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Clinical Study

Clinical, immunohistochemical, Western blot, and genetic analysis in dystrophinopathy

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ABSTRACT

Dystrophin-deficient muscular dystrophies (dystrophinopathies) are the most common form of muscular dystrophy, with variable clinical phenotypes ranging from the severe Duchenne (DMD) to the milder Becker (BMD) forms. In this study, we investigated the relationship between clinical characteristics, findings at immunohistochemistry (IHC) and Western blot, and the pattern of exon deletions in 24 male patients with dystrophinopathies. We retrospectively reviewed findings from clinical and laboratory examinations, IHC for dystrophin of muscle biopsy tissue, Western blot analysis, and multiplex polymerase chain reaction (PCR) examination of genomic DNA. All tests were performed in every patient. PCR examination revealed exon deletions in 13 patients (54.2%). At Western blot analysis, 15 patients (62.5%) were negative at all three dystrophin domains. Most of these patients had a clinical presentation consistent with the DMD phenotype. Nine (37.5%) others were weakly positive at one or more domains. Most of these patients presented clinically as BMD phenotype. One patient whose clinical presentation was consistent with BMD phenotype had normal findings at IHC and was weakly positive at all three domains on Western blot analysis; however, with the exception of this patient, the findings at IHC and Western blot were consistent for individual patients. Based on these findings, we conclude that Western blot analysis appears useful for confirmation of dystrophinopathy in BMD patients with normal staining on IHC. Exon deletion analysis by multiplex PCR using peripheral blood is also a simple and useful test for the diagnosis of dystrophinopathy, although it has limited sensitivity.

1. Introduction

Duchenne and Becker muscular dystrophy (DMD, BMD) are X-linked recessive muscle disorders characterized by progressive muscle weakness. In 1987–1988, the dystrophin gene at Xp21[1] and the corresponding cytoskeletal protein, dystrophin,[2] were discovered. Mutation of this gene was found to result in complete or partial loss of dystrophin, resulting in a repetitive cycle of muscle fiber necrosis and regeneration, clinically manifested as muscle weakness. Therefore DMD and BMD are categorized as dystrophinopathy or dystrophin-deficient muscular dystrophies.[3] DMD is the most common form of childhood muscular dystrophy, comprising nearly 90% of inherited muscle disorders, with an incidence of approximately 1 in 3300 live male births.[4] Other dystrophinopathies occur at a lower incidence, including DMD and BMD-manifesting female carriers, isolated X-linked cardiomyopathy, and isolated quadriceps myopathy.

In patients with DMD and BMD mutations, exon deletion occurs frequently, and has been detected in approximately 55% to 65% of patients with proven mutations.[5,6] In general, deletions that juxtapose exons that shift the translational reading frame (out-of-frame deletion) usually result in a severely truncated dystrophin molecule that is poorly expressed in the cell and leads to a more severe DMD phenotype.[7] Deletions that juxtapose exons but preserve the translational reading frame (in-frame deletion) result in an internally deleted but semi-functional dystrophin protein, which can persist at some quantity in the cell and thus result in a milder BMD phenotype,[7] although out-of-frame exon 45 deletions have been found in patients with the BMD phenotype. A full 92% of DMD and BMD phenotypes are explained by the "frame-shift theory".[8]

In the past, dystrophinopathies were diagnosed clinically and confirmed with routine histopathologic studies performed on muscle biopsies. However, it is now possible to confirm these disorders by exon deletion analysis using multiplex polymerase chain

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reaction (PCR) for the dystrophin gene and Western blot or immunohistochemistry (IHC) for the dystrophin protein.

The aim of this study is to investigate the relationship between the clinical characteristics of distinct dystrophinopathies and their corresponding findings on IHC and Western blot for dystrophin, and PCR for the exon deletion pattern.

2. Material and methods

2.1. Clinical materials

From September 2007 to February 2010, a total of 24 consecutive male patients with dystrophinopathy were enrolled in this study. The diagnosis of dystrophinopathy was established by clinical history and physical examination, laboratory findings, a normal nerve conduction study, a myopathic pattern on electromyography, muscle biopsy, and molecular diagnosis.[9] In practice, we first performed a multiplex PCR for patients suspected to have dystrophinopathy based on clinical and electrophysiological examination. We then performed a muscle biopsy for IHC and Western blot in all patients. We recorded the age at symptom onset, chief complaint on admission, family history, presence or absence of calf hypertrophy, EKG findings, creatine kinase (CK) levels, and cognitive function. Cognitive function was evaluated using the Korean Educational Developmental Institute Wechsler Intelligence Scale for Children (KEDI-WISC) in eight patients with ages ranging from 5 to 14 years. Echocardiography was not performed.

All study subjects provided written informed consent to allow their clinical data to be used for research purposes. The Institutional Review Board at Gangnam Severance Hospital approved this study.

2.2. Histological and histochemical procedures

All patients underwent an open biopsy from the quadriceps femoris or biceps brachii muscle under local anesthesia. In all cases, informed consent was obtained from the patients or their parents. Transverse serial frozen muscle sections (7 μm thickness) were routinely stained with hematoxylin and eosin and modified Gömöri trichrome, and a battery of histochemical techniques (nicotinamide adenine dinucleotide-tetrazolium reductase, adenosine triphosphatase pH 9.4/4.6/4.3) were applied. Routine staining for muscle tissue showed fiber size variation, fiber splitting, proliferation of endomysial and perimysial connective tissue, degenerating fibers, regenerating fibers, and necrosis and the subsequent loss of fibers, which increased internal nuclei. The findings were consistent with muscular dystrophy.

2.3. Immunohistochemical procedures

Muscle tissues obtained from the 24 subjects were sliced into 7 μm thickness serial sections, immobilized with acetone at 4 °C for 10 minutes, washed with 0.05 mol/L Tris-buffered saline (pH 7.5) for 15 minutes, and incubated for 30 minutes in a blocking solution containing 2% bovine serum albumin and 5% goat serum. The sections were then incubated at 4 °C for one day together with monoclonal IgG antibody against dystrophin (NCL-DYS 1 against rod-like domain, NCL-DYS 2 against carboxyl-terminus, NCL-DYS 3 against amino-terminus, 1:100 dilution, Novocastra Laboratories, Newcastle Upon Tyne, UK). The incubated sections were washed with Tris-buffered saline for 30 minutes, and their staining was examined by inducing the diaminobenzidine (DAB)-peroxidase reaction (Vector Laboratories, Burlingame, CA, USA) using goat anti-mouse IgG conjugated with peroxidase.

2.4. Dystrophin Western blot

Frozen muscle biopsy tissue was sliced into 10–20-μm thick sections. After 50–100 μL sodium dodecyl sulfate (SDS)-sample buffer (0.125 M Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromophenol blue) was added to 10 tissue sections, the tissue was broken completely through ultrasound fragmentation, and the fragmented specimen was denatured at 95 °C for 5 minutes. To quantify muscle protein for each specimen, 2 μL was passed through SDS-polyacrylamide gel electrophoresis (PAGE) in 6% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue, and the quantity of muscle protein was quantified by measuring the quantity of myosin heavy chain protein at 220 kD using a densitometer. After the quantity of myosin protein was standardized, SDS-PAGE was performed again in 6% polyacrylamide gel, and a nitrocellulose membrane was blotted using the Mighty Small Transphor Unit (Hoeffer, Holliston, MA, USA). The membrane was placed in primary antibody diluted at 1:200 with Tris buffer saline-T (TBS-T, 0.1% (volume/volume) Tween 20), and incubated at 4 °C overnight on an agitator. For the primary antibody, we used three antibodies against dystrophin (NCL-DYS 1 against rod-like domain, NCL-DYS 2 against carboxyl-terminus, NCL-DYS 3 against amino-terminus, 1:100 dilution, Novocastra) for immunoblotting. The membrane was washed with TBS-T at room temperature for 15 minutes, and dystrophin was detected using the Mouse ExtrAvidin Peroxidase Staining Kit (Sigma-Aldrich, St Louis, MO, USA).

2.5. Multiplex polymerase chain reaction (PCR)

Genomic DNA was isolated from peripheral blood using peripheral leukocytes by the cetyltrimetilammonium bromide method.[10] Exon analysis by multiplex PCR followed the protocol of Chamberlain et al.[11] and Beggs et al.[12] Exons 3, 4, 6, 8, 12, 13, 17, 19, 43, 44, 45, 47, 48, 49, 50, 51, 52, 60 and Pm were studied.

3. Results

Clinical evaluations, IHC, Western blot, and PCR assessment were available in all 24 patients.

3.1. Clinical and laboratory characteristics

All 24 patients were male. The age of patients at disease onset varied from 2 to 9 years (mean 4.6 ± 2.1 years), and disease duration ranged from 1 to 20 years. The initial symptoms were as follows: 18 patients (75.0%) presented with abnormal gait, such as waddling gait; four patients (16.7%) with difficulty in rising from the floor; and two patients (8.3%) with incidental findings such as glutamic oxaloacetic transaminase/glutamic pyruvic transaminase (GOT/GPT) elevation. Over 90% of patients had symmetrical lower extremity muscle weakness. Seven patients (29.2%) reported a family history of muscular dystrophy, including patients 1 and 2, who were siblings. There was symmetrical hypertrophy of the calf muscles in 21 patients (87.5%). The mean serum CK level was 14,144 IU/L (range, 181–38,200; normal, 35–232). Of 16 patients who underwent EKG evaluation, 10 (62.5%) had normal findings. The six abnormal findings (37.5%) were three cases of right ventricular hypertrophy, one case of right bundle branch block, one case of left ventricular hypertrophy, and one case of sinus tachycardia. In eight patients who were evaluated for cognitive function, the mean intelligence quotient (IQ) was 81. Three patients (37.5%) had below average IQ and two patients (25.0%) had mental retardation, with IQ scores below 69 points (Table 1).