Journal of Human Genetics (2012) 57, 807–808

www.nature.com/jhg

SHORT COMMUNICATION

Pentanucleotide repeat-primed PCR for genetic diagnosis of spinocerebellar ataxia type 31

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Spinocerebellar ataxia type 31 (SCA31) is defined by the presence of an insertion mutation containing a TGGAA repeat within the intron of the *brain-expressed, associated with NEDD4* (*BEAN*) gene. Detecting this mutation is conventionally done by southern blotting or DNA sequencing, but these methods are technically demanding and not easily implemented in clinical diagnosis. Here, we adapted repeat-primed PCR (RP-PCR) to develop a clinical genetic test for SCA31 using only the PCR process to detect the TGGAA repeat within the insertion mutation. Pentanucleotide RP-PCR and subsequent DNA fragment analysis demonstrated characteristic ladder peaks with a 5-bp periodicity, originating from the TGGAA repeat, in 100% of samples ($n = 14$) from SCA31 patients in whom the presence of the TGGAA repeat had been verified by DNA sequencing. No peaks were observed in a normal control and two non-SCA31 patients, in whom the TGGAA repeat was absent. This method is valuable for genetic diagnosis of SCA31 in clinical practice.
Journal of Human Genetics (2012) **57,** 807–808; doi:10.1038/jhg.2012.112; published online 20 September 2012

Keywords: genetic testing; repeat-primed PCR; spinocerebellar ataxia type 31

INTRODUCTION

Spinocerebellar ataxia type 31 (SCA31) is a subtype of the autosomal dominant cerebellar ataxias (ADCAs). The length of a variable insertion (2.5–3.8 kb), containing a TGGAA repeat within the intron of the *brain-expressed, associated with NEDD4* (*BEAN*) gene on chromosome 16q22, has previously been found to be inversely correlated to the age of disease onset.[1]

For genetic diagnosis of SCA31, both the presence of the long insertion and of pentanucleotide TGGAA repeats within the insertion is necessary, as some rare individuals that carry a long insertion lacking the TGGAA repeat may exist.[1,2] Typically, PCR-southern blotting[2] or DNA sequencing[1,2] are used to identify the TGGAA repeats, but these methods are technically demanding and labor intensive and require numerous steps.

Repeat-primed PCR (RP-PCR) is a standard method used to detect the expanding CAG repeat in Huntington's disease and ADCAs,[3–5] as it can detect the existence of a repeat tract using locus- and repeat-specific primers. Here, we modified this method for diagnosis of SCA31 associated with TGGAA repeats.

MATERIALS AND METHODS

Subjects and identification of the insertion mutation in SCA31

Samples of 156 patients clinically suspected to have ADCAs and who had undergone genetic testing at Chiba University Hospital and a healthy control, were analyzed. Written informed consent was obtained with appropriate genetic counseling from each individual before this study, which was approved by the Ethics Committee of Chiba University School of Medicine.

Genomic DNA was extracted from peripheral blood using a MagNA pure Compact Nucleic Acid Isolation Kit I (Roche, Basel, Switzerland). After amplification, the presence of the insertion was identified by electrophoresis, and DNA sequencing of this region was performed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), as described previously.[1]

Pentanucleotide RP-PCR and fragment analysis

To detect the TGGAA repeat, we used RP-PCR method.[3] The insertion sequence was amplified with primers shown in Table 1. Forward primer 1 was a fluorescently labeled locus-specific primer,[1] reverse primer 2 was a TGGAA repeat-specific primer with a random DNA sequence at the 5′ end, whereas reverse primer 3 contained the same 5′ sequence as primer 2. These primers enabled locus- and TGGAA repeat-specific amplification. PCR was performed in a 25 μl reaction volume containing 100 ng of genomic DNA, 1.0 U of LA Taq polymerase (TAKARA, Shiga, Japan), 1 × GC buffer I (Mg^{2+} plus; TAKARA), 400 μM of each dNTP, 1.0 μM of primer 1 and primer 3, and 0.1 μM of primer 2. Thermal cycles included an initial denaturation step at 95 °C for 5 min, followed by 32 cycles each consisting of denaturation at 95 °C for 1 min, annealing at 56 °C for 2 min and extension at 68 °C for 1.5 min; this was followed by a final extension at 68 °C for 10 min. Annealing temperature was decided in consideration of Tm values calculated from the GC content of each primer (Table 1).

For DNA fragment analysis, 2 μl of a PCR product were added to 20 μl of formamide and 0.5 μl of GS500-LIZ size standard (Applied Biosystems); this mixture was denatured at 95 °C for 2 min, and immediately cooled on ice.

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Received 6 June 2012; revised 14 August 2012; accepted 15 August 2012; published online 20 September 2012

Table 1 Sequence of primers used in RP-PCR

Primer ID	*Fluorophore*	*Sequence (5′–3′)*	*Tm value (°C)*
Primer 1[a]	6-FAM	5′-ACTCCAACTGGGATGCAGTTT CTCAAT-3′	59
Primer 2		5′-TACGCATCCCAGTTTGAGACGTTC CATTCCATTCCATTCCATTCCA-3′	55
Primer 3		5′-TACGCATCCCAGTTTGAGACG-3′	57

Abbreviation: RP-PCR, repeat-primed PCR.
[a]Primer 1 has the same sequence of 1.5k-ins-F primer.[1]

These samples were then injected into an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), and the fragments analyzed by GeneMapper software (Applied Biosystems).

RESULTS AND DISCUSSION

The long insertion was observed in 16 of the 156 samples using PCR amplification of the relevant SCA31 site and subsequent electrophoresis; representative examples are shown in Figure 1. Using DNA sequencing, the TGGAA repeat was identified in only 14 of the 16 samples containing the insertion; thus, these individuals were diagnosed with SCA31.

The samples from the 16 patients with the long insertion mutation and from the healthy control were then used for pentanucleotide RP-PCR and subsequent DNA fragment analysis. In the 14 samples with the TGGAA repeat in the long insertion, amplification products showed a characteristic ladder of peaks with a 5-bp periodicity (Figure 2: P2–5). The height of the peaks diminished gradually with increasing product size. The samples from the normal control and from the two patients who carried an insertion mutation without TGGAA repeats showed no peaks in this analysis. Thus, the ladder peaks reliably demonstrated the presence of the long insertion mutation containing the TGGAA repeat in SCA31 patients.

Using RP-PCR and subsequent DNA fragment analysis, the main peaks observed in the 14 samples from the SCA31-diagnosed patients were confined to only four sizes, differing by 5 bp (603, 608, 613 and 618 bp). The size of the initial peak depended on the number of repeats of another pentanucleotide (TAGAA) present upstream of the TGGAA repeat. The results of RP-PCR in four samples in which different numbers of TAGAA repeats were verified by DNA sequencing are shown in Figure 2. The number of these TAGAA repeats had previously been reported to correlate inversely to the total size of the insertion.[2] Although RP-PCR could not establish the repeat number of TGGAA and the size of the long insertion mutation, its size could be roughly estimated from the size of the initial peak observed on DNA fragment analysis.

The RP-PCR reported here will be efficient for SCA31 screening as TGGAA repeats have been identified at the 5′end of the insertion in all the cases reported.[1,2] It is possible, however, that this method may miss pentanucleotide repeats located in the middle of the insertion. Therefore, PCR-southern hybridization and/or direct sequencing should be considered in highly suspected SCA31 cases in which RP-PCR gives negative results.

Although SCA31 patients rarely have pathogenic insertions in both alleles, RP-PCR may not discriminate homozygote from heterozygote. This discrimination needs PCR-southern blotting and/or direct sequencing.

SCA31 is one of the major subtypes of ADCAs in Japan.[6] Without genetic testing, it remains challenging to distinguish between subtypes of ADCAs based solely on the clinical symptoms in the early stages of disease. The method presented here is easy to perform and can reliably detect the insertion mutation of SCA31, making it valuable for screening for SCA31 in clinical practice.

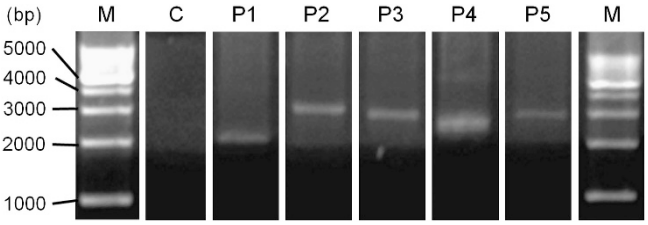

Figure 1 Agarose gel electrophoresis of *Hae*III-digested PCR products in a normal control and 5 of 16 patient samples in which the long insertion was present, are shown. These examples were selected based on the DNA sequence of the insertion. C is a sample from a normal individual, lacking the insertion mutation, whereas P1–5 are patient samples with the long insertion. DNA sequencing revealed that the long insertion of P1 contained no TGGAA repeat, whereas those of P2–5 contained the TGGAA repeat. M, 1 kb DNA ladder marker.

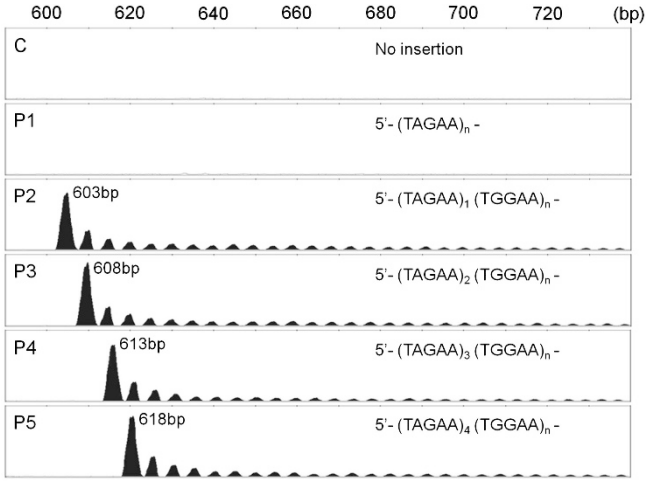

Figure 2 Results of pentanucleotide repeat-primed PCR (RP-PCR) and subsequent DNA fragment analysis. The sample numbers correspond to those in Figure 1. Characteristic ladder peaks with a 5-bp periodicity were observed only in the samples harboring the long insertion containing the TGGAA repeat (SCA31 patients P2: $[TAGAA]_1$, P3: $[TAGAA]_2$, P4: $[TAGAA]_3$, P5: $[TAGAA]_4$). No peaks were observed in a normal control sample (C) or in a patient sample (P1) harboring the long insertion without the TGGAA repeat. The calculated initial peak size of P2, P3, P4 and P5 were 603, 608, 613 and 618 bp, respectively.

1 Sato, N., Amino, T., Kobayashi, K., Asakawa, S., Ishiguro, T., Tsunemi, T. *et al.* Spinocerebellar ataxia type 31 is associated with 'inserted' penta-nucleotide repeats containing (TGGAA)n. *Am. J. Hum. Genet.* **85,** 544–557 (2009).
2 Sakai, H., Yoshida, K., Shimizu, Y., Morita, H., Ikeda, S. & Matsumoto, N. Analysis of an insertion mutation in a cohort of 94 patients with spinocerebellar ataxia type 31 from Nagano, Japan. *Neurogenetics* **11,** 409–415 (2010).
3 Warner, J. P., Barron, L. H., Goudie, D., Kelly, K., Dow, D., Fitzpatrick, D. R. *et al.* A general method for the detection of large CAG repeat expansions by fluorescent PCR. *J. Med. Genet.* **33,** 1022–1026 (1996).
4 Cagnoli, C., Michielotto, C., Matsuura, T., Ashizawa, T., Margolis, R. L., Holmes, S. E. *et al.* Detection of large pathogenic expansions in FRDA1, SCA10, and SCA12 genes using a simple fluorescent repeat-primed PCR assay. *J. Mol. Diagn.* **6,** 96–100 (2004).
5 Cagnoli, C., Stevanin, G., Michielotto, C., Gerbino Promis, G., Brussino, A., Pappi, P. *et al.* Large pathogenic expansions in the SCA2 and SCA7 genes can be detected by fluorescent repeat-primed polymerase chain reaction assay. *J. Mol. Diagn.* **8,** 128–132 (2006).
6 Nozaki, H., Ikeuchi, T., Kawakami, A., Kimura, A., Koide, R., Tsuchiya, M. *et al.* Clinical and genetic characterizations of 16q-linked autosomal dominant spinocerebellar ataxia (AD-SCA) and frequency analysis of AD-SCA in the Japanese population. *Mov. Disord* **22,** 857–862 (2007).