厚生労働科研究費補助金

こころの健康科学研究事業

デュシェンヌ型筋ジストロフィーのアンチセンス治療法の開発

平成１８年度　　総括研究報告書

主任研究者　松尾　雅文

平成１９（２００７）年　３月

目　　次

厚生労働科学研究費補助金（こころの健康科学研究事業）
研究報告書

デュシェンヌ型筋ジストロフィーのアンチセンス治療法の開発

Ⅰ総括研究報告
主任研究者：松尾　雅文　（神戸大学大学院医学系研究科　教授）

（研究要旨）
本研究では、これまでにジストロフィン遺伝子欠失のホットスポット領域にあるエクソンを対象として、そのエクソンのスキッピングを誘導する最適のRNA/ENAキメラを同定をはかった。そして、１部のエクソンにおいてそのスキッピングを誘導する最適のRNA/ENAキメラの同定に成功した。さらに、同定したRNA/ENAキメラを治療対象となるDuchenne型筋ジストロフィー（DMD）患者由来の培養筋細胞に導入し、導入細胞におけるエクソンスキッピング誘導を確認するとともに、ジストロフィンの発現も誘導されることを確認するなどの成果を挙げてきた。引き続き、ジストロフィン遺伝子の他のエクソンについても検討を行い、多くのエクソンでその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。これまで、有効なRNA/ENAキメラの同定にはRNA/ENAを合成してそのエクソンスキッピング誘導効果を1つずつ検討し、その中で有効なものを同定とするという極めて労働集約型の研究を実施してきた。そこで、各エクソンのスプライシングに関与する配列を容易に明らかにするためジストロフィン異常症患者の分子病態解析を行いエクソン内のスプライシングに必須の塩基の解明を計画した。ジストロフィン遺伝子のエクソン38内に4塩基の欠失を有し、そのためエクソン38のスキッピングを生じたDMD例を見い出した。さらに、in vitroのスプライシング系を構築し、これにDMD患者由来のエクソン38内に4塩基欠失を有する配列を挿入し、そのスプライシングを解析した。その結果、ジストロフィン遺伝子のエクソン38内の1塩基がスプライシングに極めて重要であることを明らかにすることに成功した。

Ⅱ研究報告

（分担研究者）
松尾　雅文
神戸大学大学院医学系研究科
教授

竹島　泰弘
神戸大学大学院医学系研究科
助教授

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

デュシェンヌ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。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を基盤としてエクソンのスキッピングを誘導することを応用する「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。そして、ジストロフィン遺伝子のエクソン19にスプライシング促進配列があること、こ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がエクソン19のスキッピングを有効に誘導することなどの世界的成果をあげてきた。

本研究ではこれまでに、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RNA/ENAキメラの同定とそのDMD患者由来培養筋細胞への導入によるジストロフィンの発現に成功してきた。本年度においては、さらに多くのエクソンのスキッピング誘導が可能になる様に引き続きRNA/ENAの同定を行った。また、スプライシングに必須のエクソン配列の同定に重点をおいた研究を実施した。

B. 研究方法

①RNA/ENAキメラの同定とDMD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、各種RNA/ENAキメラを合成する。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、エクソンスキッピングを誘導するRNA/ENAキメラを同定する。

さらに、ジストロフィン遺伝子の欠失のホットスポットにあ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キメラを導入する。導入筋細胞におけるmRNAの修正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確認する。

②スプライシングに必須のエクソン内の一塩基の同定

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する。多くの遺伝子異常を同定したが、その中で極めて特異な遺伝子の異常によりスプライシング異常の発生する例

を見い出した。

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エクソン内の塩基を同定する。

(倫理面への配慮)

エクソンスキッピングを誘導する治療については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、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も同様に承認されている。

C. 研究成果

①欠失のホットスポット領域のエクソンのスキッピングを誘導するRNA/ENAキメラの同定

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、その中から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。

その結果、エクソン45、51、53など多数のエクソンスキッピング誘導が可能となり、DMDを対象としてエクソンスキッピング誘導治療が大規模で実施可能となった。

②スプライシングに必須のエクソン内の一塩基の同定

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が、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。

D. 考察

1) 達成度について

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RNA/ENAキメラの同定に成功する大きな成果を挙げ

た。また、スプライシング制御機序は徐々に解明されてき、エクソン内の配列がスプライシングに関与しているとの可能性が指摘されてきた。今回の研究では、欠失のホットスポット外にあるエクソンについてもエクソン内のわずかに１塩基の異常が複雑に制御されているスプライシング反応を乱し、エクソンスキッピングを誘導することを明らかにした。

この成果は予想をはるかに超えるものでスプライシング制御機序の解明に大きな足跡を残すこととなった。また、この結果はエクソンスキッピング誘導治療が広くDMD患者の治療へ応用し得ることを示したもので、今後の治療法開発に極めて大きな貢献を示すものであった。

２）研究成果の学術的意義について

私達が提唱してきた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。

３）研究成果の行政的意義について

DMD患者は12歳で歩行不能になるなど長期にわたり、社会から介護などの支援を受けなければならない。本治療法の確立により行政の施策が支援から治療へと大きく転換するもので、従来支出されてきた介護などの行政的負担が大幅に解消することが期待される。

４）その他特記すべき事項について

私達が世界に先駆けて提唱してきたエクソンスキッピング誘導治療は、現在では世界の研究者が注目するところとなり、その確立は大競争となっている。本研究成果は、日本が世界のリーダーであることを再認識させるものである。

E. 結論

DMDの治療として最も有望視されているエクソンスキッピング誘導治療について、臨床応用研究あるいは基盤整備研究において世界の最先端の成果を挙げることができた。今後、今回の成果をより一層臨床に展開することにより、DMDのより多くの患者の治療が可能となり、DMDの治療が夢から現実へと進むものと大きく期待される。

F. 健康危険情報

特記事項なし

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

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他

なし

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
松尾雅文	クレアチンキナーゼ(CK)	五十嵐　隆, 水口　雅	小児臨床検査ガイド	文光堂	東京	2006	155-157

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
Tran, V.K., Takeshima, Y., Zhang, Z., Habara, Y., Haginoya, K., Nishiyama, A., Yagi, M., and Matsuo, M.	A nonsense mutation-created intraexonic splice site is active in the lymphocytes, but not in the skeletal muscle of a DMD patient.	Hum Genet	120	737-742	2007
Beroud, C., Tuffery-Giraud, S., Matsuo, M., Hamroun, D., Humbertclaude, V., Monnier, N., Moizard, M.P., Voelckel, M.A., Calemard, L.M., Boisseau, P., et al.	Multi-exon 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.	Hum Mutat	28	196-202	2007
Tran, V.K., Takeshima, Y., Zhang, Z., M, Y., Nishiyama, A., Habara, Y., and Matsuo, M.	Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intra-exonic four-nucleotide deletion in the dystrophin gene.	J Med Genet	43	924-930	2006
Takeshima, Y., Yagi, M., Wada, H., Ishibashi, K., Nishiyama, A., Kakumoto, M., Sakaeda, T., Saura, R., Okumura, K., and Matsuo, M.	Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy.	Pediatr Res	59	690-694	2006
Katayama, Y., Tran, V.K., Hoan, N.T., Zhang, Z., Goji, K., Yagi, M., Takeshima, Y., Saiki, K., Nhan, N.T., and Matsuo, M.	Co-occurrence of mutations in both dystrophin- and androgen-receptor genes is a novel cause of female Duchenne muscular dystrophy.	Hum Genet	119	516-519	2006

Ishibashi, K., Takeshima, Y., Yagi, M., Nishiyama, A., and Matsuo, M.	Novel cryptic exons identified in introns 2 and 3 of the human dystrophin gene with duplication of exons 8-11.	Kobe J Med Sci	52	61-75	2006

学会発表

発表者氏名	演題名	学会名	発表年
榎本真宏，起塚庸，柴田暁男，光田好寛，高寺明弘，片山義規，森沢猛，横山直樹，松尾雅文	先天性筋強直性ジストロフィーの1母児例	第238回　日本小児科学会兵庫県地方会	2006
八木麻理子，西山敦史，石橋和人，和田博子，前田貢作，竹島泰弘，松尾雅文	高CK血症を呈し筋生検を施行した女性13例の検討	第19回近畿小児科学会	2006
西山敦史，親里嘉展，石橋和人，八木麻理子，竹島泰弘，和田博子，松尾雅文	ジストロフィン遺伝子ナンセンス変異例のスプライシングパターンに関する検討	第48回　日本小児神経学会総会	2006
親里嘉展，西山敦史，石橋和人，八木麻理子，竹島泰弘，松尾雅文	Duchenne型筋ジストロフィーの合併症に関与するゲノム因子のアレイCGHを用いた探索―第一報	第48回　日本小児神経学会総会	2006
羽原靖晃，堂下真梨，八木麻理子，竹島泰弘，松尾雅文	ジストロフィン遺伝子エクソン19のスプライシング：エクソンスプライシング促進配列と上流側ポリピリミジントラクトとの関係	第8回RNAミーティング	2006
Oyazato Y, Nishiyama A, Ishibashi K, Yagi M, Takeshima Y, Uemukai K, Matsuo M.	Detection and mapping of chromosome aberrations in Japanese Duchenne muscular dystrophy patients using array CGH.	11th International Congress of Human Genetics	2006
Yagi, M., Takeshima, Y., Ishibashi, K., Nishiyama, A., Tran Van, K., Tran Thi, HT., Zhang, Z., Habara, Y., and Matsuo, M.	Maturation errors of dystrophin pre-mRNAs in 26 cases with mutations in dystrophin gene.	The 10th International Congress of Inborn Errors of Metabolism (ICIEM)	2006
Habara Y, Doshita M, Yokono Y, Yagi M, Takeshima Y, Matsuo M.	Splicing analysis cassette vector system showed that either a polypyrimidinetract or an exonic splicing enhancer is essential for correct splicing of dystrophin exon 19.	The American Society of Human Genetics 56th Annual Meeting	2006

Nishiyama A, Takeshima Y, Yagi M, Tran H. T. T., Habara Y, Matsuo M.	Splicing products of dystrophin pre-mRNA in 49 cases with intraexonic small mutations in the dystrophin gene.	The American Society of Human Genetics 56th Annual Meeting	2006
Takeshima Y, Ishibashi K, Nishiyama A, Zhang Z, Tran Thi HT, Habara Y, Yagi M, Matsuo M.	Comprehensive mutation analysis of the dystrophin gene in Japanese Duchenne and Becker muscular dystrophies.	2nd Congress of Asian Society for Pediatric Research	2006
Zhang Z, Takeshima Y, Yagi M, Matsuo M.	Dystrophin expression in myogenic converted fibroblasts from Duchenne muscular dystrophy using antisense RNA/ ENA chimera.	The American Society of Human Genetics 55th Annual Meeting	2005

Hum Genet (2007) 120:737–742
DOI 10.1007/s00439-006-0241-y

SHORT REPORT

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

Van Khanh Tran · Yasuhiro Takeshima · Zhujun Zhang · Yasuaki Habara · Kazuhiro Haginoya · Atsushi Nishiyama · Mariko Yagi · Masafumi Matsuo

Received: 18 June 2006 / Accepted: 1 August 2006 / Published online: 26 September 2006

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.

V. K. Tran · Y. Takeshima · Z. Zhang · Y. Habara · A. Nishiyama · M. Yagi · M. Matsuo (✉)
Department of Pediatrics,
Kobe University Graduate School of Medicine,
7-5-1 Kusunokicho, Chuo,
Kobe 6500017, Japan
e-mail: matsuo@kobe-u.ac.jp

K. Haginoya
Department of Pediatrics,
Tohoku University School of Medicine,
Sendai 9808574, Japan

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

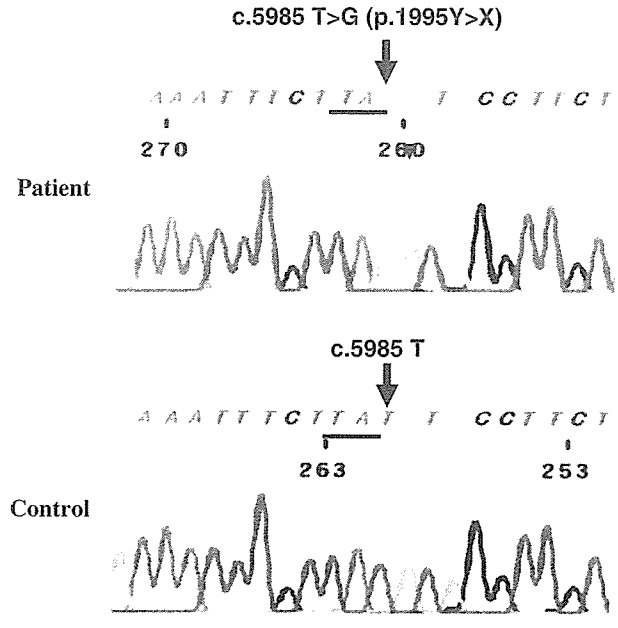

Fig. 1 Sequencing results from exon 42 are shown. The PCR products amplified from genomic DNA were directly sequenced. The 63rd nucleotide of exon 42 was a G in the index case (patient), whereas it was a T in the control sample (control). The nucleotide position corresponds to the 5,985th nucleotide of the dystrophin mRNA (c.5,985T > G). The mutation changed a tyrosine codon (TAT) to a stop codon (TAG) at the 1,995th amino acid residue of dystrophin (p.1,995Y > X)

dystrophin was consistent with the clinical diagnosis of DMD in this patient.

In order to confirm the molecular diagnosis, the gene product was examined at the mRNA level. When dystrophin mRNA extending from exons 40 to 45 was analyzed in the patient's lymphocytes by RT-nested PCR amplification, three separate products were obtained (Fig. 2a). The largest product consisted of the sequence of exons 40 to 45, and included the same nonsense mutation in exon 42 as observed in the genomic DNA. In the second largest product, 63 bp of the 5′ end of exon 42 was missing, whereas the sequences of the other exons were completely normal (dys-63). Interestingly, in the smallest product, the 3′ end of exon 41 was directly joined to the 5′ end of exon 43, which removed all 195 bp of exon 42 (dys-exon 42) (Fig. 2b). The latter two transcripts were considered natural products, because the exon boundaries were conserved and no other nucleotide changes were present in the sequenced exons. Dys-exon 42 was assumed to be a result of exon 42 skipping caused by the single nucleotide change. The exon 42 skipping observed in the lymphocytes of in the index case may have been due to the creation of a splicing silencer.

Examination of sequences near the mutation site disclosed that a novel AG dinucleotide, which is a conserved splice acceptor sequence, was introduced into the exon sequence by c.5,985T > G (Fig. 1). Therefore, the creation of the novel splice acceptor site was likely to cause the aberrant splicing that led to the production of dys-63. In order to confirm the activity of the nonsense mutation-created AG dinucleotide, experimental splicing analysis was conducted (Fig. 3). Either the wild-type or mutant exon 42 together with the flanking intron sequences were inserted into the preconstructed minigene to make hybrid minigenes and transcripts from the hybrid minigenes were analyzed by RT-PCR amplification. One PCR product containing the entire exon 42 sequence between the cassette exons A and B was obtained from the minigene encoding the wild-type exon 42 (Fig. 3b). On the other hand, two amplified products were obtained from the hybrid minigene containing exon 42 with the mutation: a major product corresponding to the normally spliced product and a minor, smaller product containing exon 42 without 63 bp of its 5′ end between exons A and B (Fig. 3c); this was the same as one of the aberrant splicing products (dys-63) identified in lymphocytes. The result indicated that the mutation-created splicing acceptor site was actually active in this hybrid minigene in HeLa cell. Therefore, dys-63 was confirmed to be a real splicing product that was transcribed from the mutated gene.

Dystrophin mRNAs obtained from lymphocytes were examined for their protein coding abilities. The authentically spliced product containing a premature stop codon in exon 42 was nonfunctional. On the other hand, dys-63 and dys-exon 42 maintained the translational reading frame and did not carry premature stop codons, and were therefore expected to produce truncated variants of dystrophin that lacked 21 and 65 amino acid residues in the rod domain, respectively. The index case, however, was diagnosed with DMD based on the lack of dystrophin in his skeletal muscle.

Considering that the dystrophin mRNA produced in muscle cells more accurately reflects the clinical phenotype than that produced in lymphocytes, muscle dystrophin mRNA from the patient was examined by RT-PCR amplification. Remarkably, the amplification of the region encompassing exons 40 to 45 produced a single PCR product (Fig. 2a). Sequencing of the product disclosed sequences of exons 40 to 45, including the nonsense mutation. It was concluded that authentic splicing was completely maintained in the skeletal muscle and no in-frame aberrant mRNA was produced in this tissue. This is compatible with the dystrophin deficiency in his muscle cells and the clinical phenotype of DMD.

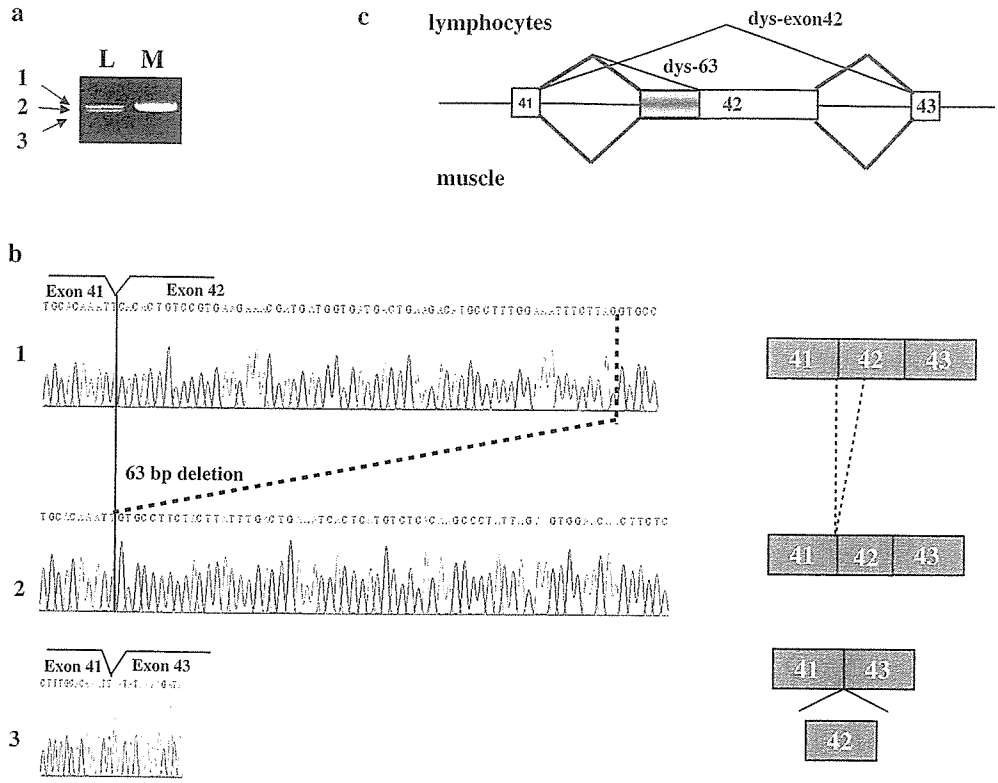

Fig. 2 Analysis of dystrophin mRNA. **a** The amplified products encompassing exons 40 to 45 are shown. Fragments encompassing exons 40 to 45 were amplified from cDNA prepared from the patient's lymphocytes and skeletal muscle. Three bands were visualized from the lymphocyte cDNA (L), whereas one clear band was visualized from the skeletal muscle (M). Numbers on the left side of the panel correspond to the numbers in panel **b** (*lower panel*). **b** The sequences of three different clones are shown. Each sequence has completely normal exons 40, 41, 43, 44, and 45. The sequence of the 3′ end of exon 41 (5′-AAATT-3′) is joined to the three different sequences in the three clones: CACAC (1), GTGCC (2), and AATAT (3). In the *top panel* (1), the normal exon structure from exon 40 to 45 is maintained, but the mutation is present. In the *middle panel* (2), 132 bp of the truncated exon 42 was followed by a completely normal exon 43. In the *bottom panel* (3), exon 41 joins directly to exon 43. The exon structure of each product is shown schematically on the right side. **c** The splicing patterns identified in the index case are represented schematically. The *diagonal lines* above and below the boxes indicate the splicing events that were observed in lymphocytes and skeletal muscle, respectively. The dys-63 and dys-exon 42 transcripts are aberrantly spliced gene products. *Boxes* and *horizontal lines* indicate exons and introns, respectively. The figure is not drawn to scale

Discussion

A novel single nucleotide change of c.5,985T > G in exon 42 of the dystrophin gene that changed a tyrosine codon to a stop codon (p.1,995Y > X) was identified in a Japanese boy diagnosed with DMD. Further molecular analysis revealed the mutation had a number of effects. In the patient's lymphocytes, the mutation caused three molecular events: (1) a premature stop codon was introduced into the authentically spliced mRNA product, (2) a mutation-created AG dinucleotide acted as a splice acceptor site, producing the aberrantly spliced dys-63 transcript, and (3) exon 42 skipping, producing the dys-exon 42 transcript (Fig. 2c). In skeletal muscle, however, only the authentically spliced product was observed. Although the patient's phenotype was expected to be mild due to the detection of in-frame dys-63 and dys-exon 42 in his lymphocytes, the patient had a typical DMD phenotype because all the dystrophin transcripts in his skeletal muscle carried the nonsense mutation.

In previous reports, the detection of aberrant splicing products in lymphocytes, which can be easily obtained, successfully led to the identification of the same transcripts in skeletal muscle (Barbieri et al. 1996; Shiga et al. 1997), thereby facilitating the molecular understanding of dystrophinopathy. Similar to previous reports (Adachi et al. 2003), however, our results showed different dystrophin mRNA splicing patterns in skeletal muscle cells and lymphocytes (Fig. 2a). This suggests that the regulators of splicing are not exactly the same in these tissues.

In vitro splicing analysis using a hybrid minigene clearly showed the nonsense mutation-created splice acceptor site was used by the spliceosome (Fig. 3). Using this hybrid minigene, a small amount of an aberrant splicing product that was produced using the novel

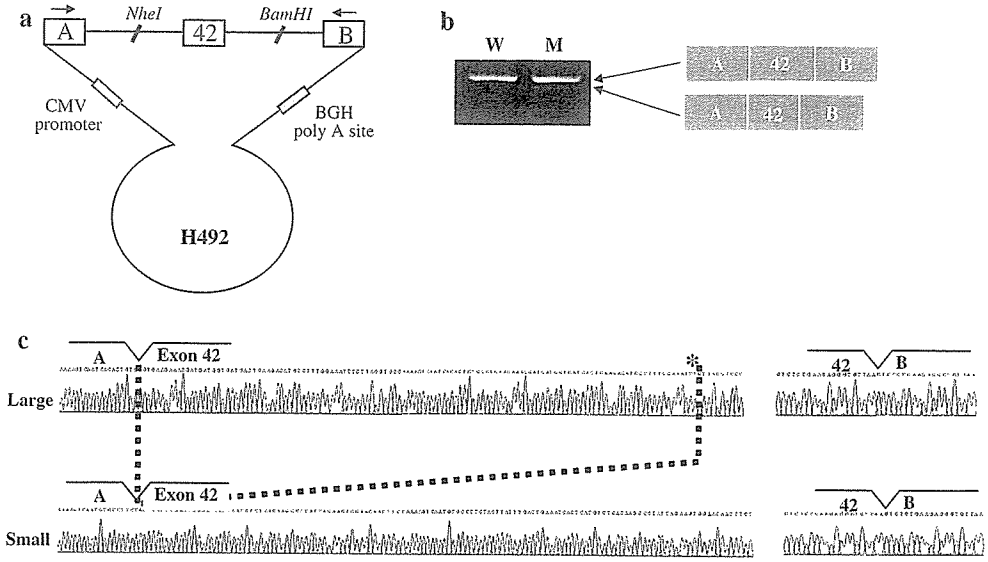

Fig. 3 Hybrid minigenes containing the indicated variants were tested in an in vitro splicing assay. **a** The hybrid minigene construct is schematically described. A minigene (H492) was constructed to encode two cassette exons (A and B) and an intervening sequence containing a multicloning site. The minigene contained a cytomegalovirus (CMV) enhancer-promoter and a bovine growth hormone gene (BGH) polyadenylation signal (*dark shaded boxes*) for complete synthesis of mRNA. The primers used in the RT-PCR assay are represented by arrows. **b** RT-PCR amplified products of hybrid minigene transcripts. A single transcript was generated from a minigene carrying the wild-type exon 42 sequence (W). From a minigene carrying the mutant exon 42, two transcripts were generated (M) and their nucleotide sequences are shown in panel **c**. A schematic description of the RT-PCR products is shown on the right. **c** Two transcripts from the mutant hybrid minigene. Nucleotide sequences at the junctions between exons are shown. The large product (*top*) consists of exon A, the complete exon 42, and exon B, whereas the small product lacked 63 bp of the 5′ end of exon 42 (*bottom*). c.5,985T > G is marked by an *asterisk*

splice acceptor site was obtained (Fig. 3). This indicates that the novel site can be recognized by the splicing machinery in HeLa cells. In contrast, the novel splice acceptor site was not used in the patient's skeletal muscle (Fig. 2). These differences in the use of the novel splice acceptor site suggest that trans-elements, such as nuclear proteins expressed in tissue-specific patterns, instead of cis-elements, such as splicing enhancer and silencer sequences, regulate the activation of the novel splice acceptor site. Future studies should clarify the trans-elements that determine whether or not the novel splice acceptor site is used.

Presently, there is no effective way to treat DMD. Recent DMD treatments have focused on converting the DMD phenotype to a BMD phenotype by changing dystrophin mRNAs from out-of-frame to in-frame. In our previous study, we showed that the induction of exon 19 skipping in a DMD patient carrying a deletion in exon 20 led to the production of in-frame dystrophin mRNA and dystrophin-positive skeletal muscle cells (Takeshima et al. 2006). Our present findings indicate that modulating the splicing of dystrophin mRNA in skeletal muscle to produce in-frame transcripts coding for truncated, semi-functional dystrophin is a potential target for treatment of this disease.

Acknowledgments We would like to thank Ms. A. Hosoda for her secretarial help. This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science; Health, and Labour Sciences Research Grants for Research on Psychiatric and Neurological Diseases and Mental Health; a research grant for Nervous and Mental Disorders from the Ministry of Health, Labour, and Welfare; and the Mitsubishi Foundation.

References

Adachi K, Takeshima Y, Wada H, Yagi M, Nakamura H, Matsuo M (2003) Heterogous dystrophin mRNAs produced by a novel splice acceptor site mutation in intermediate dystrophinopathy. Pediatr Res 53:125–131

Barbieri AM, Soriani N, Ferlini A, Michelato A, Ferrari M, Carrera P (1996) Seven novel additional small mutations and a new alternative splicing in the human dystrophin gene detected by heteroduplex analysis and restricted RT-PCR heteroduplex analysis of illegitimate transcripts. Eur J Hum Genet 4:183–187

Disset A, Bourgeois CF, Benmalek N, Claustres M, Stevenin J, Tuffery-Giraud S (2006) An exon skipping-associated nonsense mutation in the dystrophin gene uncovers a complex interplay between multiple antagonistic splicing elements. Hum Mol Genet 15:999–1013

Shiga N, Takeshima Y, Sakamoto H, Inoue K, Yokota Y, Yokoyama M, Matsuo M (1997) Disruption of the splicing enhancer sequence within exon 27 of the dystrophin gene by a non-

sense mutation induces partial skipping of the exon and is responsible for Becker muscular dystrophy. J Clin Invest 100:2204–2210

Takeshima Y, Yagi M, Wada H, Ishibashi K, Nishiyama A, Kakumoto M, Sakaeda T, Saura R, Okumura K, Matsuo M (2006) Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. Pediatr Res 59:690–694

Thi Tran HT, Takeshima Y, Surono A, Yagi M, Wada H, Matsuo M (2005) A G-to-A transition at the fifth position of intron 32 of the dystrophin gene inactivates a splice donor site both in vivo and in vitro. Mol Genet Metab 85:213–219

Tran VK, Takeshima Y, Zhang Z, Yagi M, Nishiyama A, Habara Y, Matsuo M (2006) Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intra-exonic four-nucleotide deletion in the dystrophin gene. J Med Genet (in press)

HUMAN MUTATION 28(2), 196–202, 2007

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

Christophe Béroud,[1]* Sylvie Tuffery-Giraud,[1] Masafumi Matsuo,[2] Dalil Hamroun,[1] Véronique Humbertclaude,[1] Nicole Monnier,[3] Marie-Pierre Moizard,[4] Marie-Antoinette Voelckel,[5] Laurence Michel Calemard,[6] Pierre Boisseau,[7] Martine Blayau,[8] Christophe Philippe,[9] Mireille Cossée,[10] Michel Pagès,[11] François Rivier,[12] Olivier Danos,[13] Luis Garcia,[13] and Mireille Claustres[1]

[1]*Laboratoire de Génétique Moléculaire, Institut Universitaire de Recherche Clinique (IURC), Unité de Formation et de Recherche (UFR) Médecine Site Nord Unité Pédagogique Médicale (UPM)/IURC and Centre national de la recherche scientifique (CNRS) UPR 1142, Montpellier, France;* [2]*Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan;* [3]*Laboratoire de Biochimie Génétique et Moleculaire, Centre Hospitalo-Universitaire (CHU) de Grenoble and Institut National de la Santé et de la Recherche Médicale (INSERM) U607, Grenoble, France;* [4]*INSERM U619, CHU Bretonneau, Tours, France;* [5]*Laboratoire de Génétique Moléculaire, Département de Génétique Médicale, Hôpital d'Enfants de la Timone, Marseille, France;* [6]*Laboratoire de Biochimie, Hôpital Debrousse, Lyon, France;* [7]*INSERM U533, Université de Nantes, Nantes, France;* [8]*Laboratoire de génétique Moléculaire, Rennes, France;* [9]*Laboratoire de génétique, Equipe Associée (EA) 3441, CHU Brabois, Vandoeuvre-les-Nancy, France;* [10]*Laboratoire de diagnostic génétique, Hôpitaux Universitaires de Strasbourg et Faculté de Médecine, Strasbourg, France;* [11]*Département de Neurologie, CHU de Montpellier, Montpellier, France;* [12]*Service de Neuropédiatrie, CHU de Montpellier, Montpellier, France;* [13]*Genethon and Centre national de la recherche scientifique (CNRS) and Unité Mixtede Recherche (UMR) 8115, Evry, France*

Communicated by Haig H. Kazazian

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. © 2006 Wiley-Liss, Inc.

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

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat.

Received 13 April 2006; accepted revised manuscript 21 August 2006.

*Correspondence to: Christophe Béroud, Laboratoire de Génétique Moléculaire, IURC, 641 avenue du doyen G. Giraud, 34093 Montpellier, France. E-mail: christophe.beroud@igh.cnrs.fr

Grant sponsor: Association Française contre les Myopathies (AFM).
DOI 10.1002/humu.20428
Published online 13 October 2006 in Wiley InterScience (www.interscience.wiley.com).

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.

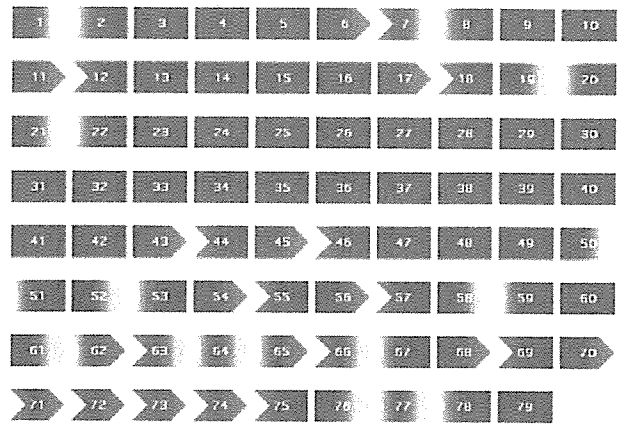

FIGURE 1. **Exon phasing of the DMD gene. Each exon is presented by a gray box. Each extremity of the box represents the specific phasing of the exon. Left end of exons: 1) dark gray vertical lane means that the exon begins by the first nucleotide of a codon; 2) light gray curve means that the exon begins 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.

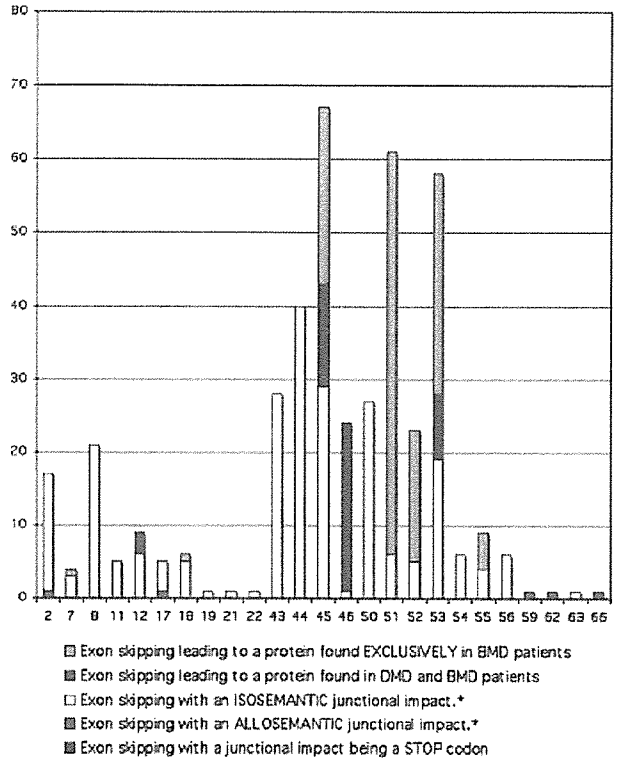

FIGURE 2. **Monoskipping of the DMD gene. For each exon is presented the number of patients from the UMD-DMD database that can be rescued by the corresponding monoskipping. Each deletion has been associated with one of the five groups. *Such protein has never been reported in patients.**

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

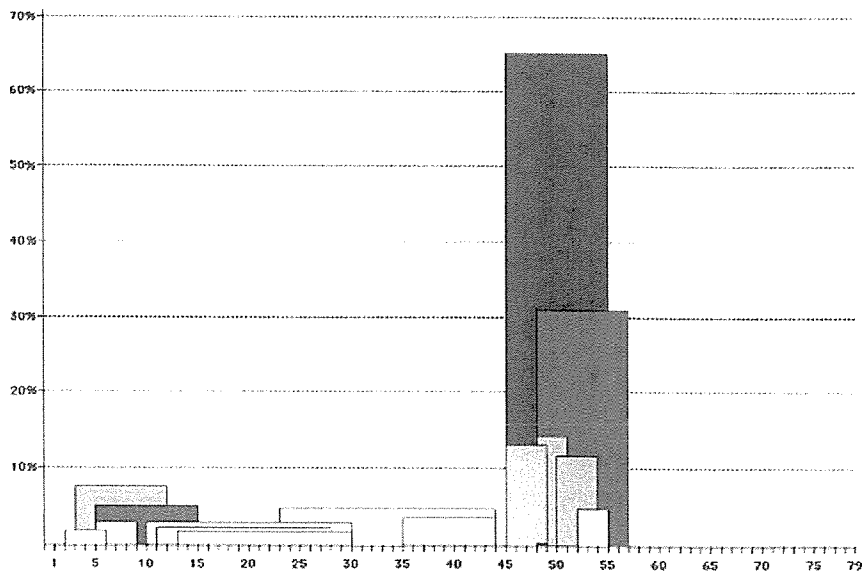

FIGURE 3. **Multiskipping of the DMD gene. X-axis: exons of the DMD gene. Y-axis: number of DMD patients rescuable by multiexon skipping. Each colored rectangle corresponds to the deletion found in BMD patients. Note the pink del45-55 deletion that can rescue 161 DMD patients with an out-of-frame deletion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