induce exon skipping should be analyzed using an in vitro splicing system. These methods have enabled us to identify optimal AOs against many exons of the *DMD* gene.

3. Molecular therapy by inducing exon skipping is applicable not only to out-of-frame deletions, but also to small mutations in in-frame exons. For example, an AO that induces the skipping of exon 41 has been reported to induce dystrophin expression in DMD myocytes with a nonsense mutation in exon 41 (20).
4. AOs are easily transfected into DMD myocytes because of the fragility of the cell membrane (21). Therefore, cultured myocytes derived from DMD cases are preferable to those from normal subjects.
5. Splicing is regulated in a tissue-specific manner; this is the reason why myocytes should be used for in vitro splicing analysis.
6. Geletin-precoated culture dish is prepared as follows: Allow 1% gelatin in PBS to stand in culture dish at room temperature for 4 h, aspirate excess gelatin, and dry the dish.
7. Eighty percent confluency is considered as semiconfluent.
8. Differentiated myocytes are recognized by the cell shape. After the differentiation, cells are elongated and fused to each other.
9. The AO which induces the exon skipping in 100% of mRNA is ideal, whereas the ideal AO cannot be identified in some exons. Therefore, the most potent AO is selected after the second screening step. Approximately 20% of the exon-skipped mRNA may be clinically effective according to the analysis of BMD case with nonsense mutation, in which the exon with nonsense mutation was skipped and in-frame mRNA was produced (22). On the other hand, there are some exons for which no efficient AO has yet been identified. For example, AOs for exon 20 are ineffective by our analysis as well as that of a colleague (18). In these cases, the method may need to be modified, for example, by the use of two or more AOs (23, 24).

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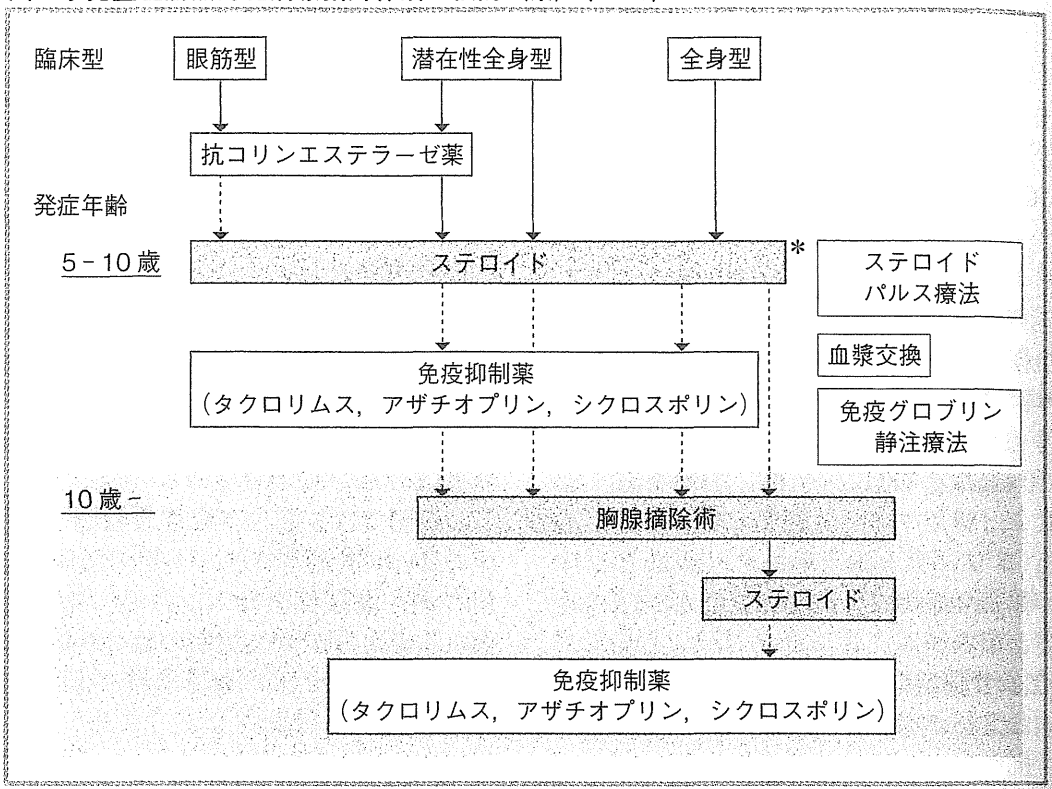
図　小児重症筋無力症治療指針作成の検討（案）（2010）



＊開始時期，維持期間，減量・終了時期に注意（本文参照）

抗例，また副作用出現時使用される．小児の経験は少ない．

℞ 処方例

プログラフカプセル（1 mg）　1カプセル　分1　夕食後

4. 胸腺摘徐術　小児では成人に比し適応は少ないが，ステロイド抵抗，10歳以後発症，G型，抗AChR抗体高値例にて適応となる場合がある．

5. 血漿交換　小児MGで血漿交換を要する例はまれである．

Ⓒ クリーゼの予防

ステロイド導入後急減したが，小児でも筋無力性（myasthenic），コリン作動性（cholinergic）の2型があり，特に抗コリンエステラーゼ薬投与時後者に注意を要する．ステロイド投与時低K血症に注意する．緊急，適切な対処が重要である．

Ⓓ 併発症・合併症の予防と治療

1. 廃用弱視の予防　3歳以下発症，一側眼瞼下垂時に注意する．

2. 眼瞼挙上術　病態の変動が完全に消失，テンシロンテストに反応しない場合，考慮されうる．

3. その他　アレルギー性疾患，甲状腺機能異常症などの合併症に注意する．

患者説明のポイント

・治療方針，治療薬の副作用，しかし寛解には十分な投与期間を要すること，クリーゼの可能性を説明する．

看護・介護のポイント

・病態の理解，それに沿った治療をすみやかに，遅滞なく行う．

筋ジストロフィー
muscular dystrophy（MD）

松尾雅文　神戸大学大学院教授・小児科

病態と診断

筋ジストロフィーは筋の壊死・再生を特徴とする疾患の総称で，小児期にあってはDuchenne型ジストロフィー（DMD）の頻度が最も高い．DMDはジストロフィン遺伝子の異常により発症する劣性遺伝病である．DMDでは骨格筋のジストロフィンが欠損するため，幼児期に筋力低下症状を示し，進行性の筋萎縮を示して，20歳代に心不全あるいは呼吸不全で死に至る．確定診断は遺伝子診断である．最近では，偶発的な血液検査でクレアチンキナーゼの著明な上昇により発見される乳児例も多くなっている．

治療方針

DMDの治療法はいまだ確立されておらず，

のがほとんどである．骨格筋でジストロフィ
発現させる根本治療として，遺伝子治療をはじ
の研究がなされてきた．現在，ジストロフィ
DNAに焦点をあてた2つの治療法が有望視さ
験レベルで進行中である．いずれの方法を選
するかは，DMDの遺伝子診断結果に依ってい

DMDでは以下の対症療法が主たるものとなる．

療法
DMDでは，幼児・学童初期には足関節，学童中
すると足関節に加え膝関節，股関節の拘縮を予
するためのリハビリテーションを実施する．歩行
持を積極的にはかるとともに，車椅子生活に
縮予防のために継続したリハビリを実施．
不全は，生命を脅かす要因の1つである．呼
を維持できるように呼吸リハビリテーション
する．さらに，血中の二酸化炭素分圧の上昇
れば呼吸補助療法を開始する．

療法
低下　筋力低下が顕著となれば，副作用の
を十分考慮したうえで，プレドニンを投与す

例)
ニン錠（5 mg）　0.75 mg/kg　分1　隔日
)
不全　心エコー検査で心筋収縮異常が出現す
重症化予防に投与を開始する．
例)　下記のいずれかを用いる．
ベース錠（2.5 mg）　5 mg　分2 [23]
チスト錠（2.5 mg）　5 mg　分2 [18]

脳性麻痺
cerebral palsy（CP）

滋賀医科大学教授・小児科学

診断

脳性麻痺は受胎から新生児期（生後4週以内）ま
生した脳の非進行性病変による運動または姿勢
で，種々の原因により生じた脳性運動障害の
あり，その症状は2歳までに出現する．原因
る脳病変は非進行性であるが，運動や姿勢の異
児の成長・発達とともに修飾を受け変容し得

脳性麻痺の原因には胎内感染，脳形成異常，胎盤
不全，新生児仮死（低酸素性虚血性脳症），分
，新生児期中枢神経感染症などが挙げら
れ，その作用時期により，出生前，周産期，出生後に分けられるが，出生前が大多数を占める．低出生体重児については脳室周囲白質軟化症の重要性が増している．

脳性麻痺の発生率は1950年代には出生1,000あたり2.5で，一時0.6まで減少したが，1981年以降，低出生体重の生存率の向上などにより，出生1,000あたり2.0を超えているとされる．

Ⓑ 早期診断

3か月までは脳性麻痺の確定診断は困難であるが，姿勢の異常，筋緊張の異常，反射の異常，異常運動が早期診断上重要な所見であり，NICU入院歴や画像診断も参考に早期診断を心がける．新生児期にはけいれん，易刺激性，過度の覚醒状態，哺乳力低下，筋緊張低下，Moro反射の減弱もしくは欠如，無呼吸がみられることが多い．乳児期以降では，筋緊張が徐々に強くなり，低筋緊張の状態から痙性が出現し，姿勢や姿勢反射の異常が出現してくる．頸定，坐位，独歩などの運動発達の遅れや，家族の「哺乳しにくい，抱きにくい，反りやすい」などの訴えによって脳性麻痺に気づかれることも多い．

治療方針

Ⓐ 早期療育と療育の流れ

早期診断，治療（薬物・手術），療育（運動療法・作業療法）により障害をより軽微にとどめることが可能である．Vojta法では，7つの姿勢反応の異常を判定し，スコア化して危険児をみつけ，中等症－重症障害児の早期治療を行う．

乳児期早期に脳性麻痺を疑われ，実際に脳性麻痺となるのは10%程度といわれているが，注意欠如・多動性障害や自閉症スペクトラム障害などの発達障害がのちに明らかになる例も多く，注意深い経過観察が必要である．

新生児－乳児期にはNICU入院中からハイリスク児に対して介入を行う．確定診断を待たずに訓練や支援を開始することは，現在注目を浴びている発達障害と同様である．

幼児期以降はQOLを最優先に，できるだけ正常児と同様の学校生活を送るためにも，地域に応じた療育の流れに乗るように助言する．理学療法士・作業療法士・言語聴覚士などと連携をとり，運動障害・視覚障害・言語障害・聴覚障害などに対処する一方，外科的手術，痙縮に対する薬物療法，ボツリヌス毒素局所注射，合併するてんかんのコントロールなど，可能性のある医学的治療について小児専門の整形外科医，脳外科医や発達障害専門医の協力を得て総合的な指導を実施する．

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Short Communication

Mutation Spectrum of *Dystrophin* Gene in Malaysian Patients with Duchenne/ Becker Muscular Dystrophy

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Abstract: We undertook the clinical feature examination and dystrophin analysis using multiplex ligation-dependent probe amplification (MLPA) and direct DNA sequencing of selected exons in a cohort of 35 Malaysian Duchenne/Becker muscular dystrophy (DMD/BMD) patients. We found 27 patients with deletions of one or more exons, 2 patients with one exon duplication, 2 patients with nucleotide deletion, and 4 patients with nonsense mutations (including 1 patient with two nonsense mutations in the same exon). Although most cases showed compliance to the reading frame rule, we found two unrelated DMD patients with an in-frame deletion of the gene. Two novel mutations have been detected in the *Dystrophin* gene and our results were compatible with other studies where the majority of the mutations (62.8%) are located in the distal hotspot. However, the frequency of the mutations in our patient varied as compared with those found in other populations.

Keywords: Becker muscular dystrophy, DNA sequencing, Duchenne muscular dystrophy, dystrophin, MLPA

INTRODUCTION

Mutation detection of *Dystrophin* gene is mostly complicated by its large gene size of 79 exons. Multiplex ligation-dependent probe amplification (MLPA) has been a widely used approach in detecting *Dystrophin* mutation among patients with Duchenne/Becker muscular dystrophy (DMD/BMD), mainly because of its overall gene coverage. Compared with the more conventional method using the Chamberlain and Beggs sets of primers (Beggs et al., 1990; Chamberlain et al., 1988) with 60% of detection rate, MLPA has increased the detection rate up to 70% of the DMD/BMD cases (Lai et al., 2006; Lalic et al., 2005; Takeshima et al., 2010). Additional direct DNA sequencing could further increase the mutation detection rate (Takeshima et al., 2010). This report described and analyzed genotype and phenotype spectrum of Malaysian patients with DMD/BMD using gene-wide coverage of MLPA coupled by direct DNA sequencing.

MATERIALS AND METHODS

Patients

Thirty-five Malaysian patients from unrelated families were referred to our laboratory with a clinical diagnosis of DMD or BMD. This study has been approved by the Ethical Review Board (Human) of the School of Medical Sciences, Universiti Sains Malaysia.

DNA Extraction

Genomic DNA was extracted from peripheral lymphocytes using a commercial kit according to the company's

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protocol (QIAamp DNA Blood Mini Kit; Qiagen, Gaithersburg, MD, USA).

MLPA

MLPA screening of all of *Dystrophin* exons employed two sets of probes (SALSA-MLPA P034-A2 and P035-A2) (MRC Holland, Amsterdam, The Netherlands).

DNA Sequencing

Direct DNA sequencing was performed using an automated DNA Sequencer (model 3130; Applied Biosystems, Forster City, CA, USA) for cases where MLPA found an apparent deletion of only one exon or no exon deletion/duplication at all.

In Silico Splicing Analysis of Nucleotide Mutations

Due to the absence of RNA specimens in our possession, we decided to perform in silico analysis to predict the effect of all nucleotide mutations on splicing. We used ESEfinder 3.0 (Cartegni et al., 2003) software (http://cb.utdallas.edu/cgi-bin/tools/ESE3/esefinder.cgi) that analyzes five exonic splicing enhancer (ESE) motifs (SF2/ASF, SF2/ASF (IgM-BRCA1), SC35, SRp40, and SRp55) to predict disruption of exonic splicing enhancer motifs by analyzing the splicing score created by the mutations as compared with the score in the wild-type exon sequence. In addition, we also analyzed the possibility of cryptic splice site using Human Splicing Finder software version 2.4.1 (www.umd.be/HSF/4DACTION/input_SSF).

RESULTS

Clinical Diagnoses and Patients Characteristics

All patients were clinically examined and diagnosed as having DMD/BMD based on their clinical features and creatine kinase (CK) levels (Table 1). Among these patients, only 18 had an identifiable family history of DMD (51.4%). All patients were positive for the Gower's sign and showed an average increase in serum CK levels by 22.8 ± 14.6 times (mean 10002.3 ± 4576.3 U/L), except those within the early stage of disease progression (65-F59 and 66-F60).

Dystrophin Mutation of the Malaysian DMD/BMD Cohort

This study included 35 DMD/BMD patients (Table 1). We identified five different types of disease-causing *Dystrophin* mutations: exonic deletions in 27 patients, exonic duplications in 2, nonsense in 3, missense in 2, and nucleotide deletions in 2. Two patients showed closely spaced combined mutations. They are patient 43-F41 with two concurrent nonsense mutations in exon 8 (c.701C→A and c.745C→T), which we reported in detail elsewhere (Rani et al., 2011), and patient 55-F51 with closely spaced missense mutation and nucleotide deletion (c.4741G→T and c.4742delA).

Mutations frequently clustered in two hotspots: 62.8% (22 out of 35) were localized within the distal hotspot. Two out of 30 (6%) deletion cases were in-frame (41-F39 and 45-F43, exons 8–19 and 3–13, respectively) but resulted in DMD phenotype.

In Silico Splicing Analyses

Mutations in our patients' cohort that underwent this analysis are the missense in patients 37-F35 and 55-F51, nonsense in patients 38-F36, 43-F41, and 49-F47, and nucleotide deletion in patients 55-F51 and 73-F63. Among them are patients with double nonsense in one exon (43-F41) and missense coupled by single-nucleotide deletion in one exon (55-F51), of which we analyzed them as combined events or separately.

We found that c.701C→A in patient 43-F41 decreases the best-hit scores in 2 out of 5 analyzed ESE motifs, SF2/ASF and SF2/ASF (IgM-BRCA1), by 0.14 and 0.16, respectively. Our in silico analyses did not show any effects of other nucleotide mutations on either ESE motifs or splice sites (raw data not shown).

DISCUSSION

A few studies have reported screening of selected *Dystrophin* exons only for deletions among Malaysian patients (Thong et al., 2005; Marini et al., 2008). This report is the first comprehensive molecular analysis of *Dystrophin* mutations among Malaysian DMD/BMD patients. We suggested that direct sequencing of additional exons could be done if MLPA could not identify any mutations. Although cDNA analyses could be an alternative, genomic DNA analyses are still preferable due to its practical reasons and use in molecular diagnosis with direct clinical relevance to patient/family counseling. Our data fit in a diverse spectrum of mutations.

We have also noted in this study that MLPA does improve the diagnostic technique, especially in detecting small mutations (patients 55-F51 and 73-F63).

Two of our patients showed DMD phenotype despite harboring in-frame deletions (41-F39 and 45-F43). The two in-frame deletions were located in the N-terminus of dystrophin. Thus, the most likely explanation for the

Table 1. Genotypes and phenotypes of Malaysian DMD/BMD patients in this study.

Patient ID	Genotypes		Age (year)	Onset (year)	Clinical information				Phenotype
	Mutations	Type			CK U/l	FH	Best current motor ability	Lower limb muscle	
1-F1	Del e43–52	Out	8	7	8540	Y	Waddling gait	Calf hypertrophy	DMD
3-F2	Del e44–51	Out	8	7	12690	N	Unstable gait	Calf hypertrophy	DMD
5-F4	Del e46–52	Out	12	5	6430	N	Bed-ridden	Contracture reflexes depressed	DMD
6-F5	Del e46–50	Out	8	3	10000	Y	Waddling gait	Calf hypertrophy	DMD
10-F9	Del e46–50	Out	7	5	4354	Y	Frequent falling, difficulties climbing stairs	Mild limb hyperextensibility	DMD
12-F11	Del e45	Out	9	5	Unknown	Y	Abnormal gait	Muscle weakness	DMD
13-F13	Del e46–53	Out	8	5	5794	Y	Gross motor development delay	Progressive muscle weakness	DMD
15-F14	Del e45–52	Out	9	6	2149	Y	Abnormal gait	Lower limb weakness, calf hyp	DMD
17-F16	Del e46	Out	8	7	8880	N	Abnormal gait and frequent falling	Weakness, reflex negative	DMD
18-F17	Del e48–54	Out	5.5	4	7355	Y	Waddling gait, not able to stand up	Wasting the muscle bulk, calf pseudohypertrophy	DMD
25-F23	Del e46–50	Out	7.5	5	8450	N	Waddling gait	Calf hypertrophy	DMD
26-F24	Del e16–17	Out	7	7	10260	N	Waddling gait	Calf pseudohypertrophy	DMD
27-F25	Del e50	Out	7	6	11770	N	Waddling gait	Calf pseudohypertrophy	DMD
28-F26	Del e18–32	Out	16	8	>10000	N	Wheelchair bound	Muscle wasting	DMD
30-F28	Del e45–54	Out	9	6	12170	N	Wheelchair bound	Small muscle bulk	DMD
33-F31	Del e8–30	Out	9	7	13325	N	Tip-toe gait	Calf pseudohypertrophy and tendon reflex absent	DMD
37-F35	c.8810A> G;p.E2937R (e59)	MS	8	5	18114	N	Waddling gait	Muscle weakness more of lower limbs, no tendons response	DMD
38-F36	c.3709A> T;p.K1237X (e27)*	NS	9	7	11456	N	Waddling gait	Progressive loss of muscles, calf pseudohypertrophy	DMD
39-F37	Del e44	Out	4.5	4	25320	Y	Frequent falling, not able to stand up	Calf pseudohypertrophy	DMD
40-F38	Del e 49–51	In	7	6	4363	Y	Still able to walk	Calf pseudohypertrophy	BMD
41-F39	Del e8–19	In	6	4	24590	Y	Wheelchair bound	Calf pseudohypertrophy and tendon reflex absent	DMD
43-F41	c.701C> A; p.S234X and c.745C> T;p.Q249X (e8)	NS	16	6	5408	N	Wheelchair bound	Calf pseudohypertrophy	DMD
45-F43	Del e3–13	In	7	5	9000	Y	Wheelchair bound	Calf pseudohypertrophy	DMD
49-F47	c.10171C> T;p.R3391X (e70)	NS	3	3	7000	Y	Tip-toe walking	Calf hypertrophy, lumbar lordosis, and hypotonic lower limbs	DMD
50-F48	Del e8–11	Out	7	6	Unknown	N	Frequent falling, difficulties climbing stairs	Calf pseudohypertrophy, oreflexia	DMD
51-F49	Del e49–50	Out	8	6	8076	Y	Waddling gait	Calf pseudohypertrophy	DMD
53-F50	Del e48–54	Out	6.5	6	18,104	N	Waddling gait, difficulty climbing stairs	Calf pseudohypertrophy	DMD
55-F51	c.4741G> T;p.M1580I and c.4742delA;p. N1581MFsX1583*	MS and Out	17	6	8440	Y	Bed-ridden	Muscle weakness	DMD
59-F54	Del e14–17	Out	10	8	6000	N	Sitting, shuttling	Weakness, calf hypertrophy	DMD
60-F55	Dup e11	Out	8	2	2466	Y	Bottom shuffling	Progressive weekness of both lower limb muscles	DMD
65-F59	Del e17–43	Out	6	5	162	N	Waddling gait	Bilateral calf swelling	DMD
66-F60	Del e3–43	Out	7	7	313	N	Waddling gait	Calf pseudohypertrophy	DMD
71-F61	Dup e45	Out	11	7	13310	N	Wheel chair bound	Weakness of lower limbs	DMD
73-F63	c.6804DelACAA	Out	8	7	11920	Y	Waddling gait	Bilateral lower limb weakness	DMD
74-F64	Del e45–50	Out	7	7	>10000	N	Not able to run or climb stairs	Calf pseudohypertrophy	DMD

e, exon; Out, out-of-frame; In, in-frame; NS, Nonsense; MS, Missense; CK, creatinine kinase level; FH, family history; Y, yes; N, no. Gower's sign was positive in all the cases. *Novel as of 18 September 2012

more severe phenotype in these two patients is a probable disruption of the actin binding domain (Muntoni et al., 1994; Cutiongco et al., 1995). Another reason might be that exon skipping has occurred at the RNA level, resulting in the skipping of these exons or creation of cryptic splice sites (Shiga et al., 1997; Melis et al., 1998; Ginjaar et al., 2000). Meanwhile, the presence of multiple mutations as possible phenotype modifiers, which have not been detected in the DNA of these two cases could not be excluded (Rani et al., 2011).

Our in silico analyses found that one mutation in exon 8 (c.701C→A) of patient 43-F41 altered the best-hit scores of two ESE motifs. However, it is difficult to postulate that this alteration may lead to changes in the splicing of exon 8. The effects of this nucleotide change on the ESE motif scores are still less than that of the classical example of exon skipping event in SMN2 exon 7 (Cartegni et al., 2003). Our analyses of SMN1/SMN2 exon 7 showed that the C→T change decreased the best-hit scores in 4 out of 5 analyzed ESE motifs: SF2/ASF, SF2/ASF (IgM-BRCA1), SC35, and SRp40, by 1.23, 0.28, 1.73, and 0.23, respectively.

Our analyses showed that exon 50 followed by exon 49 were the two most frequently deleted exons among Malaysian patients. This has further informed therapeutic studies, especially those focusing on targeted exon skipping (Takeshima et al., 2006; van Deutekom et al., 2007). In order to rescue the phenotype of at least 24% of Malaysian patients with DMD, skipping of exon 45 could be suggested.

In conclusion, we showed for the first time comprehensive clinical and molecular genetic findings in Malaysian patients with DMD/BMD. We found that MLPA coupled with further direct sequencing of 16 selected exons may increase the detection rate of *Dystrophin* mutation. Our second finding of closely spaced nucleotide changes implied that multiple *Dystrophin* mutations among DMD/BMD patients may be more frequent than previously thought.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Molecular characterization of an X(p21.2;q28) chromosomal inversion in a Duchenne muscular dystrophy patient with mental retardation reveals a novel long non-coding gene on Xq28

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Duchenne muscular dystrophy (DMD) is the most common inherited muscular disease and is characterized by progressive muscle wasting. DMD is caused by mutations in the *dystrophin* gene on Xp21.2. One-third of DMD cases are complicated by mental retardation, but the pathogenesis of this is unknown. We have identified an intrachromosomal inversion, inv(X)(p21.2;q28) in a DMD patient with mental retardation. We hypothesized that a gene responsible for the mental retardation in this patient would be disrupted by the inversion. We localized the inversion break point by analysis of *dystrophin* complementary DNA (cDNA) and fluorescence *in situ* hybridization. We used 5′ and 3′ rapid amplification of cDNA ends to extend the known transcripts, and reverse transcription-PCR to analyze tissue-specific expression. The patient's *dystrophin* cDNA was separated into two fragments between exons 18 and 19. Exon 19 was dislocated to the long arm of the X-chromosome. We identified a novel 109-bp sequence transcribed upstream of exon 19, and a 576-bp sequence including a poly(A) tract transcribed downstream of exon 18. Combining the two novel sequences, we identified a novel gene, named *KUCG1*, which comprises three exons spanning 50 kb on Xq28. The 685-bp transcript has no open-reading frame, classifying it as a long non-coding RNA. *KUCG1* mRNA was identified in brain. We cloned a novel long non-coding gene from a chromosomal break point. It was supposed that this gene may have a role in causing mental retardation in the index case.
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Keywords: dystrophin; long non-coding gene; mental retardation

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease affecting approximately one in 3500 males and is characterized by progressive muscle wasting during childhood. DMD shows muscle dystrophin deficiency because of mutations in the *dystrophin* gene that comprises 79 exons spanning >2500 kb on chromosome Xp21.2.[1] Mutations in the *dystrophin* gene range from single-nucleotide changes to chromosomal abnormalities (http://www.dmd.nl/).[2] Deletions encompassing one or more exons of the *dystrophin* gene are the most common cause of DMD and account for ~60% of mutations.[3] Disastrous mutations such as an out-of-frame deletion or nonsense mutation result in severe DMD.[4] DMD is complicated by mental retardation in one-third of patients.[5] Many studies have been conducted to elucidate the pathogenic mechanism of this complicating mental retardation. There are now several reports describing that mutations at the 3′ end of the *dystrophin* gene are related to complication with mental retardation.[6,7]

In a small portion of DMD patients, gross chromosomal rearrangements have been reported as the cause of dystrophin deficiency. In fact, a huge intrachromosomal deletion showing contiguous gene deletion syndrome was used to clone the *dystrophin* gene.[8] Intrachromosomal inversions have been identified in DMD.[9,10] X-autosome translocations involving the *dystrophin* gene have also been identified in a limited number of DMD patients.[11,12]

Disease-associated chromosomal rearrangements have been frequently used as a starting point in the elucidation of congenital

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disorders. Disrupted X-chromosomal genes are even more promising in this respect as they often represent knockouts.[10,13] In one DMD patient with complicating mental retardation, for example, an intrachromosomal inversion led to the identification of a Ras-like GTPase gene that causes mental retardation.[9] In addition, >20 genes have been identified by studying balanced X-chromosome rearrangements.[14]

The genes for X-linked mental retardation are largely unknown.[14–16] In a series of 442 Japanese mutations in the *dystrophin* gene, we have described a karyotype of 46,Y,inv (X)(p21.2;q28) to be the cause of one case of DMD.[2] This case was complicated with moderate mental retardation and it is thought very likely that the inversion disrupts one of the >40 genes responsible for mental retardation at Xq28.[17]

A diverse population of non-protein-coding RNAs has been reported in the human genome.[18,19] Long non-coding RNAs (lncRNAs), defined as greater than 200 nucleotides (nt) in length,[20] have a wide range of functions, including the regulation of transcription, RNA editing and organelle biogenesis.[19,21,22] It has been suggested that a subset of lncRNAs could contribute to neurological disorders when they become dysregulated.[23]

In this study, we characterized an intrachromosomal inversion inv(X)(p21.2;q28). We identified a novel long non-coding gene named *KUCG1* at the break point on Xq28. As this gene was expressed in the brain, we propose that disruption of the *KUCG1* gene may have a role in causing the mental retardation in the index case.

MATERIALS AND METHODS

Patient

The index patient is a 3-year-old Japanese boy. He is the first child of healthy, non-consanguineous, Japanese parents. Family history was unremarkable. When he was born at term, blood sampling was performed because of birth asphyxia. Unexpectedly, his serum creatine kinase level was highly elevated (25 510 $IU l^{-1}$; normal: <270 $IU l^{-1}$). He walked unassisted at the age of 15 months. As the high creatine kinase level persisted, a muscle biopsy was conducted at the age of 2 years to examine dystrophin expression. Dystrophin staining using monoclonal antibodies to three different domains revealed no reactive material in his skeletal muscle, confirming the diagnosis of DMD. He was referred to Kobe University Hospital for a genetic diagnosis (KUCG481). At the age of 3 years, his serum creatine kinase was 21 776 $IU l^{-1}$. His growth parameters were normal but he displayed moderate mental retardation (developmental quotient: 40). Brain magnetic resonance imaging findings were normal. His karyotype has been described in our previous report as 46,Y, inv(X)(p21.2;q28).[2] The inversion was inherited through his mother (data not shown). The protocol for the following study was approved by the ethical committee of Kobe University School of Medicine.

Dystrophin mRNA analysis

RNA was isolated from biopsied skeletal muscle and analyzed by reverse transcription-PCR as described previously.[24,25] The full-length *dystrophin* complementary DNA (cDNA) was amplified as 10 separate fragments.[26] To identify the break point within the *dystrophin* cDNA, fragments encompassing exons 18 and 19 were amplified using different sets of primers. The ends of two separate *dystrophin* cDNAs were confirmed by PCR amplification using newly designed primers; a reverse primer on exon 18 and a forward primer on exon 19, respectively (Table 1).

PCR amplification

PCR amplification was performed in a total volume of 20 µl, containing 2 µl of cDNA, 2 µl of 10 × ExTaq buffer (Takara Bio, Inc., Shiga, Japan), 0.5 U of ExTaq polymerase (Takara Bio, Inc.), 500 nM of each primer and 250 µM deoxyribonucleotide triphosphates (Takara Bio, Inc.). Thirty-five cycles of amplification were performed on a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany) using the following conditions: initial denaturation at 94 °C for 5 min, subsequent denaturation at 94 °C for 0.5 min, annealing at 59 °C for 0.5 min and extension at 72 °C for 1 min. The conditions were sometimes slightly modified for optimization. For nested or semi-nested PCR, 2 µl of the first reaction mixture was used as the template for the second amplification. The amplified PCR products were electrophoresed on 2% agarose gels with a low-molecular weight DNA standard (ϕX174-Hae III digest; Takara Bio, Inc.) and stained with ethidium bromide.

Table 1 Primers used in this study

Primer name	*Primer sequence (5′-3′)*
Dystrophin cDNA	
Exon 18r	GCAGAGTCCTGAATTTGCAATC
Exon 19f	CATTCACCATCTGTTCCACCA
5′-RACE	
c24r	CAGCCATCCATTTCTTCAGG
c21r	TTGTCTGTAGCTCTTTCTCT
c20r	ACTGGCAGAATTCGATCCAC
3′-RACE	
c16f	CTGATCTAGAGGTACCGGATCC
c18f	GCAGAGTCCTGAATTTGCAATC
KUCG1 mRNA	
Bf	GGTGAACCCCTCAATGTAAG
Cr	CTCTTGTATTCGCTGCAGTG
Cr2	CAGCAAACTTGTACAGTTGC

Abbreviations: cDNA, complementary DNA; RACE, rapid amplification of cDNA ends.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was conducted on metaphase spreads from the patients' lymphocytes with digoxigenin-labeled PCR product containing exons 18 or 19 of the *dystrophin* gene in combination with DXZ1 spectrum green probe for the X centromere (Vysis, Inc., Downers Grove, IL, USA). The exon 18 and 19 probes were detected by immunocytochemistry. This assay was carried out commercially by Mitsubishi Chemical Medience Co. (Tokyo, Japan).

5′-Rapid amplification of cDNA ends

5′-Rapid amplification of cDNA ends (RACE) was performed to obtain the 5′-end of the transcript using the 5′-RACE System Version 2 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with primers specific for the *dystrophin* mRNA (Table 1). Total RNA isolated from the patient's skeletal muscle was reverse transcribed using a gene-specific primer (c24r) and SuperScript II, a derivative of Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). PCR amplification was then performed using Taq DNA polymerase (Takara Bio, Inc.), a nested gene-specific primer (c21r), and a deoxyinosine-containing anchor primer provided with the system. A nested amplification using an inner gene-specific primer (c20r) and the anchor primer from the provider was also performed.

3′-Rapid amplification of cDNA ends

3′-RACE was performed to obtain the 3′-end of the transcript using the 3′-RACE System Version 2 (Invitrogen) with primers specific for the *dystrophin* mRNA (Table 1). First-strand cDNA synthesis was initiated at the poly(A) tail of mRNA using the adapter primer from the provider. After first-strand cDNA synthesis, the original mRNA template was destroyed with *RNase* H. Amplification was performed using a gene-specific primer (c16f) and a universal amplification primer from the provider that targets the cDNA complementary to the 3′-end of the mRNA. A nested amplification using an inner gene-specific primer (c18f) and the anchor primer from the provider was also performed.

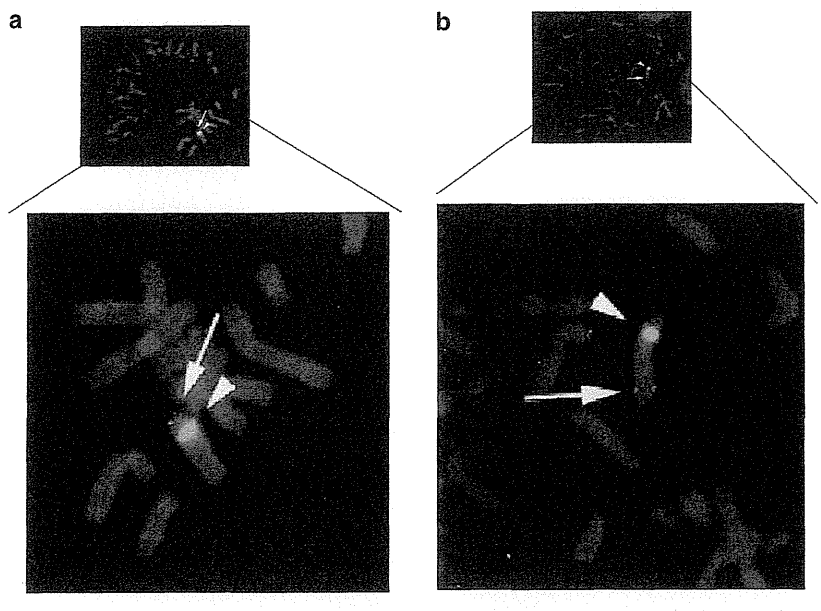

Figure 1 FISH analysis revealing disruption of the *dystrophin* gene. Results of FISH examination are shown with an enlarged panel (below). Centromeric signal is marked by arrowheads. (**a**) Exon 18 probe. Hybridization signals (arrow) are present on the short arm of the X-chromosome. (**b**) Exon 19 probe. Signals (arrow) are present on the long arm of the X-chromosome. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

DNA sequencing

PCR-amplified bands were excised from the gel with a sharp razor blade, pooled and purified using a QIAGEN gel extraction kit (QIAGEN, Inc., Hilden, Germany) according to the manufacturer's instructions. Purified products were sequenced either directly or after subcloning into the pT7 Blue T-vector (Novagen, San Diego, CA, USA). DNA sequencing was performed using a BigDye 1.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) in a Mastercycler Gradient (Eppendorf). The DNA sequences were determined using an automated DNA sequencer (ABI 310; Applied Biosystems).

mRNA expression of KUCG1

The expression of the *KUCG1* transcript was examined by reverse transcription-PCR. Human total RNA from 21 tissues (adrenal gland, bone marrow, brain, colon, fetal brain, fetal liver, heart, kidney, liver, lung, lymphocytes, placenta, prostate, salivary gland, skeletal muscle, spinal cord, testis, thymus, thyroid gland, trachea and uterus) was obtained from a human total RNA Master Panel II (Clontech Laboratories, Inc., Mountain View, CA, USA). cDNA was synthesized as described previously[27] from 2.5 μg of each total RNA. The *KUCG1* transcript spanning exon 2 to exon 3 was amplified by semi-nested PCR using primers Bf and Cr2, then Bf and Cr1 (Table 1), yielding a 314-bp fragment.

To check the integrity and concentration of the cDNA, the glyceraldehyde-3-phosphate dehydrogenase gene was also reverse transcription-PCR amplified, as described previously.[28]

Database searches and multiple sequence alignments

Homology searching was performed using the National Center for Biotechnology Information BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cloned 658-bp sequence was searched using NONCODE v3.0.[29] The core promoter of the *KUCG1* gene was analyzed using Genety X (Ver. 8.2.0) (GENETYX corporation, Tokyo, Japan).

RESULTS

We performed a molecular characterization of an intrachromosomal inversion in a DMD patient, inv(X)(p21.2;q28). We were able to amplify all 79 *dystrophin* exon-encompassing regions from the patient's genome (data not shown), indicating that the overall structure of the gene was intact. We examined the full-length *dystrophin* cDNA as 10 separate fragments. All the cDNA fragments could be obtained by PCR except one that covered exons 17 to 25 (data not shown). This suggested that the *dystrophin* cDNA was separated into two fragments; one from exons 1 to 18 and the other from exons 19 to 79 (data not shown). We used fluorescence *in situ* hybridization to confirm this. As expected, an exon 19 probe hybridized to the long arm of the X-chromosome, while an exon 18 probe hybridized to the short arm (Figure 1). We concluded that the exon 19 dislocation from the short arm to the long arm was the cause of DMD.

We were surprised the distal *dystrophin* cDNA (exons 19 to 78) could be PCR amplified, because this indicated that it formed a new fusion gene after dislocation. We, therefore, examined the full-length transcript using skeletal muscle RNA from the patient (Figure 2). We obtained a 5′-RACE product from exon 20, which contained 109 bp between the adapter and *dystrophin* exon 19 sequence (Figure 2). Homology searching of the identified sequence revealed that, although it did not match any known gene, it was identical to a portion of Xq28 (GenBank ID: NW001842413.1). The first nucleotide of the cloned sequence was 89,813 bp downstream from the melanoma antigen family A, 9 (*MAGEA9*) gene (Figure 3). Examination of the genomic sequence 3′ of the cloned 109-bp sequence revealed a GT dinucleotide, a splice donor consensus sequence (Figure 2). Although an AG dinucleotide-a consensus splice acceptor sequence was not present at the 5′-end, we did identify a TATA-box 5′-(ATATATAA CAATTTA)-3′, GC-box 5′-(TAAGGGCATACCCT)-3′ and CCAAT-box 5′-(CCTAGCCAATAG)-3′ at 168, 266 and 372 bp upstream of the cloned sequence, respectively (Figure 2). Additionally, a cap signal sequence (TCAGCAAC) was present 24 bp upstream. These characteristics indicated that the cloned sequence was the first exon of an unknown gene that is transcribed in the centromere-to-telomere direction. We concluded that, in the patient, the first exon of the

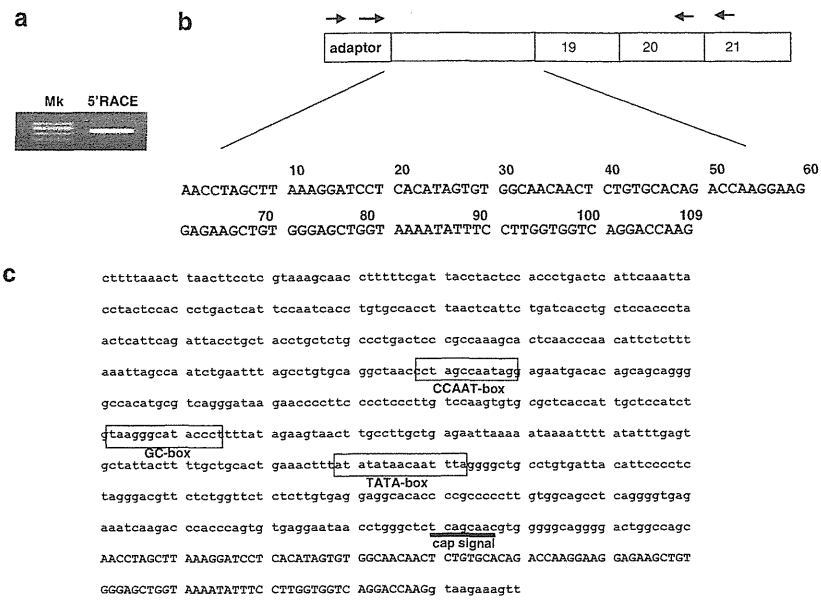

Figure 2 5′-RACE of *dystrophin* transcript. (**a**) Product of 5′-RACE of skeletal muscle RNA from the patient is shown (5′-RACE). Mk refers to ϕX174-*Hae III* molecular weight marker. (**b**) Schematic description of the amplified product. Numbered boxes indicate *dystrophin* exons. The open box indicates the novel 109-bp sequence. Arrows indicate primers used for PCR. (**c**) Part of Xq28 genomic sequence indicating the identified 109 nt (upper case). The boxed regions indicate the TATA-box, GC-box and CCAAT-box at 168, 266 and 372 bp upstream, respectively. A cap signal (thick underline) was identified 24 nt upstream of the 109-bp sequence.

unknown gene spliced to the dislocated part of the *dystrophin* gene, producing a chimeric *dystrophin* transcript.

To identify the rest of the novel gene, we conducted 3′-RACE using a primer in exon 16, and obtained one clear product (Figure 4). Sequencing of the amplified product revealed a 583-bp sequence inserted between *dystrophin* exon 18 and the adapter sequence (Figure 4). Homology searching revealed that this sequence, apart from the last seven 'A' nt, matched two separate regions of Xq28. The first 123 bp that were continuous with the 3′-end of exon 18 completely matched nt 148986563–148986685 and the last 453 bp matched nt 149008147–149008599 (NC 00023.10). The last nucleotide was located 4448 bp upstream of the melanoma antigen family A, 8 (*MAGEA8*) gene (Figure 3). Examination of the genomic sequences flanking the first 123 bp revealed consensus splice donor and acceptor sites at the 3′ and 5′ ends, respectively, indicating that it is an internal exon of an unknown gene. The last 453 bp had an AG dinucleotide immediately upstream but no GT dinucleotide downstream. Instead, a consensus polyadenylation signal (AATAAA) was identified 14 bp upstream of the 3′-end (Figure 4).[30] Considering the stretch of seven 'A's as part of a poly(A) tail, we concluded that the 453 bp sequence was the last exon of the unknown gene. The *dystrophin* promoter would produce a chimeric transcript comprising *dystrophin* exons 1–18 and two novel exons at the 3′-end.

Combining the results of 5′ and 3′-RACE, we had cloned a 685-bp-long transcript, the sequence of which we deposited in GenBank under the accession number JX283354. Homology searching did not reveal any transcript with significant similarity. The transcript had no significant open-reading frame, but because of its mRNA-like structure and length of >200 bp, we concluded that it was a novel lncRNA. We named it *KUCG1*. *KUCG1* spans nearly 50 kb on Xq28 and is located 9.0 kb downstream of *MAGEA9* and 4.4 kb upstream of *MAGEA8* (Figure 3). It has three exons separated by two introns (32 kb and 20 kb long, respectively). The site of recombination of the intrachromosomal inversion inv(X)(p21.2;q28) was intron 1. The inversion caused a head-to-tail fusion of *KUCG1* and *dystrophin* at the recombination sites. We searched for homologous lncRNAs using NONCODE v3.0,[29] but did not identify any significant matches. This indicated that *KUCG1* is a novel lncRNA. It was found that exon 3 of KUCG1 overlaps with the antisense transcript RP5-869M20.2, an lncRNA of unknown function (Figure 3).

We next examined the tissue-specific expression of *KUCG1* in humans. We amplified a fragment comprising exons 2 to 3 by reverse transcription-PCR of total RNA from 21 human tissues. The expected size product was obtained by semi-nested PCR from four tissues (lung, thyroid gland, brain and placenta), whereas no product was obtained from the other 17 tissues (Figure 5). Considering the brain expression of *KUCG1*, we consider that its disruption may be responsible for the moderate mental retardation in the index case.

DISCUSSION

In this report, we describe molecular characterization of an inverted X (p21.2;q28) chromosome in a patient with DMD and mental retardation. The inversion disrupted both the *dystrophin* gene, presumed to be the cause of the DMD, and a novel lncRNA, *KUCG1*, which may be the cause of the mental retardation. This is the third intrachromosomal inversion to be molecularly clarified in DMD,[9,10] but the first to disrupt unknown gene directly.

The *KUCG1* mRNA was detected in 4 out of 21 tissues: lung, thyroid gland, brain and placenta (Figure 5), indicating tissue-specific gene regulation despite the presence of three common consensus sequences in the promoter. The tissue-restricted expression and low expression level (semi-nested PCR was required to detect a product) could explain why this lncRNA has not been previously detected among the thousands of ncRNAs identified by high-throughput sequencing.[31]

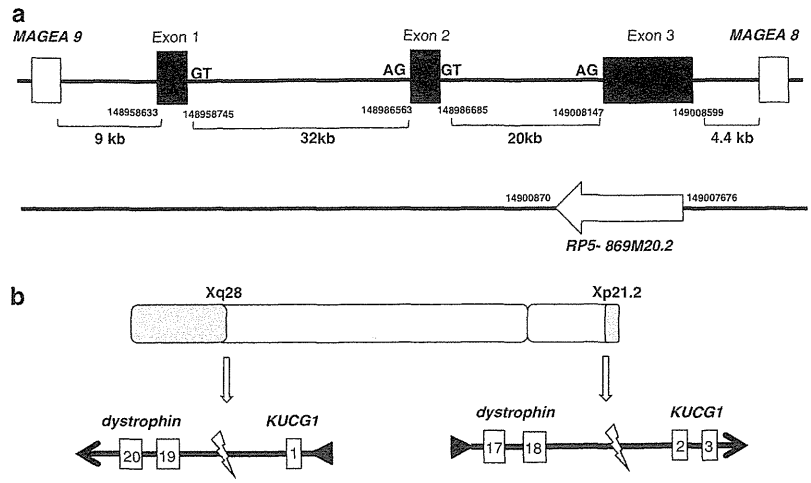

Figure 3 Schematic description of the gene and X-chromosome. (**a**) Schematic description of the *KUCG1* gene. The *KUCG1* gene that spans nearly 50 kb on Xq28 is transcribed in a centromere-to-telomere direction, and comprises three exons (black boxes) of 109, 123 and 453 bp, respectively. Numbers below the exons indicate the chromosomal nucleotide position according to GenBank NC00023.10. Introns 1 and 2 span 32 kb and 20 kb, respectively. *KUCG1* is located between *MAGEA9* and *MAGEA8* (open boxes). Another non-coding gene, *RP5-869M20.2* (ENSG00000230899.1) has been mapped to this region (nt 149007636–149009870) but is transcribed in the antisense direction (horizontal arrow). (**b**) Schematic description of the translocated X-chromosome schema of inv(X)(p21.2;q28) is described. At Xq28 intron 1 of the *KUCG 1* gene directly joined to intron 18 of the *dystrophin* gene. In contrast, intron 18 of the *dystrophin* gene joined to intron 1 of the *KUCG1* gene. Open and shaded boxes are normal and translocated parts of X-chromosome, respectively. Horizontal arrows and triangles indicate the direction and the promoter region of fused genes, respectively.

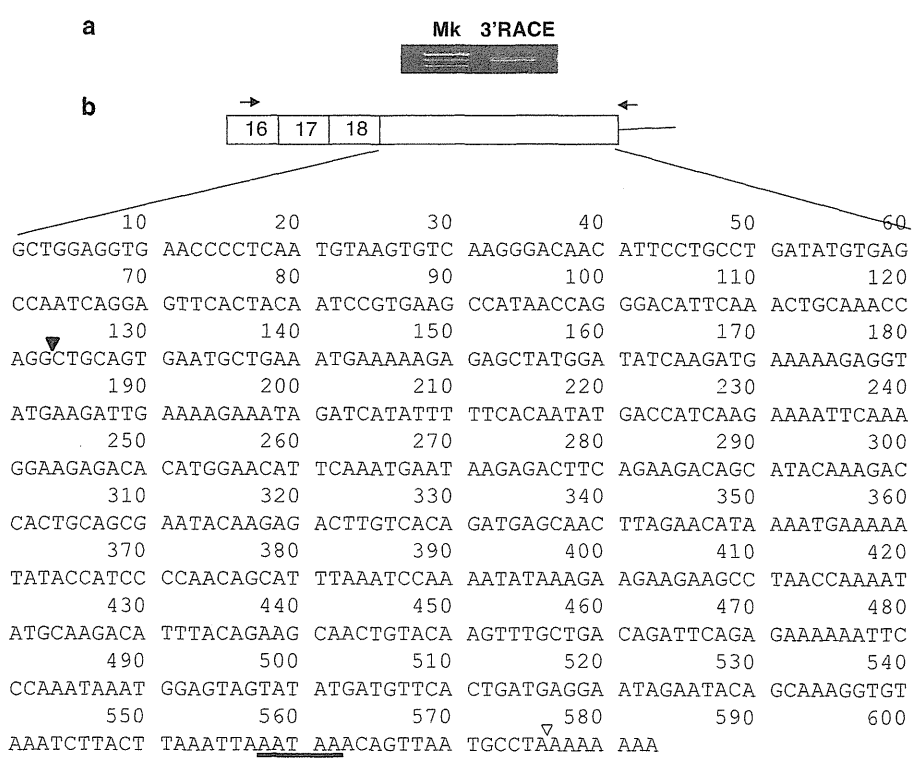

Figure 4 3′-RACE of *dystrophin* transcript. (**a**) Product of 3′-RACE of skeletal muscle RNA from the patient is shown (3′-RACE). Mk refers toϕX174-*Hae III* molecular weight marker. (**b**) Schematic description and sequence of the 3′-RACE product. *Dystrophin* exons are indicated as numbered open boxes. The product contained a 583-nt sequence (open box) downstream of *dystrophin* exon 18 (numbered box). The 583-nt sequence contains a polyadenylation signal (thick underline) followed by a short poly(A) tail (open triangle). The first 123 nt and the last 453 nt of the sequence (separated at the filled triangle) matched two separate regions on Xq28.

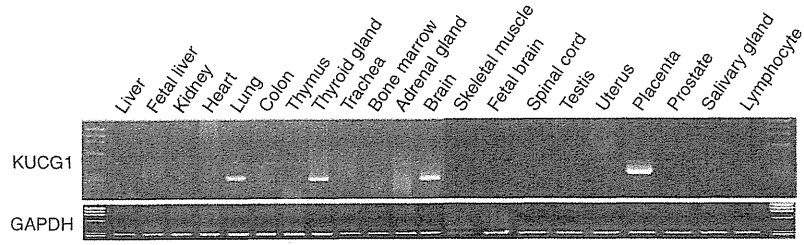

Figure 5 Tissue-specific expression of *KUCG1* mRNA. Products of reverse transcription-PCR amplification of *KUCG1* mRNA are shown. Reverse transcription-PCR amplification of 21 human tissues revealed a product in lung, thyroid gland, brain and placenta. The correct identity of the product was validated by sequencing. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were used as a reference.

What is the function of the *KUCG1* gene? As it undergoes splicing, is >200 nt long, and contains features such as a poly(A) signal/tail, *KUCG1* can be considered an mRNA-like ncRNA.[32,33] lncRNAs have been shown to have key roles in imprinting control, immune responses and human disease;[20] for instance, an ncRNA cloned from a chromosomal inversion was recently demonstrated to cause autosomal dominant hypertension and brachydactyly (OMIM 112410).[34] In the central nervous system, the increasing variety of ncRNAs shown to be expressed has suggested a strong connection between ncRNAs and the complexity of the system.[35] Hundreds of lncRNAs have been shown to localize to specific neuroanatomical regions, cell types or subcellular compartments within the brain[36] and a subset of lncRNAs is likely to contribute to neurological disorders.[23] For instance, the levels of the linc-MD1 lncRNA are strongly reduced in DMD,[37] indicating a role for this lncRNA in the disease pathology of DMD.

The mechanism of action of lncRNAs is thought to involve direct binding to target sites on proteins and RNAs.[33,37] It is interesting that exon 3 of *KUCG1* overlaps with the antisense transcript *RP5-869M20.2*, an lncRNA of unknown function. It is possible that transcripts from *KUCG1* and *RP5-869M20.2* form a double-stranded RNA that has a particular physiological role.

As *KUCG1* is expressed in the brain, we suspect that its disruption is responsible for the moderate mental retardation in the index case. Although >40 genes responsible for X-linked mental retardation have been annotated to Xq28,[17] the gene(s) responsible for many cases of X-linked mental retardation remain unidentified.[14] To test whether *KUCG1* is responsible for other cases of X-linked mental retardation, we sequenced *KUCG1* in ten Japanese families with X-linked mental retardation for which no responsible gene mutation has been identified. No mutations were identified (data not shown). Although we have not provided direct evidence linking mental retardation to mutation of *KUCG1*, further studies of its function, and mutation analysis in other X-linked mental retardation families, is warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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BRIEF REPORT

Human Mutation

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Pathogenic Orphan Transduction Created by a Nonreference LINE-1 Retrotransposon

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ABSTRACT: Long INterspersed Element-1 (LINE-1) retrotransposons comprise 17% of the human genome, and move by a potentially mutagenic "copy and paste" mechanism via an RNA intermediate. Recently, the retrotransposition-mediated insertion of a new transcript was described as a novel cause of genetic disease, Duchenne muscular dystrophy, in a Japanese male. The inserted sequence was presumed to derive from a single-copy, noncoding RNA transcribed from chromosome 11q22.3 that retrotransposed into the *dystrophin* gene. Here, we demonstrate that a nonreference full-length LINE-1 is situated in the proband and maternal genome at chromosome 11q22.3, directly upstream of the sequence, whose copy was inserted into the *dystrophin* gene. This LINE-1 is highly active in a cell culture assay. LINE-1 insertions are often associated with 3′ transduction of adjacent genomic sequences. Thus, the likely explanation for the mutagenic insertion is a LINE-1-mediated 3′ transduction with severe 5′ truncation. This is the first example of LINE-1-induced human disease caused by an "orphan" 3′ transduction.

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KEY WORDS: LINE-1; retrotransposon; 3′ transduction; dystrophin; Duchenne muscular dystrophy

Retrotransposons ("jumping genes") are highly abundant mobile genomic elements. In particular, the long interspersed element-1 (LINE-1 or L1) class comprises 17% of the human genome. A full-length human LINE-1 is about 6 kilobases and contains a 5′ untranslated region (UTR) encoding promoter activity [Swergold, 1990], two open reading frames (ORFs) separated by a spacer [Dombroski et al., 1991], a 3′ UTR, and a poly(A) tail. ORF1 encodes a protein with RNA binding [Hohjoh and Singer, 1996; Martin, 1991] and nucleic acid chaperone activity [Martin and Bushman, 2001], while ORF2 is a protein with endonuclease [Feng et al., 1996] and reverse transcriptase activities [Mathias et al., 1991]. LINE-1s are autonomous nonlong terminal repeat retrotransposons that move by a potentially mutagenic "copy and paste" mechanism via an RNA intermediate that is reverse transcribed and inserted into the genome [reviewed in Goodier and Kazazian, 2008]. LINE-1s can also cause disease indirectly, through mobilization of the nonautonomous Alu and SVA (SIVE-VNTR-Alu) retrotransposons [Dewannieux et al., 2003; Hancks et al., 2011; Ostertag et al., 2003].

Recently, the retrotransposition-mediated insertion of a new cDNA was described as a novel cause of genetic disease, Duchenne muscular dystrophy (MIM# 310200), in a Japanese boy [Awano et al., 2010]. In this work, it was presumed that the inserted sequence was derived from a nonrepetitive noncoding RNA transcribed from chromosome 11q22.3 that was reverse transcribed and integrated in the antisense orientation into exon 67 of the *dystrophin* gene on chromosome X, causing exon 67 skipping. The whole insertion was 327-bp long, of which 212 bp was identical to a sequence on chromosome 11q22.3 (chr11:105,479,198–105,479,409 of hg19/NCBI Build 37.1 Feb 2009), while the remaining 115 bp was a poly(T) stretch. The inserted sequence had hallmarks of LINE-1 retrotransposition, namely a poly(A) tail complementary to the poly(T) stretch, target site duplication flanking the insertion in *dystrophin* exon 67, and insertion at a near-consensus LINE-1 endonuclease site (TTTT/CA instead of TTTT/AA) [Awano et al., 2010]. Another LINE-1-related phenomenon is 3′ transduction, the co-mobilization of DNA sequences downstream of LINE-1s as a consequence of transcriptional read-through due to the weak LINE-1 poly(A) signal [Holmes et al., 1994; Moran et al., 1996]. Because LINE-1 insertions are often associated with 3′ transductions [Goodier et al., 2000; Moran et al., 1999; Pickeral et al., 2000], we hypothesized that the insertion in the patient might result from such an event. However, no LINE-1 was present in the DNA upstream of the single copy sequence from chromosome 11q22.3 in the human reference genome (hg19).

On the other hand, a LINE-1 directly upstream of the transduced sequence on chromosome 11q22.3 was present in one Japanese individual of 15 unrelated individuals in a LINE-1-targeted resequencing dataset generated in our laboratory [Ewing and Kazazian, 2010]. Based on bioinformatic analysis, this LINE-1 was absent from the 185 HapMap phase I individuals, including 30 individuals with self-reported Japanese ancestry, whose genomes were sequenced by the 1000 Genomes Consortium [1000 Genomes

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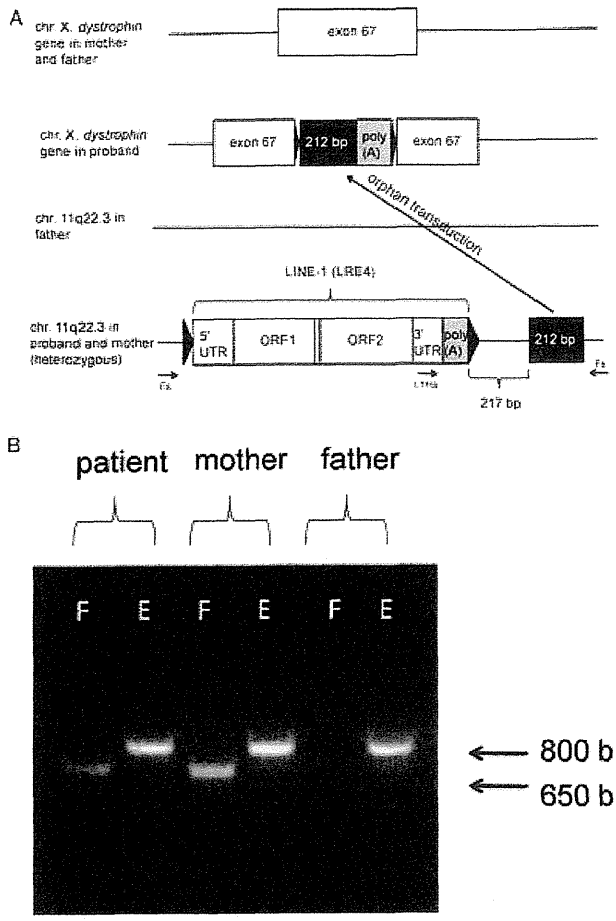

Figure 1. **A:** Orphan 3′ transduction event in the patient. The transduced sequence is in black. Black triangles indicate target site duplications (3 bp in exon 67, 21 bp at chromosome 11q22.3). **B:** PCR analysis of the 3′ junctions of the presumed progenitor (LRE4) of the DNA sequence inserted into the *dystrophin* gene with the primers indicated in A. Genotyping primer pairs L1Hs (5′-GGGAGATATACCTAATGCTAGATGACAC-3′) and Fs (5′-CGTTACATTTCACCACAGATTG-3′) amplify the filled (F) site (around 686 bp), while primers Es (5′-AGCACAATACCTTGCACATTAG-3′) and Fs amplify only the empty (E) site (828 bp) due to short primer extension time. Forty Japanese individuals (80 chromosome 11s) were also genotyped for the LRE4 allele and five were positive (data not shown). Thus, this allele is an uncommon variant in the Japanese population. PCR conditions are available upon request.

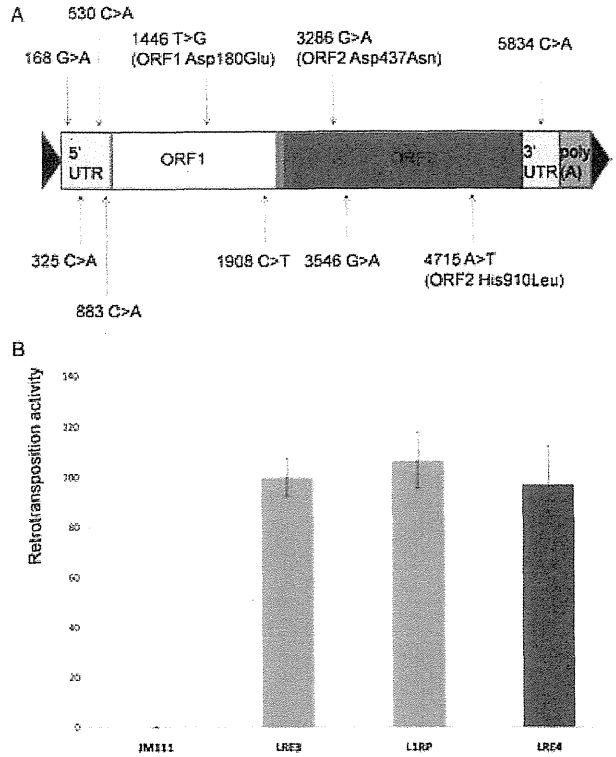

Figure 2. **A:** Schematic diagram of LRE4. Nucleotide changes differing from LRE3 [Brouha et al., 2002] are indicated. LRE4 was PCR amplified from the patient's blood DNA with iProof High Fidelity DNA Polymerase (Bio-Rad, Hercules, CA) and sequenced. LRE4 belongs to the Ta-1d class of human-specific LINE-1s [Boissinot et al., 2000], and the Ta class elements cause most de novo pathogenic human insertions [reviewed in Chen et al., 2005]. **B:** Retrotransposition activity of LRE4 (99-LRE4-EGFP-Puro) in HEK293T cells. LRE4 was PCR amplified with iProof High-Fidelity DNA Polymerase (Bio-Rad) using the primers 5′-CGTTACATTTCACCACAGATTG-3′ and 5′-AAGTAAAATAGAGGTTTTGGGGG-3′. The PCR product was cloned with the TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA) and was subsequently cloned to replace L1RP in the 99-RPS-EGFP-Puro reporter construct with *Bsf*Z17I and *Not*I-HF double digestion and ligation. HEK293T cells were transfected with 1 μg of each plasmid in a 6-well plate using Fugene 6 (Roche, Indianapolis, IN). Transfected cells were selected with puromycin, and retrotransposition events were evaluated 4 days later by FACS analysis of EGFP expressing cells. 99-RPS is the highly active L1RP cloned into a modified pCEP4 plasmid lacking the CMV promoter. Retrotransposition activity of 99-LRE3-EGFP-Puro is set to 100%. JM111 has the same sequence as 99-RPS, except that it contains two point mutations in ORF1 that abolish retrotransposition in cis [Moran et al., 1996] and serves as a negative control. Retrotransposition activity of L1RP and LRE3 was similar in HEK293T cells. Standard deviation of two independent experiments done in triplicate is shown.

Project Consortium, 2010; Stewart et al., 2011]. Additionally, it was not detected in our independent whole-genome analysis of individuals included in the 1000 Genomes project [Ewing and Kazazian, 2011]. The lack of evidence for this insertion in multiple analyses indicates that it may represent a population-restricted variant present at low allele frequency in the general population. To evaluate further its gene frequency in the Japanese population, we analyzed 80 Japanese chromosome 11q22.3 sites by PCR and found five sites containing the LINE-1, corresponding to a gene frequency of 6% (data not shown). Here, we demonstrate through PCR-based analysis that this nonreference LINE-1 is full length and is situated in the maternal and proband genomes at chromosome 11q22.3, 217 bp 5′ of the retrotransposed 212-bp sequence that was inserted into the *dystrophin* gene (Fig. 1A and B). Thus, the most likely explanation for the mutagenic insertion is a LINE-1-induced 3′ transduction event from chromosome 11q22.3 with severe 5′ truncation upon insertion, such that only part of the 3′ transduced sequence was inserted (Fig. 1A). What is less clear is in which cell lineage and at what time-point the LINE-1 RNA was reverse transcribed and inserted into the genome. Integration into the *dystrophin* gene most likely occurred in one or more of the mother's germ cells or early during the proband's development, because the mother's blood was negative for the presence of the 3′ transduced sequence.

To characterize the LINE-1 progenitor element on chromosome 11q22.3, hereafter referred to as LRE4 (LINE-1 Retrotransposable Element 4; BankIt1482137 LRE4 JN698885 [GenBank, http://www.ncbi.nlm.nih.gov/Genbank/]), we PCR amplified it

from the patient's blood DNA and sequenced it from multiple independent PCR products, all of which had the identical sequence. LRE4 is a full length Ta-1d element and contains 10 nucleotide alterations relative to LRE3, the most active LINE-1 isolated to date, and the only LINE-1 that is an exact match to the active LINE-1 amino acid consensus sequence [Brouha et al., 2002, 2003]. Three of the nucleotide substitutions in LRE4 resulted in amino acid changes relative to LRE3 (Fig. 2A). All LRE4 nucleotide changes were identified in other LINE-1s present in the hg19 assembly. However, no reference LINE-1 contained all 10 nucleotide alterations, nor was it present in a recent dataset of active LINE-1s [Beck et al., 2010]. To determine the retrotransposition activity of LRE4, we cloned it into a plasmid with an enhanced green fluorescent protein (EGFP) retrotransposition indicator cassette, creating 99-LRE4-EGFP-Puro. This cassette was designed so that translation of the EGFP reporter gene occurs only after LINE-1 reverse transcription and integration of its cDNA copy into the genome—that is, after a retrotransposition event [Moran et al., 1996; Ostertag et al., 2000]. Upon transfection of HEK293T cells with 99-LRE4-EGFP-Puro, and selection for transfected cells with puromycin, retrotransposition events were evaluated by FACS analysis of EGFP expressing cells [Ostertag et al., 2000]. 99-LRE4-EGFP-Puro demonstrated a retrotransposition activity comparable to plasmids containing LRE3 or L1RP (Fig. 2B), indicating that it is a highly active or "hot" retrotransposon [Brouha et al., 2003].

This is the fifth case of LINE-1-driven insertional mutagenesis of the *dystrophin* gene [Narita et al., 1993; Yoshida et al., 1998; Musova et al., 2006; and Awano et al., 2010 together with the current study]. Therefore, mutation analyses of this gene should take into account large insertions mediated by LINE-1s. Although, LINE-1s are often truncated at their 5′ end, this is the first example of LINE-1-induced human disease caused by an orphan 3′ transduction, that is, a LINE-1-mediated insertion lacking LINE-1 sequence. Two nondisease causing retrotransposition events of gene fragments have also been described that may have arisen by LINE-1-mediated 3′ transduction, with the transducing LINE-1 being lost [Ejima and Yang, 2003; Rozmahel et al., 1997]. In a previous report, we showed that a mutagenic insertion into the *α-spectrin* gene was the result of an SVA-mediated orphan 3′ transduction [Ostertag et al., 2003]. Therefore, any insertion of a nonrepetitive sequence bearing the hallmarks of retrotransposition should be further investigated for a LINE-1- or SVA-mediated transduction event, as previously postulated by Moran et al. [1999]. Our results indicate that LRE4 is a highly active, polymorphic retrotransposon with a pathogenic history.

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