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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に関する研究

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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に関する研究

Ⅰ総括研究報告

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（研究要旨）

デュシェンヌ型筋ジストロフィー(DMD)の治療法としてジストロフィン遺伝子のエクソンのスキッピングをスプライシング時に誘導する方法が注目されている。これは、DMD患者にみられるジストロフィンmRNAのアミノ酸読み取り枠のずれをエクソンのスキッピングを誘導することにより修正し、ジストロフィンを発現させるものである。私達はアンチセンスオリゴヌクレオチドを用いて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することに世界で初めて成功するなど、本治療法開発において世界をリードしてきた。最近、新しく開発された修飾核酸(ENA)からなるアンチセンスオリゴヌクレオチドのRNA/ENAキメラが、従来のアンチセンスオリゴヌクレオチドに比較して飛躍的に高いエクソンスキッピング誘導能を有することを示す結果を世界で初めて得た。このためRNA/ENAキメラは、DMDの患者の治療に最適な核酸として大きく注目されている。

本研究は、RNA/ENAキメラの実用化を世界に先駆けて図るもので以下の研究を行うものである。①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キメラを同定する。そのため、培養筋細胞に様々な合成RNA/ENAキメラを導入し、そのエクソンスキッピング誘導能を解析する。②同定した最適のRNA/ENAキメラをDMD患者培養筋細胞に導入し、導入細胞におけるジストロフィン発現を免疫組織化学的方法などを用いて検討する。初年度においては①の研究を重点的に実施し、研究２，３年目には②に比重をおいた研究を実施する。そして、各DMD患者がそれぞれに有するエクソン欠失の型に対応した治療用のRNA/ENAキメラを決定する。

本研究は、RNA/ENAキメラを用いたエクソンのスキッピング誘導により、DMD患者培養筋細胞でジストロフィンの発現を世界で初めて実証するもので、DMD治療の確立を大きく促進するものである。本研究内容全般については、すでに本学部の医学倫理委員会にて承認を得ているものである。

Ⅱ分担研究報告

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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

本研究では、デュシェンヌ型筋ジストロフィー(DMD)患者が有する遺伝子欠失に対応したエクソンのスキッピングを誘導するのに最適な RNA/ENA キメラの探索を培養筋細胞を用いた系を用いてまず行う。ついで、RNA/ENA キメラを患者筋細胞に導入しジストロフィン mRNA が修正され、ジストロフィンが発現されることを検証する。

B. 研究方法

1) 培養筋細胞を用いた最適なRNA/ENAキメラの探索

ジストロフィン遺伝子の欠失のホットスポット領域にあるエクソンを標的として、そのエクソンのスキッピングを誘導する能力を有するRNA/ENAキメラを探索する。そのために、エクソンの配列と相補的な配列からなる候補RNA/ENAキメラを多数合成する。合成したRNA/ENAキメラを培養筋細胞に導入し、導入した培養細胞からRNAを抽出し、RT-PCR法を用いてジストロフィンmRNAを解析する。そして、標的とするエクソンのスキッピングを最も強く誘導するRNA/ENAキメラを同定する。この作業を繰り返し実施し、各エクソンについてそれぞれに最も有効なRNA/ENAキメラを同定する。

2) 患者由来培養筋細胞でのジストロフィン発現

先に明らかにした最適のRNA/ENAキメラの患者筋細胞でのジストロフィン発現誘導能を検証する。それぞれのDMD患者の遺伝子欠失に対応したエクソンのスキッピングを誘導するRNA/ENAキメラを、患者培養筋細胞に導入する。導入した筋細胞で標的としたエクソンがスキッピングしたジストロフィンmRNAの産生をRT-PCR法で確認する。ついで、ジストロフィンmRNAでのエクソンのスキッピングを確認したのち蛍光免疫染色法を用いて培養細胞レベルでジストロフィンの発現を確認するとともに、ウエスタンブロットでもジストロフィンの発現を確認する。

C. 研究成果

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RNA/ENAキメラの同定を重点的に実施した。エクソン45の配列に相補的な配列からなる18塩基のRNA/ENAキメラを各種合成した。そして、それぞれのRNA/ENAキメラを培養筋細胞に導入し、導入後48時間にジストロフィンmRNAを解析した。RT-PCRでジス

トロフィン遺伝子のエクソン45をはさむ領域を増幅した。増幅したPCR産物を電気泳動で分離し、エクソン45のスキップした産物の検出を行った。多くのRNA/ENAキメラが程度の差はあれ、エクソン45スキッピングを誘導した。しかし、最高能力を発揮するものではすべてのmRNAからエクソン45の配列が消失していた。そこで、このRNA/ENAキメラを治療に応用できる様に検討した。

ジストロフィン遺伝子のエクソン45のスキッピングを誘導することにより治療し得る欠失のタイプは様々である。その中でジストロフィン遺伝子のエクソン44を欠失したDMD、46から47を欠失したDMD例で本治療による効果を検討した。その結果、両例ともにジストロフィンmRNAからエクソン45のスキッピングを誘導することが100%のmRNAで生じさせることに成功した。これは、ジストロフィンmRNAがインフレームに転換されたことを示すものでジストロフィンの産生能が得られることを示唆した。そこで、培養筋細胞のジストロフィン染色を行うとジストロフィン陽性細胞の検出が出来た。このことは、本RNA/ENAが患者の治療に応用し得ることを示した。

D. 考察

本年度の研究においては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治療に用いられ得るRNA/ENAキメラの同定に成功した。したがって、今後本研究を推進することにより、より一層多くの症例で治療し得るRNA/ENAキメラの同定が可能になると考えられた。

本研究の成果は当初の予想通りに得られた。したがって、本研究課題は今後も当初の計画通りの成果が得られるものと考えられる。

E. 結論

本年度においてはジストロフィン遺伝子の欠失のホットスポット領域に存在するエクソンス45のスキッピング誘導能について検討した。その結果、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同定に成功するとともに、DMD患者由来筋細胞でのジストロフィンの発現を誘導することに成功した。このように、本研究は順調に成果を挙げてきており今後継続して実施することにより、より一層大きな成果が挙げられるものと強く期待される。

F. 健康危険情報

特記事項なし

G. 研究発表

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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

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Becker Muscular Dystrophy

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INTRODUCTION

Becker muscular dystrophy (BMD) is an X-linked recessive inherited disease with a worldwide incidence of 1 in 35,000 male births. Becker muscular dystrophy is characterized by progressive muscle wasting but is distinguished by delayed onset, later dependence on wheelchair support, and longer life span from Duchenne muscular dystrophy (DMD) that follows severe progressive muscle wasting.

Dystrophin, the gene defective in not only BMD but also DMD, was isolated in 1986. Since then, genetic diagnosis of BMD has been done leading to better understanding of the disease process, and the difference between DMD and BMD can be explained at the molecular level by the reading frame rule.

CLINICAL DIAGNOSIS

Becker muscular dystrophy patients show normal growth and development in their early childhood. In BMD, affected men start to show disturbance of walking due to muscle weakness at 20s or over. He maintains to walk but his muscle strength gradually decreases. Dilated cardiomyopathy is sometimes an initial clinical sign for the diagnosis of BMD. The muscle weakness involved in BMD follows a mild downward course with patients living near normal lives.[1]

Serum creatine kinase (CK) is markedly increased. This marked elevation of serum CK is the most important hallmark for the diagnosis of BMD, but the level of elevation of serum CK is not so high compared to DMD. During the asymptomatic period, elevation of serum CK is the sole sign for BMD. Some BMD patients are identified accidentally because of elevations of AST or ALT which are commonly examined for liver function as serum CK elevation is accompanied with elevations of AST and ALT.

Becker muscular dystrophy should be included in the differential diagnosis of moderately elevated serum CK in males. Pathological examination of biopsied muscle consolidates the diagnosis of BMD.

GENE DIAGNOSIS

Becker muscular dystrophy is caused by mutations of the dystrophin gene that is also mutated in DMD.[2] Therefore, both DMD and BMD are sometimes called as dystrophinopathy. Furthermore, not only types but also locations of mutations identified in both BMD and DMD are quite similar.[3] For gene diagnosis of BMD both multiplex PCR and Southern blot analysis have been employed as in the genetic diagnosis of DMD.[4–6] Nearly two-thirds of mutations identified on the dystrophin gene are deletions or duplications occupying a single or multiple exons. Every mutation identified in BMD cases would be examined based on the following reading frame rule.[3] In the rest of the BMD cases it is rather difficult to identify the responsible mutations on the dystrophin gene as a single nucleotide change is supposed to be present. However, some point mutations that induced exon skipping have been reported.[7,8]

READING-FRAME RULE OF DMD/BMD

Although both DMD and BMD patients have been shown to have deletion or duplication mutations of the dystrophin gene, the extent of the deletion does not always correlate with the severity of the disease: some BMD patients with mild symptoms have deletions encompassing numerous exons, whereas some DMD patients with severe symptoms lack only a few exons. In some cases, the long deletions resulting in BMD and the short deletions resulting in DMD may even overlap. The reading-frame rule explains the difference between DMD and BMD as follows: in DMD the translational reading frame of the dystrophin mRNA is shifted after a deletion or duplication mutation whereas it is maintained in BMD.[3] According to the reading-frame rule, BMD patients with long deletions are able to produce dystrophin mRNA that would still direct the production of an internally truncated semifunctional protein. Shorter deletions harbored by severe DMD patients, on the other hand, would bring together exons that, when spliced, would change the translational reading frame in the mRNA, such that a

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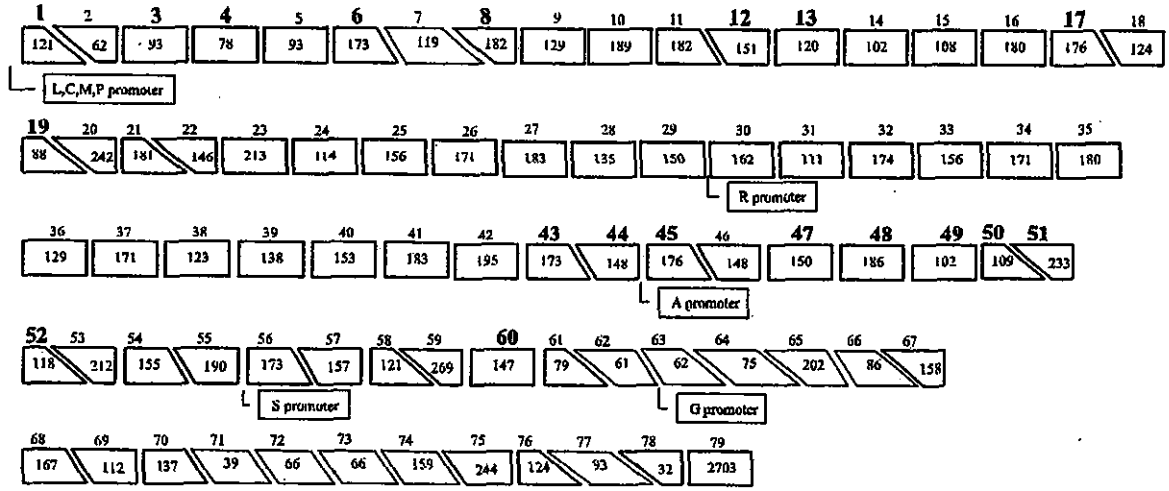

Fig. 1 Schematic description of the exon structure of the dystrophin gene. The dystrophin gene consisting of 79 exons (boxes) and at least eight alternative promoters (boxes under the lined boxes) is schematically described. Numbers over the box indicate exon number, the bold numbers being the exons that are examined by multiplex PCR. Quadrilaterals and parallelograms indicate in-frame exons (type 0 exons). Trapezoids indicate out-of-frame exons (type 1 or type 2 exons).

premature stop codon is created. This rule predicts that milder BMD patients would produce a smaller semifunctional protein whereas DMD patients would either produce a severely truncated dystrophin lacking the entire C-terminal region or would not produce dystrophin at all.

Subsequent gene analyses have shown that over 90% of the deletion–duplication mutations that cause BMD maintain the dystrophin mRNA reading frame whereas those causing DMD are frameshifts.[9] Accordingly, point mutations identified in DMD are nonsense mutations[10] except in rare DMD cases with missense mutations.[11,12]

Considering that molecular therapy for DMD to change the reading frame from out-of-frame to in-frame has been proposed,[13,14] it is important to see the resulting translational reading frame of dystrophin mRNA after the identification of a deletion or duplication mutation. Exons of the dystrophin gene are classified into three types according to the number of nucleotides encoded in the exon (Fig. 1): 1) in-frame exon that encodes nucleotides of multiples of 3 (type 0 exon); 2) two out-of-frame exons that have nucleotides of multiples of 3+1 or 2 (type 1 exon or type 2 exon, respectively). Among the 79 exons, 40, 18, and 21 exons are classified into types 0, 1, and 2 exons, respectively. In cases with deletion/duplication of the dystrophin gene the reading frame can be determined as described in Fig. 1. Cases having a deletion of a type 2 exon, e.g., exon 45, should be DMD based on the reading frame rule. Although gene diagnosis of DMD/BMD has been conducted, not all DMD/BMD cases have been examined for its reading frame.

In other types of mutations, nonsense mutations are expected to be identified in DMD. However, nonsense mutation that should result in DMD phenotype has been identified in BMD cases,[8,15] where exon skipping is shown as a mechanism that modified clinical phenotype. Furthermore, BMD has been shown to have a nonsense mutation in in-frame exons.[16–18] Detailed analysis of genotype–phenotype correlation would lead a better understanding of molecular mechanism of dystrophinopathy.

PATHOLOGICAL DIAGNOSIS

The pathological examination of biopsied skeletal muscle confirms the diagnosis of BMD. Immunohistochemical analyses of normal muscle demonstrate that dystrophin is present along with muscle cell membranes. Muscle from BMD patients contains reduced amounts of dystrophin that is stained discontinuously and patchy along the muscle cell membranes.[19] Western blot analysis using dystrophin antibody reveals a band corresponding to 427 kDa, close to the predicted size of dystrophin, in extracts of normal muscle tissue. Shorter or lower amount of dystrophin is detected in muscle extracts from patients with BMD.

Dystrophin contains 3685 amino acids organized in four domains: N-terminal actin binding, triple helical rod, cystein-rich, and C-terminal domains. The internally truncated dystrophin identified in BMD maintains both N-terminal and C-terminal domains, but lacks some of the 24 repeat sequences of triple helical rod domain. Therefore, dystrophin is stained when antibody recognizing either N-terminal or C-terminal domains is used, but in

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some cases no dystrophin is stained as in DMD when antibody recognizing rod domain is employed.[20]

TREATMENT

For BMD patients, supportive therapies such as rehabilitation or ventilator support are clinically employed, but no effective way to improve the clinical course is available. Gene therapy has been considered a cure for BMD but no clinically applicable way has been established.

CONCLUSION

Becker muscular dystrophy is a mild muscle wasting disease and characterized by dystrophin abnormality in skeletal muscle. Currently, no effective treatment is available although a molecular understanding of BMD developed well.

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Duchenne Muscular Dystrophy

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a common inherited disease with a worldwide incidence of 1 in 3500 male births. DMD is a lethal disorder of childhood characterized by progressive muscle wasting. Affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in their mid to late 20s.

Dystrophin, the gene defective in DMD was isolated in 1986. Since then, genetic diagnosis of DMD has been done leading to better understanding of the disease process. Based on the molecular pathogenesis of DMD, molecular therapies for DMD have been proposed.

CLINICAL DIAGNOSIS

Patients with DMD show normal growth and development in their early childhood. In DMD, affected boys start to show disturbance of walking and frequently fall because of muscle weakness at 4 to 5 years of age. Patients are shown to have a positive Gower's sign wherein the child climbs up his thighs to extend the hips and push his trunk up. He manages to walk but his muscle strength gradually decreases. He loses the ability to climb up stairs. Lumbar lordosis becomes more exaggerated and the waddling gait increases. Patients usually are wheelchair-bound by the age of 12. Muscle wasting progresses as the patients get older until finally respiratory or cardiac failure develops due to muscle wasting.

Serum creatine kinase (CK) is markedly increased 50 times more than the normal range in infantile DMD. This marked elevation of serum CK is the most important hallmark for the diagnosis of DMD. During the asymptomatic period, elevation of serum CK is the sole sign for DMD. Some DMD patients are accidentally identified due to elevations of AST or ALT, which are commonly examined for liver function, because serum CK elevation is accompanied with elevations of AST and ALT.

GENE DIAGNOSIS

Duchenne muscular dystrophy is caused by mutations of the dystrophin gene.[1] The dystrophin gene is 3000 kb in size and consists of 79 exons encoding a 14-kb mRNA.[2,3] At least eight alternative promoters that are regulated in a tissue- or development-specific manner have been identified on the dystrophin gene. The unusually high incidence of DMD in all human populations could be simply a reflection of the enormous mutation target size of the gene, but the recombination rate is reported to be four times the rate expected for a gene of this size.[4] Nearly two-thirds of mutations identified on the dystrophin gene are deletions or duplications occupying a single or multiple exons, with the rest of the DMD cases having other types of mutations including point mutations. Remarkably, deletion mutations have been localized to two deletion hot spots of the dystrophin gene, the 5′ and the central regions.

Multiplex PCR Analysis

Currently, multiplex PCR analyses that amplify deletion-prone exons are used as the first step for gene diagnosis. Two sets of PCR amplification are used to screen 19 deletion-prone exons (exons 1, 3, 4, 6, 8, 12, 13, 17, 19, 43–45, 47–52, and 60).[5,6] Using this method of examination, one finds that nearly half of the DMD cases are shown to have deletion mutations. Therefore, the rest of the cases need further examination to identify the responsible mutation in the dystrophin gene.

Southern Blot Analysis

To examine the deletion/duplication in every exon of the dystrophin gene, Southern blot analysis is used, using segments of the dystrophin cDNA as probes.[7] Two-thirds of DMD patients are shown to have recombination events of deletions or duplications spread in one or more exons at the genomic DNA level.[8] However, Southern blot analysis not only needs high-quality DNA and radioisotope, but it is also time-consuming.

Detection of Fine Mutation

In DMD cases that have no large recombination event, identification of the causative mutation remains a laborious goal because of the difficulty in detecting a single point mutation in the 3000-kb-sized gene. To

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facilitate the identification of mutations in the dystrophin gene, more than 99% of which is made up of introns, dystrophin mRNA that is 100 times smaller than the dystrophin gene has been analyzed.[9] Analysis of dystrophin mRNA expressed in lymphocytes leads to not only identification of rare genomic mutations, but also to disclosures of nonauthentic alternative splicing.[10–12] In addition, several ways to identify small mutations have been proposed.[13–17] In the advent of recent advances in mutation analysis techniques, more than 90% of DMD cases are shown to have mutations in the dystrophin gene.[18,19]

PATHOLOGICAL DIAGNOSIS

The pathological examination of biopsied skeletal muscle confirms the diagnosis of DMD. Immunohistochemical analyses of normal muscle demonstrate that dystrophin is present along with muscle cell membranes. In DMD, dystrophin is missing from skeletal muscle.[20] Western blot analyses using dystrophin antibody reveals a band corresponding to 427 kDa, close to the predicted size of dystrophin, in extracts of normal muscle tissue, whereas no protein can be detected in DMD.

TREATMENT

For DMD patients, supportive therapies such as rehabilitation or ventilator support are clinically employed but no effective way to improve the clinical course is available. Since the discovery of the dystrophin gene, gene therapy is now considered an attractive way to cure the disease. The main aim of DMD gene therapy is to establish a way to inject constructed dystrophin genes consisting of partial- or full-length cDNA joined to an appropriate promoter. Although much progress has been made in this field of study, we still seem to be a long way from achieving a clinically significant result. As an alternative for gene transfection, molecular therapies have been studied including antisense oligonucleotide treatment[21–23] or translational readthrough treatment using gentamicin.

Antisense Oligonucleotide Treatment

An alternative strategy for DMD treatment is to retard the progression of the clinical symptoms, i.e., to convert DMD into the BMD phenotype. Theoretically, this therapy can be done by changing a frame-shift mutation causing DMD into an in-frame mutation characteristic of BMD by modifying the dystrophin mRNA. Artificial induction of exon skipping with antisense oligonucleotides is a way to make the out-of-frame dystrophin mRNA in-frame. Artificial induction of exon 19 skipping using an antisense oligonucleotides against the splicing enhancer sequence has been reported,[24] and this treatment was shown to produce dystrophin expression in exon-20-deleted DMD myocytes.[23] Disruption of the splicing enhancer sequence to induce exon skipping was further evidenced by the fact that in the nonsense mutation of exon 27 the dystrophin gene resulted in exon 27 skipping, producing an in-frame dystrophin mRNA.[25] In addition, another natural example causing conversion of DMD to BMD was identified in a nonsense mutation in exons 25 and 29.[26,27] Furthermore, BMD has been shown to have a nonsense mutation in in-frame exons.[28,29]

Antisense oligonucleotides against a purine-rich sequence have been used to induce skipping of exons 44, 45, 46, 49, 50, 51, or 53.[22,30,31] In these studies, induction of exon skipping led to the expression of dystrophin in their respective dystrophin-deficient myocytes by correcting the translational reading frame. Recently, double exon skipping of exon 43 and 44 or exon 45 and 51 has been induced.[32] This extends the application of the antisense oligonucleotide treatment to more varieties of deletion mutations of the dystrophin gene.

Phosphorothioate DNA has been the standard choice for the clinical application of antisense technology.[33–37] However, phosphorothioate DNA is associated with a variety of potentially toxic non-antisense effects.[38] In order to develop less toxic antisense oligonucleotides, nucleic acids have been modified in various ways.[39,40] Recently, morpholino modified oligonucleotides were shown to be delivered to muscle cells efficiently.[41] Furthermore, the chimera of 2′-*O*-methyl RNA and 2′-*O*, 4′-*C*-ethylene-bridged nucleic acid (ENA) was shown to induce exon 19 skipping of the dystrophin gene 40 times stronger than the conventional phosphorothioate oligonucleotides.[42]

Translational Readthrough of Stop Codon

Aminoglycoside antibiotics have been suggested as possible therapeutic interventions for treating patients who carry a nonsense mutation because of the ability of these antibiotics to lead translational readthrough of stop codons. To evaluate whether aminoglycosides can be used to suppress the nonsense mutation in a human DMD case, four DMD/BMD cases with various stop codon sequences were tested once daily with intravenous gentamicin at 7.5 mg/kg/day for 2 weeks. However, the full-length dystrophin protein was not detected in posttreatment muscle biopsies.[43] The possible reason for the failure of gentamicin treatment in human cases is the difference in efficiency of aminoglycoside-induced readthrough among the different types of nonsense mutations.

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CONCLUSION

Duchenne muscular dystrophy is a fatal disease without any effective treatment. Recent studies opened a door to the establishment of molecular therapy for DMD.

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C-Terminal Truncated Dystrophin Identified in Skeletal Muscle of an Asymptomatic Boy with a Novel Nonsense Mutation of the Dystrophin Gene

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ABSTRACT

Mutations that cause premature stop codons in the dystrophin gene lead to a complete loss of dystrophin from skeletal muscle, resulting in severe Duchenne muscular dystrophy. Here, a C-terminally truncated dystrophin resulting from a novel nonsense mutation is shown for the first time to be localized to the muscle plasma membrane. An asymptomatic 8-y-old boy was examined for dystrophin in skeletal muscle because of high serum creatine kinase activity. Remarkably, no dystrophin labeling was seen with an MAb against the C-terminal domain, suggesting the presence of an early stop codon in the dystrophin gene. Labeling with an antibody specific to the N-terminal domain, however, revealed weak, patchy, and discontinuous staining, suggesting limited production of a truncated form of the protein. Molecular analysis revealed a novel nonsense mutation (Q3625X) as a result of a single nucleotide change in the patient's genomic DNA (C10873T), leaving 1.6% of dystrophin gene product unsynthesized at the C terminus. Dystrophin mRNA analysis did not show rescue of the nonsense mutation as a result of exon-skipping by an alternative splicing mechanism. This is the first report of an asymptomatic dystrophinopathy with a nonsense mutation in the dystrophin gene. (***Pediatr Res* 56: 739–743, 2004**)

Abbreviations
BMD, Becker muscular dystrophy
CK, creatine kinase
DMD, Duchenne muscular dystrophy

The severe Duchenne muscular dystrophy (DMD) and the more benign Becker muscular dystrophy (BMD) are allelic conditions characterized by progressive muscular degeneration and wasting accompanied by an elevation of serum creatine kinase (CK). DMD is a rapidly progressive disease, with those affected starting to show muscle weakness at ~4–5 y of age and losing the ability to walk independently before the age of 12 y. BMD has a slower rate of progression; affected individuals remain ambulatory beyond the age of 16 y, and a few may lead near-normal lives (1).

DMD and BMD are caused by mutation of the dystrophin gene, which encodes a 14-kb mRNA that consists of 79 exons. The gene is the largest in humans and covers >3000 kb on the X chromosome (2,3). DMD and BMD are the most common genetic muscle diseases, affecting >1 in 3,500 male births. Two thirds of DMD/BMD patients have deletion or duplication mutations of the dystrophin gene, and their clinical progression can be predicted by whether the deletion or duplication maintains (in-frame) or disrupts (out-of-frame) the translational reading frame (the reading-frame rule) (4). Dystrophin is absent from skeletal muscle of DMD, because the dystrophin that is produced is truncated as a result of the premature stop codon and therefore is unstable, whereas in BMD, dystrophin that contains internal in-frame deletions produces protein that can be detected (5).

Single-base nonsense mutations have been suspected in DMD patients who do not show deletion/duplication mutations. However, detection of such defects in individual DMD patients is very difficult as a result of the large size of the gene. More than 100 nonsense mutations have been reported at various points over a 14-kb length of the dystrophin mRNA (http://www.dmd.nl). Despite this wide variation in coding potential (0–98.6% of the full-length protein), these truncating mutations are associated with a surprisingly uniform severity of the DMD phenotype (6). However, a limited number of single-base nonsense mutations have been reported in patients with mild BMD that showed skipping of the exon encoding the mutation, thus producing an in-frame mRNA (7–11).

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Dystrophin is a cytoskeletal protein that is implicated in membrane stability and in communication between the extracellular matrix and the inner cytoskeleton (12,13). The protein, which consists of 3685 amino acids, is divided into four distinct domains: an N-terminal domain, a large rod-like domain of 24 spectrin-like repeats that occupies >70% of its length, a cysteine-rich domain, and, finally, a C-terminal domain (2,14). Studies conducted on DMD/BMD patients suggest that the N-terminal, cysteine-rich, and C-terminal domains are essential for dystrophin's function (15,16). Notably, the C-terminal domain, which consists of 416 amino acids encoded by 13 exons, shows sequence similarity with only two other dystrophin-related proteins and is considered to exert dystrophin's specific function (14,17,18). In fact, in-frame deletions that extend into the C-terminal domain have been reported to result in DMD, whereas large in-frame deletions of the rod domain result in BMD (16).

Here we report a C-terminally truncated dystrophin caused by a mutation in an asymptomatic boy with high CK activity. We propose that nonsense mutations of the dystrophin gene can result in a wide variety of clinical phenotypes.

METHODS

Case. The proband (KUDN 02765682) was an 8-y-old boy. His family history disclosed no neuromuscular disease. He started to walk independently at 1 y of age, and his motor development was normal. He had a history of transient muscle weakness. At the age of 3 y, he complained of pain in the lower legs without any predisposing signs or symptoms and lost the ability to stand up and walk by himself. His serum CK was found to be 4901 IU/L (normal <169 IU/L). The muscle weakness persisted for 1 wk but disappeared spontaneously and completely.

During the following period, his serum CK remained elevated but showed a strong fluctuation in value, ranging from 1,607 to 21,100 IU/L. Despite his high CK, he did not show any muscle weakness. At the age of 5 y, he was referred to Kobe University Hospital for examination of his elevated serum CK activity. His mental development was normal. On physical examination, there was no Gower's sign, walking abnormality, or pseudohypertrophy of the legs. An electromyogram disclosed myogenic changes. A chest x-ray, electrocardiography, and echocardiography failed to reveal cardiac abnormalities. To clarify the cause of the elevation in serum CK and myogenic pattern in electromyogram, a quadriceps muscle biopsy was carried out after obtaining informed consent. The protocols of this study were approved by our ethical committee.

Immunohistochemical analysis. The muscle biopsy sample was examined pathologically and immunohistochemically. An indirect immunofluorescence analysis was performed using three dystrophin antibodies that recognize the N-terminal (NCL-Dys3), the rod (NCL-Dys1), and the C-terminal (NCL-Dys2) domains of dystrophin (Novocastra Laboratories, Newcastle upon Tyne, UK) (5,19). Furthermore, utrophin, β-dystroglycan, γ-sarcoglycan (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), laminin α2 (Chemicon International Inc., Temecula, CA), and α-dystroglycan (Upstate Biotechnology, Lake Placid, NY) were also stained using their respective antibodies. Control skeletal muscle tissue was obtained with informed consent and was simultaneously stained with the same panel of antibodies. Western blot analysis of dystrophin using an MAb that recognizes the C-terminal domain was performed by Athena Diagnostics (Worcester, MA).

Analysis of the dystrophin gene. For mutational analysis of the dystrophin gene, blood samples were obtained from the index case and family members after obtaining informed consent. DNA was isolated by standard phenol-chloroform extraction methods. For screening for deletion mutations, 19 deletion-prone exons were amplified from the genomic DNA by PCR essentially according to methods described previously (20). Southern blot analysis using dystrophin cDNA as a probe was performed with *Hin*dIII restriction enzyme–digested DNA as a template, as described by Koenig *et al.* (21). For analyzing genomic mutations, the region that encompasses exon 76 was amplified by PCR with g76F:5'-GGAGGGCTTCTAAAGTAGG-3' as the forward primer and g76r:5'-ATGTCCCTGTAATACGACTCTACC-3' as the reverse primer under conditions described elsewhere (20).

Analysis of dystrophin mRNA. Reverse-transcription PCR (RT-PCR) was used to analyze the dystrophin mRNA expressed in lymphocytes or skeletal muscle as described by Roberts *et al.* (22,23). Full-length dystrophin cDNA was amplified as 10 separate, partially overlapping fragments and sequenced directly. For obtaining a fragment showing aberrant splicing, including exon 76 skipping, a region that encompasses exons 70–79 was amplified using a forward primer corresponding to a segment of exon 70 (70f:5'-CAGGAGAAGATGTTCGAGAC-3') and a reverse primer complementary to a segment of exon 79 (5f:5'-ATCATCTGCCATGTGGAAAAG-3').

Sequencing of the amplified product. The amplified product was purified and subjected to sequencing either directly or after subcloning into a pT7 blue T vector (Novagen, Madison, WI) (24). The DNA sequence was determined using an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA).

RESULTS

For elucidating the cause of the elevation in serum CK, the biopsied muscle sample was examined pathologically. Microscopic examination disclosed slight dystrophic changes such as size variation in muscle fibers, fibers with central nuclei, and degenerated and regenerated fibers. Immunofluorescence staining for dystrophin revealed a complete absence of C-terminal domain labeling (Fig. 1). In contrast, both N-terminal and rod-domain staining was weak, patchy, and discontinuous along the plasma membrane (Fig. 1). These findings clearly indicated dystrophinopathy, but the patterns of dystrophin staining were not typical for either DMD or BMD. Western blot analysis of dystrophin using an antibody that recognizes the C-terminal domain of dystrophin revealed no significant bands (data not shown). These staining patterns indicate that a nonsense mutation in dystrophin is present in this patient, leading to production of a protein truncated somewhere up-

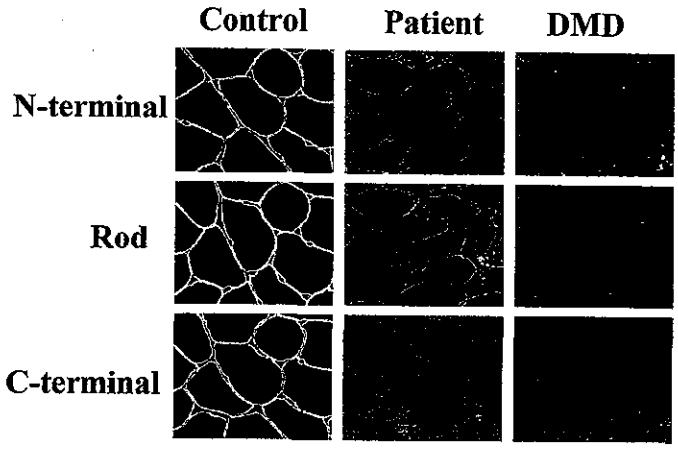

Figure 1. Immunofluorescence staining of biopsied muscle for dystrophin. The biopsied muscle was stained for dystrophin using antibodies against the N-terminal, rod, and C-terminal domains. Staining of N-terminal and rod domains was weak, patchy, and discontinuous. In contrast, no reactive material was visualized for the C-terminal domain (patient). In unaffected individuals, all domains were clearly stained along the plasma membrane (control) but not in DMD.

stream of the C-terminal epitope recognized by the aforementioned antibody.

For clarifying the molecular pathogenesis of the abnormal dystrophin, the dystrophin gene was scanned for mutations. Neither PCR amplification of 19 selected exons nor Southern blot analysis of the dystrophin gene revealed any gross gene rearrangements. The possibility of deletion mutation therefore seemed unlikely. To find a single-base mutation, we analyzed dystrophin mRNA extracted from peripheral lymphocytes using RT-PCR as described previously (22). Ten fragments covering the full-length dystrophin cDNA could be amplified as normal-sized products. Direct sequencing of a fragment that encompasses exons 70–79 disclosed a single nucleotide change: a transition from a cytosine to a thymine at nucleotide 10873 (C10873T) in exon 76 (14). The same nucleotide change (C10873T) was present not only in his muscle dystrophin mRNA (Fig. 2) but also in his genomic DNA (data not shown) (25). His mother was found to be a carrier of the same mutation (data not shown). Because sequencing of other fragments of dystrophin cDNA disclosed no other significant nucleotide changes, it was concluded that this mutation (C10873T) is the cause of the dystrophinopathy. The nucleotide change converted a CAG codon, which encodes glutamine at the 3625th amino acid position, to a stop TAG codon (Q3625X; Fig. 2). Therefore, a truncated dystrophin lacking 60 amino acids at its C terminus (1.6% of the total dystrophin sequence) was expected to be produced.

This truncation of dystrophin is compatible with the failure of the C-terminus–specific antibody to label the protein (Fig. 1), because this antibody recognizes amino acids 3669–3685, an epitope that is downstream of the premature stop codon (Q3625X). However, that dystrophin did stain positively with antibodies against its N-terminal and rod domains (Fig. 1) does not seem consistent with this truncation mutant, because other truncated dystrophin mutants have been found to be very unstable and undetectable immunohistochemically (4,6). In

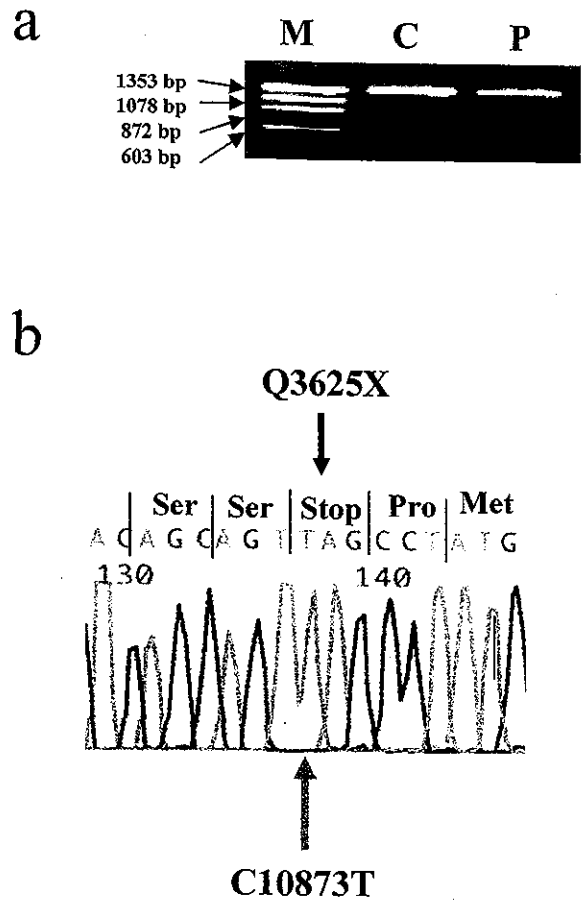

Figure 2. Analysis of dystrophin mRNA prepared from muscle. (*A*) The amplified product encompassing exons 70–79 is shown. One clearly visible product was obtained from the index case (P), and the size of the amplified product is the same as that of the control (C). M refers to a DNA size marker, *Hae*III-digested ϕX174 DNA. (*B*) Nucleotide sequence around the mutation site. Direct sequencing of the amplified product disclosed a single nucleotide change from C to T at nucleotide 10873 (C10873T). This nucleotide change converted a CAG codon to a TAG stop codon (Q3625X). Nucleotide and amino acid numbering are based on those presented by Koenig *et al.* (14).

other cases, it has been hypothesized that the positive staining of dystrophin is the result of rescue of nonsense mutations by exon skipping or aberrant splicing (8,26). However, the RT-PCR–amplified product encompassing exons 70–79 disclosed only one visible band upon agarose gel electrophoresis (Fig. 2). In addition, both direct sequencing and sequencing after subcloning the product confirmed the presence of normal exon structure, indicating that only one mRNA was produced from the mutated gene. These observations do not support the possibility of exon-76 skipping or aberrant splicing.

Although the patient in our case harbored a Q3625X nonsense mutation, his clinical phenotype was unusually mild. Utrophin, a dystrophin-related protein, has been proposed to compensate for the function of dystrophin (27). Therefore, overexpression of utrophin might account for the clinical phenotype seen in the present case. The expression of utrophin was studied in muscle (Fig. 3) and was not found to be elevated in the index case in comparison with that typically seen in DMD. Therefore, enhanced expression of utrophin does not seem, to be modifying the clinical phenotype. Furthermore, the dystro-

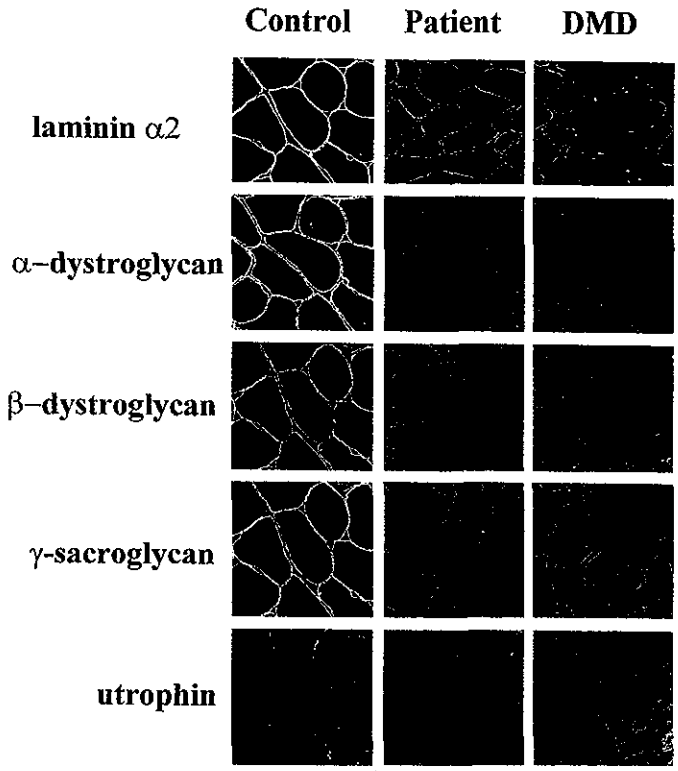

Figure 3. Immunofluorescence staining for dystrophin-associated and dystrophin-related proteins. The dystrophin-associated proteins α- and β-dystroglycan, γ-sarcoglycan, and laminin α2 were labeled with immunofluorescence. The staining patterns seen in the patient's muscle were similar to those observed in DMD. Also as in DMD, no labeling was seen for utrophin, a dystrophin-related protein.

phin-dystroglycan axis was examined (Fig. 3). Laminin α2, an extracellular matrix protein, stained weakly. Neither α-dystroglycan, an extracellular β-dystroglycan–binding protein, nor β-dystroglycan, a transmembrane dystrophin-binding protein, was stained. γ-Sarcoglycan, a member of the sarcoglycan complex, was stained very weakly. All of these staining patterns were similar to those found in DMD (Fig. 3), indicating no difference in the stabilization of the dystrophin-dystroglycan axis from that observed in DMD (Fig. 3). Therefore, no explanation for the mild phenotype was obtained through studies of protein staining.

DISCUSSION

A novel nonsense mutation (Q3625X) in the dystrophin gene was identified in a Japanese boy who was as yet asymptomatic at the age of 8 y. Although a severe DMD phenotype would be expected to develop from his mutation type, his clinical course has been extraordinarily mild. The case has raised an important question to be answered: What is the mechanism that determines the severity of the dystrophic phenotype?.

A somatic mosaic for a nonsense mutation has been shown to attenuate the clinical phenotype (28). However, this possibility seems to be excluded in the index case for the following reasons: *1*) the mutation was inherited through the mother, and *2*) a single genomic clone harboring C10873T was obtained not only from his lymphocytes but also from his muscle (data not shown). To rule out this possibility unequivocally, it is necessary to examine other muscle tissues, but this has not yet been done.

Another possible attenuating mechanism would be the modification of mRNA by either exon skipping or aberrant splicing, which would remove the nonsense mutation and produce a more complete dystrophin mRNA. Examples of exon skipping have been reported in nonsense mutations identified in exons 25, 27, 29, and 72 of the dystrophin gene (7–11), and aberrant splicing has been reported in intermediate dystrophinopathy (26). In our case, however, not only the RT-PCR product of dystrophin mRNA but also subcloning sequencing disclosed the existence of only one kind of mRNA consisting of a normal exon structure (Fig. 2). The possibility of either exon skipping or aberrant splicing thus was ruled out. Therefore, the discrepancy between genotype and phenotype could not be explained at the mRNA level.

Elevated expression of utrophin has been speculated to convert a severe phenotype to a mild one without affecting dystrophin expression (27). However, level of utrophin expression was the same in our case as in DMD (Fig. 3). Furthermore, the staining of proteins encompassing the dystrophin-dystroglycan axis was identical to that seen in DMD (Fig. 3). These similarities show that protein-level changes do not underlie the observed phenotypic differences.

The truncated dystrophin produced in the index case seems to be unusually stable, as demonstrated by the weak but significant staining of the N-terminal and rod domains (Fig. 1). This may be because the truncated dystrophin retains functionally important binding sites, such as actin binding sites in the N-terminal and rod domains (29,30), a β-dystroglycan binding site in the cysteine-rich domain (31), and syntrophin and dystrobrevin binding sites and a phosphorylation site in the C-terminal domain (Fig. 4) (32–35). In fact, it has been dem-

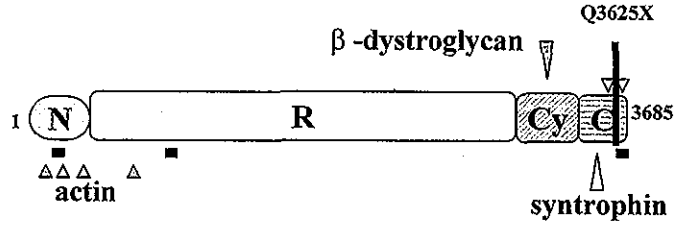

Figure 4. Dystrophin domain structure. Dystrophin consists of 3685 amino acids and is divided into four domains: the N terminus (box N), the rod (box R) and cysteine-rich (box Cy) domains, and the C terminus (box C). Three actin-binding sites have been identified in the N terminus (ABI 18–37 amino acids, ABS3 86–120 amino acids, and ABS2 128–149 amino acids), and one site (amino acids 1416–1880) has been found in the rod domain (all four sites indicated by arrowheads). In the cysteine-rich domain (amino acids 3115–326), dystrophin interacts with transmembrane β-dystroglycan (inverted triangle), which in turn binds to α-dystroglycan. Syntrophin binds to a region in the C-terminal domain (3446–3481; open triangle), and a serine at the 3552nd residue and threonine at the 3675th residue serve as phosphorylation sites (open arrowheads). Antibodies that recognize the N-terminal, rod, and C-terminal domains react to amino acid residues 321–494, 1181–1388, and 3669–3685, respectively (bars). The novel nonsense mutation (Q3625) is located at the end of the C-terminal domain (vertical bar). Numbers at both ends of the boxes indicate amino acid residues. The figure is not drawn to scale.

onstrated that dystrophin lacking the amino acids encoded by exons 71–78 is stable in muscle membranes of the *mdx* mouse, an animal model of DMD (36). However, this hypothesis is not supported by a previous report that Q3625X, a nonsense mutation just 10 amino acids downstream of the one reported here (Q3635X), gave rise to clinically typical DMD (6) (Fig. 4). Furthermore, examination of laminin alpha 2, α- and β-dystroglycans, and γ-sarcoglycan disclosed no difference in their staining patterns between our case and DMD (Fig. 3), indicating that augmented stabilization of these proteins does not contribute to the mildness of the phenotype. Clearly, further study is required to clarify these complex results.

Activation of transcription of the dystrophin gene may lead to overproduction of dystrophin mRNA. It has been proposed that an abnormality in a transcription factor(s) or in its binding site in the promoter of the dystrophin gene is a factor in phenotypic severity. In fact, mutation of the MYF6 gene results in a severe phenotype of BMD (37). However, modifier(s) that make the phenotype mild have not been reported to date (38,39), although *mdx* mice characterized by dystrophin deficiency do not show a severe DMD phenotype (40). We are now following up the index case, and a future study analyzing not only the dystrophin gene but also other genes may clarify the molecular mechanism explaining his mild phenotype.

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