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Detection of Large Expansions in SCA8 Using a Fluorescent Repeat-Primed PCR Assay

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ABSTRACT

Spinocerebellar ataxia type 8 (SCA8) is a neurodegenerative disorder characterized by slowly progressive cerebellar ataxia. It is caused by bidirectional expression of (CUG)n expansion in the *ATXN8OS/ATXN8* gene and (CAG)n expansion transcripts in *ATXN8*. The diagnosis of SCA8 must be confirmed by the presence of a (CTG)n trinucleotide repeat expansion in the ATXN8OS gene. On the other hand, there are many human genetic diseases that are caused by expansion of short tandem repeats. Since Werner et al proposed a repeat-primed fluorescent PCR to detect large CTG-repeats in myotonic dystrophy, Friedreich ataxia, SCA2, SCA7, SCA10 and SCA12 have been reported. In this study, we applied a fluorescent PCR method for detection of expanded repeats in the *ATXN8OS/ATXN8* gene. Although this test cannot give a precise estimate of the size of the expansion, it proved useful for confirming the presence of expansions in SCA8.

Key words: *SCA8, Repeat-Primed PCR*

Spinocerebellar ataxia type 8 (SCA8) is a neurodegenerative disorder characterized by slowly progressive cerebellar ataxia. Koob et al first reported this neurodegenerative disorder as autosomal dominant spinocerebellar ataxia in a large kindred[6]. It is caused by bidirectional expression of (CUG)n expansion in the *ATXN8OS/ATXN8* gene, which interferes with the normal antisense function of this transcript, and (CAG)n expansion transcripts in *ATXN8*, which is translated into a nearly pure polyglutamine protein that forms 1C2-positive inclusions in Purkinje cells and other neurons[4,8]. In general, the diagnosis of SCA8 must be confirmed by molecular genetic testing. The presence of a (CTG)n trinucleotide repeat expansion in the *ATXN8OS* gene is important.

Routine testing for the expansions is usually performed by Southern blot analysis for larger expansions and long-range PCR for smaller allele sizes. However, these are not suitable for screening tests. Performing a combination of these examinations as a screening test is complex, cumbersome and time-consuming.

On the other hand, there are many human genetic diseases that are caused by expansion of short tandem repeats. Since Warner et al proposed using a repeat-primed fluorescent PCR to detect large CTG-repeats in myotonic dystrophy[13], Friedreich ataxia, SCA2, SCA7, SCA10 and SCA12 have been reported[1-3].

In this study, we applied a fluorescent PCR method for detection of expanded repeats in the *ATXN8OS/ATXN8* gene. Normal alleles have 15 to 50 repeats, and a repeat range from 80 to 250 is most often associated with ataxia. We used these parameters to distinguish normal and expanded repeats.

MATERIALS AND METHODS

Genomic DNA was extracted from peripheral blood leukocytes using the conventional method. We analyzed the DNA of 17 SCA8 patients and 258 controls from our collection of DNA from patients with ataxia. Each sample had known expansions tested by Southern blot analysis.

The research protocol was approved by the Ethics Committee of Hiroshima University. Written consent was obtained for all blood samples used in the study. The patient genotypes at the repeated regions are shown in Table 1[5,7].

PCR analyses were based on the method of Warner et al as well as those of other researchers[1-3,13]. We designed a locus-specific primer region upstream of the unstable repeat. The

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Table 1. Patients with large SCA8 repeat alleles

Patient	Sex	Age of Onset	Repeat Length
1	M	35	144/101
2	M	13	172/27
3	F	60	82/19
4	F	53	100/82
5	M	43	110/24
6	M	65	100/28
7	F	36	89/28
8	F	10	144/30
9	F	60	73/30
10	M	36	86/28
11	F	75	110/29
12	F	31	140/28
13	M	17	126/29
14	F	29	100/28
15	M	40	105/34
16	F	34	160/19
17	F	61	126/101

reverse primer consists of 5 units of the repeat and a 5′ tail that is used as an anchor for a second reverse primer, which prevents progressive shortening of the PCR products during subsequent cycles.

PCRs were performed with the following general conditions: 200 to 1000 ng of genomic DNA, 800 mmol/liter of a locus-specific forward primer (5′- FAM- ctgggtccttcatgttagaaaacct), 40 mmol/liter of a repeat-specific reverse primer (5′-tacgcatcccagtttgagacg cagcagcagcagcag) and 800 mmol/liter of the "common" flag primer (5′-tacgcatcccagtttgagacg), 200 µmol/liter dNTPs, buffer and 1U Expand High Fidelity (Roche). PCR conditions were performed with the following cycling parameters: initial denaturation for 10 min at 95°C, 40 cycles consisting of 45 sec at 95°C, 1 min at 60°C, 4 min at 72°C, and a final extension of 10 min at 72°C.

Analysis of fluorescent PCR products was performed with an ABI-Prism 3100 automatic sequencer using a 36-cm capillary and a GeneScan™ 500 ROX size standard (Applied Biosystems). Data were examined using Genescan 3.1 software. Samples were considered reliable in the range of 200 to 6000 fluorescence units.

RESULTS

The test was validated in 17 SCA8 patients carrying 73 to 221 repeats and in 258 subjects with ataxia (range of normal repeats: 19 to 36). The expanded allele could be detected by fluorescent repeat-primed PCR. The trace of pathogenic patterns was identified only in patients with SCA8 who had pathogenic expansions (Fig. 1).

DISCUSSION

The fluorescent repeat-primed PCR, for which we present an application to detect expansions in the SCA8 locus, is a valuable tool, though this test is unable to give an accurate size of the expansion.

Using this method, we were able to detect expansions (73-221) that had already been diagnosed. No controls showed pathogenic patterns. There were no false-positive or false-negative results. This method appears to be sensitive, specific, reliable, and easy to handle with results quickly obtained.

We detected more than 73 repeats that covered a repeat range from 80 to 250, which is most often associated with ataxia. We therefore consider this method to be useful for detecting the presence of a pathogenic (CTG)n trinucleotide repeat expansion, especially for the screening of a large population.

By using our method, we can detect the presence of a (CTG)n trinucleotide repeat expansion in the ATXN8OS/ATXN8 gene, and we can proceed to the use of other techniques, such as Southern blot analysis, for positive cases. To make a diagnosis fairly, we recommend a two-step protocol.

In the first step, both standard PCR and fluorescent repeat-primed PCR must be performed. Standard PCR is suitable for the detection of normal range repeats and 50 to 70 repeats. On the other hand, fluorescent repeat-primed PCR can detect an expansion of more than 70 repeats. This method is easier and quicker than using long PCR and Southern blot analysis. For positive cases, we can proceed to the next step. The second step involves determination of the accurate size of the expansion by using long PCR or Southern blot analysis.

It is not clear whether repeat sizes ranging from 50 to 70 repeats can be pathogenic, and it is known that not all expanded alleles are pathogenic. Moreover, expansions have been found in individuals with positive gene test results for other SCDs[5,9,10] and other diseases[5,9,11,12]. Therefore, we believe it is possible to obtain new insights by increasing the number of screening tests and confirming expansions by the use of our method.

In conclusion, the fluorescent repeat-primed PCR is useful as a screening test or predictive test for detecting the presence of a (CTG)n trinucleotide repeat expansion.

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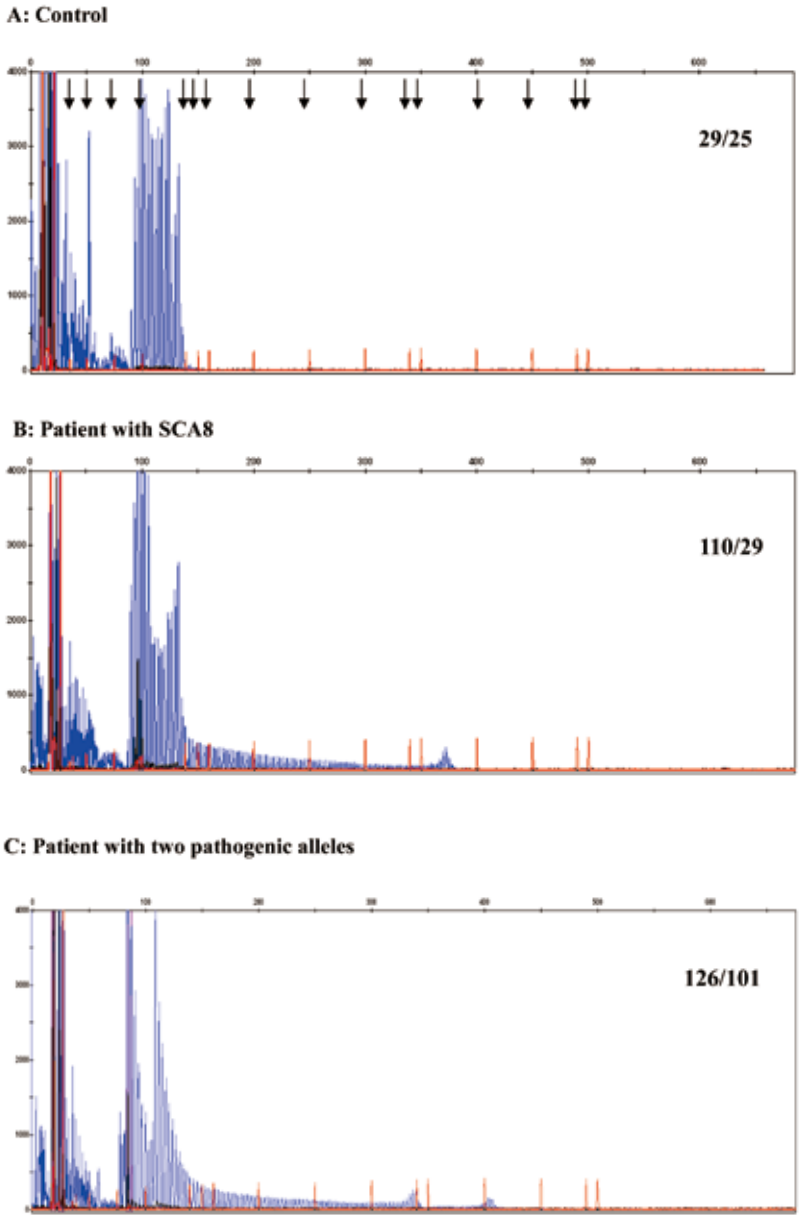

Fig. 1. Fluorescent repeat-primed PCR analysis in the SCA8 locus
The ordinate shows fluorescence intensity in arbitrary units. The abscissa shows size in bp. Arrows show size markers. A: The profile is consistent with the absence of expansion. The ladder demonstrates amplification of a small number of repeats in the normal size range. B: The profile is consistent with the presence of expansion. The 'tail-like' ladder shape shows amplification of the repeat expansion. A small peak can be seen at the end of the tail. C: Patient who has two pathogenic alleles. Two small peaks can be seen at the end of the tail.

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