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BRIEF REPORT

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Detecting the polymorphism of *TERF1* gene by an improved PCR-RFLP method

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Background: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a common and mature method of detecting the single nucleotide polymorphism (SNP). But, for the polymorphism site rs3863242 of telomeric repeat binding factor 1(*TERF1*) gene, there is no appropriate restriction enzyme to recognize it, which limits the research between the variants of rs3863242 and human diseases.

Methods: The reverse primer was designed based on turning the 3rd base T into the mismatch base G. After PCR amplification, a new restriction enzyme site was introduced into the *TERF1* gene amplification products. Two hundred forty samples from Chinese Han individuals were genotyped to evaluate this method.

Results: A new restriction enzyme site for *Cvi*QI was introduced into the PCR products. The genotype frequencies of 240 samples from Chinese Han individuals were 4.17% for A/A, 29.58% for A/G, 66.25% for G/G respectively. The allele frequencies were 18.96% for A and 81.04% for G respectively. The genotyping results of PCR products were consistent with the gene sequencing result.

Conclusions: We developed a simple, direct and economical technique for analyzing the polymorphism of *TERF1* rs3863242. It may be applied to the colony screening of other SNPs, mutation-screening of tumor-related gene or mutations in some specific genes on a large scale, in the future.

KEYWORDS
PCR-RFLP, rs3863242, Single nucleotide polymorphism, *TERF1*

1 | INTRODUCTION

Telomere is a DNA-protein complexes located in the terminus of eukaryotic chromosome, comprising with repetitive DNA sequence and shelterin.[1] And the shelterin is composed by six subunit protein complex, including TERF1, TERF2, POT1, TPP1, TIN2, and Rap1.[2] TERF1 (Telomeric repeat binding factor 1), one important telomeric DNA-binding protein of the shelterin,[3] plays crucial roles in telomere protection,[4] the resolution of sister telomeres and the alteration of telomere length.[5-7] Related studies suggested that the *TERF1* gene polymorphism is implicated in some diseases, such as: osteosarcoma,[8] aplastic anemia[9] and so forth.[10] And the polymorphism of rs3863242 in *TERF1* showed the association with T2D risk.[11]

The genotyping of SNP could be detected by about four types of approaches: primer extension, ligation amplification, hybridization, and enzyme cleavage. The primer extension is based on common primer extension and could detect multiple SNPs, which needs the expensive instrument and apparatus for discrimination, such as: matrix assisted

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laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The specificity of ligase enzymes is required in the ligation amplification to achieve genotyping. The method of hybridization is according to the differences in thermal stability of double-stranded DNA to distinguish genotyping,[12] which also relies on the expensive instrument and apparatus or GeneChip® array technology.[13] The approach of enzyme cleavage, for instance: PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) is used to detect the genotypes commonly.[14]

Although the PCR-RFLP is a simple, direct and economical technology to detect polymorphisms, it is not perfect. For the SNP site rs3863242, there is no sites recognized by restriction enzymes available now, thus it could not be detected by PCR-RFLP. In order to circumvent this problem, we introduced a new restriction site into *TERF1* amplification products by mismatching primer and verified the possibility of colony screening polymorphism with this technique on a large scale in the Han nationality. The technique is based on distinguishing restriction sites which are artificially created by PCR site-directed mutagenesis of allele specific site.[15,16]

The "*TERF1* rs3863242" information was searched from NCBI official website (http://www.ncbi.nlm.nih.gov). The sequence analysis by NEB cutter program (http://nc2.neb.com/NEBcutter2/) showed that no restriction enzymes of New England Biolabs can distinguish the single nucleotide polymorphism site (rs3863242) in the *TERF1* gene. Therefore, in order to detect the *TERF1* rs38636242 genotypes, the restriction enzyme site of *Cvi*QI was designed by mismatching primer.

A pair of primers was designed with the primer premier 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). And the PCR primer sequences for *TERF1* rs3863242 were as below: forward primer 5′-AATGCTTAGTTTCTTAAAGACGGAAGAC-3′ and reverse primer 5′-CAGTAATAATAATGACCCCTAAAAATG***G***TA-3′. The bold italic letter with underline was the mismatched base which replaced natural base T. The mechanism that created *Cvi*QI restriction enzyme site by mismatching primer was demonstrated in Figure 1A. The corresponding amplification sequence was as follows: AATGCTTAGTTTCTTAAAGACGGAAGACttctctaaacatttcttagatttttaaaagattttacatttatc***R***TACCATTTTTAGGGGTCATTATTATTACTG.

Genomic DNA was extracted from leukocytes in human peripheral blood using genomic DNA isolation kit (Bio Teke Corporation, Beijing, China). And then, the PCR was performed in a final volume of 15 μL, containing 100 ng of genomic DNA, 0.3 μmol/L each

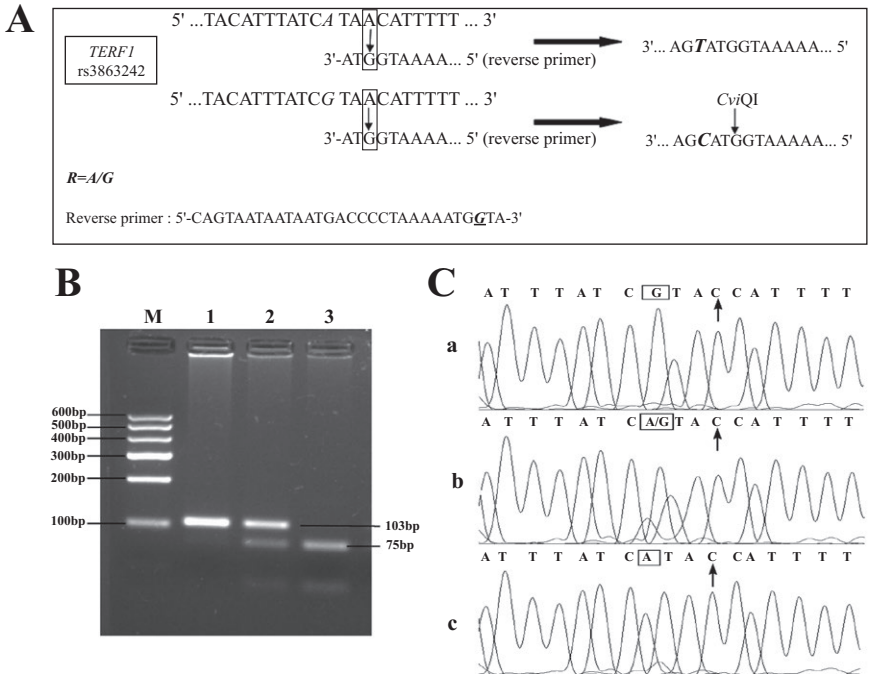

FIGURE 1 (A) The mechanism of creating *Cvi*QI restriction enzyme site by mismatching primer. The polymorphism bases *R* was shown with the letter "***A***" and "***G***" in bold italic. The way of mismatching bases were shown in frames. The PCR product was shown on the right. The recognition site GTA***T*** with wave line could not be cut by restriction enzyme *Cvi*QI, but GTA***C*** could. The ***G*** base in bold italic with underline represented the mismatching base of reverse primer. (B) PCR-RFLP for *TERF1* rs3863242 polymorphism by 3% agarose gel. After digestion with *Cvi*QI, the PCR products with homozygous A/A yielded one uncut band (103 bp), homozygous G/G yielded two bands of 75 bp and 28 bp, and the heterozygous A/G allelic variations yielded three bands of 103 bp, 75 bp and 28 bp. The length of the digestion products were shown on the right of the figure. Genotype analysis (from lane 1 to lane 3), Lane1: *TERF1*-A/A; Lane2: *TERF1*-A/G; Lane3: *TERF1*-G/G. Lane M: molecular weight markers. (C) DNA sequences of the *TERF1* PCR products. The C base with vertical arrows corresponds to the mismatched base of reverse primer. The polymorphic site (A/G) was marked with frames. The G base in frame of C1: corresponds to the genotype "G/G"; The A and G bases in frame of C2: correspond to the genotype "A/G" and the A base in frame of C3: corresponds to the genotype "A/A".

primer (Sangon Biotech, Shanghai, China), 2×PCR Mix (Lifefeng Biotech, Shanghai, China) and adjusted with double-distilled water to 15 μL/reaction. The PCR thermal cycles: lid temperature 96°C; Pre-degeneration at 95°C for 5 minutes; 35 cycles of 30 seconds at 95°C, 30 seconds at 53.1°C and 30 seconds at 72°C for 30 seconds each, postcycle at 72°C for 5 minutes and at 4°C ended. The PCR products were separated by electrophoresis on the 3% agarose gel stained with EB (ethidium bromide) for 30 minutes, and then investigated the results under the "Gel Doc 2000" UV transilluminator (Bio-Rad, California, CA, USA).

Enzyme digestion was conducted in a 20 μL final volume and consisted of 5 U of *Cvi*QI enzyme (New England Biolab, Cambridge, UK) and 10 μL of PCR product. The reaction was performed overnight at 37°C and the digested products were separated by electrophoresis on the 3% agarose gel containing EB for 40 min, and then observed under the UV transilluminator.

Based on the created restriction enzyme site theory, the *TERF1* gene polymorphism could be identified directly. As shown in Figure 1B, the PCR products were separated by electrophoresis on the 3% agarose gel. Apparently, the PCR amplified the fragment of 103 bp. After incubation at 37°C with *Cvi*QI, the PCR products with homozygous A/A was not cut and yielded one band (103 bp), homozygous G/G was cleaved into two bands of 75 bp and 28 bp, and the heterozygous A/G allelic variations yielded three bands of 103 bp, 75 bp, and 28 bp.

The polymorphism of *TERF1* rs3863242 in 240 Chinese Han individuals were genotyped by the above method. The genotype frequencies of homozygous A/A, G/G or heterozygous A/G allelic variations were 4.17%, 66.25%, and 29.58%. The allele frequencies were 18.96% for A and 81.04% for G. The Genotype distribution for the *TERF1* polymorphism conformed to the Hardy–Weinberg equilibrium expectation ($\chi^2 = 0.333$, $P > .05$). To verify the accuracy of our method, we compared the result of enzyme digestion with DNA sequencing (Sangon Biotech, Shanghai, China). The results of DNA sequencing were shown in the Figure 1C. Three figures (from 1 to 3) represent the genotype of *TERF1*-G/G, *TERF1*-A/G, and *TERF1*-A/A respectively. The genotyping results of PCR products proved to be consistent completely with the method of gene sequencing.

Polymerase chain reaction-restriction fragment length polymorphism, as a rapid and sensitive method has been applied in detecting the gene polymorphism since 1988.[17] However, there are still some limitations. For the SNP rs3863242 in *TERF1* locus, there are no specific recognition sites for restriction enzymes. In this study, a new PCR-RFLP assay was created by mismatching the primer to detect the SNP rs3863242 in *TERF1* locus. As a result, the DNA amplification carried out successfully though without a perfect primer matched, and the polymorphisms of *TERF1* were genotyped accurately.

In conclusion, we developed a precise, direct and economical technique for analyzing the polymorphism of *TERF1* rs3863242 accurately. It could be applied to the colony screening of other SNPs, mutation-screening of tumor-related gene or mutations in some specific genes et al., in the future.

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COMPLIANCE WITH ETHICAL STANDARDS

All authors have declared that they have no potential conflicts of interest about this article. The research work was approved by Institutional Ethics Committee of Zhengzhou University. All participants provided written informed consent.

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