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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に関する研究

平成１９年度　総括研究報告書

主任研究者　松尾　雅文

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目　　次

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主任研究者：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）の治療法の確立を目指して、世界中で多くの研究がさまざまな方向からなされている。最近では、我々が提唱してきたＤＭＤ患者が有するアウトオブフレーム変異をインフレームへと変換するジストロフィン遺伝子のエクソンスキッピング誘導による治療が最有力治療として世界的にも大きく注目されている。本研究は、エクソンスキッピング誘導治療をジストロフィン遺伝子の欠失のホットスポットのエクソンに欠失を有する例へ適応を拡大するために強力なエクソンスキッピング誘導作用を有するRNA/ENAキメラを臨床に応用しようとする画期的なものである。そのため、臨床に応用するための基本であるGMPグレードでのRNA/ENAキメラ合成が本研究進展の鍵であった。今回、エクソン45のスキッピング誘導能を有するRNA/ENAキメラの合成が我が国の核酸合成の現場においても可能なことが確認された。そして、合成されたRNA/ENAキメラが計画通りに合成され、また、エクソンスキッピング誘導効果も有し、さらに患者筋細胞でジストロフィン発現誘導作用も発揮することも確認した。 これらのことから、新たに合成したRNA/ENAキメラが十分に治療に応用できるものであることが確認された。また、合成したRNA/ ENAキメラをマウスに投与し、形態学的・生化学的変化について検討し、合成RNA/ENAキメラには急性毒性は見られないことを明らかにした。今回得られた結果は、RNA/ENAキメラの臨床応用の可能性を強く示した。このように、本年度の研究においてほぼ当初の計画通りの研究が進行した。DMD患者を対象としたジストロフィン遺伝子のエクソン45のスキッピング誘導による治療法の確立に対して大きく前進した。

今後、本年度の成果をさらに発展させエクソン45のスキッピング誘導治療の実践に向けた基盤整備をはかり、当初の研究成果を挙げる予定である。

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本研究は神戸大学大学院医学系研究科の松尾雅文と竹島泰弘が共に分担研究者として共同して研究を推進してきた。本報告書は重複を避けるため2名の研究内容をまとめて記したものである。

A. 研究目的

Duchenne 型筋ジストロフィー（DMD）は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。申請者は、ジストロフィン遺伝子のエクソンのスキッピングを誘導し、mRNAのアミノ酸読み取り枠を修正してインフレームにする独自のＤＭＤ治療法を着想した。そして、ジストロフィン遺伝子のエクソン 19 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、エクソン 20 を欠失した DMD 患者でエクソン 19 のスキッピングを誘導させ、着想通りに患者骨格筋にジストロフィンを発現させることに世界で初めて成功した。

その成果は、DMD の最も有効な治療法として「Pediatric Research」誌の表紙に紹介されるなど、世界中から大きな注目を集めた。こうしたことから、本エクソンスキッピング誘導治療をジストロフィン遺伝子の欠失のホットスポットのエクソンに欠失を有する DMD 例へ適応を拡大し、多数の患者が治療し得る方法の確立を望む声が世界中の DMD 患者から殺到している。

本研究はエクソンスキッピング誘導治療をジストロフィン遺伝子の欠失のホットスポットのエクソンに欠失を有する例へ適応を拡大するもので、神戸大学小児科を受診中の DMD 患者の遺伝子診断の結果から最も多数の患者が治療しうるジストロフィン遺伝子のエクソン 45 のスキッピング誘導に焦点を当て、その臨床応用を最終ゴールとした研究を行うものである。

B. 研究方法

対象

神戸大学小児科を受診中の DMD 患者でジストロフィン遺伝子の遺伝子診断を実施し、200 名を超える患者でエクソンの欠失の異常を同定している。そのエクソンの欠失異常のパターンで、アウトオブフレームをインフレームへとするためにはそれぞれの欠失に対応して異なったエクソンスキッピングを誘導しなければならない。したがって、効率的な治療法の確立は 1 つのエクソンのスキッピングを誘導して最も多くの患者が治療できることである。神戸大学の DMD 患者で同定したエクソン欠失の型から、

エクソン４５のスキッピングを誘導することにより最も多くの患者が治療し得ることが判明した。そこで、エクソンスキッピング誘導治療をジストロフィン遺伝子の欠失のホットスポットのエクソンに欠失を有する例へ適応を拡大する最も有効な方策として、エクソン４５のスキッピング誘導を導くアンチセンスオリゴヌクレオチドを開発し、臨床応用することとした。

アンチセンスオリゴヌクレオチド

アンチセンスオリゴヌクレオチドとしてRNAとENA (ethylene-bridgednucleic acid)の2種の核酸からなるキメラが強力なエクソンスキッピング誘導効果を発揮することを見出しているので、このRNA/ENAキメラを臨床応用することとした。RNA/ENAキメラは世界的に見て全く臨床応用に向けた基盤整備がなされていない研究者らが独自に注目している化合物である。そのため、世界的にGMPグレードでのRNA/ENAキメラ合成の施設は見当たらず、そのため世界に先駆けたGMPグレードでのRNA/ENAキメラ合成の基盤整備について検討した。基盤整備を図っている施設において新たに合成されたRNA/ENAキメラがデザインされたとおりの配列であり、また、ヒトに応用できるほどの純度であるか否かの検討を行った。そのために、高速液体クロマトグラフィーおよびTOF/MS分析をおこない、RNA/ENAキメラの組成についての詳細な検討を実施した。

RNA/ENAキメラのエクソンスキッピング誘導とジストロフィン発現誘導能

合成したRNA/ENAキメラをキャリアーと混合したのちに培養筋細胞の培養液に混じた。導入後培養をさらに続けたのち、細胞を採取した。細胞から全RNAを抽出した。そして、ジストロフィンmRNAの標的領域をRT-PCR増幅した。増幅産物を電気泳動により分離した。分離した増幅産物の同定はその塩基配列を決定することによって行った。

また、ジストロフィン発現能については DMD 患者由来の筋細胞にRNA/ENAキメラを導入して検討した。対象となる細胞は、エクソン45をスキップさせることにより、アウトオブフレームの変異がインフレームに転換されるエクソンの欠失を有しているDMD患者から得た。この筋細胞では、エクソンスキッピング誘導によりジストロフィンの発現が期待された。そして、導入した筋細胞をジストロフィンのモノクローナル抗体により染色し、ジストロフィンの発現の有無を解析した。

急性毒性解析

今回合成したRNA/ENAキメラの臨床応用の可能性が強く支持された。しかし、RNA/ENAキメラはRNAとENA(Ethylenebridged nucleic acid)という2種の異なった核酸の混成化合物である。特にENAは我が国独自に開

発された新規の修飾核酸であるため、臨床応用に向けた基礎的データの集積は少ない。そのため、RNA/ENA キメラという全く新たに合成された化合物についての毒性に関する調査報告はない。そこで、今回合成した RNA/ENA キメラが毒性を有するか否かの急性毒性試験を実施した。合成 RNA/ ENA キメラをヒトに投与を想定している量の大過剰量をマウスに投与し、形態学的・生化学的変化について検討した。

スプライシング異常例の解析

エクソンスキッピングを最も効率的に誘導するアンチセンスオリゴヌクレオチドの標的部位の同定は、ジストロフィン異常症患者が自然に発症したエクソンスキッピングを見い出すことが最も有効な手段であると考えられる。そこで、今後のエクソンスキッピング誘導治療の適応範囲を拡大するためとさらに有効性の高いアンチセンスオリゴヌクレオチドを得るため、DMD 患者でジストロフィン遺伝子のスプライシング異常例の探索を行った。特にナンセンス変異は一塩基の置換でありながら、スプライシングに大きな変化来たす可能性があることから、ナンセンス変異を持つ症例を中心にそのスプライシング異常に焦点をおいた解析を行った。対象とする患者のジストロフィン mRNA を RT-PCR 解析を実施した。そして、スプライシング異常の同定された例ではスプライシング促進配列の解析などの詳細な構造配列を検討した。

C.研究結果

ジストロフィン遺伝子のエクソン 45 のスキッピング誘導に焦点をあてた研究を遂行した。これまでの予備的な研究によりエクソン 45 のスキッピングを強力に誘導する RNA/ENA キメラを同定していた。今回、RNA/ENA キメラオリゴヌクレオチドの臨床への応用を前提とした合成について検討した。そして合成した RNA/ENA キメラが当初の目論見通りの効果を発現するか否かを検討した。また、動物投与により有毒性がないことを検討した。さらにより強力なエクソンスキッピング誘導作用を発揮するアンチセンスオリゴヌクレオチドの標的部位の探索を DMD 患者で探索した。

1. RNA/ENA キメラの合成

ジストロフィン遺伝子のエクソン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 キメラの配列についてはすでに明らかにしてきた。臨床に応用できるRNA/ENAキメラの合成について検討した。RNAとENAとの異なった性質の核酸からなるアンチセンスオリゴヌクレオチドを合成する特殊な技術と、臨床応用にあったってはＧＭＰ基準に沿った合成を実施する基盤が必要であった。我が国の核酸合成の現場においては、こうした2点を即座に解決する基盤整備がいまだなされておらず、臨床応用を前提にした合成を推進するのは困難であった。幸いにも、合成の受託を検討してもよいという企業が見出された。そこで、試験的な合

成を実施した。
試験的にRNA/ENAキメラの合成をおこなったところ、回収率もよく合成できることが判明した。そして、合成産物の高速液体クロマトグラフィー分析あるいはTOF-MS分析においては、計画した配列をもったRNA/ENAキメラが高純度で合成されたことが確認できた。臨床応用を考慮した核酸合成の現場において比較的スムーズにRNA/ENAキメラの合成がわが国でも可能なことが確認された。さらに、この合成について、GMP基準に合致する方法での可能性について検討中である。

2. 合成 RNA/ENA キメラのエクソンスキッピング誘導能解析

合成した RNA/ENA キメラを培養筋細胞に導入し、細胞内に発現するジストロフィン mRNA を RT-PCR 解析した。ジストロフィン mRNA のエクソン 45 を含む領域を増幅したところ、一本の増幅産物を得た。この増幅産物のサイズからエクソン 45 の消失した mRNA が得られたものと推察された。これを確認するために、その塩基配列を解析したところ、エクソン 45 の配列が完全に消失していることが明らかとなり、エクソンスキッピングが誘導されたことを確認した。また、増幅産物が 1 種のみであることからすべての mRNA でエクソンスキッピングが誘導されたことを示した。この結果は、今回新たに合成した RNA/ENA キメラが予想通りにエクソンスキッピング誘導効率を極めて高く発揮するものであることを示した。

3. 患者由来筋細胞でのジスロフィン発現

新たに合成した RNA/ENA キメラが強力にエクソン 45 のスキッピングを誘導することを確認したので、このエクソン 45 のスキッピング誘導がジストロフィン発現をも誘導するかを DMD 患者由来筋細胞を用いて検討した。エクソン４５のスキッピング誘導によりをアウトオブフレームからインフレームへと変換されるジストロフィン遺伝子のエクソン欠失を有する患者から得た培養筋細胞に、合成した RNA/ENA キメラを導入した。導入後,ジストロフィン mRNA をＲＴ－ＰＣＲ解析するとやはりエクソン 45 の消失した増幅産物を得た。このエクソン 45 のスキップした産物は、一部のアミノ酸配列を欠いたジストロフィンを産生する能力を回復するところとなった。したがって、筋細胞のジストロフィン産生が強く期待された。
そこで、ジストロフィンに対するモノクローナル抗体を用いて筋細胞を染色をしたところ、ジストロフィン陽性の筋細胞を検出した。一方、RNA/ENA キメラを導入しない細胞ではジストロフィン陽性細胞は検出されなかった。
このことから、RNA/ENA キメラ導入により、エクソンスキッピングの誘導、ジストロフィンの発現という DMD の治療戦略が機能することが再確認された。

4. RNA/ENA キメラの急性毒性試験

先の結果は RNA/ENA キメラの臨床応用の可能性を強く支持した。しかし、RNA/ENA キメラは全く新たに合成された混成化合物であるため、その毒性に関して調査した報告はない。そこで、新たに合成した RNA/ENA キメラが毒性を有するか否かの急性毒性試験を実施した。合成 RNA/ ENA キメラをマウスに投与し、形態学的・生化学的変化について検討した。RNA/ENA キメラ投与後のマウスにおいて形態学的ならびに生化学的、いずれの方法においても異常は誘発されなかった。

この結果から、RNA/ENA キメラはヒトにも安全に投与できるものと考えられた。

5. スプライシング異常例の解析

エクソンスキッピングを最も効率的に誘導するアンチセンスオリゴヌクレオチドの標的部位の同定は、ジストロフィン異常患者が自然に発症したエクソンスキッピングを見い出すことが最も有効と考えられる。そこで、DMD 患者でジストロフィン遺伝子のスプライシング異常例の探索を行った。特にナンセンス変異は一塩基の置換でありながら、スプライシングに大きな変化来たす可能性であることから、ナンセンス変異を持つ症例を中心にそのスプライシング異常に焦点をおいた解析を行った。そして、多くの症例でエクソンスキッピングが 2 次的に誘導されていることを確認した。さらに、1 部の例では、エクソンでスプライシング促進配列の解明に成功した。

今後、同定した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デザインすることによりエクソンスキッピング誘導に効果的な核酸医薬の開発が可能と考えられた。

D. 考察

Duchenne型筋ジストロフィー（DMD）の治療法の確立を目指して、世界中で多くの研究がさまざまな方向からなされている。最近では、DMD患者が有するアウトオブフレーム変異をインフレームへと変換するジストロフィン遺伝子のエクソンスキッピング誘導による治療が最有力治療として世界的にも大きく注目されている。本研究は、エクソンスキッピング誘導を行うためにRNA/ENAキメラを臨床に応用しようとする画期的なものである。これまで、世界でこうした研究をした例はなく、先進的である。そのため、臨床に応用するための基本であるGMPグレードでのRNA/ENAキメラ合成が本研究進展の鍵であった。今回の本研究の成果によりほぼ我が国の核酸合成の現場においても合成が可能なことが確認された。

そして、合成されたRNA/ENAキメラが計画通りに合成され、また、エクソンスキッピング誘導効果も有していることが明らかになったことから、臨床に応用できるものであることが確認された。また、合成RNA/ ENAキメラをマウスに投与し、形態学的・生化

学的変化について検討し合成RNA/ENAキメラには急性毒性は見られなかった。これらの結果は、RNA/ENAキメラの臨床応用の可能性を強く示した。
このように、本年度の研究においてほぼ当初の計画通りの研究が進行した。DMD患者を対象として、ジストロフィン遺伝子のエクソンスキッピング45のスキッピング誘導治療することに対して大きく前進した。

E. 結論

今後、本年度の成果をさらに発展させエクソン45のスキッピング誘導治療の実践に向けた基盤整備をはかり、当初の研究成果を挙げる予定である。

F. 健康危険情報

本研究の実施中に生じた報告すべき危険情報は全くありませんでした。

G. 研究発表

1. 論文発表
   一覧表参照

2. 学会発表
   一覧表参照

H. 知的財産権の出願・登録状況
(予定を含む)

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他

研究成果の刊行に関する一覧表

雑誌

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発表者氏名	タイトル名	学会名	日程
Yagi M, Nishiyama A, Oyazato Y, Takeshima Y, Ogura T, Matsuo M.	Gene mutations and intellectual impairment in Duchenne Muscular Dystrophy patients.	Pediatric Academic Societies' Annual Meeting	2007.5.3-8 Toronto
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RESEARCH ARTICLE

Multiexon Skipping Leading to an Artificial DMD Protein Lacking Amino Acids from Exons 45 Through 55 Could Rescue Up to 63% of Patients With Duchenne Muscular Dystrophy

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Approximately two-thirds of Duchenne muscular dystrophy (DMD) patients show intragenic deletions ranging from one to several exons of the *DMD* gene and leading to a premature stop codon. Other deletions that maintain the translational reading frame of the gene result in the milder Becker muscular dystrophy (BMD) form of the disease. Thus the opportunity to transform a DMD phenotype into a BMD phenotype appeared as a new treatment strategy with the development of antisense oligonucleotides technology, which is able to induce an exon skipping at the pre-mRNA level in order to restore an open reading frame. Because the DMD gene contains 79 exons, thousands of potential transcripts could be produced by exon skipping and should be investigated. The conventional approach considers skipping of a single exon. Here we report the comparison of single- and multiple-exon skipping strategies based on bioinformatic analysis. By using the Universal Mutation Database (UMD)-DMD, we predict that an optimal multiexon skipping leading to the del45-55 artificial dystrophin (c.6439_8217del) could transform the DMD phenotype into the asymptomatic or mild BMD phenotype. This multiple-exon skipping could theoretically rescue up to 63% of DMD patients with a deletion, while the optimal monoskipping of exon 51 would rescue only 16% of patients. Hum Mutat 28(2), 196–202, 2007.

KEY WORDS: multiple-exon skipping; DMD; BMD; dystrophin; muscular dystrophy

INTRODUCTION

DMD (MIM# 310200) and BMD (MIM# 300376) are two X-linked recessive allelic disorders characterized by mutations in the dystrophin gene (MIM# 300377; NM_004010.1; Xp21.2) [Hoffman et al., 1987; Koenig et al., 1987]. DMD affects approximately 1 out of 3,500 live male newborns, while BMD is five times less frequent. The phenotypes of these two diseases are quite different. In DMD, clinical symptoms resulting from progressive muscle fiber degeneration are observed between 2 and 3 years old (yo) and the muscle wasting will lethally affect heart and lungs in adulthood. In contrast, BMD has a slower

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disease progression and a wider spectrum of phenotypes ranging from mild DMD to almost asymptomatic forms [Morrone et al., 1997]. The dystrophin Dp427 muscular isoform (3,685 amino acids) harbors four domains [Koenig et al., 1988]: the N-terminal actin binding domain (amino acids [aa] 12–240); a large central domain with 24 spectrin motif repeats (aa 253–3112); a cysteine-rich domain (aa 3113–3299) that interacts with various proteins of the dystroglycan complex; and a C-terminal complex (aa 3300–3685). Dystrophin plays a critical role in the preservation of the structure and function of muscle fibers while interacting with proteins of the associated dystrophin-glycoprotein complex (DAG), thus establishing a bond between the extracellular matrix and the cytoskeletal actin. The absence of dystrophin in humans leads to disorganization and slow degeneration of the muscular cells. Approximately two-thirds of patients show intragenic deletions ranging from one to several exons of the *DMD* gene. The remaining cases arise from genomic duplications or micro-rearrangements (nonsense mutations, small deletions or insertions, intronic mutations, and rare missense mutations). The reading frame rule [Monaco et al., 1988] explains the two different phenotypes resulting from mutations in the same gene. Mutations that change the translational reading frame of the gene elicit formation of premature stop codons and consequent abortion of the translation process that results in dystrophin deficiency and the DMD phenotype, whereas mutations that conserve the translational reading frame of the mRNA result in the BMD phenotype. The reading frame hypothesis explains the phenotypic differences observed in approximately 92% of the DMD/BMD cases [Koenig et al., 1989]. Nevertheless, several exceptions to this rule have been described such as BMD with the out-of-frame exon 3–7 deletion. Thus, dystrophin can be detected in patients with out-of-frame mutations, theoretically leading to absence of protein while large in-frame deletions can be detected in DMD patients. In parallel, in many DMD patients as well as in animal models (X-linked muscular dystrophy [*mdx*] and Golden Retriever muscular dystrophy [GRMD] dogs), rare dystrophin-positive fibers have been reported [Crawford et al., 2001; Wilton et al., 1997]. It has been suggested that restoring the reading frame by exon skipping is the most likely cause of this natural phenomenon. In fact, it is well known that the phenotype is directly correlated with the functional importance of the deleted protein domains, the repeated central domain being more tolerant to deletions than the N- and C-terminal domains because of its modular structure [Beggs et al., 1991]. Thus the opportunity to transform a DMD phenotype into a BMD phenotype appeared as a new therapeutic strategy with the development of the antisense oligonucleotide (AO) technology, which is able to induce an exon skipping at the pre-mRNA level in order to restore an open reading frame. Genomic deletions of one or more exons being the most frequent mutations among DMD patients and mainly localized in the repeated central domain, exon skipping in this area should allow the production of a partially functional dystrophin. Such exon skipping exists among patients and can explain exceptions to Monaco's rule. This has prompted many groups to investigate the possibility of designing strategies for gene repair/modulation based on the use of compounds interfering with splicing, thus inducing exon skipping [Aartsma-Rus et al., 2004b; Goyenvalle et al., 2004; Kapsa et al., 2003]. Because the DMD gene contains 79 exons, thousands of potential transcripts can be produced by exon skipping and should be investigated. Here we report the prediction of an optimal exon skipping to transform the DMD phenotype into the asymptomatic or mild BMD phenotype. This work was performed by using the UMD-DMD mutation database.

PATIENTS AND METHODS

Database of Mutations

We used the UMD® software [Beroud et al., 2000, 2005] to build the UMD-DMD database of mutations from dystrophin gene–mutated patients identified in French reference diagnostic laboratories. For each patient, data have been collected at the molecular, protein, and clinical levels. On July 1, 2006, the UMD-DMD database included 602 records from 409 DMD patients, 160 BMD patients, and eight patients with an intermediate phenotype. Phenotype for the remaining 25 patients was not assessed because of their young age. In order to evaluate the impact of various exon skipping events, specific tools have been developed. The "exon-phasing" tool gives access to a graphical presentation of all exons of the DMD gene according to their phasing. Thus the direct consequence of the deletion of one or more exons on the gene's reading frame can be easily evaluated. When deletions result in a new junctional codon (the first or the two first nucleotides of the junctional codon comes from the 5′ exon while the remaining nucleotide(s) come(s) from the 3′ exon) this could theoretically lead to an allo- or an isosemantic impact and ultimately to a stop codon. In this latter situation the apparent reading frame preservation based on the exon phasing is invalidated at the nucleotide level. We therefore created the "AA junction after exon skipping" tool, which displays for each artificial deletion generated by exon skipping its consequences both at the reading frame and at the junctional codon levels. As mentioned previously, the dystrophin protein is tolerant to internal deletions. Therefore, to restore the reading frame of an out-of-frame deletion found in a DMD patient, one can imagine additionally deleting one or more exons either on the 5′ and/or 3′ side of the deletion. More than 3,000 artificial deletions can theoretically be generated by exon skipping of one or more exons. As it is impossible to evaluate the therapeutic potential of all the possible resultant dystrophins, various groups have designed mini-, micro-, or quasidystrophins [Kapsa et al., 2003; Kobinger et al., 2003; Li et al., 2005; Liu et al., 2005; Nonaka, 2004; Takeda, 2004; Yoshimura et al., 2004]. In addition, microdystrophin has been designed as an alternative to the incorporation of a full-length dystrophin cDNA (14 kb) into an AAV vector because of the limited size of the cassette. Using a 4.9-kb rod-truncated microdystrophin CS1, Takeda [2004] has almost completely ameliorated the dystrophic phenotypes in transgenic *mdx* mice. Concomitantly, Liu et al. [2005] have used a C-terminal truncated DeltaR4-R23/DeltaC microgene (DeltaR4/DeltaC). These are promising approaches to rescue muscular dystrophy in young *mdx* skeletal muscle. Nevertheless, if the percentage of centrally nucleated myofibers was reduced to approximately 22% in microdystrophin-treated muscle [Liu et al., 2005], one can expect that a larger functional dystrophin could give better results. We designed an automatic tool that presents, for each mutation, the largest in-frame artificial dystrophin that can be generated by exon skipping. All other alternative exon skippings restoring the frame are also accessible. In addition, we created the "in-frame correction table" module, which summarizes the number of patients with a deletion that could be rescued by a skipping of one, two, or three exons either in the 5′ or 3′ side of the deletion. Direct access to data of corresponding patients is also provided. Because patients with an in-frame deletion are frequently associated with the milder form of the disease (BMD), they are natural mutants that give valuable information about the phenotypic consequence of various minidystrophins. We therefore created the "Large rearrangements rescuable by mono-exon skipping" tool. It evaluates all monoexon

skippings that can rescue out-of-frame deletions for patients described in the UMD-DMD database. For each monoexon skipping, it lists the number of patients eligible for this skipping, their deletion, the amino acid junctional impact, and the patients or individuals naturally harboring the artificial deletion. Furthermore, each deletion rescuable by monoexon skipping is associated with one of the following groups: 1) exon skipping with a junctional impact being a stop codon; 2) exon skipping with an allosemantic junctional impact (such protein has never been reported in patients); 3) exon skipping with an isosemantic junctional impact (such protein has never been reported in patients); 4) exon skipping leading to a protein found exclusively in DMD patients; 5) exon skipping leading to a protein found both in DMD and BMD patients; and 6) exon skipping leading to a protein found exclusively in BMD patients. The goal of the exon skipping strategy in the context of the DMD being to transform a DMD phenotype into a BMD phenotype, deletions belonging to groups 2, 3, or 6 above are the best target for such strategies. In parallel to this monoskipping analysis, we wished to evaluate multiexon skipping. We then designed a specific routine to display the full set of correcting events for each mutation. In addition, to evaluate the potential use of multiexon skipping to rescue the largest set of DMD patients, we developed the "Exon skipping leading to BMD" algorithm. This routine selects all in-frame deletions found in BMD patients and searches for all deletions found in DMD patients that can be transformed into in-frame deletions through various multiexon skipping. The list of all corresponding cases is available.

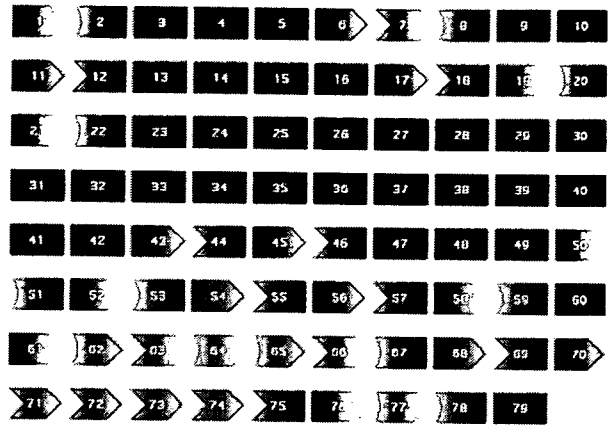

FIGURE 1. **Exon phasing of the DMD gene. Each exon is presented by a gray box. Each extremity of the box represents the specific phasing of the exon. Left end of exons: 1) dark gray vertical lane means that the exon begins by the first nucleotide of a codon; 2) light gray curve means that the exon begins by the second nucleotide of a codon; and 3) gray arrow means that the exon begins by the third nucleotide of a codon. Right end of exons: 1) dark gray vertical lane means that the exon ends by the last nucleotide of a codon; 2) light gray curve means that the exon ends by the first nucleotide of a codon; and 3) gray arrow means that the exon ends by the second nucleotide of a codon. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

Patients

Using UMD-DMD tools, we identified 11 male individuals carrying the deletion of exons 45 to 55 (abbreviated as "del45-55"; approved mutation nomenclature c.6439_8217del based on GenBank NM_004010.1 and using the A of the ATG initiation codon as +1) of the DMD gene. We also collected four individuals with this mutation from Japan. For each patient, clinical data were collected from the caring physicians. These data included age and symptoms of onset, age of ambulation loss, age of last examination, neuromuscular, cardiological, respiratory and behavioral data, creatine phosphokinase (CPK) level, and muscle biopsy results. When muscle biopsy samples were available, dystrophin analysis was performed by conventional techniques [Anderson and Davison, 1999; Bornemann and Anderson, 2000].

RESULTS

Junctional Impact of Exon Skipping

The DMD gene is composed of 79 exons that are distributed in various phases, as shown in Figure 1. When a deletion occurs, it can result into a frameshift and therefore lead to a premature stop codon. This is achieved when two exons in different phases are joined end to end (for example, exons 51 and 53). When two exons in the same phase are involved, it is usually assumed that no premature stop codon is generated as no frameshift occurs as predicted by the DMD exonic deletions/duplications reading frame checker 1.6 from the Leiden database (www.dmd.nl) [Fokkema et al., 2005]. Nevertheless, this does not take into account the impact at the junctional codon. The automatic tool developed in the UMD-DMD database allowed us to demonstrate that among the 3,081 possible deletions, seven deletions that are not predictive of a frameshift in fact lead to a junctional stop codon. These are del2-19 (c.32_2380del), del2-58 (c.32_8668del), del 2-77 (c.32_11014del), del51-58 (c.7310_8668del), del51-77 (c.7310_11014del), del65-77 (c.9362_11014del), and del67-77 (c.9650_11014del). Only del51-58 is of interest in the context of exon skipping.

Monoexon Skipping

To evaluate if mono-, bi-, or triexon skipping are equally efficient to rescue out-of-frame deletions described in patients, we used the "in-frame correction table" module. For 71 patients, the skipping of one exon was the only available approach, while for six patients their deletion could be rescued by deletion of one, two, or three exons and for 111 patients by deletion of one or three exons. Interestingly, monoskipping could theoretically restore the frame of deletions found in 215 patients, biskipping in 76 patients, and triskipping in 160 patients. If only patients exclusively rescued by one of these approaches are evaluated, monoskipping is required for 71 patients, biskipping for eight patients, and triskipping for two patients (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Therefore, monoskipping is the most efficient approach in this cohort. The rescue by monoexon skipping of various deletions will lead to individualized therapeutic approaches. To evaluate if some exons could rescue various types of mutations and therefore could be the best targets for clinical trials, we used the "Large rearrangements rescuable by mono-exon skipping" tool. It reveals that only 11 monoexon skipping events could potentially rescue more than 10 patients (Fig. 2). If we consider that some deletions can be rescued by monoskipping of either the 5′ or the 3′ exons, only nine monoskipping events are relevant. Indeed, the nine patients with deletion of exon 44 can be rescued alternatively by monoskipping of exons 43 or exon 45. Similarly, the 15 patients with deletion of exon 51 can be rescued either by monoskipping of exons 50 or 52.

The monoskipping of exons 51 is the best choice, as it can rescue deletions of exons 48–50 (12 patients), leading to artificial deletions found exclusively in BMD patients, i.e., exons 48–51 (four patients). Deletions of exons 43–50 (one patient), 45–50 (13 patients), 47–50 (one patient), 49–50 (eight patients), 50 (four patients), 52 (five patients), and 52–63 (one patient) that can be rescued by monoskipping of exon 51 result in artificial dystrophins that so far have not been reported in the UMD-DMD database.

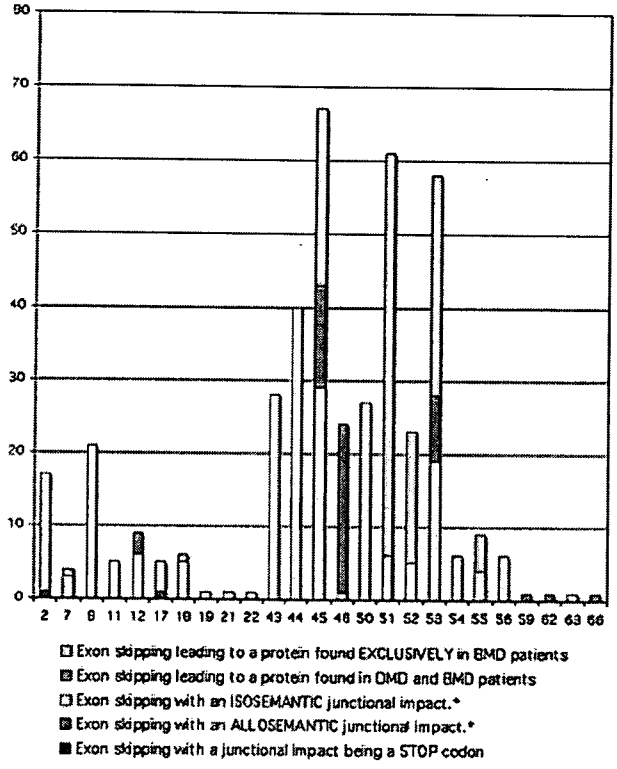

FIGURE 2. **Monoskipping of the DMD gene. For each exon is presented the number of patients from the UMD-DMD database that can be rescued by the corresponding monoskipping. Each deletion has been associated with one of the five groups. *Such protein has never been reported in patients.**

Multiexon Skipping

The work from J.S. Chamberlain's group (Department of Human Genetics, University of Michigan Medical School) led to the description of the microdystrophin and to the recognition of dispensable spectrin repeats in the dystrophin protein [Phelps et al., 1995]. Today it is recognized that repeats four to 23 (DeltaR4-R23) can be removed leading to a functional protein. Therefore, it is possible to use the exon skipping approach to generate artificial dystrophins including deletions of various sizes. This could be achieved by targeting multiple exons with various antisense oligonucleotides or modified small nuclear RNA unit #7 (U7 snRNA). In this situation, various deletions could be rescued by the same approach and therefore only one procedure will have to be developed. To evaluate which is the artificial dystrophin that can be generated by exon skipping and could rescue the largest number of patients, we developed a specific tool. As expected, such approach should invariably lead to the largest functional deletion (i.e., DeltaR4-R23). Because we have collected phenotypic data for most patients, we decided to limit this analysis to artificial dystrophin already reported in BMD individuals and therefore for which functional in vivo data were available. We developed the so-called "Exon skipping leading to BMD" algorithm. It evaluates, for each in-frame deletion reported only in BMD patients, how many deletions found in DMD patients could be rescued by multiexon skipping leading to this in-frame deletion. Figure 3 summarizes available data. This tool allowed us to identify deletion from exons 45 to 55 (c.6439_8217del) as the best solution as it could rescue deletions described in 161 DMD patients among the 254 DMD patients with a large deletion (63%) (Supplementary Table S2). This deletion could also rescue small

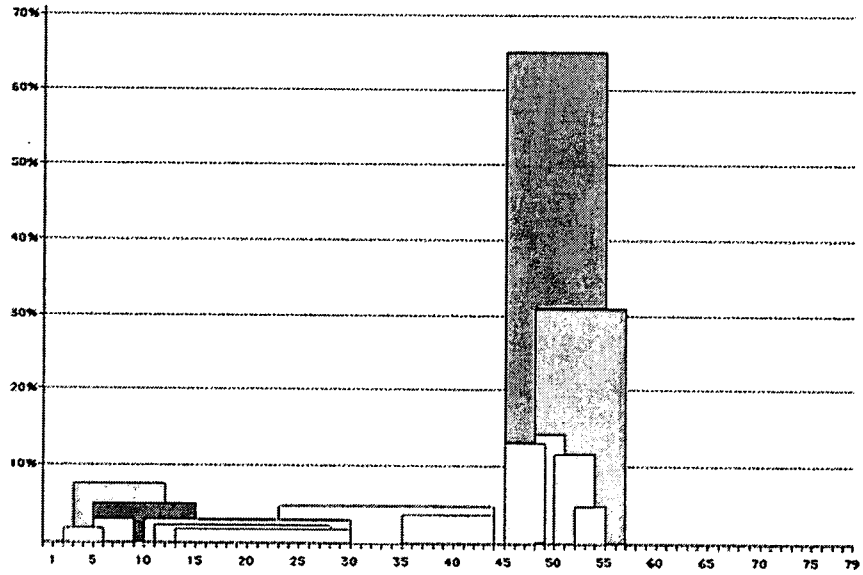

FIGURE 3. **Multiskipping of the DMD gene. X-axis: exons of the DMD gene. Y-axis: number of DMD patients rescuable by multiexon skipping. Each colored rectangle corresponds to the deletion found in BMD patients. Note the pink del45-55 deletion that can rescue 161 DMD patients with an out-of-frame deletion of the DMD gene.**

TABLE 1. Clinical Features of the 15 Documented Patients Carrying the del45-55 Dystrophin Gene Mutation

Patient	Symptoms of onset/age (years)	Age at last examination (years)	Clinical status	CPK/age (years)[a]	Ambulation (if no, then age of loss)	Cardiac involvement/ age (years)
1	Muscle pain, myoglobinuria/13	14	Mild BMD	6 times/14	Y	N
2	Chance high CK levels/8	9	AS	17 times/8	Y	N
3	Chance high CK levels/6	7	AS	6 times/7	Y	N
4	Chance high CK levels/13	18	Mild BMD	5 times/18	Y	N
5	Myalgia/12	14	Myalgia	Elevated/14	Y	—
6	Weakness/45	55	Mild BMD		Y	—
7	Weakness/4	40	Mild BMD	4 times/22	Y	—
8	Weakness/4	40	Mild BMD	4 times/22	Y	—
9	Toe walking, weakness/2	46	Mild BMD	—	Y	—
10	Weakness (very mild)/3.5	13	Exercise intolerance	19 times/3.5	Y	N
11	Chance (related to Patient 10)/57	63	AS	1.3 times/57	Y	Cardiomyopathy (LVEF 51%)/57
12	Scoliosis, weakness/19	40	Mild BMD	7 times/40	Y	N
13	Calf hypertrophy/40	54	Mild BMD	6 times/41	Y	Cardiomyopathy (LVEF 57%)/54
14	Exercise intolerance/12	19	Mild BMD	41 times/12	Y	N
15	Weakness/49	50	Mild BMD	1.5 times/50	Y	—

[a]CPK levels are expressed as number of times normal level.
–, not available; N, No; Y, Yes; BMD, Becker muscular dystrophy; AS, asymptomatic; LVEF, left ventricular ejection fraction; Chance, individual discovered by chance without any clinical sign.

rearrangements found in six DMD patients out of 129 (4.7%) and large duplications found in nine patients out of 41 (22%).

Clinical Study

According to neuromuscular features, the 15 individuals were classified as asymptomatic, exercise intolerant and/or myalgia, and mild BMD phenotypes (Table 1). Mild BMD was defined as a mild weakness with or without calf hypertrophy and autonomous ambulation. None of them had evidence for respiratory involvement or mental retardation.

Overall phenotypes of the 15 documented patients associated with del45-55 are heterogeneous ranging from asymptomatic (three individuals, ages: 7 to 63 yo), exercise intolerance and/or myalgia (two patients, ages: 13 and 14 yo) and mild BMD (10 patients, ages: 14 to 55 yo). The age of onset when available was ranging from 2 to 49 yo (mean = 19.5 ± 19.6 yo, median = 12, interquartile range = 24.5). The most frequent symptoms of onset were weakness; defined as walking, running, and stair-climbing difficulties (seven patients), exercise intolerance (three patients), and calf hypertrophy (one patient). CPK level was highly elevated (>5 times) for seven individuals (mean age = 17 ± 15 yo, median = 12, interquartile range = 19) while it was moderately elevated for others (≤5 times) (mean age = 34 ± 19 yo, median = 22, interquartile range = 28). All patients, including nine individuals at least 39 yo, were still ambulatory at last examination (ages: 7 to 63 yo). Two patients had a mild cardiac involvement identified at echocardiography without clinical manifestation.

Muscle Biopsy Results

When tissues were available (two cases with exercise intolerance and/or myalgia , one asymptomatic case, and one mild BMD), the microscopy revealed mild (two patients) or moderate (two patients) muscular dystrophy. Dystrophin immunostaining was moderately reduced with Dys-2 antibody (Novocastra Laboratories, Newcastle, United Kingdom; www.novocastra.co.uk) (aa 3668–3684) in two patients and with the C5G5 antibody (aa 1173–1728) [Fabbrizio et al., 1993] in four patients. It was negative with the Do antibody (aa 1840–2226), whose epitope overlap the deleted portion of the dystrophin, in two patients (data not shown).

The Western blot analysis was performed in three samples and a reduced amount of dystrophin was detected in all samples (data not shown). In all analyzed cases dystrophin displayed a reduced molecular weight.

Other patients with a deletion of exons 45 to 55 are reported in the Leiden database [Fokkema et al., 2005]. Only one of these patients was previously published [Sironi et al., 2001]. This patient was 60 yo and classified as a mild BMD without cardiomyopathy. The level of expression of the deleted dystrophin was estimated to 80% by Western blot analysis.

Overall, these data suggest that deletion of exons 45 to 55 is associated with mild BMD (10 patients), exercise intolerance and/or myalgia (two patients) or even no phenotype (three patients).

DISCUSSION

Many teams worldwide have been engaged in monoexon skipping to rescue the dystrophic *mdx* mouse bearing a nonsense mutation in exon 23 by skipping of this exon. Recent very promising results [Goyenvalle et al., 2004; Lu et al., 2005] open the way to human DMD exon skipping. One approach aims at inducing skipping of an exon carrying a nonsense mutation (such as R1967X in exon 41) in human cultured myocytes [Surono et al., 2004]. Another approach has been to perform double skipping [Aartsma-Rus et al., 2004a]. As a broad pattern of deletions is observed in DMD patients (today 163 different deletions are recorded in the UMD-DMD database), the set of monoexon skipping events would have to be large with some being indicated for only one patient while others would correct many. In order to better define the most efficient artificial dystrophins resulting from monoexon skipping, we developed various dedicated tools. We have been able to demonstrate that only nine exons could be used to rescue deletions found in more than 10 patients (254 patients harbor a large deletion of the DMD gene). The best target is exon 51 that could rescue 45 patients (16%). We also developed tools to evaluate artificial deletions

produced by exon skipping that would lead to deletions reported in DMD patients. For example, deletion of exon 45 is associated with DMD as it results in a premature stop codon. This deletion can theoretically be rescued by monoskipping of exon 44 or 46. The resulting del44-45 has been described once in a DMD patient [Herczegfalvi et al., 1999] and the del45-46 three times, in two DMD and one BMD patients. Therefore the deletion of exon 45 could not be efficiently rescued by monoexon skipping. Furthermore, we also evaluated the consequences of the skipping on the junctional codon. We demonstrated that seven deletions apparently restoring the frame would indeed result in a premature stop codon: del2-19 (c.32_2380del), del2-58 (c.32_8668del), del2-77 (c.32_11014del), del51-58 (c.7310_8668del), del51-77 (c.7310_11014del), del65-77 (c.9362_11014del), and del67-77 (c.9650_11014del).

As suggested by Aartsma-Rus et al. [2004a], the targeting of 20 monoskipping events would theoretically be beneficial for at least 75% of patients. Nevertheless, this approach will be time consuming and many clinical trials will be required, as each monoskipping has to be addressed individually. We believe that multiexon skipping is a better solution, as we can expect that only a limited number of different multiskipping events would be required. We therefore developed a set of dedicated tools to address the multiskipping. We used the phenotypes of various individuals included in the UMD-DMD database as an in vivo control of the functional impact of artificial dystrophins generated by the multiskipping. The "Exon skipping leading to BMD" algorithm allowed us to select the del45-55 (c.6439_8217del) artificial dystrophin as the most efficient multiskipping as it would theoretically be beneficial to 63% of patients harboring a deletion. In order to assess the exact phenotypes of individuals harboring a del45-55 deletion, we used the clinical data provided by the corresponding caring physicians. After reviewing detailed clinical data, we can conclude that for all patients older than 40 yo (eight patients) the phenotype is either asymptomatic (one patient) or a mild BMD (eight patients) with cardiac involvement of variable severity (two patients). The observed clinical heterogeneity could at least partially result from genetic heterogeneity in the *DMD* gene. In fact the deletions have only been characterized in term of missing exons in the DNA but not at the intronic level (deletion breakpoints). Therefore we can expect that asymptomatic patients have a fully functional neointron 44–56 and therefore an almost normal level of del45-55 (c.6439_8217del) dystrophin, while patients with mild BMD phenotype have different breakpoints that lead to partially functional neointron 44–56. In a family with a del45-54 (c.6439_8027del) mutation, an asymptomatic female carrier with an altered dystrophin mRNA fragment generating a mRNA deletion of exon 44 has been reported [Lenk et al., 1993]. It was concluded that this fragment could be generated by an alternative splicing of exon 44. In one patient from our study, we detected a weak signal corresponding to a del44-55 (c.6291_8217del) fragment. We can therefore hypothesize that various levels of alternative splicing of exon 44 could partially explain the observed heterogeneity as del44-55 (c.6291_8217del) leads to nonsense mediated decay.

This is so far the largest series of patients harboring a large deletion of the DMD gene either asymptomatic or with mild phenotype. It reinforces the bioinformatic predictive value of the del45-55 (c.6439_8217del) deletion as being the "target of choice" for exon skipping. Furthermore, the number of individuals harboring this deletion is probably under evaluated as asymptomatic individuals exist and one of them has been identified by chance in our series. Indeed Patient 11 has been detected at 57 yo during the course of a family analysis. His grandson (Patient 10) was under investigation because the pediatrician had noticed a slight toe walking and a very mild weakness in the lower limbs. The molecular investigations revealed that he had inherited the mutated dystrophin gene from his grandfather who was asymptomatic.

To evaluate the efficiency of the exon skipping leading to the del45-55 artificial dystrophin for a specific patient, it could be necessary to use his own cells for in vitro testing prior to clinical trial. Alternatively, the precise definition of intronic breakpoints could lead to a better understanding of functional or partially functional neointrons. The identification of exonic splicing silencer (ESS) [Sironi et al., 2004; Wang et al., 2004; Zhang and Chasin, 2004] and exonic splicing enhancer (ESE) [Cartegni et al., 2003; Fairbrother et al., 2004] patterns can also give valuable information.

A major concern about this approach is the feasibility of multiexon skipping at the technical level. Various reports lead us to believe that this could be performed in the near future. So Aartsma-Rus et al. [2004a] have shown that double skipping of exons 43 and 44 or 45 and 51 can be successfully achieved. More importantly, a recent study by Fall et al. [2006] showed that a cocktail of AOs can be used to efficiently induce the skipping of exons 19 to 25 in the *mdx* mouse.

In conclusion, we demonstrated that use of dedicated algorithms from the UMD software could predict the best multiexon skipping to rescue DMD patients. We conclude that the del45-55 (c.6439_8217del) could rescue up to 63% of DMD patients harboring a deletion of one or more exons in our series, while the best monoskipping approach (monoskipping of exon 51) will only rescue 16% of these patients. This approach will also be useful for patients with small rearrangements (small insertions/deletions, splice mutations, etc.) or large duplications. Overall, the multiskipping of exons 45 to 55 could rescue 41.5% of DMD patients from our series. This "general" as opposed to "specific" approach may therefore simplify the development of clinical trials in the context of DMD.

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SHORT REPORT

A nonsense mutation-created intraexonic splice site is active in the lymphocytes, but not in the skeletal muscle of a DMD patient

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Abstract Production of semi-functional dystrophin mRNA from the dystrophin gene encoding a premature stop codon has been shown to modify the severe phenotype of Duchenne muscular dystrophy (DMD). In this study, we report the tissue-specific production of semi-functional dystrophin mRNA via activation of a nonsense mutation-created intraexonic splice acceptor site. In a DMD patient a novel nonsense mutation was identified in exon 42. In his lymphocytes semi-functional dystrophin mRNA with a 63-nucleotide deletion in exon 42 (dys-63) was found to be produced. In vitro splicing assay using hybrid minigenes disclosed that the mutation-created intraexonic splice acceptor site was activated. In his skeletal muscle cells, however, only the authentically spliced dystrophin mRNA was found. This finding identifies the modulation of the splicing of muscle dystrophin mRNA in cases of DMD as a potential target for therapeutic strategies to generate a milder phenotype for this disease.

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Introduction

The severe Duchenne muscular dystrophy (DMD, MIM 310200) and the more benign Becker muscular dystrophy (BMD, MIM 300376) are caused by mutation of the dystrophin gene. More than 100 nonsense mutations located at various positions along the 14-kb dystrophin mRNA have been reported (http://www.dmd.nl). Despite the wide variation in the coding potentials of the mutated mRNAs (0–98.6% of the full-length protein), these truncating mutations are surprisingly associated with a uniformly severe DMD phenotype. A limited number of nonsense mutations, however, have been reported to result in a mild phenotype. In some of these cases, the production of semi-functional in-frame mRNA due to skipping of the exon containing the mutation has been shown as the cause of the phenotype modification (Barbieri et al. 1996; Disset et al. 2006; Shiga et al. 1997).

The production of semi-functional mRNA via activation of a mutation created-splice site has never been reported for nonsense mutations in the dystrophin gene. In this report, semi-functional mRNA was shown to be produced in the lymphocytes of a DMD patient via activation of a mutation created-splice site; this patient was found to have a novel nonsense mutation in the dystrophin gene. Only authentic splicing products containing a nonsense codon, however, were obtained from the patient's skeletal muscle, which explained his severe DMD phenotype. Because in vitro splicing analysis of a hybrid minigene carrying the nonsense mutation disclosed the production of in-frame mRNA, modulation of the splicing of dystrophin mRNA in muscle cells is a potential target for therapeutic strategies that aim to make the DMD phenotype milder.

Case and methods

Case

The proband (KUCG 593) was a 5-year-old boy. He was born at 36 weeks of gestation and was admitted to a neonatal care unit. During a routine blood examination, his serum creatine kinase (CK) level was found to be 4,050 IU/l (normal < 169 IU/l). When the subject was 3 months old, his CK level was elevated to 25,550 IU/l. He started to walk independently at 1 year of age and his motor development was normal. During the following period his serum CK remained elevated. A muscle biopsy when the boy was 3 years old disclosed no dystrophin staining, confirming a DMD diagnosis. At 5 years old, he was referred to Kobe University Hospital to examine his dystrophin gene for a mutation. These studies were approved by our ethics committee.

Methods

Mutation analysis

DNA was isolated from blood samples by standard phenol-chloroform extraction methods. The region encompassing exon 42 was amplified by the PCR with g42F (5′-CAATTGTCAGCTGTAGAATGAGACC-3′) as the forward primer and g42R (5′-TGAAGCCAACCAC ACTATCAAGTA-3′) as the reverse primer.

Total RNA was isolated from peripheral lymphocytes that were collected from whole blood using Ficoll–Paque density gradients (Amersham Biosciences AB, Uppsala, Sweden) or from thin-sliced (6 μm) muscle sections of frozen muscle samples. Reverse-transcription PCR (RT-PCR) and RT-nested PCR were employed to analyze the dystrophin mRNA expressed in skeletal muscle and lymphocytes, respectively. For dystrophin mRNA from lymphocytes, a region encompassing exons 36 to 45 was first amplified using a forward primer corresponding to a segment of exon 36 (3E: 5′-CCCAGCAA AAAGAAGACGTG3–3′) and a reverse primer complementary to a segment of exon 45 (3B: 5′-ACTGGC ATCTGTTTTTGAGGAT-3′). The PCR product was then used as a template for a second PCR amplification using a nested set of primers (forward-c40F: 5′-CAGC CTACCTGAGCCCAGAGATG-3′ and reverse-3F: 5′-CTTCCCCAGTTGCATTCAAT-3′). For dystrophin mRNA from skeletal muscle, a region encompassing exons 40 to 45 was amplified using the inner set of primers.

The amplified products were purified and sequenced either directly or after subcloning into the pT7 Blue-T vector (Novagen, Madison, WI). The DNA sequences were determined using an automated DNA sequencer (model 310; Applied Biosystems, Foster City, CA).

In vitro splicing assay

Using a minigene (H492) constructed from the pcDNA 3.0 mammalian expression vector (Invitrogen, Carlsbad, CA) (Thi Tran et al. 2005; Tran et al. 2006), hybrid minigenes were created by inserting a test sequence consisting of exon 42 and its flanking introns into the multicloning site (Fig. 3a). The region encompassing exon 42 was amplified from both the control sample and the patient's genomic DNA by the PCR with primers that correspond to introns 41 and 42 and included *Nhe*I and *Bam*HI restriction enzyme recognition sites, respectively (In42F-Nhe: 5′-GCCGCTA GCAGCCCAGTTTGTAGATTC-3′ and In42R-Bam: 5′-CGGGATCCGTCAAAATGCCATCATGATG C-3′). Amplified products were digested with *Nhe*I and *Bam*HI (New England Biochem, UK), and inserted into the minigene that had been digested with the same restriction enzymes. In this way, we constructed both wild-type (TVK-42) and mutant (TVK-42 m) hybrid minigenes that carried wild-type exon 42 and exon 42 with the nonsense mutation, respectively. After checking their sequences, these hybrid minigenes were transfected into HeLa cells for splicing assays as described before (Thi Tran et al. 2005; Tran et al. 2006). Cells were harvested 24 h after the transfection and total RNA was extracted as using an Isogen Kit (Nippon Gene Co., Toyama, Japan).

Five microgram of total RNA was subjected to reverse transcription using random hexamer primers and the PCR was performed using a forward primer corresponding to a segment of upstream exon A and a reverse primer complementary to a segment of the downstream exon B as previously described (Thi Tran et al. 2005) (Fig. 3a). PCR products were analyzed by electrophoresis on an 8% polyacrylamide gel.

Results

In the index case, a novel disease-causing mutation was identified in exon 42; a transition mutation from a thymine to a guanine was located at the 63rd nucleotide of exon 42, corresponding to the 5,985th nucleotide of the dystrophin mRNA (c.5,985T > G) (Fig. 1). The nucleotide change converted a TAT codon, which encodes a tyrosine residue at the 1,995th amino acid position of the dystrophin protein, to a TAG stop codon (p.1,995Y > X) (Fig. 1). The predictive truncation of