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SHORT COMMUNICATIONS

An Improved Polymerase Chain Reaction Method for Genetic Testing of Spinocerebellar Ataxia Type 3[1]

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Abstract—The development of a reliable PCR assay for genetic testing of spinocerebellar ataxia type 3. Touchdown PCR conditions were tested and different primer sets were evaluated with genomic DNA from blood sample of patients suffering from spinocerebellar ataxia type 3 (SCA3). An improved PCR assay was developed with a new set of primers and using the optimized touchdown PCR protocol. This new assay had been successfully employed in the screening of one identificated SCA3 family. Results from the present study document a simple and reliable PCR assay for genetic testing of SCA3. Strategies used in the present study may find applications in the optimization of PCR assay for triplet expansion with GC rich in the sequence context.

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A number of neurological diseases are caused by the abnormal expansion of the CAG trinucleotide repeats in relevant genes [1]. Spinocerebellar ataxias (SCAs) are a group of chronic degenerative diseases of the central nervous system characterized by the progressive balance and coordination disorder [2]. The main affected regions are the spinal cord, cerebellum and brain stem, but abnormalities would also involve in other systems. Various types of SCAs overlapping one another in their clinical symptoms can only be carried out to their initial diagnosis, and confirmed diagnosis requires genetic analysis [3].

SCA3, known as Machado-Joseph disease (MJD) [4], is the most common SCA subtype among different races and origins in the world [5]. The related *ATXN3* gene locates at 14q24.3–q32.2 encodes a gene product of protein ataxin-3 consisting of 960 amino acid residues [6]. The CAG repeat of *ATXN3* is located in exon 4 and the normal allele carries 10 to 51 CAG repeat, while the mutant allele carries 55 ~ 87 repeats [7]. A PCR method with specific primers visualized by agarose gel electrophoresis was previously reported to to detect the abnormal expansion of CAG repeats of *ATXN3* of patient [4]. Although it worked, however, this reported assay was found to be inefficient and even yield false negatives when employed in our laboratory in screening SCA3 patients. Here we report an improved method with new designed primers and "Touchdown" PCR program, which achieved high efficiency and specificity of amplification of the CAG repeats in the *ATXN3* gene. This new primer set and amplification method could provide reliable, quick and unambiguous genetic analysis in a clinical laboratory for the diagnosis of spinocerebellar ataxia type.

The genomic DNA was extracted from the blood samples using the UNIQ-10 genomic extraction kit from Sangon (Shanghai, China). Blood samples from clinically diagnosed SCA3 patients and their family members were collected in the First Affiliated Hospital of Soochow University in Suzhou, China. This study was carried out following the Research Ethics Board approval of the First Affiliated Hospital of Soochow University.

Three primers were used, including one forward primer: SCA3F: 5'–CCAGTGACTACTTTGATTCG–3'; two reverse primers: SCA3R1: 5'–TGGCCTTTC ACATGGATGTGA A–3', and SCA3R2: 5'–CATGATGAATGGTGAGCAGG–3'. SCA3F and SCA3R1 were reported previously. All primers are synthesized by the Sangon. PCR reaction was carried out in a 20 μl mixture, containing 80 ng of genomic DNA, 200 nM of forward and reverse primers, 200 μM each dNTP (dATP, dCTP, dGTP and dTTP), 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KC1 and 1 unit of *Taq* DNA polymerase (Sangon, Shanghai). "Regular PCR" was carried out by 50 cycles of denaturing at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extending at 72°C for 40 seconds. "Touchdown PCR" consisted of phase I and phase II. In phase I, the PCR reaction was carried out by pre-denaturing at 94°C for 5 minutes followed by 36 cycles of denaturing at 94°C for 30 s, annealing temperature at initial 70°C with 1°C decrease every two cycles for 30 s and extending at 72°C for 40 s. In phase II: the PCR reaction was performed by 15 cycles of denaturing at 94°C for 30 s,

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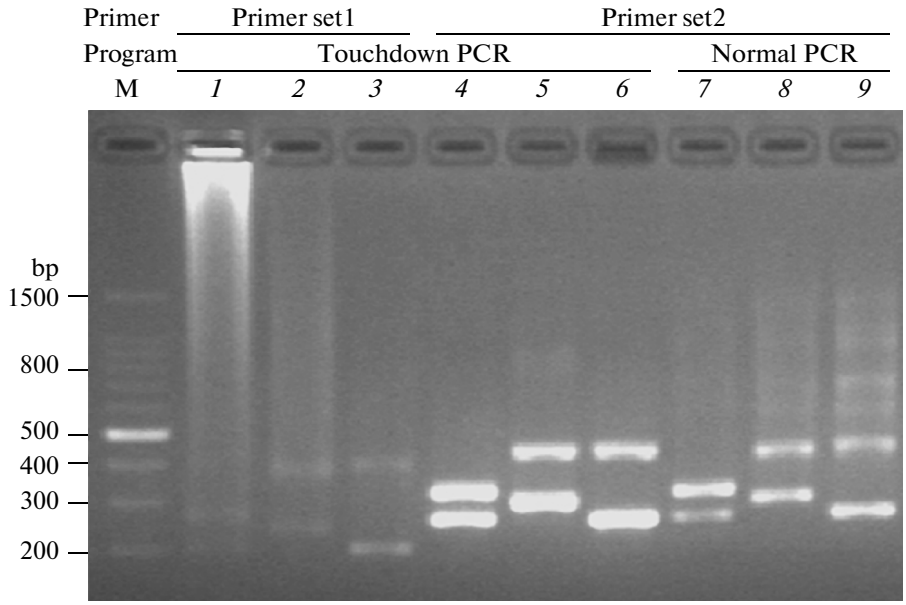

Fig. 1. Comparison of the CAG repeats amplification with different primers and PCR methods. Lanes *1, 4* and *7*: normal control; lanes *2, 5* and *8*: patient 1; lanes *3, 6* and *9*: patient 2.

annealing at 54°C for 30 s and extending at 72°C for 40 s. Four μl of PCR products were loaded and run on the 2% agarose gel stained with 0.5 μg/ml ethidium bromide. The amplified PCR products separated by the agarose gel were cut out and purified with AxyPreP™ DNA Gel Extraction Kit from AXYGEN (Union, USA). The purified DNA fragments were directly sequenced (Invitrogen, Shanghai).

The efficiency and specificity between reported primer pair, primer setl (SCA3F and SCA3R1) and the new primer pair, primer set2 (SCA3F and SCA3R2) using different PCR program were evaluated by amplifying DNA samples from a normal control and two clinically diagnosed patients. Under the "regular PCR" condition, while primer setl produced faint bands on agarose gel, primer set2 produced bright bands corresponding to normal and mutant allele of *ATXN3* (Fig. 1, lanes *7*, *8* and *9*). PCR assay using the primer setl also amplified some non specific products (Fig. 1, lanes *9*). Those nonspecific bands sometimes caused incorrect call for mutant allele.

Two strategies were applied in the optimization of the PCR assay for the CAG repeat expansion in the present study: PCR primer selection and amplification protocol modification. The combinatory effect of a new PCR primer set 2 and the touchdown protocol significantly improved the assay's specificity and efficiency. These results were confirmed by DNA sequencing analysis (Fig. 2). Direct sequencing of both PCR products of normal control (Fig. 1, lane *4*) and mutant allele of patient (Fig. 1, lane *5*) showed the both allele of normal control with 8 and 1 27 CAG repeats (data not shown) and the mutant allele with 66 CAG repeats (Fig. 3).

The newly developed PCR assay was used in the screening of the CAG repeats of *ATXN3* gene in a family of a genetically diagnosed SCA3 patient (Fig. 3). Direct sequencing has showed that the patient carries an abnormal expansion with 66 CAG repeats of *ATXN3*. With the use of this newly developed PCR assay, four more family members were identified as they showed similar abnormal expansion of the CAG repeats (Fig. 2, lane *4, 7, 8* and *9*) to the previously diagnosed patient (Fig. 2, lane *10*). All the relevant bands were cut out for sequencing analysis (data not shown). The larger bands from lanes *4, 7, 8* and *9* had the same CAG repeats as the patient (Fig. 2, lane *10*). The mutant allele carriers are all second generation of the patient. Due to their young age, they have not shown ataxia manifestation by the time of this genetic testing.

Genetic testing is mandatory in the confirmation of SCA3. The commonly used methods are direct sequencing, and PCR fragment sizing with capillary electrophoresis or agarose gel. Albeit the resolution of agarose gel electrophoresis is relatively low, it is usually sensitive enough to distinguish PCR fragments with 10 nucleotides when 2–2.5% gel is used. The present study described a simple PCR method with agarose electrophoresis in detecting abnormal CAG repeats of *ATXN3*. This new PCR assay is especially useful for the purpose of large scale screening. In case of marginal expansion of the CAG repeats with 3–5 triplets longer than normal, the suspected PCR amplified product could be better sized by running between known size of markers with specific number of CAG repeats, and direct sequencing could be an optional choice whenever needed in rare situations.

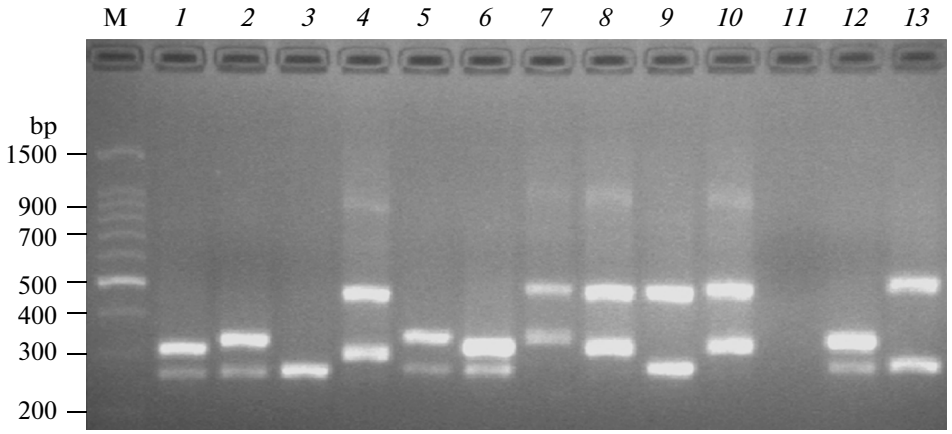

Fig. 2. Genetic analysis of a SCA3 patient family using primer set2 with "Touchdown" PCR program. Lanes *1–10* are the amplified products from members of the testing family, while the lane *10* is from the proband, lane *11* is the blank control, the lane *12* is from a normal sample and the lane *13* is positive control from a previously confirmed SCA3 patient not related to the testing family.

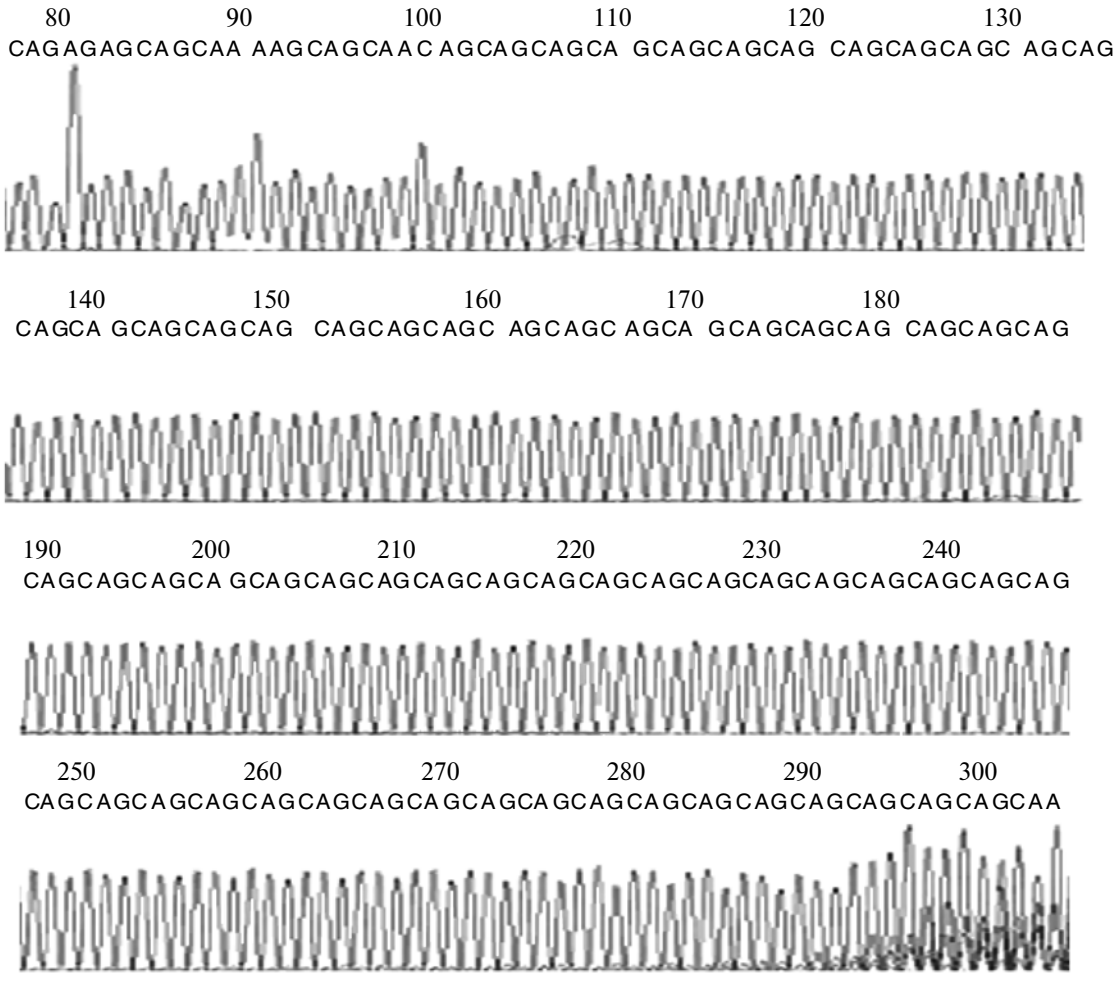

Fig. 3. Sequencing data of a patient's DNA sample. There 66 CAG repeats found in the patient diagnosed by the new PCR assay presented in the present paper.

In summary, with the combination of optimized primer set used and modified amplification protocol of touchdown PCR, a simple, quick and reliable PCR assay for detecting abnormal CAG repeats in *ATXN3* gene has been developed. This assay provides a genetic test for the diagnosis of spinocerebellar ataxia type 3. Furthermore, the significant improvement of the new PCR assay over previous reported assay suggests that the strategies used in this study may find applications in situations when GC rich fragments are difficult or failed in amplification.

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