An improved polymerase chain reaction-restriction fragment length polymorphism assay for the detection of a *PON2* gene polymorphism

XIAORAN DUAN[1], YONGLI YANG[2], TUANWEI WANG[1], XIAOLEI FENG[1], WU YAO[1], ZHEN YAN[1] and WEI WANG[1]

Departments of [1]Occupational and Environmental Health, and [2]Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, Zhengzhou, Henan 450001, P.R. China

Received February 2, 2016; Accepted April 27, 2016

DOI: 10.3892/br.2016.676

Abstract. In recent research, it has been shown that there have been variants of rs12026 within the paraoxonase 2 (*PON2*) gene, which have been associated with cardiovascular disease, cerebrovascular disease, diabetes and other diseases. The isochizomers, such as the *Bso*FI enzyme, required for the detection of this polymorphism are expensive. Therefore, an improved and less expensive polymerase chain reaction (PCR)-restriction fragment length polymorphism method was established for the detection of the single-nucleotide polymorphism rs12026 in the exon 5 of chromosome 7 of the human *PON2* gene using the method of amplification-created restriction site. Subsequent to assessing 302 individuals, the genotype frequencies were 68.9% for CC, 29.8% for CG and 1.3% for GG, and the allelic frequencies were 83.8% for C and 16.2% for G. The PCR results were confirmed by DNA sequencing. The χ^2 test showed that the genotype and allele frequencies of *PON2*-148 do not deviate from Hardy-Weinberg equilibrium, and the sequences of amplified products were consistent with the sequence published in GenBank with the exception of a mismatched base.

Introduction

Paraoxonase 2 (*PON2*) is a member of the *PON* gene family located on chromosome 7q21.3, which also includes the homologous genes for *PON1* and *PON3* (1,2). In contrast to *PON1*, which is mainly expressed in the liver, *PON2* is expressed in a variety of tissues. It is known that *PON1* has been shown to have antioxidant properties (3). However, the function of ubiquitously expressed *PON2* remains to be elucidated (1).

A G/C single-nucleotide polymorphism (SNP) rs12026 in exon 5 of the *PON2* gene introduces the coding variant of alanine/glycine at position 148 (*PON2*-148). There have been associating variants of rs12026 within the *PON2* gene with cardiovascular disease (4-6), cerebrovascular disease (7), diabetes (8,9) and other diseases (10). For this SNP detection, several assays have been suggested, such as the fluorescently labeled probes technique (11), dynamic allele-specific hybridization technique (7), TaqMan assay (5) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (6). Among them, PCR-RFLP is the most frequently used technique due to its high sensitivity and reliability. However, the current PCR-RFLP assay is facing practical challenges and is not suitable for high throughput screening. The present study demonstrated an improved PCR-RFLP assay for the detection of a polymorphism of the *PON2* gene.

Materials and methods

Primer design. The information regarding the *PON2* gene and polymorphism rs12026 was obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The method of the amplification-created restriction site was used to introduce a new enzyme site to recognize the variant.

The sequences of the primer pair were designed by primer premier 5.0 software as follows: Forward, 5' AGGTCCCGT AGTTATGTCTTGT 3'; and reverse, 5' TCAGATGCA ACAGAGAATTGTCT 3'.

The natural occurring DNA sequences according with positions of primers were: AGGTCCCGTAGTTATGTCTTGT aaattaactctgttctttcaattctttagatgacacagtttatctctttgttgtaaaccaccc agaattcaagaatacagtggaaatttttaaatttgaagaag*S*AGA<u>A</u>AATTCT CTGTTGCATCTGA. Capital bases A, C, G and T represent the sequences according to the positions of primers, and *S* represents the position for the polymorphism G/C. The length of the base sequences was 150 base pairs (bp). The natural

Correspondence to: Professor Wei Wang, Department of Occupational and Environmental Health, College of Public Health, Zhengzhou University, 100 Kexue Road, Zhengzhou, Henan 450001, P.R. China
E-mail: ww375@126.com

Key words: *PON2*, polymorphism, polymerase chain reaction-restriction fragment length polymorphism, *Bsm*AI enzyme, rs12026

occurring 'T' nucleotide that matches with A (underlined) in the DNA sequences was substituted by a 'G' in the reverse primer (underlined) in order to introduce a recognized site for the *Bsm*AI restriction endonuclease.

Genotype of DNA. Genomic DNA was extracted from the peripheral blood leukocytes of 302 Chinese Han individuals followed by standard procedures. The research objects were obtained from one enterprise in Zhengzhou in 2014. The study protocol was approved by the Ethics Committee of the University of Zhengzhou (Zhengzhou, Henan, China). The PCR was performed in a volume of 25 μl containing 100 ng of genomic DNA, 1X PCR Master mix (Tiangen, Beijing, China) and 5 pmol of each primer. The DNA was denatured at 95˚C for 3 min, and temperature cycles were set at 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 30 sec for 30 cycles, followed by a final extension at 72˚C for 10 min. PCR products underwent electrophoresis on 3% agarose gels and were stained with ethidium bromide and exposed to ultraviolet illumination.

Enzyme digestion was conducted in a 20 μl final volume using 5 units of *Bsm*AI enzyme (New England Biolabs, Ipswich, MA, USA) and 10 μl of the PCR product. The reaction was conducted at 37˚C overnight. The digested products were visualized on 3% agarose gels stained with ethidium bromide.

Statistical analysis. The basic data were analyzed using SPSS 21.0 software (IBM, Corp., Armonk, NY, USA). Methods of representation and examination were based on the distribution of quantitative data. All the statistical tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Polymorphisms of PON2. Based on the PCR designed with the mismatched primers, the *PON2* polymorphisms could be identified simultaneously. The PCR products that were separated on 3% agarose gel are shown in Fig. 1 (*Bsm*AI PCR-RFLP analysis of the *PON2* biallelic polymorphism). From the gel, that the PCR amplification yielded a product of 150 bp. Following incubation with *Bsm*AI, the individual homozygous for the C allele yielded one uncut band (150 bp). The homozygous G allele yielded two bands of 121 and 29 bp.

Polymorphisms of *PON2* were detected in 302 Chinese Han individuals. The genotype frequencies were 68.9% for CC, 29.8% for CG and 1.3% for GG. The allelic frequencies were 83.8% for C and 16.2% for G. The PCR results were confirmed by DNA sequencing. The χ^2 test showed that the genotype and allele frequencies of *PON2*-148 do not deviate from Hardy-Weinberg equilibrium ($\chi^2=2.80$, $P>0.05$).

Examples of DNA sequencing of the PCR product of the *PON2* gene are shown in Fig. 2. As expected, the sequences of the amplified products were consistent with the one published in Genbank (NM_000446), with the exception of the mismatched base.

The *Bsm*AI recognition site (GAGAC) is indicated by a marked symbol in Fig. 2A and C, and the vertical arrow corresponds to the mismatched base. The genotypes of GC, CC and GG are shown in Fig. 2, respectively, and the bases representing the polymorphism sites are denoted in squares.

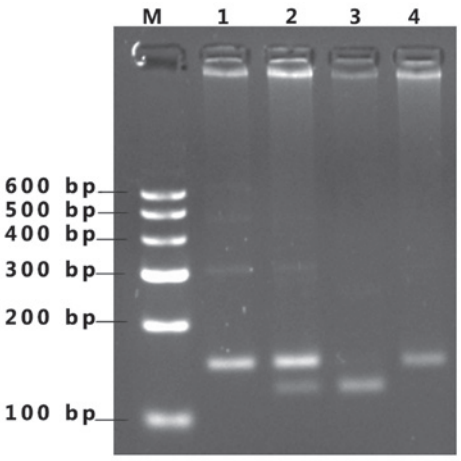

Figure 1. *Bsm*AI polymerase chain reaction-restriction fragment length polymorphism analysis of the *PON2* biallelic polymorphism, M, DNA ladder; lanes 1 and 4, CC; lane 2, CG; lane 3, GG. *PON2*, paraoxonase 2; bp, base pair.

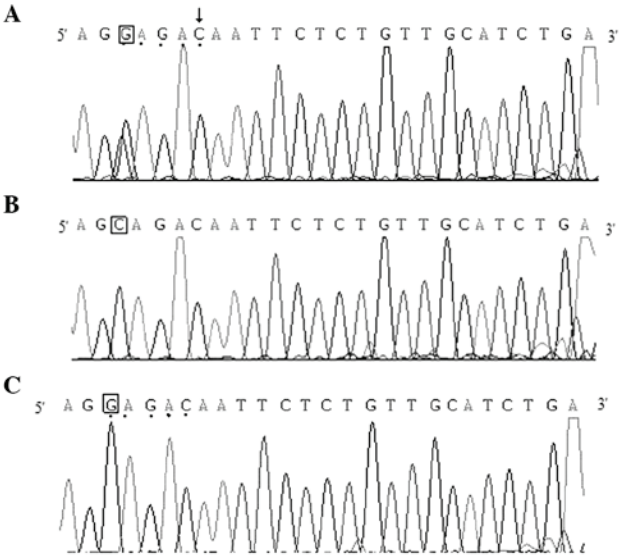

Figure 2. Examples of DNA sequencing of the polymerase chain reaction product of the *PON2* gene. (A and C) The *Bsm*AI recognition site (GAGAC) is indicated by a mark symbol, and the vertical arrow corresponds to the mismatched base. Three figures represent the genotype of (A) GC, (B) CC and (C) GG are shown, and the bases (G and C) representing the polymorphism sites are in the squares. *PON2*, paraoxonase 2.

Discussion

The commonly used detection methods for SNP analysis include DNA sequencing, TaqMan assay, fluorescently labeled probes, molecular beacons and PCR-RFLP. DNA sequencing is an accurate method for genotyping; however, it is labor-intensive and not suitable for high-throughput analysis. The most prominent advantage for the TaqMan probe technique is that it does not require a PCR post-treatment process, such as separation or elution, which improves the detection rate. To ensure accurate classification is difficult, as the probe must be designed and can be expensive (12). Molecular beacons are marked by fluorescent dyes of different colors to achieve the simultaneous detection of multiple SNPs. However, dyes are

less for the suitable fluorescent dye-labeled, which significantly limits the detection of flux. In addition, a fluorescent molecular beacon probe must be designed and these are expensive (13). The abovementioned methods do have their own advantages; however, the equipment used is expensive and the experiment methods are complex, therefore, they are difficult to popularize and apply for SNP detection. The PCR-RFLP technique is widely used in the detection of SNP as the detection method is simple, and it has a high sensitivity and reliability. However, certain assays can be employed to detect rs12026 polymorphism of PON2 using common equipment. The PCR-RFLP method has been used since 1997 (14), but the *Bso*FI enzyme or its isoschizomers required are expensive. In order to overcome this, PCR-RFLP with mismatched primers and the inexpensive *Hin*fI enzyme was developed to detect rs12026 (15). However, the polyacrylamide gel electrophoresis in this developed method was inconvenient compared with the agarose gel electrophoresis. The present study introduced the inexpensive *Bsm*AI enzyme and fast agarose gel electrophoresis to detect *PON2* polymorphism rs12026 using the PCR-RFLP method. In conclusion, a simple and economical technique for analysis of the *PON2* polymorphism rs12026 was reported. This may be used widely in the future study of the genetics of diseases, including cardiovascular disease, cerebrovascular disease and diabetes.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. NSFC81001239 and 30972457).

References

1. Mochizuki H, Scherer SW, Xi T, Nickle DC, Majer M, Huizenga JJ, Tsui LC and Prochazka M: Human PON2 gene at 7q21.3: Cloning, multiple mRNA forms, and missense polymorphisms in the coding sequence. Gene 213: 149-157, 1998.
2. Shiner M, Fuhrman B and Aviram M: A biphasic U-shape effect of cellular oxidative stress on the macrophage anti-oxidant paraoxonase 2 (PON2) enzymatic activity. Biochem Biophys Res Commun 349: 1094-1099, 2006.
3. Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM and Reddy ST: Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. J Biol Chem 276: 44444-44449, 2001.
4. Saeed M, Perwaiz Iqbal M, Yousuf FA, Perveen S, Shafiq M, Sajid J and Frossard PM: Interactions and associations of paraoxonase gene cluster polymorphisms with myocardial infarction in a Pakistani population. Clin Genet 71: 238-244, 2007.
5. Thameem F, He X, Voruganti VS, Nath SD, Fanti P, Blangero J, Maccluer JW, Comuzzie AG, Arar NH and Abboud HE: Evaluation of polymorphisms in paraoxonase 2 (PON2) gene and their association with cardiovascular-renal disease risk in Mexican Americans. Kidney Blood Press Res 32: 200-204, 2009.
6. Chi DS, Ling WH, Ma J, Xia M, Hou MJ, Wang Q, Zhu HL, Tang ZH and Yu XP: Relationship between paraoxonase 1 55 Met/Leu, paraoxonase 2 148 Ala/Gly genetic polymorphisms and coronary artery disease. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 23: 289-293, 2006 (In Chinese).
7. Pasdar A, Ross-Adams H, Cumming A, Cheung J, Whalley L, St Clair D and MacLeod MJ: Paraoxonase gene polymorphisms and haplotype analysis in a stroke population. BMC Med Genet 7: 28, 2006.
8. Calle R, McCarthy MI, Banerjee P, Zeggini E, Cull CA, Thorne KI, Wiltshire S, Terra S, Meyer D, Richmond J, *et al*: Paraoxonase 2 (PON2) polymorphisms and development of renal dysfunction in type 2 diabetes: UKPDS 76. Diabetologia 49: 2892-2899, 2006.
9. Pinizzotto M, Castillo E, Fiaux M, Temler E, Gaillard RC and Ruiz J: Paraoxonase2 polymorphisms are associated with nephropathy in Type II diabetes. Diabetologia 44: 104-107, 2001.
10. Chen D, Hu Y, Chen C, Yang F, Fang Z, Wang L and Li J: Polymorphisms of the paraoxonase gene and risk of preterm delivery. Epidemiology 15: 466-470, 2004.
11. Shin BS: Paraoxonase gene polymorphism in south-western Korean population. J Korean Med Sci 24: 561-566, 2009.
12. de Kok JB, Wiegerinck ET, Giesendorf BA and Swinkels DW: Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). Hum Mutat 19: 554-559, 2002.
13. Steemers FJ, Ferguson JA and Walt DR: Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. Nat Biotechnol 18: 91-94, 2000.
14. Hegele RA, Connelly PW, Scherer SW, Hanley AJ, Harris SB, Tsui LC and Zinman B: Paraoxonase-2 gene (PON2) G148 variant associated with elevated fasting plasma glucose in noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 82: 3373-3377, 1997.
15. Zhang Y, Zheng F, Du H, Krepinsky JC, Segbo JA and Zhou X: Detecting the polymorphisms of paraoxonase (PON) cluster in Chinese Han population based on a rapid method. Clin Chim Acta 365: 98-103, 2006.