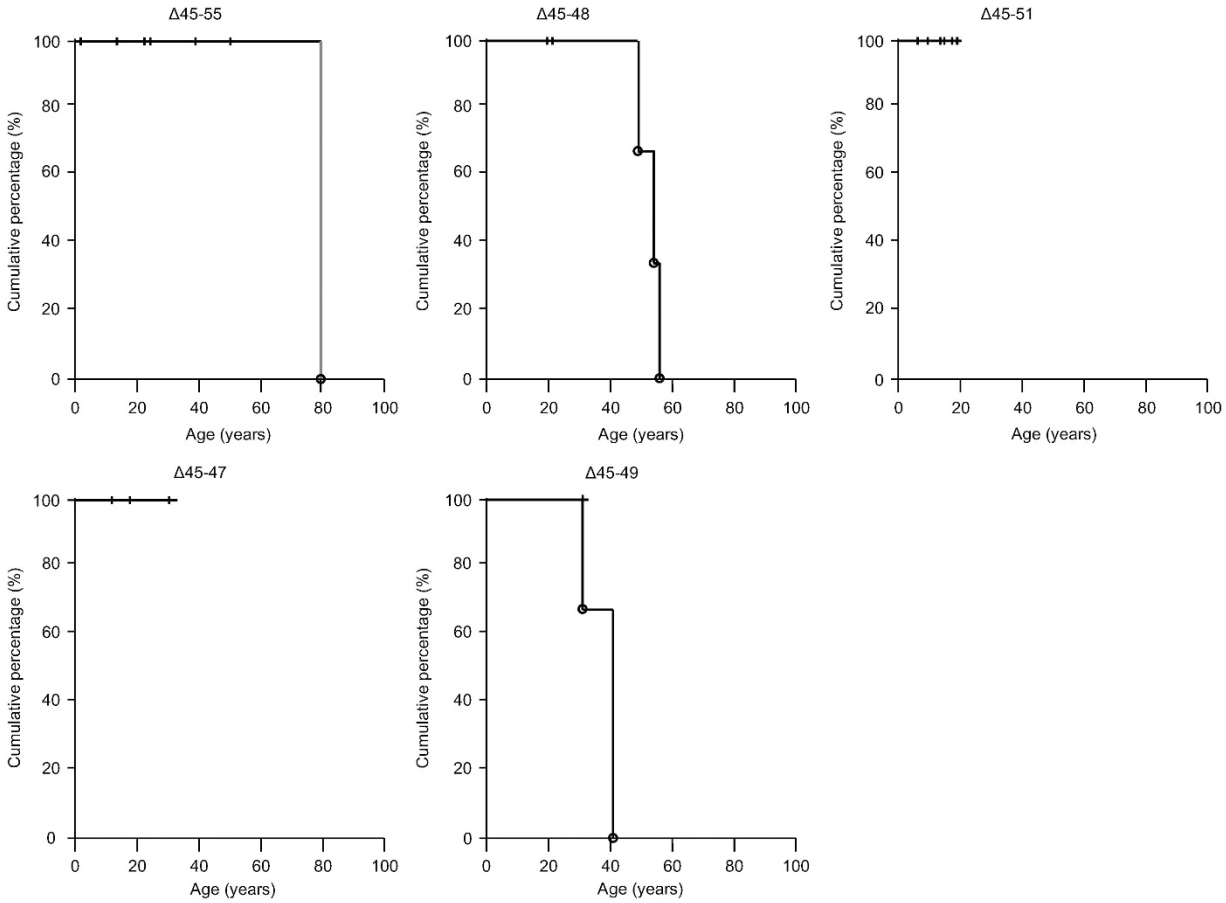

Figure 1 Kaplan–Meier analysis of the age at which patients became wheelchair-bound in each group: Δ45–55 ($n=7$), Δ45–48 ($n=5$), Δ45–49 ($n=3$), Δ45–47 ($n=3$), and Δ45–51 ($n=6$). The open circles show the age at which patients became wheelchair-bound; crossbars show the age at the non-wheelchair boundary but censored for this study. The log-rank test analyzed potential differences in the cumulative percentage of ambulatory patients between the two groups. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

this knowledge will better inform clinical physicians when treating BMD.

Based on our results, in order to develop therapy focused on correcting the leading frame, the clinical phenotype that will result from the treatment should be considered. According to another report, Δ45–46 patients show typical DMD and the conformation of the resultant protein may result in protein instability or altered binding of critical partners.[13] Thus, in DMD patients with Δ45, skipping exon 44 and multiexon skipping of exons 46 and 47 (or exons 46–48) are better potential therapies than skipping exon 46 alone; this report emphasizes that the development of exon skipping therapy must consider the genotype–phenotype correlation in dystrophinopathy.

Recently, not only exon skipping but also a CRISPR/Cas9 genome-editing strategy have received much attention as a way of correcting the leading frame. The CRISPR/Cas9 system can restore the *DMD* reading frame by targeting the mutational hot spot at exons 45–55 in DMD patient myoblasts.[14] The CRISPR/Cas9 system can be used for a single large *DMD* deletion. Therefore, we would like to emphasize that larger cohort studies of BMD are necessary in order to provide a strategic rationale for the development of these therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by an Intramural Research Grant (26-6) for Neurological and Psychiatric Disorders of the National Center of Neurology and Psychiatry (to AN). We thank Editage (www.editage.jp) for English language editing.

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