厚生労働科学研究費補助金

こころの健康科学研究事業

Duchenne型筋ジストロフィーのエクソンスキッピング誘導治療

平成１９年度～２１年度　総合研究報告書

研究代表者　松尾　雅文

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目　　次

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研究代表者：松尾　雅文（神戸大学大学院医学研究科・教授）

【研究要旨】
Duchenne 型筋ジストロフィー（DMD）のエクソンスキッピング誘導治療の臨床応用に向けた研究を行った。ジストロフィン遺伝子のエクソン欠失のホットスポット領域にあるエクソンのスキッピングを有効に誘導する RNA/ENA キメラの同定に成功した。また、GMP グレードの RNA/ENA キメラの合成体制に目__をつけた。さらに、治療効果判定の指標となる DMD のバイオマーカーとして、血清クレアチンキナーゼの自然歴を明らかにした。一方、RNA/ENA キメラにかわる低分子化合物によるエクソンスキッピング誘導についても検討し、有望な結果を得た。その結果、RNA/ENA キメラの臨床応用へ大きく前進した。

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A. 研究目的

デュシェンヌ型筋ジストロフィー（DMD）はジストロフィン欠損を呈する最も頻度の高いかつ重篤な遺伝性筋疾患である。しかし、未だ有効な治療法は確立されていない。DMD の多くはジストロフィン遺伝子のエクソン単位の欠失により、ジストロフィン mRNA のアミノ酸読み取り枠にずれを生ずるアウトオブフレーム変異を有している。そのため、欠失の下流でストップコドンが出現し、骨格筋でジストロフィン欠損を呈している。

私たちは、「ジストロフィン神戸」に関する分子病態の詳細な解析結果から「DMD を mRNA レベルで治療する」という独自の治療法を着想した。これは、ジストロフィン遺伝子のスプライシング時にエクソンのスキッピングを誘導することにより、アウトオブフレームをインフレームにかえてアミノ酸読み取り枠を修正し、ジストロフィンを産生させるものである。そして、ジストロフィン遺伝子のエクソン 19 内のスプライシング促進配列に対するアンチセンスオリゴヌクレオチドを用いて、着想通りにエクソンスキッピングの誘導及びジストロフィンの発現に成功し、本 DMD 治療法が有効なことを世界で初めて報告した。

本研究ではこのアンチセンスオリゴヌクレオチドを用いた治療をさらに多くの患者に応用できる様にするため、ジストロフィン遺伝子の欠失のホットスポット領域内にあるエクソンを対象として、そのスキッピングを誘導するアンチセンスオリゴヌクレオチドの確立をはかった。神戸大学小児科でフォローしている DMD 患者のエクソン欠失型の検討から、エクソン 45 のスキッピング誘導が多くの患者を治療できることが判明し、エクソン 45 のスキッピングをアンチセンスオリゴヌクレオチドを用いて誘導する治療法を確立する研究を行った。そして、より有効性の高い治療法とするためアンチセンスオリゴヌクレオチドとして ethylenebridged nucleic acid（ENA）という新規修飾核酸を用い、ENA と RNA とが入り混じった RNA/ENA キメラを用いた。また、臨床治験へと進めるた

めに、GMP グレードでの RNA/ENA キメラ合成の可能性についても検討した。

さらに、ジストロフィン遺伝子のエクソンスキッピング誘導を効率的に行う方針を確立するため、DMD 患者でジストロフィン遺伝子のスプライシング産物を解析したところ、エクソン 45 のスプライシング時の特性を明らかにした。同時に、治療効果を判定するためのバイオマーカーとしての血清クレアチンキナーゼについて検討を行った。一方、本治療確立の鍵となるスプイシング促進配列に関する検討をジストロフィン遺伝子異常例から加え、低分子化合物によるエクソンスキッピング誘導という新たな治療法の展開の可能性を検討した。

B. 研究方法

①RNA/ENA キメラの同定と DMD 患者由来培養筋細胞でのジストロフィン発現

欠失のホットスポットにあるエクソンのスキッピングを誘導するアンチセンスオリゴヌクレオチドを同定するため、標的とするエクソンの決定を行ったところ、エクソン 45 のスキッピング誘導により治療できる症例数が神戸大学小児科受診中の DMD 例の中で多いことが判明した。そこでエクソン 45 を標的とする各種 RNA/ENA キメラを合成した。そして、それらを導入した培養筋細胞の mRNA を解析することにより、標的となるエクソン 45 のスキッピングを最も強く誘導する RNA/ENA キメラを同定した。

さらに、エクソン 45 のスキッピング誘導の治療対象となるエクソンの欠失を有する DMD 患者から培養筋細胞株を樹立し、これに先に同定した RNA/ENA キメラを導入した。導入筋細胞におけるエクソンスキッピングの確認ならびにジストロフィン発現をそれぞれ RT-PCR および免疫染色法により行った。

②エクソンスキッピング誘導に関する研究

エクソン 45 のスプライシング時にスキッピングを誘導するための検討を重ねた。そして、DMD 患者で、スプライシングのコンセンサス配列のスプライスドナーサイトの 1 番目の塩基の G が A に置換した異常を有しながらも、スプライシングの様式がエクソンにより異なることを見い出した。エクソン 25 では、エクソンスキッピングが誘導され、エクソン 45 では、潜在的スプライスサイトの活性化がもたらされた。これらの 2 つのエクソンに見い出された変異を in vitro のスプライシング系を用いて解析した。正常と変異のあるエクソン 25 あるいは 45 の配列とその両側のイントロン配列のそれぞれを発現ベクターに挿入し、pre-mRNA を産生させた。この pre-mRNA を核抽出液と混じ、そのスプライシング産物を RT-PCR 解析した。また、文献報告の中でジストロフィン遺伝子の+1G>A 変異例を検索し、そのスプライシング様式を検討した。

③RNA/ENA キメラの GMP グレードで合成の可能性について検討した。

④スプライシング促進配列の解明

DMD 患者の遺伝子診断を実施し、そのジストロフィン mRNA を解析することにより遺伝子の異常から 2 次的に発生するスプライシング異常を明らかにした。さらに、スプライシング異常を来たしたジストロフィン遺伝子のエクソン内の異常をハイブリッド型ミニ遺伝子に導入し、in vitro のスプライシング反応系を用いてスプライシングに必須のエクソン内のスプライシング促進配列を明らかにした。

⑤治療効果判定のためのバイオマーカー検索

従来、DMD の診断には血清中のクレアチンキナーゼ（CK）が極めて有力なバイオマーカーとして知られていた。この CK が治療効果判定に生かせるか否かを DMD 患者のデータを集積し解析した。

また、DMD 患者の血清アミノ酸分析を行い、血中アミノ酸のバイオマーカーとしての意義を検討した。

⑥スプライシング促進配列を標的とする新規治療法の開発

in vitro スプライシング系を用いたスプライシングを変化させる低分子化合物の探索を行った。ある特定の遺伝子異常を対象としたスプライシング系に様々な低分子化合物を加え、そのスプライシング産物を解析することにより、低分子化合物の効果を判定した。

（倫理面への配慮）

DMD 患者の遺伝子診断並びに患者細胞でのジストロフィン発現の検討について神戸大学医学部医学倫理委員会での審査を経て承認されており、エクソンスキッピングを誘導する治療についても同様に承認を得た経験がある。

C. 研究結果

①欠失のホットスポット領域のエクソンスキッピング誘導に関する研究

（1）欠失のホットスポット領域にあるエクソンのスキッピングを誘導するアンチセンスオリゴヌクレオチドを同定するため多数の RNA/ENA キメラを合成し、それぞれの標的とするエクソンのスキッピング誘導能を順次解析した。その結果、標的となるエクソンに対して有効にエクソンスキッピングを誘導する RNA/ENA キメラの同定に成功した。

DMD 患者の持つジストロフィン遺伝子の欠失のホットスポットでエクソン欠失の型を解析していったところ、その欠失パターンからエクソン 45 のスキッピングを誘導することにより多くの患者が治療対象となることが判明した。すなわち、エクソン 45 のスキッピングを誘導するアンチセンスオリゴヌクレオチドは、神戸大学の DMD 患者のデータベース内では約 40 例が治療可能である。

エクソン 45 のスキッピングを誘導するアンチセンスオリゴヌクレオチドの RNA/ENA キメラによる治療の対象となる欠失を有する患者で治療の効果検討を行った。キメラを導入した患者細胞においてエクソンスキッピングが誘導が可能となり、ジストロフィンの発現も確認された。キメラを用いた治療の有効性が患者培養筋細胞で確認された。一方、このエクソンスキッピング誘導の効果は症例により反応性に個人差があることが判明した。

②in vitro のスプライシング系での検討

患者で発見されたスプライシング異常について検討した。エクソン 25 の＋1G＞A 変異はエクソンスキッピングを誘導した。一方、エクソン 45 の変異ではスプライシング産物として、3 種のものが得られた。1 つはエクソン 45 内の潜在的スプライシスサイトが活性化されたものであり、1 つはイントロン内の同じく潜在的スプライスサイトが活性化されたもの、もう 1 つはイントロンの配列がすべてあるものであった。

in vitro のスプライシングにおいてもエクソン 25 と 45 は異なるスプライシング様式を示した。現在まで、こうしたスプライシングのコンセンサス配列の異常では、遺伝子の異常に近接した潜在的スプライス部位の有無がスプライシング様式を決定するとの説が有力であった。実際エクソン 45 の異常の場合はこの説を支持した。しかし in vitro では、エクソン 25 の変異は潜在的スプライシス部位が存在するにもかかわらず、その活性化を起こしていなかった。このことは、スプライス部位の決定は、潜在的スプライスサイトの有無によるのではなく、エクソン自体の性質によることを示した。

そこで、ジストロフィン遺伝子の+1G＞A 変異の報告例を文献的に調べ、それらの例でのスプライシング様式とエクソンの特性について検討した。その結果、エクソンのスプライシングアクセプターサイトのスコアが高くかつエクソンのサイズが長い特性をもつ 4 つの強いエクソンが潜在的スプライス部位の活性化をもたらすことが明らかとなった。それ以外の弱いエクソンではエクソンスキッピングをおこすことが判明した。

そこで、DMD のエクソンスキッピング誘導治療の当面の標的となる欠失のホットスポットにあるエクソンをこの基準にあてはめて、強いエクソンと弱いエクソンに分けてみた。ジスト

ロフィン遺伝子のエクソン40から60の中で、エクソン45をはじめとして4つのエクソンが強いエクソンに分類された。こうしたエクソンでは、人工的なスプライシング誘導において潜在的スプライス部位の活性化が発生する可能性が指摘された。

③GMPグレードでの合成

RNA/ENAキメラのGMPレベルで合成することについて、その可能性を検討していった。その結果、GMPレベルでの合成が可能となりエクソンスキッピング誘導治療の治験はRNA/ENAキメラ供給面から可能となった。

④スプライシングに必須のエクソン内の配列

一塩基の置換とエクソンスキッピング誘導との関連を明らかにすべく、ナンセンス変異例でエクソンスキッピング誘導の可能性を解析した。ジストロフィン遺伝子にナンセンス変異を持つ例を57例に見い出し、その38例についてそのジストロフィンmRNAを解析した。38例中8例でエクソンスキッピングの異常が見い出され、ナンセンス変異で高率にエクソンスキッピングが誘導されることが判明した。また、これらの異常を発生したナンセンス変異ではスプライシング促進配列の破壊をもたらしたことが示唆された。一方、同じナンセンス変異でもDMD患者によりスプライシングの影響に差があることが判明した。

ジストロフィン遺伝子のエクソン38に4塩基欠失を有する例がエクソンスキッピングを誘発することを見い出した。ハイブリッド型ミニ遺伝子のin vitroのスプライシング反応系に様々な配列を有するエクソン38を挿入し、欠失した4塩基中のどの塩基が正常なスプライシングを決定する必須の配列であるかを検討した。

その結果、4塩基中の3番目の塩基の置換がエクソンスキッピングを誘導することを明らかにした。これは、この3番目の塩基がスプライシングに必須であること示した。

この結果は、今後エクソンスキッピングを誘導するアンチセンスオリゴヌクレオチドの同定に当たってはその標的部位をこの必須の塩基に焦点を当てることにより容易に決定できることを示す極めて重要な知見であった。

⑤血清バイオマーカーの探索

(1) 血清CK

DMDを治療するに際して、その評価法が大きな課題である。治療に際して運動能力の改善をはかることは必須の要件ではあるが、小児においては筋力の評価は極めて困難である。これまでに神戸大学小児科に受診中のDMD患者の年令毎のCKの変動についての解析を行った。その結果、CKの年令変化には3つのフェーズが存在していた。まず、第一フェーズの6歳まででは、血清CKの値は極めて高い値をとっている。そのCK値はおおよそ10,000IU/ℓ以上である。

こうした極めて高いCK値をとる時期がヒト発生のどの段階からであるかは不明である。そこで、未熟児で生まれた男児でDMDを発症した例の全国調査を実施した。全国の主要な小児科にアンケートを発送した。その結果、回答のあった施設から2例の報告があり、神戸大学の1例を合わせて計3例の未熟児発症例を見い出した。3例の未熟児発症例について今後周産期のCKの変化について検討してゆく予定である。この結果から、DMD患者でCKの上昇する時期を明確にし、DMDの治療法の確立の暁には、これを指標とすることが可能である。

また、DMD患者の血清CKは6歳を過ぎて急激に低下するフェーズと、10歳を過ぎて比較的低いレベルを維持するフェーズもあることが判明した。今後、DMDの治療開始の際にはこの様なフェーズの変化も効果判定に有用と考えられた。

(2) アミノ酸

DMD患者の血中アミノ酸分析を行い、病態の解明に有用なバイオマーカーの探索を行った。33種のアミノ酸を一挙に解析し、その病態との関連について検討した。その結果、1つのアミノ酸がDMDのバイオマーカーになり得る可能性が判明した。今後、さらに検討を加え、新し

いバイオマーカーとしての意義を明らかとする。

⑥スプライシングを制御する治療薬の探索

スプライシング促進配列の解析をすすめる中、先に示した結果の様に特定の塩基がスプライシングを制御していることが判明した。そこで、アンチセンスオリゴヌクレオチドを用いる以外の手法でエクソンスキッピングを誘導できる可能性を明らかにするため、その部位を標的とした低分子化合物の探索を行った。その結果、ある特定の化学物質がスプライシングを制御していることが明らかになり、これが患者由来筋細胞でその効果を発揮することが確認された。これは、DMD の新しい治療法となることが大きく期待される。

D. 考察

1）達成度について

ジストロフィン遺伝子の欠失のホットスポットにある欠失に対する RNA/ENA キメラの決定が出来、多くの DMD 患者の治療開始への道筋が確立できた。また、エクソン 45 を標的とした RNA/ENA キメラを用いたエクソンスキッピング誘導療法については、DMD 患者細胞で有効性が示され、また GMP レベルでの合成体制もでき、治験開始へと道が開かれた。

さらに、新しい DMD の治療薬の探索を可能とする方法を見い出し、DMD 治療にニューフロンティアーを開拓している。

この様に本研究計画の成果は順調に得られた。

2）研究成果の学術的意義について

私達が提唱した DMD に対するエクソンスキッピング誘導治療の成功は、世界から大きな注目を集めた。そのため、この治療法の確立を目指した全世界的な研究が極めて活発化した。一方、私達は本研究において多数の DMD 患者が治療できる RNA/ENA キメラの同定に成功し、本分野の世界のリーダーとしての地位を引き続き確保した。

また、エクソン内のわずか一塩基がスプライシングの制御に大きく関与することを明らかにした。これは、スプライシング制御機序の解明に大きく貢献するもので基礎分子生物学の世界にも少なからずインパクトを与えた。

さらに、この成果から低分子化合物を用いたエクソンスキッピング誘導治療の可能性を世界で初めて示した。

3）研究成果の行政的意義について

DMD の治療法の確立は世界の DMD 患者が切望されているものである。DMD 患者の医療費の削減をはかり、国民の負担を大きく緩和される。

4）その他特記すべき事項について

2009 年 11 月 9 日には神戸大学において国際シンポジウムを開催した。講演者は世界の本領域の 3 大拠点研究から 1 名ずつで、DMD のエクソンスキッピング誘導治療について世界の最先端の知識の交流を行った。

E. 結論

DMD のエクソンスキッピング誘導治療が多数の症例で可能となる方法の確立が大きく前進し、近々臨床的治験が開始できる体制が出来上がった。また、スプライシングを制御する低分子化合物の探索も可能となり、DMD 治療の確立へ新しい世界を切り開くことに成功した。

F. 研究発表

1. 論文発表
   一覧表参照
2. 学会発表
   一覧表参照

G. 知的所有権の出願・取得状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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III. 研究成果の刊行物・別刷

Journal of Human Genetics (2009), 1–8

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ORIGINAL ARTICLE

Insertion of the *IL1RAPL1* gene into the duplication junction of the dystrophin gene

Zhujun Zhang, Mariko Yagi, Yo Okizuka, Hiroyuki Awano, Yasuhiro Takeshima and Masafumi Matsuo

Duplications of one or more exons of the dystrophin gene are the second most common mutation in dystrophinopathies. Even though duplications are suggested to occur with greater complexity than thought earlier, they have been considered an intragenic event. Here, we report the insertion of a part of the *IL1RAPL1* (interleukin-1 receptor accessory protein-like 1) gene into the duplication junction site. When the actual exon junction was examined in 15 duplication mutations in the dystrophin gene by analyzing dystrophin mRNA, one patient was found to have an unknown 621 bp insertion at the junction of duplication of exons from 56 to 62. Unexpectedly, the inserted sequence was found completely identical to sequences of exons 3–5 of the *IL1RAPL1* gene that is nearly 100 kb distal from the dystrophin gene. Accordingly, the insertion of *IL1RAPL1* exons 3–5 between dystrophin exons 62 and 56 was confirmed at the genomic sequence level. One junction between the *IL1RAPL1* intron 5 and dystrophin intron 55 was localized within an Alu sequence. These results showed that a fragment of the *IL1RAPL1* gene was inserted into the duplication junction of the dystrophin gene in the same direction as the dystrophin gene. This suggests the novel possibility of co-occurrence of complex genomic rearrangements in dystrophinopathy.
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Keywords: duplication; dystrophin; insertion

INTRODUCTION

Duchenne and Becker muscular dystrophies (DMD/BMD) are the most commonly inherited muscle diseases that are caused by mutations in the dystrophin gene. This gene consists of 79 exons and is the second largest human gene, spanning more than 2.5 Mb on Xp21.2. It is characterized by a number of large introns and at least eight alternative promoter/first exons scattered among the introns.[1,2] Duplications involving one or more exons of the dystrophin gene are the second most common type of mutation in humans, accounting for approximately 5–10% of dystrophinopathy patients, whereas deletions occupy nearly 60% of mutations[3–5] Though the exact organization of the duplicated exons remains uninvestigated in most cases, it is believed that such duplicated rearrangements consist of a simple tandem head-to-tail duplication within the dystrophin gene. This hypothesis has been adopted to explain DMD or BMD phenotypes based on the reading frame rule that is widely accepted.[6]

However, one duplication that is not in tandem has been reported,[5] suggesting that the above hypothesis is not always correct. In addition, duplicated exons were shown to exist in separate regions of the dystrophin gene.[5,7] Recently, two separate duplicated dystrophin exons were found to consist of two tandem head-to-tail duplications by analysis of dystrophin mRNA.[8] These observations suggest that duplication mutations are sometimes complex. So far, all duplications have been reported as intragenic events.

The *IL1RAPL1* (interleukin-1 receptor accessory protein-like 1) gene, responsible for X-linked mental retardation, spans 1.36 Mb on Xp22.1, nearly 100 kb distal from the dystrophin gene.[9,10] Both dystrophin and *IL1RAPL1* genes were found to reside in an identical common fragile site (FRAXC).[10] However, little attention has been paid to *IL1RAPL1* gene mutation in dystrophinopathy, even though mental retardation is observed in nearly one-third of DMD patients. The co-occurrence of mutations in these two genes has been reported in a contiguous gene deletion syndrome, in which both dystrophin and *IL1RAPL1* genes are affected, the deletion beginning and ending within these genes[11–13] Therefore, it is supposed that an unknown mechanism mutates these two separated genes concurrently. Recently, a mechanism of the fork stalling and template switching (FoSTeS) was proposed as a novel mechanism to mutate two separated genes.[14–16] However, any mutation in the dystrophin gene has not been claimed to be caused by FoSTeS, as far as we know.

Here, we report a previously undescribed duplication of the dystrophin gene that is complicated by the insertion of a duplicated part of the *IL1RAPL1* gene into the dystrophin duplication site. This complex rearrangement raises the novel possibility that there is a co-occurrence of two gene mutations even in dystrophinopathy.

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Determination of insertion breakpoints

In order to define rearrangement junctions in intron sequences, the ends of the duplications were determined by quantitating PCR amplified products from regions of each intron as described earlier.[21] The dystrophin and *IL1RAPL1* gene sequences were obtained from the human genome sequence (GenBank NT_011757). Five distributed regions of introns 55 and 62 of the dystrophin gene (120 and 63 kb, respectively) and four regions of introns 2 and 5 of the *IL1RAPL1* gene (494 and 269 kb, respectively) were each amplified to yield 150–400-bp-long PCR products (data available on request). The genomic dosage of the amplified intron regions was assessed by semiquantitative multiplex PCR as described earlier.[20] The insertion breakpoints were assumed roughly in the region between fragments with single and double amounts of the PCR products. Again, distributed fragments within the breakpoint region assumed from the preceding PCR amplification study were PCR amplified to localize the limit between single and double amounts of the PCR products. This process was repeated to narrow down the breakpoint until the breakpoint junction was presumed to be less than 1000 bp (data available on request). Finally, two junction fragments were obtained: one using a forward primer located in dystrophin intron 62 and a reverse primer in *IL1RAPL1* intron 2, and the other using a forward primer from *IL1RAPL1* intron 5 and a reverse primer from dystrophin intron 55 (Table 1). The amplified products were then purified and directly sequenced.

Genomic sequence analysis

Four genomic regions corresponding to the junctions in dystrophin introns 55 and 61 and *IL1RAPL1* introns 2 and 5, and genomic regions encompassing duplicated exons in both dystrophin and *IL1RAPL1* genes were amplified using primers derived from the flanking sequences (Table 1). Amplified products were directly sequenced using an automated DNA sequencer (model 310; Applied Biosystems). Dinucleotide repeat markers from intron 59 (DMD1-2c/DMDI) and intron 62 (DI623) were also analyzed to try to disclose allelic differences as described earlier.[22,23]

Examination of the insertion junction sequences

DNA sequences encompassing the insertion junction were analyzed for repetitive elements and low-complexity sequences by use of the RepeatMasker web server (www.repeatmasker.org). *Alu* sequences were annotated according to Batzer's report.[24]

RESULTS

In 15 duplications in the dystrophin gene, actual exon junctions were disclosed by sequencing of the reverse transcription-PCR product encompassing the junction region and all but one showed junction sequences compatible with genomic results (data not shown). In the index patient, multiplex ligation-dependent probe amplification analysis of the dystrophin gene showed twice the amount of products for 7 exons consisting of exons 56–62, whereas other exons remained normal (data not shown). On the basis of the conventional understanding of multiplex ligation-dependent probe amplification analysis, exons 56–62 were thought to be involved in a tandem head-to-tail duplication, with the 7 duplicated exons inserted between exons 62 and 63 in the patient's dystrophin mRNA. Applying this assumption to the translational reading frame, a premature stop codon appeared in the sequence of the inserted exon 56. Therefore, the patient's genotype matched with the severe DMD phenotype.

To determine the orientation and location of the duplicated region, the exon structure of dystrophin mRNA was examined. A fragment encompassing the junction between exons 62 and 56 was reverse transcription-PCR amplified using a pair of primers, forward and reverse primers for exon 61 and complementary to exon 59, respectively (Table 1). As a result, the amplification product was obtained from the patient's skeletal muscle RNA, but not from a normal control individual (Figure 1). Unexpectedly, the size of the product (1287 bp) was larger than the expected size of 666 bp. Direct sequencing of the product revealed a novel transcript: sequences of exons 61 and 62 were followed by an unknown 621 bp long sequence, after which sequences of exons 56, 57, 58 and 59 appeared (Figure 1). Both sequences and junctions of dystrophin exons were maintained completely intact. From the order of identified exons in dystrophin mRNA, we concluded that exons 56–62 were tandem head-to-tail duplications, leaving the 621-bp insertion unidentified.

A BLAST search of the 621 bp sequence revealed that the sequence was completely identical to a part of the cDNA sequence of the *IL1RAPL1* gene (bases 713–1333 of GenBank NM_014271). The inserted 621-bp sequence was found to consist of exons 3, 4 and 5 of the *IL1RAPL1* gene, and was inserted in the same direction as the dystrophin exons (Figure 1). These results showed that a novel chimeric dystrophin-*IL1RAPL1*-dystrophin transcript consisting of dystrophin exons 1–62, *IL1RAPL1* exons 3–5 and dystrophin exons 56–79 was produced in the index patient. This transcript encoded a TGA stop codon at the fifth codon in the inserted sequence, consistent with the severe DMD phenotype (Figure 1).

The patient's *IL1RAPL1* mRNA was examined by reverse transcription-PCR amplification of a fragment encompassing exons 1–9 of the *IL1RAPL1* gene. Surprisingly, this amplification yielded a product with an expected size (1289 bp) and completely normal sequence, including exons 3–5 (Figure 2a). Therefore, we concluded that the *IL1RAPL1* gene was intact in the patient's genome.

From the above findings, we supposed that an extra copy of exons 3–5 of the *IL1RAPL1* gene was present in the patient's genome. We therefore examined the copy number of exons of the *IL1RAPL1* gene by semiquantitative PCR. The amount of amplified products from exons 3, 4 and 5 of the *IL1RAPL1* gene was nearly twice that of the control, but the same for exons 2 and 6, indicating two copies of exons 3–5 of the *IL1RAPL1* gene in the patient's genome (Figure 2b). Taken together with the above results, we concluded that the dystrophin gene acquired an extra copy of the *IL1RAPL1* gene between exons 62 and 56 in the same direction as the dystrophin gene.

To confirm the insertion of the *IL1RAPL1* gene into the dystrophin gene at the genomic level, insertion breakpoints were examined. At first, the junction between dystrophin intron 62 and *IL1RAPL1* intron 2 was narrowed down by repeating semiquantitative PCR amplifications of intron regions (Supplementary Information). Finally, we attempted to PCR amplify the junction fragment between dystrophin intron 62 and *IL1RAPL1* intron 2. Although the primer binding sites would be too distant for amplification to occur in the control, the primers should have been in sufficient proximity for amplification to occur in the patient (Figure 3). From the patient, one 995-bp product was obtained. As expected, sequencing of the product revealed the junction sequence between dystrophin intron 62 and *IL1RAPL1* intron 2; a multiple sequence alignment revealed that the sequence of intron 62 ended at nt 29066747 (GenBank NT_011757) and joined to nt 26840485 (GenBank NT_011757) of intron 2 of the *IL1RAPL1* gene (Figure 3). This showed that dystrophin intron 62 was shortened to 56.7 kb from 62.6 kb, and *IL1RAPL1* intron 2 to 242.3 kb from 251.3 kb.

Remarkably, a single C-nucleotide that did not match with either intron sequence was found inserted at this intron junction (Figure 3). Although intron sequences nearby the junction were further examined, neither significant sequence homology between two introns nor specific structures, such as a topoisomerase consensus cleavage site were found. These characteristics indicated that the rearrangement was caused by nonhomologous recombination. Remarkably, it was found that the breakpoint in intron 62 was actually within the first exon of G-dystrophin, a dystrophin isoform transcribed from alternative promoters.[25] Thereby, the 194-bp long first exon was disrupted

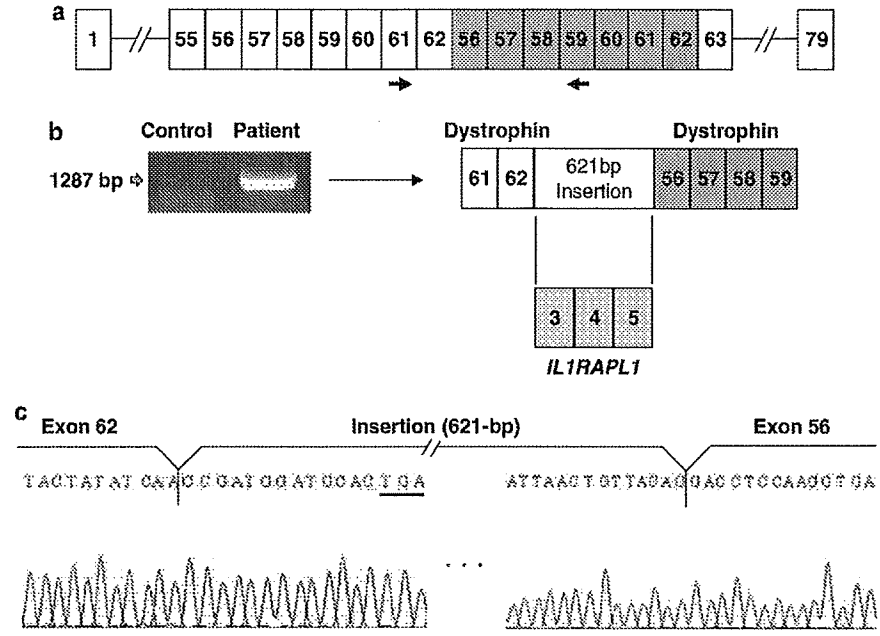

Figure 1 Examination of the duplication junction between dystrophin exons 62 and 56. (**a**) Schematic description of the examined region of the predicted dystrophin transcript. The boxes represent exons and the numbers inside the box indicate the exon number. Duplicated exons (exons 56–62) are marked by shaded boxes. Horizontal arrows show the locations and directions of the primers that were used for reverse transcription (RT)-PCR amplification. (**b**) RT-PCR amplified product. RT-PCR product encompassing dystrophin exons 61 to 59 is shown. The product (1287 bp) was obtained from the index patient (right), but the size of the product was larger than the expected 666 bp. However, no amplified product was obtained from the control (left). On the right side, a schematic representation of the exon organization of the amplified fragment is shown. An unknown 621-bp insertion is present between dystrophin exons 62 and 56 (upper); the unknown sequence was found to consist of *IL1RAPL1* exons 3 to 5 (lower). (**c**) Sequence of the junctions between dystrophin exons and the inserted fragment. A part of the sequence of the amplified product is shown. The 3′ terminal sequence of exon 62 (CAA) joined to the 5′ end of the unknown sequence (CCG), and the 3′ end of the unknown sequence (CAG) then joined to the 5′ end of the sequence of exon 56 (GAC). The underlined represents a stop codon (TGA).

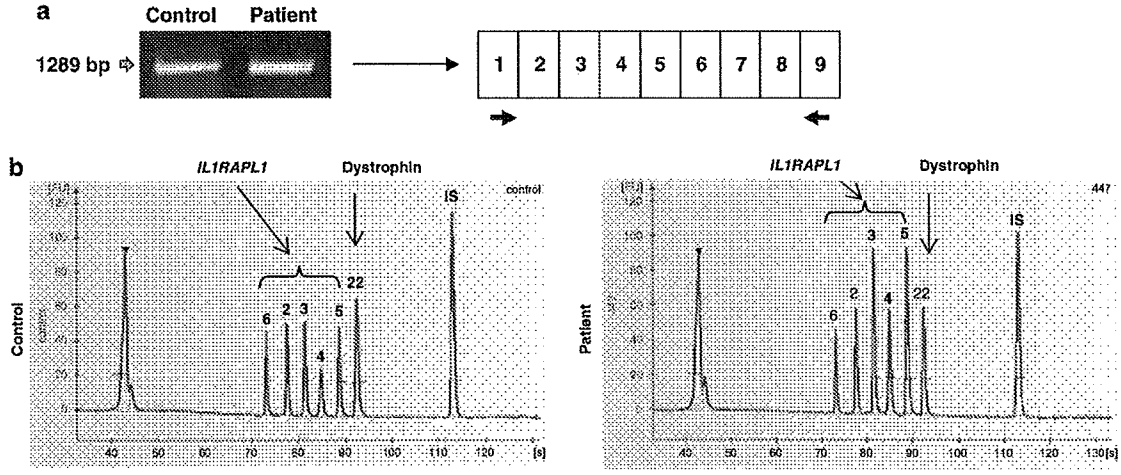

Figure 2 Analysis of the *IL1RAPL1* gene. (**a**) Reverse transcription (RT)-PCR amplification of the *IL1RAPL1* transcript. RT-PCR products encompassing *IL1RAPL1* exons 1–9 are shown. One clear product of the same size (1289 bp) was identified in both the index patient and control (left panel). The exon composition of the amplified product is described schematically on the right. Horizontal arrows show the locations and directions of the primers that were used for RT-PCR amplification. (**b**) Quantification of *IL1RAPL1* exons. Capillary electrophoretic patterns of PCR products are shown. Six genomic regions coamplified in one PCR reaction were separated using capillary electrophoresis. Numbers over the peaks indicate exons 2, 3, 4, 5 and 6 of the *IL1RAPL1* and exon 22 of the dystrophin gene. The peak area of exons 3, 4 and 5 is nearly doubled in the patient, but normal for exons 2 and 6 (right). The inverted triangle and IS refer to 15- and 1500-bp markers, respectively.

at the 40th nucleotide (Figure 3). In addition, a total of 15 bases (5′-CGGAGCCCGACACGG-3′) (nt 29066825 to 29066810) were found deleted from intron 62 at 65 bp upstream from the junction. Instead, a single T-nucleotide was inserted at this deletion site. The 15-bp deletion removed 10 bp of the 3′ part of one GC-box, constituting a promoter region of G-dystrophin (Figure 3).

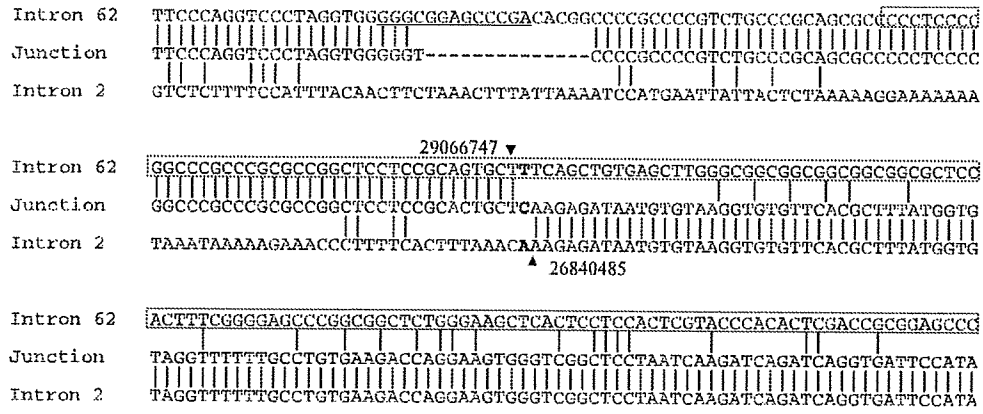

Figure 3 The junction between dystrophin intron 62 and *IL1RAPL1* intron 2. Normal sequences of dystrophin intron 62 and *IL1RAPL1* intron 2 are aligned in a 5′ to 3′ direction (left to right) at the top and bottom lines, respectively. In the middle, the junction sequence is shown. Vertical lines indicate nucleotide matches. Gaps are indicated by dashes and the inserted nucleotide at the junction is indicated in bold. Vertical arrowheads indicate nucleotide numbers on the X-chromosome (GenBank NT_011757). No significant sequence homology was found around the breakpoint. Exon 1 of G-dystrophin in dystrophin intron 62 is boxed. A GC-box is underlined.

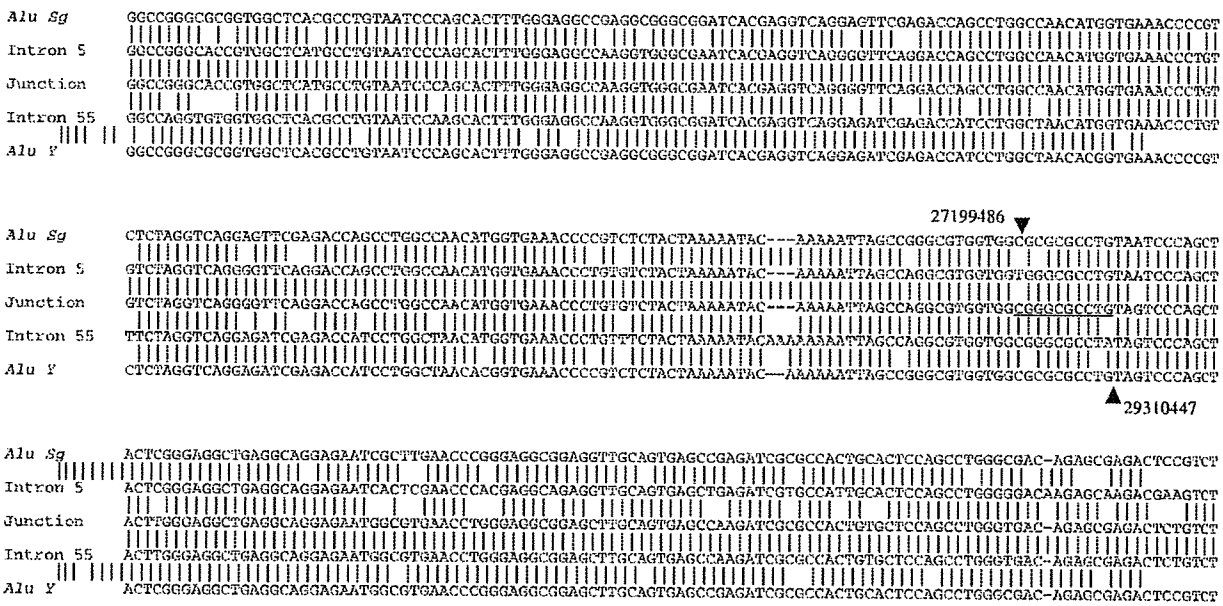

Figure 4 The junction between *IL1RAPL1* intron 5 and dystrophin intron 55. Normal sequences of *IL1RAPL1* intron 5 and dystrophin intron 55 are shown on the second top and second bottom lines, respectively. In the middle, the sequence across the insertion junction between *IL1RAPL1* intron 5 and dystrophin intron 55 is shown. The *Alu* consensus sequences of *Alu Sg* and *Alu Y* are shown on the top and bottom lines, respectively. Vertical lines indicate nucleotide matches. Gaps are indicated by dashes. Before mismatches appear, the *IL1RAPL1* intron 5 and dystrophin intron 62 are completely identical to the junction sequences. However, 10 bp, including two mismatches, are not found in either sequence. Vertical arrowheads indicate the rearrangement site with numbers on the X-chromosome (GenBank NT_011757).

Regions covering the junctions in dystrophin intron 62 and *IL1RAPL1* intron 2 were amplified. One PCR amplified product specific to each region was obtained from the case as well as from a normal control. Sequencing of the products disclosed completely intact sequences, indicating no genomic changes predisposing the rearrangement. The 15-bp deletion identified upstream of the recombination site in intron 62 was present only in the junction site and not in non-recombined intron. Therefore, the deletion seemed to be an additional genomic rearrangement complicating the duplication mutation. In fact, a G-dystrophin transcript with normal exon content was detected in this patient as in the control, indicating intact intron 62 (data not shown).

As one junction was clearly revealed, we supposed that the junction between *IL1RAPL1* intron 5 and dystrophin intron 55 should be present. Using the same strategy as described above (Supplementary Information), the junction fragment was cloned. Finally, the junction

fragment was PCR amplified using primers on *IL1RAPL1* intron 5 and dystrophin intron 55 and one amplified product was obtained (Figure 4). Sequencing of this product showed a recombination site between the *IL1RAPL1* and dystrophin genes; a multiple sequence alignment revealed that the *IL1RAPL1* intron 5 sequence ended at nt 27199486 (GenBank NT_011757) and joined to nt 29310447 (GenBank NT_011757) of the dystrophin intron 55 sequence (Figure 4). Between these two sites, 10 nucleotides remained uncategorized in either sequence because of nucleotide mismatches. Therefore, the exact breakpoint of the junction could not be identified. Remarkably, the junction was found to be within an *Alu* repetitive element in both introns; an *Alu Sg* element in intron 5 of the *IL1RAPL1* gene and an *Alu Y* element in intron 55 of the dystrophin gene (Figure 4). These two *Alu* elements were different from each other by 17.7% in their sequences. We decided that this junction was due to a homologous recombination between two *Alu* repeat sequences. To see any genomic change at junctions in intact introns, regions covering the junction site on *IL1RAPL1* intron 5 and dystrophin intron 55 were PCR amplified from the patient's genomic DNA. The sequences of the amplified products were completely normal around the junction in the two introns.

These results clearly indicated that a part of the *IL1RAPL1* gene was inserted at the junction of the duplication mutation of the dystrophin gene in the same direction as the dystrophin gene. The duplication size of the dystrophin gene was calculated to be 243.7 kb. The size of the inserted fragment of the *IL1RAPL1* gene was calculated to be nearly 359.0 kb. Both the patient's mother and maternal grandmother were found to have these two junctions in their genome by PCR amplification of the junction fragment (data not shown), suggesting that the complex duplication had been stably inherited. A total of 30 normal female controls were negative for each junction, suggesting that no part of the rearrangement is polymorphic.

Examination of duplicated exons

As we identified an extra copy of three exons of the *IL1RAPL1* gene (Figure 2), we looked for sequence differences between the extra copy and the wild type. The region encompassing the three exons was PCR amplified and directly sequenced. The sequences were completely normal and no heterozygosity was found (data not shown). In addition, all duplicated exons and polymorphic markers in introns 59 and 62 of the dystrophin gene were also analyzed. Their sequences were also completely normal and no heterozygosity was detected (data not shown). These sequencing results strongly suggested that the duplicated fragments were derived from the same genomic source.

In summary, we found the most complex dystrophin duplication mutation yet reported, consisting of a duplication/insertion mutation. The recombination event consisted of two different mechanisms of nonhomologous and *Alu*-mediated recombination and produced a chimeric transcript.

DISCUSSION

Here, we report a novel complex rearrangement in the dystrophin gene discovered by analyzing the dystrophin transcript. We identified a chimeric dystrophin-*IL1RAPL1*-dystrophin transcript with *IL1RAPL1* exons 3–5 inserted between dystrophin exons 62 and 56. This rearrangement at the genomic level can be roughly summarized as a duplication of exons 56–62 of the dystrophin gene that was accompanied by an insertion of exons 3–5 of the *IL1RAPL1* gene. In one study on duplications of the dystrophin gene, one of 118 duplications was reported to be complex, complicating an exon deletion.[5] As far as we know, our case is the second example of a complex duplication in the dystrophin gene but the most complicated one. In particular, all duplications have been reported as intragenic events.[5,8,26] This was the first study to show commitment of other genetic elements to cause duplication in the dystrophin gene. Although the identical duplication of dystrophin exons 56–62 has been reported in two cases (Leiden Muscular Dystrophy pages, www.dmd.nl), analysis of their mRNA has not been carried out. Further study is needed to see whether or not our complicated duplication is an exceptional event.

Although duplications are the second most common mutation in the dystrophin gene, the sequence of the duplication junction has rarely been clarified at the genomic DNA level.[5,27] In this case, a small deletion and several nucleotide changes complicated a large insertion event (Figures 3 and 4). It has been supposed that the sequence or structure of intron 62 may be vulnerable to complex rearrangement.[5] Considering that an alternative promoter for G-dystrophin is present within intron 62, this intron should be accessed easily by transcription factors (Figure 3). It is conceivable that unknown structural characteristics promote complex rearrangement in this intron.

It was remarkable that a 359.0-kb fragment of the IL1RAP1 gene located 100 kb apart from the dystrophin gene was found inserted into the duplication junction (Figure 5). An example of transposition of a large fragment from a distant site into the recombination site was reported in the coagulation factor *F8* gene located on chromosome Xq28, and the 263-kb duplicated region was shown to have originated 5.2 Mb apart from the inserted site.[28] These suggest that the insertion of a large genomic fragment into a far distant region can be a cause of disease.

Considering that both dystrophin and *IL1RAPL1* genes are present in the same common fragile site (FRAXC),[10] it is reasonable to consider co-occurrence of mutations in these two genes[11–13] Production of a chimeric dystrophin-*IL1RAPL1* transcript, however, has been shown in one DMD patient who had a deletion encompassing ~1.6 Mb from the *IL1RAPL1* gene to the dystrophin gene.[12] Our case is the second to show the production of a chimeric dystrophin-*IL1RAPL1* transcript (Figure 1). Remarkably, both rearrangements producing a chimeric dystrophin-*IL1RAPL1* transcript occurred in the *Alus* of *IL1RAPL1* intron 5 located only 50 kb apart.

Recombination mechanisms, such as nonallelic homologous recombination and nonhomologous end joining, have been shown to underlie rearrangements causing genomic disorders,[16,29] with the former accounting for the majority. It has been suggested that duplications within the dystrophin gene are primarily caused by unequal sister-chromatid exchange.[30,31] However, synthesis-dependent nonhomologous end joining has been proposed as a cause of duplications, resulting in a tandem duplication at the site of a double-strand break.[5] Large tandem duplications are well-known causes of human genetic disease.[32] Especially in Pelizaeus–Merzbacher disease, an X-linked dysmyelinating disorder, nonrecurrent duplication of the dosage-sensitive *PLP1* gene is known as the most common cause.[33] Recently, it was proposed that complex duplications of the proteolipid protein 1 (*PLP1*) gene are due to the replication-based mechanism of 'replication folk stalling and template switching (FoSTeS),' as these duplications could be explained by neither nonallelic homologous recombination nor a simple nonhomologous end joining recombination mechanism.[14] In our case, it is better to consider this possibility as the underlying mechanism for the complex genomic rearrangement (Figure 5).

As mutations of the *IL1RAPL1* gene have been identified in X-linked mental retardation,[34–36] our finding that three exons of the *IL1RAPL1* gene were transposed into the dystrophin gene suggested the possibility of X-linked mental retardation caused by their deletion.

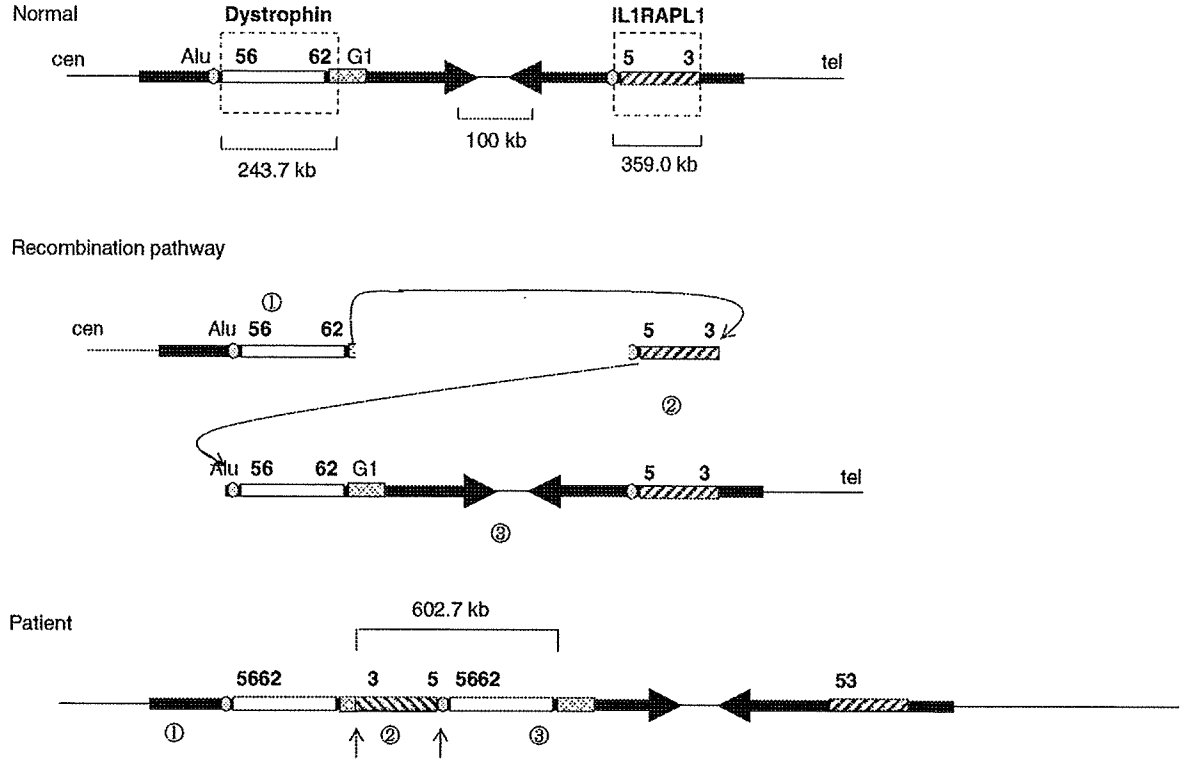

Figure 5 Schematic description of the genomic rearrangement in the index patient. The dystrophin and *IL1RAPL1* genes involved in this rearrangement are described schematically. These two genes are separated by nearly 100 kb on the short arm of the X chromosome (top). The dystrophin gene is transcribed from the centromere (cen) to the telomere (tel), whereas the *IL1RAPL1* gene is transcribed in the opposite direction. A 243.7-kb fragment encompassing dystrophin exons 56–62 is duplicated, and at the recombination site another 359.0-kb-long region encompassing exons 3–5 of the *IL1RAPL1* gene is inserted (bottom). The rearrangements between dystrophin intron 62 and *IL1RAPL1* intron 2 and between *IL1RAPL1* intron 5 and dystrophin intron 55 are through nonhomologous and *Alu*-mediated recombination, respectively. The rearrangement is illustrated by its predicted order and origins (middle). The bold horizontal arrows indicate the dystrophin and *IL1RAPL1* genes and their transcriptional direction. The duplicated region is boxed with dotted lines. Open, shaded and dotted boxes indicate dystrophin exons 56–62, *IL1RAPL1* exons 3–5 and the alternative G-dystrophin promoter/first exon (G1), respectively. Numbers over bars indicate the exon number of the respective genes. Ovals represent *Alu* repeat sequences (*Alu*). Parentheses indicate the size of the regions. Size is not to scale. Vertical arrows indicate junctions.

Accordingly, there is one case of a three-exon deletion described in the literature resulting in mental retardation.[34] Therefore, we strongly suspect that concomitant mutations in the *IL1RAPL1* gene and dystrophin gene account for both DMD and mental retardation. Further studies will elucidate the co-occurrence of mutations in both these genes and help explain why mental retardation is a complicating factor in DMD patients.

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Small Mutations Detected by Multiplex Ligation–Dependent Probe Amplification of the Dystrophin Gene

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Currently, multiplex ligation–dependent probe amplification (MLPA) has been recognized as the most powerful and convenient method to identify exon deletions or duplications in the dystrophin gene, the mutation of which causes Duchenne and Becker muscular dystrophies (DMD/BMD). The mutation diagnosis is easily done by assessing the amounts amplified by MLPA (loss, single, or double). However, an ambiguous amount of amplified product has never been reported. When 77 Japanese DMD/BMD patients were examined by MLPA from MRC-Holland (Amsterdam, The Netherlands), deletions/duplications in the dystrophin gene were identified in 64.8%. Ten male patients showed loss of a single exon by MLPA, but one of them was found to have not an exon deletion, but a four-nucleotide deletion (c.3347–3350delAGAA) within the exon. Remarkably, two patients showed ambiguous amounts of product with less than half of that of a single copy, making the genetic diagnosis impossible. In one patient, a novel single-nucleotide change (c.4303G>T) leading to a nonsense mutation was identified. In another patient, a novel five-nucleotide deletion (c.4536–4540delGAGTG) was identified. It was considered that these two mutations partially disturbed MLPA amplification, resulting in ambiguous amplification. These results show that MLPA can serve as a tool for screening small mutations, as well as for detecting exon deletions or duplications.

Introduction

Duchenne and Becker muscular dystrophies (DMD/BMD) [OMIM 310200/300376], the most common inherited muscle diseases affecting more than 1 in every 3500 live born male child, are caused by mutations in the dystrophin gene, which consists of 79 exons spreading more than 3000 kb on Xp21.2 (Ahn and Kunkel, 1993). 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 patients may be affected by small mutations, including point mutations, microdeletions, and microinsertions.

A reading frame rule explains the difference between DMD and BMD; mutations that create premature stop codons in the reading frame of dystrophin mRNA usually result in a severe DMD phenotype, whereas mutations that maintain the original reading frame cause the milder BMD phenotype (Monaco *et al.*, 1988). Therefore, it is essential to identify gene mutations in clinically diagnosed DMD/BMD patients not only for the establishment of diagnosis (Shiga *et al.*, 1997) but also for application of exon skipping induction treatment of DMD (Aartsma-Rus *et al.*, 2006; Takeshima *et al.*, 2006).

It has been difficult to identify mutations in the dystrophin gene because of its huge size and large exon number. Therefore, many methods have been employed for the detection of mutations in the dystrophin gene. The most commonly used multiplex PCR technique offers simple screening for the deletion of 19 exons, allowing for detection of mutations in only half of DMD/BMD patients (Chamberlain *et al.*, 1988; Beggs *et al.*, 1990). Multiplex ligation–dependent probe amplification (MLPA) allows for the simultaneous hybridization and ligation of several probes in a single-reaction tube, followed by PCR amplification and analysis by capillary electrophoresis, making multiplex quantitation of copy numbers at specific target sequences possible. MLPA that examines all 79 exons of the dystrophin gene has been developed and has been found to be a simple, rapid, and reliable tool for detecting exon deletions or duplications (Janssen *et al.*, 2005; Lalic *et al.*, 2005).

Currently, the MLPA kits from MRC-Holland (Amsterdam, The Netherlands) (Lalic *et al.*, 2005) are the most widely employed. MLPA analysis easily discriminates between deletion and duplication from the amount of amplified product, which is absent or doubled, respectively. However, caution has to be exercised in discriminating a single-exon deletion, because small mutations have been found to completely abolish amplification, thereby leading to apparent loss of a single exon (Schwartz and Duno, 2004; Janssen *et al.*, 2005; Todorova *et al.*, 2008). In patients with loss of a single exon by MLPA, it is necessary to examine the exon structure by

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