

Discordance in the paraoxonase (Pon1)-192QR polymorphism

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Abstract

Paraoxonase (PON1) has been implicated to have a cardioprotective role, due to its physical attachment with high-density lipoprotein. *PON1*_{192QR} is a variation of the *PON1* gene, the *R* allele being a risk factor for cardiovascular disease. Kinetic studies resulting in a plot of paraoxon versus diazoxon hydrolysis rates may be used to accurately predict *PON1*₁₉₂ genotype. In this study, paraoxonase and diazoxonase activities in plasma were measured spectrophotometrically using plasma while PCR-based *PON1*₁₉₂ genotyping was performed using polymerase chain reaction followed by restriction digestion. The two-substrate assay-derived genotypes were cross-referred with those determined by PCR-based genotyping. When results did not concur, sequencing of the 99-bp region spanning codon 192 of *PON1* was performed to verify the genotype. Concordant samples with high or low activities were sequenced for comparison. In addition, the rare *PON1*_{194WX} polymorphism was examined as a source of discordance. The frequency of the *PON1*_{192Q} allele in a Malaysian population comprising three ethnic groups (Malays, Chinese and Indians) was estimated to be 0.46, and that of the *PON1*_{192R} allele, 0.54. Discordance between the genotype and phenotype was observed for two samples. One of the two subjects genotyped as *PON1*_{192QR} and phenotyped as *PON1*_{192QQ}. The sample showed low paraoxonase and diazoxonase activities. Sequencing confirmed that the genotype was *PON1*_{192QR}. The other subject was genotyped as *PON1*_{192RR} but phenotyped as *PON1*_{192QR}. Sequencing showed that the genotype was in fact *PON1*_{192RR}, with the subject showing relative high activities. The *PON1*_{194WX} mutation was not detected in the sequenced samples and was not the source for discordance.

Introduction

Paraoxonase1 (PON1) is an esterase belonging to a family of proteins that includes PON2 and PON3 [1]. The genes coding for the PON family are located on human chromosome 7 (q21.22). PON1 has two exonic amino acid polymorphisms, one at position 55 (methionine/leucine; M/L) and another at position 192 (arginine/glutamine; R/Q). PON1 is associated with high-density lipoprotein (HDL) particles, thus linked to the prevention of oxidative damage to low-density lipoprotein (LDL) particles. The protective effect of HDL has been implicated in arresting the development of coronary heart disease (CHD), as well as other vascular complications of diabetes [2]. Several studies describe the *PON1*_{192R} allele as a significant risk factor for heart disease although a meta-analysis of 43 such studies have shown a weak association whereby the estimated relative risk of *PON1*_{192R} for coronary heart disease was 1.12 [3]. Thus genotyping the diabetic individuals for both the PON1 192QR and 55LM polymorphisms updates

available data and would be useful in identifying those individuals at the highest risk of developing complications. The characteristics of *PON1*_{192QQ}, *-QR* and *-RR* genotypes are such that the plasma activity differs towards the substrates paraoxon and diazoxon. The *PON1*_{192Q} polymorphism causes a ten-fold decrease in paraoxonase activity. The *PON1*_{192R} mutation possibly reshapes the active site thereby improving the positioning of paraoxon [4]. In addition, hydrophobicity is common to most of PON1's effective substrates, probably due to the hydrophobicity and depth of PON1's active site. Therefore, the poor and effective substrates bind at the active site with similar affinity, except that the mode of binding differs; the poor substrates would be inadequately positioned, resulting in a lower activity.

Material and Methods

Outpatients who attended the Diabetic Clinic, University of Malaya Medical Centre, Kuala Lumpur, and healthy

volunteers residing in Selangor and Kuala Lumpur were screened for the study. This study was approved by the

University of Malaya Medical Ethics Committee (MEC) and informed consent was obtained from all subjects.

Table 1: Characteristics of subjects with discordant *PON1*₁₉₂ genotypes in comparison with selected Concordant samples

Group	Discordant	Discordant	Low POase activity	Low DZOase activity	High POase activity	High DZOase activity
PON1 ₁₉₂ genotype	QR	RR	QR	RR	RR	QQ
PON1 ₁₉₂ phenotype	QQ	QR	QR	RR	RR	QQ
PON1 ₁₉₂ genotype (sequenced)	QR	RR	QR	RR	RR	QQ
POase activity (U/L)	143	599	107	765	1049	315
DZOase activity (U/L)	10030	14037	3819	6770	4985	27571
PON1 ₅₅ genotype	LL	LL	LL	LL	LL	LL
Age (years)	29	43	52	58	53	45
Sex	Male	Male	Male	Female	Female	Female
Ethnicity	Malay	Malay	Chinese	Malay	Malay	Malay
Diabetes/Cardiovascular complications	None	None	Diabetic/ Hypertensive	Diabetic/ Hypertensive	None	None
HDL-C (mmol/L)	1.16	0.93	0.71	0.82	1.63	1.10

Demographic characteristics of each subject were recorded. All subjects were screened for the variations in the *PON1* gene, including unmatched cases or controls. Venous blood samples were obtained by a trained phlebotomist under sterile conditions from subjects. Genomic DNA was extracted from whole EDTA blood using a Gene All Blood DNA Purification kit (General Biosystem, Seoul, South Korea). *PON1* genotypes were determined by the PCR-based methods [5]. To assess polymorphism at position 192, the sense primer (5' TAT TGT TGC TGT GGG ACC TGA G 3') and the antisense primer (5' CAC GCT AAA CCC AAA TAC ATC TC 3') which encompass the codon 192 of the human *PON1* gene were used. For the polymorphism at position 55, the sense primer (5' AGA GGA TTC AGT CTT TGA GGA AA 3') and the antisense primer (5' CTG CCA GTC CTA GAA AAC GTT 3') were used. PCR reactions were carried out using a Hybaid Omnigene thermal cycler (Hybaid, Middlesex, UK). The hot-start PCR reaction mixture for the PON1-192 polymorphism contained 100 ng of DNA template, 0.5 μM of each primer, 1.5 mM MgCl₂, 200 μM of each dNTP and 1 unit of *Taq* DNA polymerase and hot-start antibody mixture (Biotherm PCR kit, Genecraft, Germany). The reaction mixture was denatured for 3 min at 93°C, followed by 35 cycles of 1 min of denaturation at 93°C, 30 s of annealing at 61°C and 1 min of extension at 72°C. The final extension was 7 min at 72°C. The resulting 99 bp PCR product was then digested with 5 units of *AlwI* restriction endonuclease (New England Biolabs, Cambridge, MA, USA) overnight at 37°C, followed by size fractionation on 4% agarose gels with 10 μl

matched undigested and digested samples loaded alternately into sample wells. Visualization was carried out with ethidium bromide staining. The R genotype (arginine) contains a unique *AlwI* restriction site which results in products of 63 and 36 bp, whereas the Q genotype (glutamine) is not cut.

For the PON1-55 polymorphism, the PCR reaction mixture contained 100 ng of DNA template, 0.5 μM of each primer, 1.5 mM MgCl₂, 200 μM of each dNTP and 1 unit of *Taq* DNA polymerase (Biotherm). After denaturation for 5 min at 94°C, the reaction mixture was subjected to 35 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 61°C and 30 sec of extension at 72°C. The final extension time was 7 min at 72°C. The 386 bp PCR product was digested with *NlaIII* (New England Biolabs) in the presence of BSA overnight at 37°C. Digested products were separated in 3% agarose gels. The L genotype (leucine) does not contain an *NlaIII* site, whereas the M genotype (methionine) contains an *NlaIII* site, giving rise to products of 296 and 90 bp [5]. For two-substrate assays, paraoxonase and diazoxonase activities of corresponding heparinised plasma samples were measured spectrophotometrically (Varian Cary 50, Varian Inc. Scientific, Australia). A two-dimensional plot of diazoxon versus paraoxon hydrolysis rates allowed simultaneous PON1-192 genotyping and activity phenotyping of each individual [6]. The genotypes obtained from both methods were compared. Sequencing was performed to validate genotypes in cases of discordance. Subjects with low activities for their *PON1*₁₉₂ genotype were chosen for sequencing

purposes as follows: $PON1_{192QQ}$ subjects with diazoxonase activities under 5000 U/L, and $PON1_{192QR}$ and $-RR$ subjects with paraoxonase activities less than 200 U/L and 400 U/L, respectively. Reference to figure 1 shows that paraoxonase and diazoxonase activities are low for the subjects identified. The 99-bp PCR product was purified by using a kit (MinElute, Qiagen, The Netherlands) and sequenced on an ABI 3730 DNA Analyzer (Applied Biosystem, California, USA). The sequenced products were compared using the nucleotide blast program against a nucleotide sequence database with reference to Genbank Accession #AF539592 [7]. The presence of the $PON1_{194WX}$ genotype was also examined based on the results from sequencing.

Results and Discussion

Examination of demographic characteristics showed that a greater proportion (60%) of the study population con-

sisted of women. Malays constituted 47% of the study population, followed by Indians (33%) and Chinese (20%). The pooled age range was 17-97 years, with the mean age being 52 ± 13.5 years old. The subjects who were diabetic made up 52 % while the remaining 48 % were the non-diabetic controls. The observed frequencies of L , M , Q and R alleles in the pooled population were 0.912, 0.088, 0.459 and 0.541, respectively. Discordance between the genotype and phenotype was observed for two samples, 29- and 43-year-old, both healthy males. The 29-year-old subject was genotyped as $PON1_{192QR}$ and activity phenotyped as $PON1_{192QQ}$. The sample had low paraoxonase and diazoxonase activities (Table 1). DNA sequencing confirmed that the genotype was $PON1_{192QR}$. This subject had PON1 activity (<200 U/L) in the lowest tertile within its genotype group (Fig. 1). This low activity is consistent with the inactive allele hypothesis whereby the $PON1_{192R}$ allele is predicted to be inactive in this heterozygote.

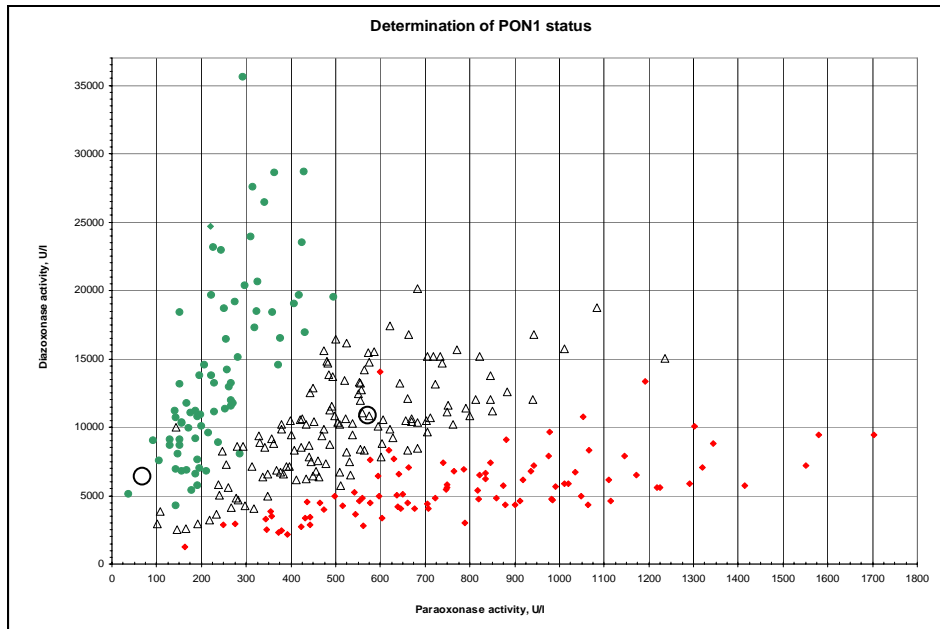


Figure 1: Determination of the functional genomics of plasma PON1 using the $PON1_{192}$ bivariate phenotype versus genotype plot. The $PON1_{192QQ}$, $-QR$ and $-RR$ genotypes are shown as closed circles, triangles and diamonds, respectively. The two discordant subjects are indicated by open circles.

The 43-year-old subject was genotyped as $PON1_{192RR}$ but phenotyped as $PON1_{192QR}$. Sequencing showed that the genotype was $PON1_{192RR}$, with the subject showing relative high activities. For both samples, the presence of the rare $PON1_{194stop}$ polymorphism ($PON1_{WX}$) which coded for the stop codon was assessed. All the selected samples were confirmed to be of the common $PON1_{194WW}$ genotype. This subject showed comparatively high paraoxonase and diazoxonase activities. As the placement of activity on the plot reflects the differential expression of the $PON1_{192Q}$ and $PON1_{192R}$ alleles, $PON1_{192}$ homozygotes is

genotype. In this case, the subject not only displayed discrepant genotypes despite being homozygous, it also displayed high activities.

Repeated genotyping and phenotyping were performed to eliminate the possibility of error. In fact, other reports have noted the incidence of error when the salt-stimulation assay was used. Eckerson *et al.* [8] noted that one of the two individuals misclassified had low paraoxonase activity. The percentage of error was less than 1%. Browne *et al.* [9] reported a slightly higher error or ana-

lytical imprecision of 1.0-3.0%. These errors were resolved when the genotypes were compared using various techniques. The rare PON1 protein truncation mutation *PON1_{trp194stop}* may result in diminished PON1 activity. This alteration has been associated with the *PON1_{192R}* allele (Jarvik *et al.*, 2003). In the present study, all subjects sequenced for the mutation carried the *PON1_{194WW}* genotype which was not the stop codon, thereby ruling out the effect of the stop codon on the loss of function of the particular allele. This is to be expected as the allele frequency of such a mutation occurring is relatively low at 1/458 *PON1_{192R}* alleles in the population studied by Jarvik *et al.* (2003).

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