AO against exon 19 resulted in dystrophin expression in the skeletal muscle of one DMD patient with an exon 20 deletion by producing in-frame mRNA lacking the exon 19 sequence (1). More recently, it has been reported that local administration of AOs against exon 51 induced exon skipping and dystrophin expression in the muscle of DMD patients (2, 3). These findings strongly support the rationale for the clinical application of AO therapy.

The exon skipping approach is mutation specific because different mutations require skipping of different exons. Genomic deletions of one or more exons of the *DMD* gene cluster in proximal (exons 2–20) or distal (exons 45–55) hot spots are categorized into various patterns (4). Therefore, a series of AOs inducing the skipping of various exons of the dystrophin transcript are required to treat the majority of DMD patients.

To develop the broad therapeutic applicability of this exon skipping strategy, the identification of an AO that causes efficient skipping of a particular exon is a critical step. There are presumed target sequences to which AOs bind: exonic splicing enhancer (ESE) sequences and splicing consensus sequences. Many exons contain ESEs, which facilitate the inclusion of genuine exons (5). We found an ESE in exon 19 by analyzing one DMD patient in whom an intraexonal 52-bp deletion in exon 19 resulted in exon skipping (6). In vitro and in vivo splicing analysis revealed that the deleted sequence in the index case functioned as an ESE, and the skipping of exon 19 was induced by an AO against this ESE sequence in cultured cells and in a patient (1, 7–9). The mechanism of AO-mediated exon skipping is considered to be blocking the binding of splicing factors to the ESE.

An alternative mechanism is to use AOs to block the function of splicing consensus sequences. However, this approach runs the risk of unintentionally inducing the skipping of other exons because highly conserved sequences are present in most splice sites. Therefore, ESEs are favorable targets for AOs.

ESEs can, in theory, be identified by Web-based algorithms (10–12), but none has 100% predictive accuracy. Therefore, in general, a trial and error procedure is still required to identify potent AOs. This must be a reliable and simple process. In this chapter, we describe a method for AO optimization using an in vitro splicing system with cultured DMD myocytes. As examples, we show the optimization of AOs against exons 44 and 6. In this method, AOs are synthesized that cover the whole region of the target exon sequence. The AOs are then individually transfected into cultured DMD myocytes, and dystrophin mRNA expression is analyzed to determine the ability of each AO to induce exon skipping.

To select optimized AOs using in vitro splicing analysis with cultured DMD myocytes, the chemical characteristics of the AOs must also be considered. In antisense technology, phosphorothioate oligonucleotides have been most widely used, but these have a

number of limitations, such as a low affinity for RNA. Therefore, many studies have focused on developing modified oligonucleotides; phosphorodiamidate morpholino oligomers (PMOs) and 2′-*O*-methyl phosphorothioate oligomers (2OMePS) have been used in recent clinical trials (2, 3). A novel nucleotide with an ethylene bridge between 2′-*O* and 4′-*C* ribose (2′-*O*, 4′-*C*-ethylene-bridged nucleic acid [ENA]) was chemically synthesized and has been shown to be highly nuclease resistant and have a high binding affinity for the complementary RNA strand (13). Recently, we showed that the exon skipping ability of an AO consisting of 2′-*O*-methyl RNA and ENA (an RNA/ENA chimera) was more than 40 times higher than that of the corresponding phosphorothioate oligonucleotide (14). Therefore, for optimization of AOs, we have used RNA/ENA chimera oligonucleotides.

2. Materials

2.1. AO Design

No materials needed.

2.2. Myocyte Culture

1. Trypsin 250, 5% in phosphate-buffered saline (PBS). Stored in single-use aliquots at −20°C.
2. Sterilized scissors (to cut the muscle biopsies).
3. 100-μm nylon cell strainers (to make single-cell suspensions).
4. Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS).
5. Growing medium: DMEM supplemented with 20% FBS, 2% Ultroser-G, and 1% Antibiotics–Antimycotics.
6. Differentiation medium: DMEM supplemented with 2% horse serum (HS) and 1% Antibiotics–Antimycotics.
7. Gelatin (to coat tissue culture dishes).

2.3. Transfection of AO

1. Medium for transfection: OptiMEM (Invitrogen).
2. Reagents for transfection: Plus Reagent (Invitrogen) and Lipofectamine (Invitrogen).
3. Horse serum.
4. AOs.

2.4. mRNA Analysis

1. RNA isolation reagent, e.g., from ISOGEN.
2. PBS.
3. Reagents for reverse transcription (RT)-PCR: e.g., moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen) and random hexamer primers.
4. Plasmid for subcloning of RT-PCR products, e.g., pT7 Blue T vector (Novagen).

3. Methods

3.1. Design of AOs for the First Screening Step

Design a series of 15–20-mer AOs to cover the whole target exon sequence (see Notes 1–3). AOs consist of 2′-*O*-methyl RNA and ENA residues, and ENA residues are incorporated at cytosines and thymines or at both the 5′-end and 3′-end. They are synthesized using a DNA synthesizer as described previously (15).

3.2. Myocyte Culture

The procedure for myocyte culture is shown in Fig. 1.

1. Obtain the muscle biopsy sample from the biceps or quadriceps muscle of DMD patients with informed consent (see Notes 4 and 5).

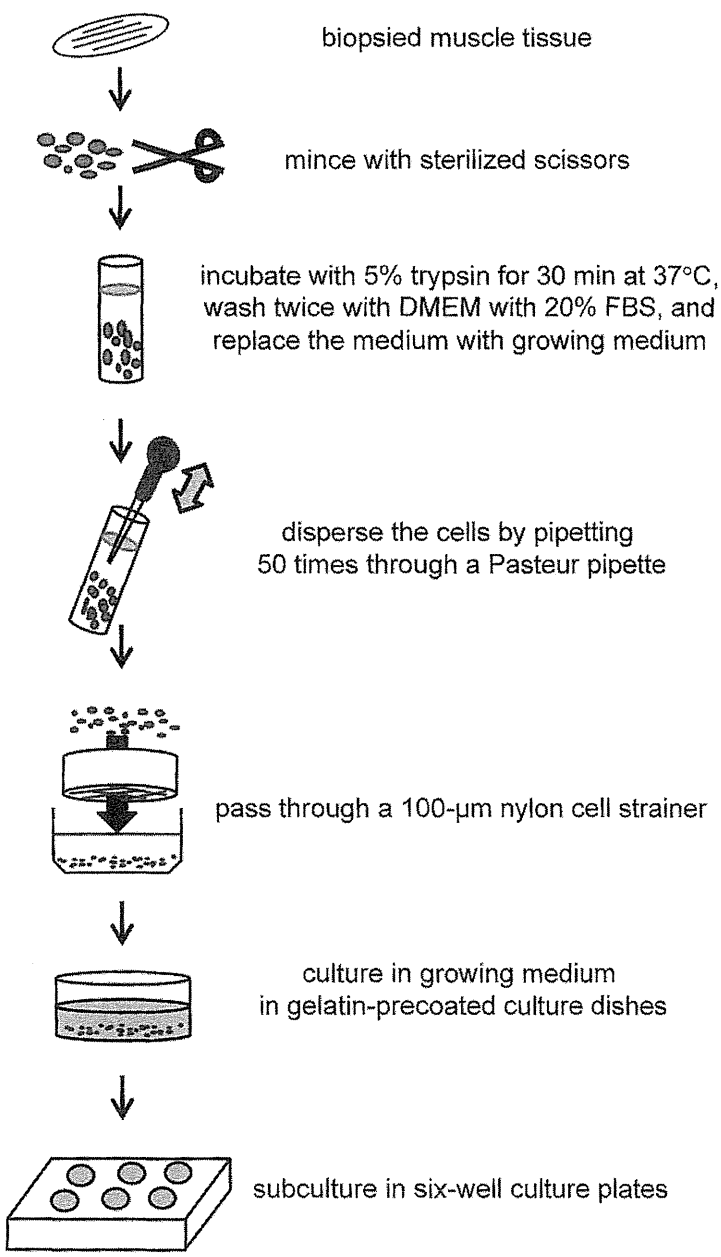

Fig. 1. The procedure for myocyte culture preparation.

2. Mince the muscle biopsy sample with sterilized scissors and wash three times with PBS.
3. Incubate minced tissue with 5% trypsin in PBS for 30 min at 37°C.
4. Collect dissociated cells by centrifugation at 200 × *g* for 2 min, wash twice with DMEM supplemented with 20% FBS, and then replace this medium with growing medium. Disperse cells by pipetting 50 times through a Pasteur pipette and then pass through a 100-μm nylon cell strainer to generate a single-cell suspension. Culture cells in growing medium in culture dishes precoated with gelatin (see Note 6).
5. When the number of primary myoblasts is sufficient, subculture by seeding in 6-well culture plates precoated with gelatin.
6. When the myoblasts reach semiconfluency, replace the medium to differentiation medium to induce cell differentiation (see Notes 7 and 8).

3.3. Transfection of AO

The procedure for the transfection of AOs is shown in Fig. 2.

1. On day 7, after induction of the differentiation, transfect DMD primary myotubes with an AO.
2. Dissolve the AO in 100 μl OptiMEM mixed with 6 μl Plus Reagent and incubate for 15 min at room temperature. Mix the incubated solution with 8 μl Lipofectamine dissolved in 100 μl OptiMEM and incubate for a further 15 min.
3. Add the mixture to culture medium (800 μl OptiMEM) to a final AO concentration of 200 nM.
4. After 3 h of incubation, add HS to a final concentration of 2% and incubate for another 2 days before RNA isolation.

3.4. mRNA Analysis

1. Two days after transfection of the AO, wash cultured myocytes twice with PBS, dissolve in 500 μl RNA isolation solution, and extract total RNA according to the manufacturer's instructions.

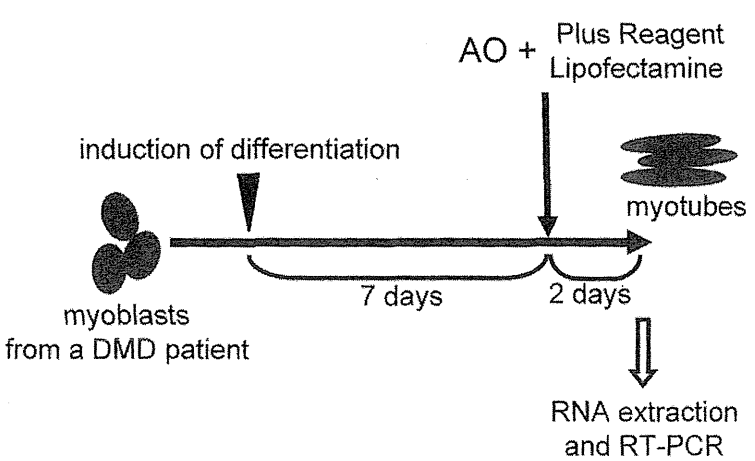

Fig. 2. The procedure for AO transfection.

2. Prepare cDNA from 2 μg total RNA using, e.g., MMLV-RT and random hexamer primers according to the instructions of the RT enzyme supplier.
3. Perform a PCR amplification on cDNA spanning the AO-targeted exon, and confirm the nucleotide sequence of the amplified product by direct sequencing or after subcloning of the product into a plasmid vector, e.g., pT7 Blue T.
4. Examine the potency of each AO by comparing the amount of PCR product with and without the targeted exon, and assess the most effective AO for each exon.

3.5. The Second Screening Step

When an optimal AO cannot be selected in the first screening step (see Note 9), design another set of AOs based on the results of the first screening, in which a potential target region has been roughly identified. Analyze the ability of the new AOs to induce exon skipping using the same procedure, until an optimal AO is identified.

Examples of the optimization for exon 44 and exon 6 are shown in Figs. 3 and 4.

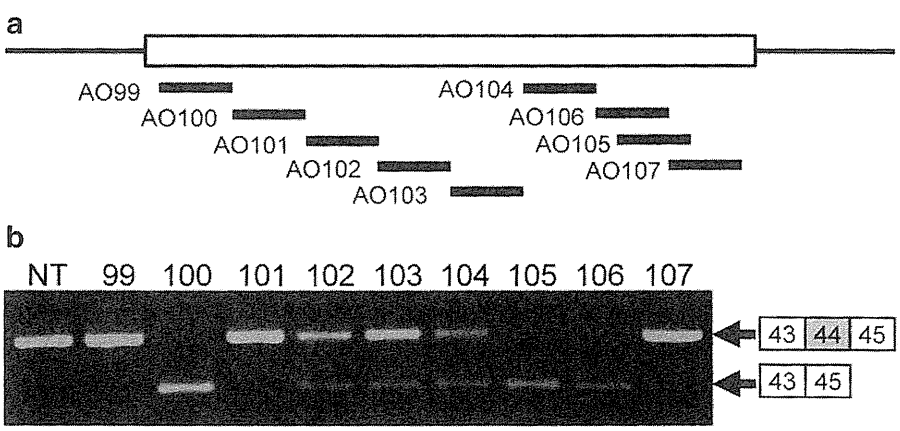

Fig. 3. Analysis of AOs designed to induce exon 44 skipping. (**a**) *Bars* represent the location of the AOs targeting exon 44, in which ENA residues are incorporated at cytosines and thymines, and 2′-*O*-methyl RNAs are incorporated at the other positions. The length of each AO is 18 mer, and the location is as follows: AO99 (6293–6310), AO100 (6311–6328), AO101 (6329–6346), AO102 (6347–6364), AO103 (6365–6382), AO104 (6383–6400), AO105 (6406–6423), AO106 (6400–6417), and AO107 (6418–6435), according to the cDNA reference sequence in GenBank, accession number NM_004006.1, in which the "A" of the start codon is nucleotide 1. The *box and lines on either side* represent exon 44 and its flanking introns, respectively. (**b**) RT-PCR results of AO-induced dystrophin exon 44 skipping. The RT-PCR products shown span exons 43–45 of the *DMD* gene after transfection of AOs designed to induce exon 44 skipping. *Lane NT represents* nontreated myocytes, and the numbers above each lane represent the name of the AO. The exons in the amplified products are shown schematically *on the right*, and the *shaded box represents* the AO target exon. AO100 induced exon skipping in 100% of the mRNAs, whereas AO99, AO101, and AO107 did not induce exon skipping. For the other AOs, both skipped and unskipped products were detected. AO102, AO103, and AO104 resulted in less skipped product than unskipped product, whereas AO105 and AO106 induced exon 44 skipping in the majority of mRNAs. Because AO100 induced complete exon skipping, further screening was not necessary and AO100 was selected as the optimal AO for exon 44.

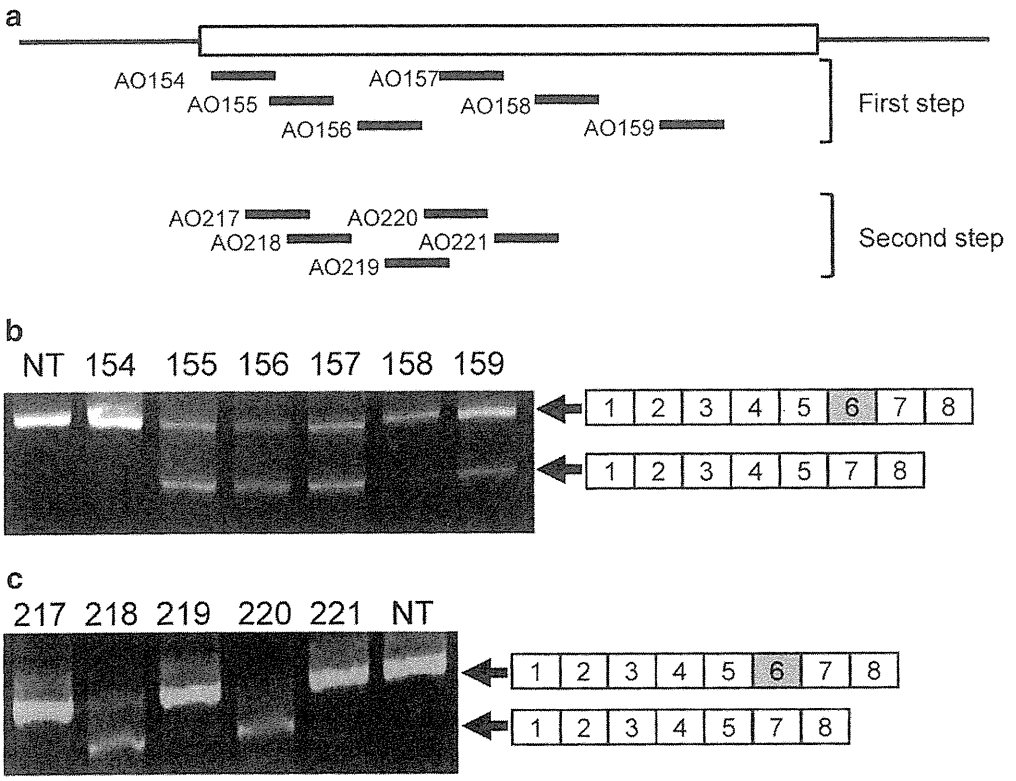

Fig. 4. Analysis of AOs designed to induce exon 6 skipping. (**a**) *Bars* represent the location of AOs targeting exon 6, in which ENA residues are incorporated at cytosines and thymines, and 2′-*O*-methyl RNAs are incorporated at the other positions. The AOs shown in the upper and the lower half were used in the first and second screening steps, respectively. The length of each AO is 18 mer, and the location is as follows: AO154 (360–377), AO155 (376–393), AO156 (399–416), AO157 (425–442), AO158 (449–466), AO159 (485–502), AO217 (369–386), AO218 (383–400), AO219 (407–424), AO220 (418–435), and AO221 (437–454), designated as described in the legend for Fig. 3. The *box and lines on either side* represent exon 6 and its flanking introns, respectively. (**b**) RT-PCR results of the first screening step for AO-induced dystrophin exon 6 skipping. The RT-PCR products shown span exons 1–8 of the *DMD* gene after transfection of AOs designed to induce exon 6 skipping. *Lane NT* represents nontreated myocytes, and the *numbers above each lane* represent the name of the AO. The exons in the amplified products are shown schematically *on the right*, and the *shaded box* represents the AO target exon. AO155, AO156, and AO157 induced exon skipping in half of the mRNAs, but an optimal AO could not be determined. (**c**) RT-PCR results of the second screening step for AO-induced dystrophin exon 6 skipping. For further screening, AOs 217–221 were designed around AO155, AO156, and AO157. AO220 induced exon skipping in almost all of the mRNAs, and was therefore selected as the best AO for exon 6.

Briefly, for exon 44, nine 18-mer AOs were designed to cover the whole exon sequence as shown in Fig. 3a. Representative examples of AO-induced exon 44 skipping are shown in Fig. 3b. AO100 induced complete exon skipping in the first screening step, and then AO100 was selected as the optimal AO for exon 44. In the case of exon 6, six 18-mer AOs were designed as shown in Fig. 4a (first step); however, an optimized AO could not be determined after first screening step (Fig. 4b). Therefore, another set of AOs were designed as shown in Fig. 4a (second step), and AO220 was selected as the best AO for exon 6 as shown in Fig. 4c (see legends to Figs. 3 and 4 for more details).

3.6. Modification of the Position of the ENA Residues (See Fig. 5)

For exon 44 and exon 6, the optimal AO induced exon skipping in almost all the mRNAs (Figs. 3 and 4). For other exons, such as exon 46, unskipped mRNA still remained even after transfection

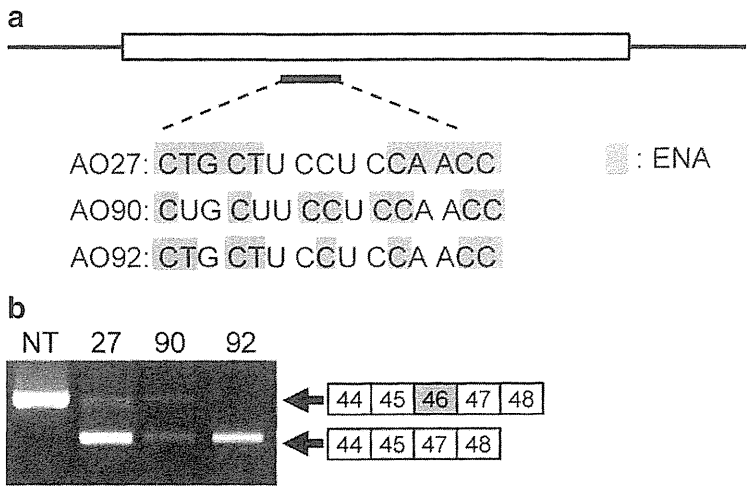

Fig. 5. The ability of modified AOs to induce exon skipping of exon 46. (**a**) The location and nucleotide sequence of each AO. AO27, AO90, and AO92 were designed at the same position (nt. 6677–6691), but with differing patterns of ENA residue incorporation. *Shaded letters* represent ENA-modified residues; *nonshaded letters* represent 2′-*O*-methyl RNAs. The *box and lines on either side* represent exon 46 and its flanking introns, respectively. (**b**) RT-PCR results of the modified AO-induced dystrophin exon 46 skipping. The RT-PCR products shown span exons 44–48 of the dystrophin mRNA after transfection of the modified AOs. *Lane NT* represents nontreated myocytes and the numbers above each lane represent the name of the AO. AO92 induced the skipping of exon 46 in almost 100% of the mRNAs, whereas unskipped mRNA still remained after AO27 transfection. The exons in the amplified products are shown schematically *on the right*, and the *shaded box* represents the AO target exon.

of the best AO, AO27 (Fig. 5b, lane 27). The position of the ENA residues within the AO might affect exon skipping ability. Therefore, the potency of the modified AOs in which nucleotide sequence was same but the ENAs were incorporated at different positions was examined when the optimal AO could not be determined. Modified AOs are shown in Fig. 5a (AO90 and AO92). Representative examples of modified AO-induced exon 46 skipping are shown in Fig. 5b. AO92 induced exon skipping in almost mRNA, and then AO92 was selected as the optimal AO for exon 46.

4. Notes

1. AOs that are complementary to ESEs are thought to be most effective at inducing exon skipping. There are Web-based algorithms to identify ESEs, such as ESEfinder (10), RESCUE-ESE (11), and SpliceAid (12), but none has 100% predictive accuracy (16–19). Therefore, using these Web-based programs alone runs the risk of missing the optimal AO.
2. The nucleotide sequences of AOs should be designed to cover the whole target exon sequence, and the ability of each AO to

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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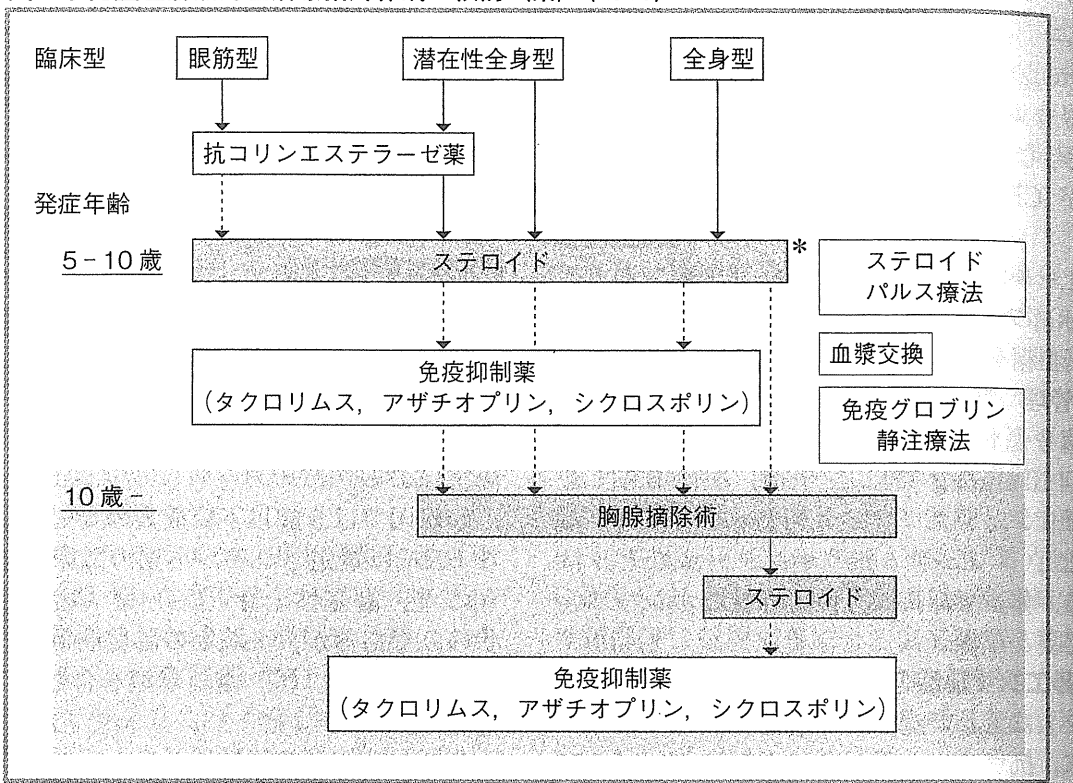
図　小児重症筋無力症治療指針作成の検討（案）（2010）



＊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，継続に行う．

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が多くなっている．

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…細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…

…MDでは以下の対症療法が主たるものとなる．

…理学療法

…MDでは，幼児・学童初期には足関節，学童中…な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…維持を積極的にはかるとともに，車椅子生活に…は脊弯予防のために継続したリハビリを実施．

…機不全は，生命を脅かす要因の1つである．呼…機能を維持できるように呼吸リハビリテーション…施する．さらに，血中の二酸化炭素分圧の上昇…られれば呼吸補助療法を開始する．

…薬物療法

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

…使用例）

…ドニン錠（5 mg）　0.75 mg/kg　分1　隔日…

…使用例）

…心不全　心エコー検査で心筋収縮異常が出現す…ば，重症化予防に投与を開始する．

…使用例）下記のいずれかを用いる．

…ナベース錠（2.5 mg）　5 mg　分2 [23]

…ーチスト錠（2.5 mg）　5 mg　分2 [18]

…性麻痺

…ebral palsy（CP）

…博　滋賀医科大学教授・小児科学

…診断

…概念

…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…障害で，種々の原因により生じた脳性運動障害の…群であり，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…常児の成長・発達とともに修飾を受け変容し得…

…性麻痺の原因には胎内感染，脳形成異常，胎盤…機不全，新生児仮死（低酸素性虚血性脳症），分…外傷，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．

Treatment of Duchenne muscular dystrophy with antisense oligonucleotides

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Abstract

Duchenne muscular dystrophy (DMD) is a lethal disorder of childhood caused by deficiency of muscle dystrophin. Interestingly, a milder form of the disease called Becker muscular dystrophy (BMD) is distinguished form DMD by delayed onset, later dependence on wheelchair support and longer life span. Both DMD and BMD are caused by mutations in the dystrophin gene and their clinical difference can be explained by the reading frame rule. Many attempts have been made to express dystrophin in DMD patients, but an effective treatment has not yet been established. Identification of dystrophin Kobe promoted the understanding of the regulatory mechanism of dystrophin pre-mRNA splicing. As a result, the antisense oligonucleotides therapy whereby the correction of the translational reading frame of dystrophin mRNA is provided by inducing exon skipping has been proposed to transform severe DMD into the milder form. Consequently, a 31-mer phosphorothioate oligonucleotides against the splicing enhancer sequence of exon 19 of the *dystrophin* gene (antisense oligonucleotides : AO) was shown to be able to induce exon 19 skipping. Furthermore, the transfection of AO into cultured myocytes from a DMD case with an out-of -frame deletion of exon 20 promoted expression of dystrophin successfully. The peripheral infusion of the AO solution into a DMD case harboring an out-of-frame deletion of exon 20 produced in-frame dystrophin mRNA and led dystrophin expression in skeletal muscle.

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal disorder of childhood usually associated with a functional deficiency of dystrophin. Until now no effective treatment for DMD has been established. Currently much attention has been paid for antisense oligonucleotides

(AO) therapy that converts severe DMD to milder Becker muscular dystrophy (BMD) by inducing exon skipping. Present progress made in this area of research with particular reference to dystrophin Kobe is presented.

2. Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is the most common inherited muscular disease with a worldwide incidence of 1 in 3500 male births. DMD is a lethal disorder of childhood associated with a functional deficiency of dystrophin. The affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in the mid to late 20s. Interestingly, a milder form of the disease called Becker muscular dystrophy (BMD) is distinguished from DMD by delayed onset, later dependence on wheelchair support and longer life span. Both DMD and BMD are caused by mutations in the *dystrophin* gene. The *dystrophin* gene is 3,000 kb in size and consists of 79 exons encoding a 14 kb mRNA. Nearly two-thirds of identified mutations in the *dystrophin* gene are deletions with a loss of one or more exons. Duplications with acquisition of one or more exons have been observed in 5-10% of DMD/BMD patients. The remaining 20-30% of cases are caused by small mutations including point mutations, microdeletions, microinsertions, and chromosomal abnormalities.

3. Reading-frame rule

Although both DMD and BMD patients have been shown to have deletion mutations, the extent of the deletion does not always correlate with the severity of the disease : some BMD patients with mild symptoms have deletions encompassing numerous exons, whereas some DMD patients with severe symptoms lack a single exon. According to the reading-frame rule[1], the BMD patents with the deletions might be able to produce an in-frame dystrophin mRNA that would still direct the production of an internally truncated semi-functional protein. The deletions harbored by severe DMD patients, on the other hand, would bring together exons that, when spliced, would shift the translational reading frame in the mRNA, such that premature stop codon is created. This rule predicts that milder BMD patients would produce a smaller semi-functional protein while DMD patients would not produce a protein at all. Based on this rule phenotype of dystrophinopathy can be explained by their genotype in more than 90% of cases.

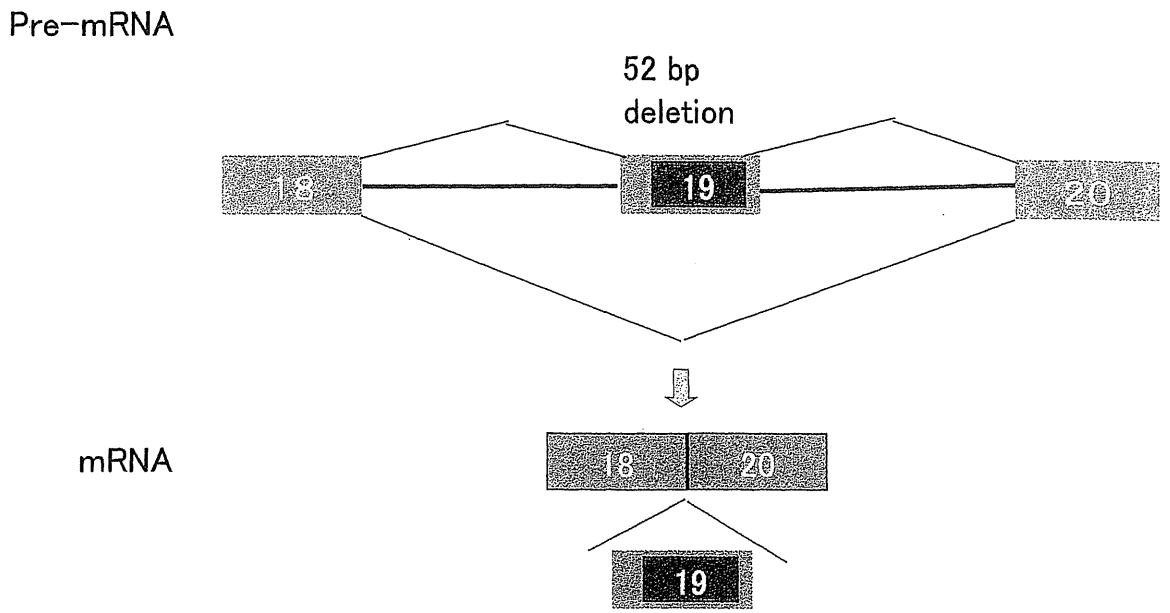

Fig. 1 Dystrophin Kobe

Splicing pathway of dystrophin Kobe is schematically described. In normal exon 19 is incorporated into dystrophin mRNA. In dystrophin Kobe the whole sequence of exon 19 disappeared from dystrophin mRNA. This exon skipping was induced even though all consensus sequences around the exon/intron junctions were maintained intact. Boxes and lines indicate exon and intron, respectively. Numbers inside the boxes indicate exon number. Diagonal lines indicate splicing pathways.

4. Dystrophin Kobe

In one particular *dystrophin* gene mutation named dystrophin Kobe[2], we found that exon skipping during splicing was induced by the presence of intra-exon deletion mutation in the genome, although all of the consensus sequences known to be required for splicing were unaffected. In dystrophin Kobe 52 bp out of 88 bp of exon 19 were found deleted and this 52 bp deletion was considered to cause DMD by shifting the reading frame[3]. Unexpectedly, his dystrophin mRNA of dystrophin Kobe was found smaller than predicted when it was analyzed by reverse-transcription PCR amplification. Sequence analysis of the product disclosed that the whole 88 bp of exon 19 was missing from the dystrophin cDNA, instead of the shortened exon 19[3]. This indicated that exon 19 of the dystrophin Kobe was skipped out during pre-mRNA splicing.

Taken it consideration that consensus sequences for splicing locating around the exon/intron junctions were maintained intact in the dystrophin Kobe[3], it was hypothesized that the deleted sequence of exon 19 in dystrophin Kobe may function as a cis-acting element for

exact splicing for the upstream and downstream introns. In order to exemplify this hypothesis, an *in vitro* splicing system using artificial dystrophin pre-mRNAs was constructed and the role of deleted sequence was examined. It was disclosed that splicing of intron 18 was almost completely abolished when the wild-type exon 19 was replaced by the dystrophin Kobe exon 19[4]. This indicated that the deleted region functioned as a splicing enhancer sequence. It was hypothesized that the antisense oligonucleotides may induce exon skipping by blocking the function of splicing enhancer. In fact, the 31-mer-phosphorothioate oligonucleotides (5'-GCCTGAGCTGATCTGCTGGCATCTTGCAGTT-3') (AO19) covering a splicing enhancer sequence of exon 19 was found to inhibit splicing in *in vitro* splicing[4]. Since the aforementioned result suggested a possibility of artificial induction of exon 19 skipping, AO19 was then transfected to normal lymphoblastoid cells. Remarkably exon 19 sequences disappeared from all dystrophin mRNAs at 24 hours after the transfection[5]. AO19 was thus proved to be a powerful tool with the ability to induce exon 19 skipping.

5. Production of dystrophin in DMD derived muscle cells

Induction of exon 19 skipping with AO19 was assessed for DMD treatment. An alternative strategy for DMD treatment might be to retard progression of the clinical symptoms, i. e., to convert DMD into BMD phenotype. Theoretically, this therapy can be done by changing a frame-shift mutation causing DMD into an in-frame mutation characteristic of BMD by modifying the dystrophin mRNA. Since transfection of the AO19 has been shown to induce exon 19 skipping[5], we subsequently investigated whether AO19 can be used to treat a DMD case with a 242 nucleotide deletion of exon 20 (**Fig. 2**). If exon 19 (88 bp) skipping is induced in this case, the translational reading frame of dystrophin mRNA will be restored by enlarging the deletion from 242 to 330 bp. As a result, this modulation of splicing should lead to the production of internally deleted dystrophin in muscle cells from the case. A Japanese DMD patient was identified to have a deletion of exon 20 of the *dystrophin* gene. A primary muscle culture cell established from his muscle biopsy sample was treated with AO19. Introduction of AO19 into the nuclei of cultured cells led to skipping of exon 19 in a proportion of total mRNA. As expected dystrophin-positive cells were identified. The percentage of dystrophin positive cells was nearly 20% at the 10th day after the transfection[6]. These results showed that dystrophin expression was promoted by editing dystrophin mRNA and strongly indicated a possibility of DMD treatment with

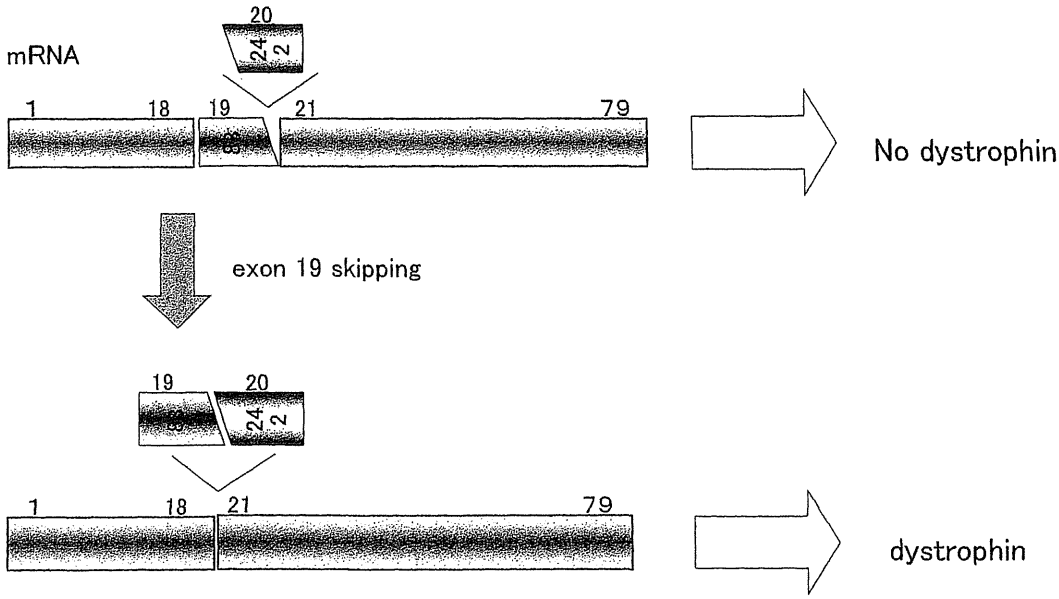

Fig. 2 Correction of translational reading-frame using antisense oligonucleotides

In DMD with a deletion of exon 20 (242 bp), splicing of dystrophin pre-mRNA proceeds to produce out-of-frame mRNA lacking exon 20 and no dystrophin is produced (top). In the presence of the antisense oligonucleotide against the splicing enhancer sequence, recognition of exon 19 (88 bp) is inhibited and splicing proceeds unrecognizing exon 19 to produce in-frame mRNA (bottom). Boxes and number in boxes indicate exon and exon number, respectively.

AO19.

However, delivery of AO19 into skeletal muscle was puzzled. When injection of labeled AO19 into peritoneum of *mdx* mouse was conducted, nucleus of muscle cells became fluorescent positive. Remarkably exon 19 skipping was observed in cardiac and skeletal muscle[7]. It thus can be concluded that AO19 can be delivered to muscle cells by directly injecting into venous system.

6. Treatment with antisense oligonucleotides

After obtaining the permission from the Ethical Committee of Kobe University Graduate School of Medicine and the informed consent from parents, a 10-year-old DMD patient possessing an out-of-frame, exon 20 deletion of the *dystrophin* gene received a 0.5 mg/kg intravenous infusion of AO19 that was synthesized under the GMP guideline. AO19 was administered at one-week intervals for 4 weeks. No side effects attributable to infusion were

observed. Remarkably, exon 19 skipping appeared in a portion of the dystrophin mRNA in peripheral lymphocytes after the infusion. In a muscle biopsy one week after the final infusion, the novel in-frame mRNA lacking both exons 19 and 20 was identified and found to represent approximately 6% of the total reverse transcription PCR product. Dystrophin was identified histochemically in the sarcolemma of muscle cells after oligonucleotide treatment. These findings demonstrate that phosphorothioate oligonucleotides may be administered safely to children with DMD, and that a simple intravenous infusion is an effective delivery mechanism for oligonucleotides that lead to exon skipping in DMD skeletal muscles[8].

7. Exon skipping therapy for DMD

Correction of the dystrophin reading frame from out-of-frame into in-frame by induction of exon skipping is now considered as the most promising treatment for DMD[9]. Therefore, this strategy has been applied for treatment of DMD with deletion in the deletion hot-spot[10]. In addition, this strategy can be applied to DMD cases with a nonsense mutation as natural rescue by exon skipping has been reported in some nonsense mutations[11,12]. Therefore, exon skipping by AO is considered widely applicable than expected. However, it should be careful in assessing the effect of AO, because unanticipated splicing error may ensue the altered splicing by AO[13].

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J. Neurogenetics, Early Online: 1–5

ISSN: 0167-7063 print/1563-5260 online
DOI: 10.3109/01677063.2012.762580

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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

Received 26 November 2012; accepted 21 December 2012
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