Application of Created Restriction Site PCR-RFLP to Identify *POT1* Gene Polymorphism

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Abstract—Protection of telomeres protein 1 (POT1) plays pivotal roles in protection of chromosome ends and regulation of telomere length with other telomere binding proteins; its genetic polymorphisms are associated with many diseases. In this study, we explored a novel PCR-RFLP method for typing the single nucleotide polymorphism (SNP) rs1034794 of the human *POT1* gene. A new restriction enzyme site was introduced into a *POT1* gene amplification product by created restriction site PCR (CRS-PCR). One primer was designed based on changed sequence; after PCR amplification, a new restriction enzyme site for AluI was introduced into the PCR products. One hundred and seventy eight samples from Han Chinese individuals were tested to evaluate this new method. The 3′-end of the forward primer was next to the polymorphic site, and the third base from the 3′-end was the mismatched base A. The final PCR product contained the AGCT sequence (AluI recognition site) when the ancestral *POT1* alleles were amplified. The data obtained with the new method perfectly matched those obtained with the sequencing method. Thus, CRS-PCR is a new low-cost and high-efficiency alternative for rs1034794 typing.

Telomere-related proteins play an important role in maintaining the integrity of telomeric structure through protecting chromosome ends [1]. Protection of telomeres protein 1 (POT1) has been shown to be involved in telomere elongation and to bind telomerase [2]. These proteins are able to bind the G-rich domains in the telomeric repeat sequences to protect chromosome ends [3-5]. Recently, some studies regarding *POT1* gene polymor-

phism have been published. Interestingly, certain types of polymorphism have been linked to diseases [6, 7].

PCR-RFLP (PCR restriction fragment length polymorphism) is a commonly used method for detecting polymorphisms. This method is simple, fast, and effective. Many restriction enzymes are commercially available and inexpensive. However, some enzymes are very expensive, which limits their application for large-scale sample screening. For instance, such is the restriction enzyme HpyCH4V that could be used for *POT1* gene rs1034794 typing. To circumvent this problem, we introduced a new restriction site into *POT1* amplification products by use of a mismatched primer and examined the possibility of the application of this method for a large-scale screening in the Han Chinese population.

Abbreviations: CRS-PCR, created restriction site PCR; PCR-RFLP, PCR restriction fragment length polymorphism; POT1, protection of telomeres protein 1; SNP, single nucleotide polymorphism.
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MATERIALS AND METHODS

Gene information. The *POT1* gene and rs1034794 single nucleotide polymorphism (SNP) data were obtained from the NCBI website (http://www.ncbi.nlm. nih.gov). The sequence analysis shows that the sequence TGCA in polymorphism site rs1034794 can be cleaved by expensive restriction enzyme HpyCH4V. To find a lower cost PCR-RFLP method, a new AluI enzyme site was created to detect the *POT1* genotypes.

Primer design. The primers designed with Primer Premier 5.0 software are shown below. Forward: 5′-TTCAAACTTCAGGATCA<u>A</u>GC-3′; reverse: 5′-ATCA-TAGATGGAATAGACC-3′. In the forward primer sequence, the underlined capital A is the mismatch base, which replaces the natural base T. The strategies of base conversion are shown on the Fig. 1. The designed primers and corresponding amplification sequence are given below:

TTCAAACTTCAGGATCA<u>A</u>GC(5′-3′)

TTCAAACTTCAGGATCA<u>A</u>GC*W*actatcttaagaaaattgtcc tgctttgttgtcattttaacctcttttggtgagtagtttagttcatttggacataaatc- taaaatttatgtttgaactgcttgaatcttatagatcatcttcttgtttttgctcta

GGTCTATTCCATCTATGAT

CCAGATAAGGTAGATACTA(3′-5′).

In the sequence, the capital bases A, T, C, and G represent the same sequences as primers, and *W* indicates polymorphic locus A/T. Underlined is an artificially introduced A residue in the DNA sequence to produce a recognition site AGCT for restriction endonuclease AluI in the ancestral genotype allele *POT1*-AA (A − in the reverse *POT1* gene encoding strand). The price of AluI is given in the table. As can be seen from the table, the price of AluI is nine times less than that of HpyCH4V.

Genotyping of DNA. Genomic DNA was extracted from peripheral blood leukocytes using genomic DNA isolation kit (Bio Teke Corporation, China). PCR reactions were performed in a final volume of 15 µl contain-

Recognition sites and prices of respective endonucleases in NEB Co.

Endonuclease	Recognition sequence	$/500 units
HpyCH4V	TGCA	483
AluI	AGCT	55

ing 100 ng of genomic DNA, 0.3 µM of each primer (Sangon Biotech, China), 2× PCR mix (Lifefeng Biotech, China), and double-distilled water as follows: at 95°C for 3 min; at 95°C − 52°C − 72°C for 30 s each, 30 cycles; and then at 72°C for 5 min.

RFLP conditions. Enzyme digestion was carried out in a 20 µl final volume reaction mix consisting of 5 units (U) of AluI enzyme (New England Biolabs, USA), 2 µl 10× Buffer Tango, 7.5 µl double-distilled H_2O, and 10 µl of PCR reaction mixture. The reaction was performed at 37°C overnight, and the digested products were electrophoresed on 3% agarose gel containing ethidium bromide (0.5 µg/ml) for 40 min. Agarose gels were observed under an UV transilluminator.

DNA sequencing. PCR products were sequenced using the reverse PCR primer provided by Sangon Biotech Company (China).

RESULTS

Enzyme digestion results. As can be seen in Fig. 2, the size of undigested PCR product is 177 bp as expected (lane *1*). After digestion with AluI, the PCR product from homozygous alternative variant genotype (*POT1*-TT) allele could not be cut (lane *4*). However, the homozygous ancestral genotype (*POT1*-AA) allele was cut into bands of 158 and 19 bp (lane *2*), and the heterozygous genotype yielded three bands of 177, 158, and 19 bp (lane *3*).

Consistency of the results. Polymorphisms of *POT1* were typed in 178 Chinese Han individuals. The genotype frequencies were 116 for AA, 56 for AT, and six for TT. We

HpyCH4V
↓
5′ ···AGGATCA|TGC *W*| ACTATCTTAA··· 3′   →   5′ ···AGGATCA|AGC *W*| ACTATCTTAA···3′
3′ ···TCCTAGTACG *W* TGATAGAATT··· 5′   3′ ···TCCTAGTACG *W* TGATAGAATT···5′
AluI
↓
W=A/T,
|TGCA| cut by restriction enzyme HpyCH4V converted into |AGCT| cut by restriction enzyme AluI
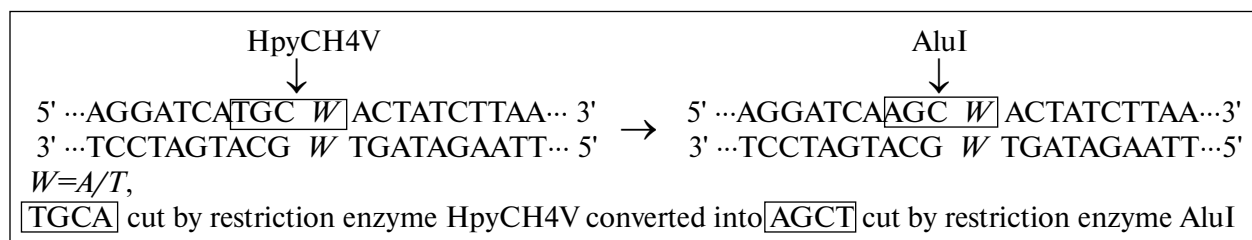
Fig. 1. Strategies of base conversion. In this base conversion strategy, W (A/T) was the polymorphic site, and the natural T base is replaced by mismatched A base. The recognition site TGCA cut by restriction enzyme HpyCH4V is converted into recognition site AGCT cut by restriction enzyme AluI.
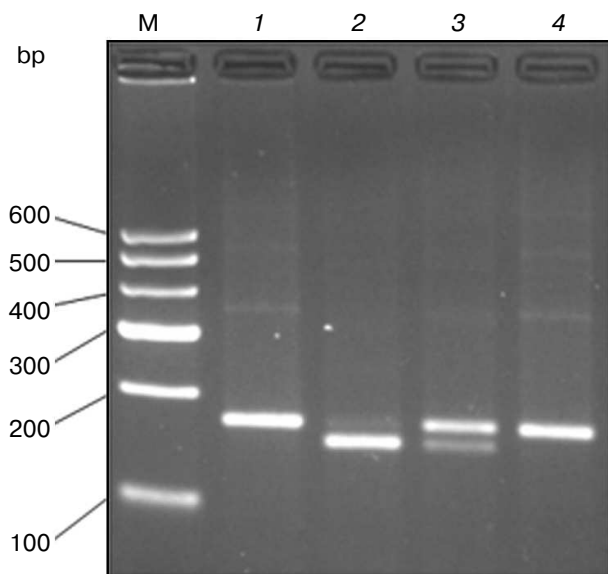
Fig. 2. PCR-RFLP for *POT1* separated on a 3% agarose gel. The 177 bp *POT1* product was digested with AluI, which does not cut the wild-type sequence but cleaves the T sequence into two smaller fragments of 158 and 19 bp, respectively. Lanes: *1*) PCR product; *2*) homozygous ancestral genotype (*POT1*-AA); *3*) heterozygous genotype (*POT1*-AT); *4*) homozygous alternative variant genotype (*POT1*-TT); M, molecular weight markers.

Fig. 3. Sequencing results of three rs1034794 SNP genotypes of *POT1* gene. The T underlined is the mismatched base corresponding to the A base in the forward primer. The T base in-frame in Fig. 2a corresponds to *POT1*-AA genotype, and A base in-frame in Fig. 2c corresponds to *POT1*-TT genotype, respectively. The W in-frame in Fig. 2b corresponds to *POT1*-AT genotype.

also genotyped the 178 PCR products by Sanger sequencing, which is the gold standard for genotyping. The genotypes of the 178 samples obtained with the new method matched the gene sequencing data completely (the DNA sequencing results of the *POT1* gene are shown in Fig. 3). Since we used the reverse primer to sequence the PCR products, the sequence from the sequencing map was the complementary sequence of PCR products. In Fig. 3, the underlined T was the mismatched base, which was consistent with mismatched A base in the forward primer; the T base in frame in Fig. 3a corresponds to *POT1*-AA genotype, and A base in frame in Fig. 3c corresponds to *POT1*-TT genotype. The W in frame in Fig. 3b surely corresponds to *POT1*-AT genotype. With the exception of the mismatched base, the sequences of the PCR products were consistent with those published in GenBank.

DISCUSSION

PCR-RFLP method has been widely used since 1988 [8]. It proved to be a rapid and sensitive method for detection of gene polymorphisms [9]. However, this method has some limitations, for instance, some restriction enzymes, such as HpyCH4V or its isoschizomers, are very expensive. This circumstance has hindered applicatio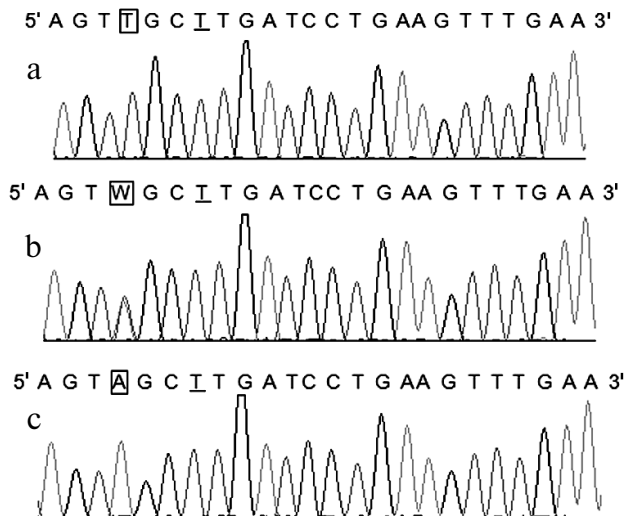n of the method, especially in developing countries. In this study, we introduced a new restriction enzyme site for AluI to type the *POT1* polymorphism rs1034794. Restriction enzyme AluI is much less expensive than HpyCH4V; $428 would be saved when 500 U of enzyme is used to screen 100 samples. Besides, the new method proved quite reliable compared with sequencing.

In conclusion, here we report a simple and economical technique for typing of the *POT1* gene polymorphism rs1034794, which allows for large-scale sample analyses.

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