Unequal Crossing-over in Unique PABP2 Mutations in Japanese Patients

A Possible Cause of Oculopharyngeal Muscular Dystrophy

Mika Nakamoto, MD, PhD; Satoshi Nakano, MD, PhD; Shingo Kawashima, MD, PhD; Masafumi Ihara, MD; Yo Nishimura, MD; Akiyo Shinde, MD; Akira Kakizuka, MD, PhD

Background: Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset autosomal dominant muscle disease with a worldwide distribution. Recent findings reveal the genetic basis of this disease to be mutations in the polyA binding–protein 2 (PABP2) gene that involve short expansions of the GCG trinucleotide repeat encoding a polyalanine tract. The underlying mechanism causing the triplet-expansion mutation in PABP2 remains to be elucidated, although the DNA slippage model is thought to be a plausible explanation of that.

Methods and Results: We analyzed PABP2 using polymerase chain reaction analysis and DNA sequencing in Japanese patients with pathologically confirmed OPMD, and found mutated (GCG)3GCA(GCG)3(GCA)3GCG and (GCG)6(GCA)3GCG alleles instead of the normal (GCG)6(GCA)3GCG allele. These mutated alleles could be explained by the insertions or duplications of (GCG)6, (GCA)3(GCG)3, and (GCG)2(GCA)3, respectively, but not by the simple expansion of GCG repeats. The clinical features of our patients were compatible with those of other Japanese patients carrying PABP2 that encodes a polyalanine tract of the same length, but were not compatible with those of Italian patients.

Conclusions: The mutated alleles identified in our Japanese patients with OPMD were most likely due to duplications of (GCG)6(GCA)3GCG and (GCG)3GCA(GCG)3(GCA)3GCG but not simple expansions of the GCG repeats. Therefore, unequal crossing-over of 2 PABP2 alleles, rather than DNA slippage, is probably the causative mechanism of OPMD mutations. All mutations that have been reported in patients with OPMD so far can be explained with the mechanism of unequal crossing-over. On the other hand, comparison of the clinical features of our patients with those of other patients in previous reports suggests that specific clinical features cannot be attributed to the length of the polyalanine tract per se.

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SUBJECTS AND METHODS

REPORT OF CASES

The first patient (patient A) was a 65-year-old man born in Hyogo Prefecture, Japan. At 53 years of age, a swallowing disturbance developed. At 60 years of age, he noticed ptosis on both sides, which gradually worsened. Results of an examination showed moderate ptosis and mild weakness of the facial and pharyngeal muscles. Extraocular muscles showed no weakness. He exhibited moderate symmetrical weakness of the proximal lower limb muscles. Results of laboratory examination showed a 6-fold increase in serum creatine kinase (CK) level (843 U/L; reference value, <141 U/L). His father had had dysphagia and dysarthria since the sixth decade of life. A sister of patient A had had dysphagia since her sixth decade of life, whereas a brother had no neuromuscular symptoms.

The second patient (patient B) was a 69-year-old woman born in Shiga Prefecture, Japan. She had no neuromuscular symptoms until 61 years of age, when she noticed ptosis on both sides. The symptoms progressed gradually, and she underwent an operation for it at 65 years of age. At 66 years of age, easy fatigability on walking, swallowing difficulty, and speech disturbance developed. Results of an examination disclosed ptosis that completely covered the pupils. Medium grade of ophthalmoparesis was noted in all directions. Eye and mouth closures were moderately impaired. She had moderate symmetrical muscle weakness of the proximal limb and neck muscles. Her serum CK level was 300 U/L. She was separated from her parents in early childhood, which hindered confirmation of parental symptoms. She had a sister with ptosis and a child without neuromuscular symptoms.

In patients A and B, results of muscle biopsy revealed the occasional occurrence of muscle fibers containing rimmed vacuoles. Results of electron microscopic studies showed intranuclear tubulofilamentous inclusions with a diameter of 8.5 nm, which is a characteristic of OPMD.

METHODS

We analyzed PABP2 in both index patients and the 2 siblings of patient A after obtaining informed consent.

Biopsy specimens of muscle tissue from patients A and B were stored at −70°C and were then cut into 5- to 10-mg sections using a razor. The sections of muscle tissue were incubated in a solution containing 50mM Tris hydrochloride (pH, 8.0), 10mM EDTA, 100mM sodium chloride, 0.3% sodium dodecyl sulfate, and 1-mg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) for 5 hours at 50°C. After phenol extraction, genomic DNA was precipitated using 99% ethanol, then dissolved in a solution consisting of 10mM Tris hydrochloride (pH, 7.5) and 0.1mM EDTA. Peripheral blood samples were obtained from the 2 siblings of patient A and Japanese control subjects. Genomic DNA was extracted from the samples with the use of a blood kit (QiAamp; QiAGEN, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction analysis was performed in 50-mL volumes containing 100 ng of genomic DNA, 250mM of each dNTP (ie, deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxyxytosine triphosphate, and deoxycytidine triphosphate), 1µM of the forward 5’-GCGATGCTCCCCGCTTAAAGGTG-3’ and backward 5’-ACAAGATGGCGCCGCCGCCC-3’ primers, 1.25 U of Taq DNA polymerase (Takara LA Taq; Takara, Kyoto, Japan) and its specific reaction buffer (GC Buffer II; Takara). After initial denaturation at 95°C for 5 minutes, amplification was performed in 30 cycles consisting of denaturation at 95°C for 18 seconds, annealing at 70°C for 30 seconds, and extension at 74°C for 40 seconds. The final extension proceeded at 74°C for 10 minutes. Products were separated on a 5% nondenaturing polyacrylamide gel. After electrophoresis, the gels were stained using ethidium bromide. Allele-specific bands were excised from the gels, eluted, and subcloned into a plasmid vector (pGEM-T Easy Vector; Promega, Madison, Wis). Sequencing of the normal and mutated fragments was performed bidirectionally using a dideoxy sequencing kit (Amplicyc; Applied Biosystems, Foster City, Calif) on at least 6 clones for every allele, for validation. The sequences obtained were compared with the genomic sequence of the human PABP2 gene (accession number AF026029; GenBank, Bethesda, Md; available at: http://www.ncbi.nlm.gov/entrez/query).

Sequence analyses disclosed a 12-base pair (bp) elongation in patient A and his affected sister and a 15-bp elongation in patient B (Figure 1) within the (GCG)n(GCA), GCG normal sequence in exon 1 of PABP2. The mutated sequence detected in patient A and his sister was (GCG)n(GCA)nGCG, and that in patient B was (GCG)n(GCA),GCG, (GCA),GCG(GCG)n(GCA),GCG. Both sequences could be explained by the insertions or duplications of (GCG)n(GCA),GCG and (GCG)n(GCA), respectively, into the normal sequence. The normal sequence in PABP2 is translated into a series of 10 alanine residues. The mutations in our patients increase the number of alanine residues encoded from 10 to 14 in patient A and his sister, and to 15 in patient B. All of the patients were homozygous for the mutated and the normal alleles. The unaffected brother of patient A was homozygous for the normal allele.

In addition to the duplications, we observed in all patients and 10 Japanese controls a CG-to-GGGC change in position 1146, a G deletion in position 1215, a GG insertion in position 1229, and an ATC-to-CAT change in position 1250 (Figure 2). The latter 2 changes have previously been described in an Italian population. All 4
changes are located in the 5'-untranslated region of PABP2 and were present in all healthy and affected Japanese subjects studied. This indicates that they may be polymorphisms; the former two are characteristic to date of the Japanese population and the latter two are shared to date by the Japanese and Italian populations.

## MECHANISM OF REPEAT EXPANSIONS IN OPMD

Except for the mutation found in Cajun patients who had a (GCG)₆GCA (GCG)₃GCG mutated allele, all of the PABP2 mutations reported so far in patients with OPMD were expansions of the (GCG)₆ repeat. These expansions make a (GCG)₆₋₁(GCA)₆₋₁GCG allele from the (GCG)₆₋₁(GCA)₆₋₁GCG normal sequence. We found 2 novel mutations, (GCG)₆₋₁(GCA(GCG)₃(GCA)₆₋₁GCG and (GCG)₆₋₁(GCA)₆₋₁(GCG)₆₋₁(GCA)₆₋₁GCG, in Japanese patients with OPMD.

The molecular mechanism causing the repeat expansion in PABP2 has not been determined. In general, the following 2 types of mechanisms leading to the generation of longer DNA sequences have been proposed: replication- and recombination-associated expansion.

Repeat expansions that involve the process of DNA replication may originate from slipped mispairing between repeated sequences, as has been described for the slippage model. It has been shown recently that expanded triplet repeats are responsible for a number of hereditary neuromuscular diseases. These pathologic repeat expansions can be explained by the slippage model. However, it has been proposed that tracts of approximately 25 to 35 perfect trinucleotide repeats are required for instability and expansion via slippage. The heterogeneous sequences of the mutated alleles of PABP2 detected in the Cajun patients and our Japanese patients and the fact that even the longest perfect repeat reported (13 repeats) is less than 25 repeats argue against the slippage model.

Recombination-associated repeat expansion may result from homologous recombination, which occurs in germ cells during meiosis and sometimes during mitosis. The mutations that we found suggest that the molecular mechanism resulting in generation of longer DNA sequences in PABP2 is unequal crossing-over (Figure 3), which is a kind of homologous recombination. The (GCG)₆₋₁(GCA)₆₋₁GCG normal allele was reported to be found in 98% and 99% of French Canadian and Japanese control chromosomes, respectively, whereas the rest of both populations carried a (GCG)₆₋₁(GCA)₆₋₁GCG polymorphism (Figure 3A). Unequal pairing with variable degrees of...
overlapping expansions of polyalanine tracts, or of the altered properties of the mutated PABP2 protein. In both cases, the length of the polyalanine tract may be a key determinant of the effect. The (GCCG)6(GCA)3GCC sequence in the normal PABP2 gene is translated into 10 alanine residues in the protein. The mutated alleles in our patients encode 14 and 15 alanine residues instead of 10. In that case, the number of alanine residues in our patients is equivalent to those reported to be generated by the (GCCG)6(GCA)3GCC and (GCCG)7(GCA)3GCC expanded alleles, respectively.

All of our patients were heterozygous for the mutations. To our knowledge, only 1 Japanese family and 2 Italian families heterozygous for the (GCCG)6(GCA)3GCC allele and 2 Japanese families heterozygous for the (GCCG)11(GCA)3GCC allele have been described clinically and genetically. According to the reports, dysphagia with moderately increased CK levels and proximal myopathy of the lower legs initially developed in the Japanese patients heterozygous for the (GCCG)6(GCA)3GCC allele. Ptoasis first developed in the 2 Japanese families heterozygous for the (GCCG)11(GCA)3GCC allele, whereas their CK levels were generally within normal limits or only mildly increased. These clinico-genetic correlations are compatible with those of our patients, especially with respect to the initial symptoms and CK levels. However, both Italian families heterozygous for the (GCCG)6(GCA)3GCC allele presented with ptosis as the first symptom, with later development of dysphagia and severe weakness of limb and pelvic girdle muscles. Therefore, the specific clinical feature perhaps cannot be attributed to the length of the polyalanine tract of PABP2 per se. Although these observations argue that the pathologic characteristics of OPMD may be the result of nucleotide configuration, intrapopulational similarities of phenotype among the Japanese and among the Italian patients are more likely to be due to the genetic background of each race. Further case accumulation is needed to clarify the relationship between genotype and phenotype in OPMD.

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Corresponding author and reprints: Akira Kakizuka, MD, PhD, Laboratory of Functional Biology, Graduate School of Biostudies, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan (e-mail: kakizuka@lif.kyoto-u.ac.jp).

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16. Kunsta CB, Warren ST. Cryptic and polar variation of the fragile X repeat could overlap can generate each of the (GCCG)11(GCA)3GCC mutant alleles by crossing-over of the 2 (GCCG)6(GCA)3GCC normal alleles (Figure 3B). At most, a (GCCG)11(GCA)3GCC allele can be derived by unequal crossing-over of the (GCCG)6(GCA)3GCC normal allele and the (GCCG)6(GCA)3GCC polymorphic allele. This mechanism can also explain the (GCCG)6(GCA)3GCC (GCCG)7(GCA)3GCC GCG mutation reported in Cajun patients (Figure 3C) and the (GCCG)6(GCA)3GCC (GCCG)7(GCA)3GCC (Figure 3D) and (GCCG)6(GCA)3GCC (GCCG)7(GCA)3GCC GCG mutations found in our patients (Figure 3E). Since the (GCCG)6-10(GCA)3GCC pathologic expansions in PABP2 are reported to be stable with no variation among family members and between such different tissues as blood and skeletal muscle in the same individual, we suspect that unequal crossing-over occurred once at meiosis in an ancestor of each patient with OPMD. Similar expansions of cryptic repeats composed of mixed synonymous codons causing a polyalanine expansion in the homeobox D13 (HOXD13) protein has been found to cause synpolydactyly. The HOXD13 mutation has been explained by unequal crossing-over. The muscle degeneration seen in OPMD may be a consequence of the toxic effects of the aggregates caused by the expanded polyalanine tracts, or of the altered properties of the mutated PABP2 protein. In both cases, the length of the polyalanine tract may be a key determinant of the effect. The (GCCG)6(GCA)3GCC sequence in the normal PABP2 gene is translated into 10 alanine residues in the protein. The mutated alleles in our patients encode 14 and 15 alanine residues instead of 10. In that case, the number of alanine residues in our patients is equivalent to those reported to be generated by the (GCCG)6(GCA)3GCC and (GCCG)7(GCA)3GCC expanded alleles, respectively.

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