Ethnic variations in paraoxonase1 polymorphism in the Malaysian population

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ETHNIC VARIATIONS IN PARAOXONASE1 POLYMORPHISM IN THE MALAYSIAN POPULATION

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Abstract. The role of high-density lipoprotein associated paraoxonase (PON1) in protection against oxidative stress associated with the development of complications in diabetes mellitus has been reported. Variations in the PON1 gene, 55LM and 192QR have been described in different populations. These variations are known to be risk factors for heart disease, especially the L and R alleles. We have investigated the prevalence of both polymorphisms in the Malaysian population comprising the three major ethnic groups: Malay, Chinese and Indian, using polymerase chain reaction followed by restriction endonuclease digestion. The results show the pooled frequencies of L and R alleles were 0.91 and 0.54, respectively, similar to those in the Asian region. The frequency of the M allele was higher in Indians (p < 0.05), whereas the R allele was higher in both the Chinese and Malays compared to Indians (p < 0.05), indicating ethnic group-dependent genetic differences. The most common genotypic combination was LL/QR, followed by LL/RR. The genotype frequencies for the total Malaysian population showed a significant departure from Hardy-Weinberg equilibrium for the 55LM (p = 0.013) but not the 192QR (p = 0.056) polymorphisms. A strong linkage disequilibrium between L/55 and R/192 alleles was also observed. In the Malaysian population as a whole, Malays and Chinese showed a higher frequency of the R allele which is a risk factor for cardiovascular diseases.

INTRODUCTION

Paraoxonase1 (PON1) is an esterase belonging to a family of proteins that includes PON2 and PON3 (Costa et al, 2005). 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) (Durrington et al, 2001). PON1 is associated with high-density lipoprotein (HDL) particles, connotating its role in preventing oxidative damage to low-density lipoprotein (LDL) particles (Mackness et al, 1998). Purified PON1 was significantly more efficient than other enzymes effects on HDL in preventing oxidation of LDL (Arrol et al, 1996). The protective effect of HDL has been well described in arresting the development of coronary heart disease (CHD), as well as other vascular complications of diabetes (Mackness et al, 2002).

A number of studies have found the R allele of PON1 to be associated with heart disease in some populations, whereas others have not found such an association (Santos et al, 2005).

Little is known of the prevalence of mutations of the PON1 gene. Furthermore, there is a lack of information regarding the association between allele frequencies and risk factors for heart disease, especially in diabetics in the Malaysian population. Thus genotyping the diabetic individuals for both the PON1 192QR and 55LM polymorphisms may provide a method for identifying those individuals at the highest risk for developing complications. Previous studies have focused on Caucasians and some Asian populations, includ-
ing the Japanese (Odawara et al, 1997) and Thai populations (Phuntuwate et al, 2005).

The effects of the two polymorphisms on PON1 activity and concentration in diabetic patients have previously been reported (Mackness et al, 1998; Ikeda et al, 1998) with PON1 activity showing an increase in the order of the RR>QR>QQ genotypes and LL>LM>MM genotypes in patients.

The present study evaluates variations in PON1 polymorphisms in the Malaysian population in order to identify specific risk factor alleles for cardiovascular complications in type 2 diabetes mellitus.

MATERIALS AND METHODS

Subjects

A group of subjects drawn from outpatients who attended the Diabetic Clinic University Malaya Medical Center, Kuala Lumpur, as well as healthy volunteers residing in Selangor and Kuala Lumpur were selected. Some demographic characteristics of these subjects were recorded.

This study was approved by the Medical Ethics Committee (MEC) and informed consent was obtained from each subject in the study.

Blood sampling and DNA extraction

Venous blood was 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).

Determination of PON1 genotypes

PON1 genotypes were determined by PCR-based methods according to previously published protocols (Richter et al, 2004). For the polymorphism at position 192, sense primer 5´ TAT TGT TGC TGT GGG ACC TGA G 3´ and antisense primer 5´ CAC GCT AAA CCC AAA TAC ATC TC 3´ which encompass the 192 polymorphic region of the human PON1 gene were used. For the polymorphism at position 55, sense primer 5´ AGA GGA TTC AGT CTT TGA GGA AA 3´ and 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 minutes at 93°C, followed by 35 cycles of 1 minute of denaturation at 93°C, 30 seconds of annealing at 61°C and 1 minute of extension at 72°C. Final extension was performed for 7 minutes 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 with 10 µl matched undigested and digested samples loaded alternately into wells of 4% 0.05 M Tris-borate-EDTA pH 8.0 horizontal agarose gel containing 0.5 µg/ml ethidium bromide at a voltage of 5 V/cm. The R genotype (arginine) contains a unique AlwI restriction site which resulted in products of 63 and 36 bp, whereas the Q genotype (glutamine) was 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 minutes at 94°C, the reaction mixture was subjected to 35 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 61°C and 30 seconds of extension at 72°C. The final extension time was 7 minutes at 72°C.
The 386 bp PCR product was digested with 3 units of NlaIII (New England Biolabs) in the presence of BSA overnight at 37°C, and the digested products were separated in 3% 0.05 M Tris-borate-EDTA agarose gels containing 0.5 µg/ml ethidium bromide at a voltage of 5 V/cm as above. The L genotype (leucine) did not contain an NlaIII site, whereas the M genotype (methionine) contained an NlaIII site, giving rise to products of 296 and 90 bp (Richter et al, 2004).

Statistical analysis
Analysis was performed using SPSS 13.0 for Windows statistical software. One-way analysis of variance (ANOVA) was used to test for differences in parameters between ethnicity and genotypes (Mackness et al, 2000). Computation of allele frequencies and estimation of Hardy-Weinberg equilibrium and linkage disequilibrium were performed using GENEPOP 3.4 software (Raymond and Rousset, 1995). The Markov chain method was employed at 1,000 dememorization steps, 100 batches and 1,000 iterations per batch to obtain an unbiased estimate of the p-value. Haplotype frequencies were computed by Arlequin software (Schneider et al, 2000) using the expectation-maximization (EM) algorithm (Slatkin and Excoffier, 1996).

RESULTS
Demographic characteristics of the study population
The study population had a higher percentage of women (59%). Malays formed almost half the study population at 47%, 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 diabetics made up 52% while the remaining 48% were non-diabetics.

Genotype and allele frequencies of the PON1 polymorphisms
The frequency of the M allele was higher in Indians compared to Malays and Chinese (p < 0.05), whereas the R allele was higher in the Chinese (R = 0.621) and Malays (R = 0.587).

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>N (%)</th>
<th>LLQQ</th>
<th>LLQR</th>
<th>LLRR</th>
<th>LMQQ</th>
<th>LMQR</th>
<th>LMRR</th>
<th>MMQQ</th>
<th>L</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malay</td>
<td>133 (47)</td>
<td>19</td>
<td>48</td>
<td>52</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0.944</td>
<td>0.587</td>
</tr>
<tr>
<td>Chinese</td>
<td>58 (20)</td>
<td>9</td>
<td>20</td>
<td>25</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.957</td>
<td>0.621</td>
</tr>
<tr>
<td>Indian</td>
<td>94 (33)</td>
<td>18</td>
<td>35</td>
<td>15</td>
<td>7</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>0.839</td>
<td>0.426</td>
</tr>
<tr>
<td>Total</td>
<td>285 (100)</td>
<td>46</td>
<td>103</td>
<td>92</td>
<td>16</td>
<td>21</td>
<td>1</td>
<td>6</td>
<td>0.912</td>
<td>0.541</td>
</tr>
</tbody>
</table>

Table 1
Demographic profile and breakdown of PON1 genotype and allele frequencies observed in three major Malaysian ethnic groups.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PON1-192</th>
<th>100%</th>
<th>PON1-55</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ (n =68):</td>
<td>24%</td>
<td>LL (n =241):</td>
<td>85%</td>
<td></td>
</tr>
<tr>
<td>QR (n =124):</td>
<td>43%</td>
<td>LM (n =38):</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>RR (n =93):</td>
<td>33%</td>
<td>MM (n =6):</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td>Q</td>
<td>0.459</td>
<td>L</td>
<td>0.912</td>
</tr>
<tr>
<td>R</td>
<td>0.541</td>
<td>M</td>
<td>0.088</td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Pooled genotype and allele frequencies of the PON1 gene polymorphisms in the study population.
compared to Indians (R = 0.426, p < 0.05) (Table 1). Only seven out of the expected ten possible genotypic combinations of the two polymorphisms were seen in this population. The most common genotype was LL/QR (36%) followed by LL/RR (32%). Moreover, the LM/RR genotype, the least frequent of the seven genotypes, was found in only one subject of Malay origin. Both MMQR and MMRR genotypes were absent in the study population. Of particular significance was the frequency of the MM/QQ genotype, which was found predominantly in Indians (67%), but generally of low frequency in the Malaysian population at large.

In the pooled population, the observed frequencies for L, M, Q and R alleles were 0.912, 0.088, 0.459 and 0.541, respectively (Table 2).

The genotype frequencies for the total Malaysian population showed a significant departure from HWE for the 55LM (p=0.013) but not the 192QR (p=0.056) polymorphisms. A strong linkage disequilibrium between L/55 and R/192 alleles was observed (p<0.0001) characterized by an excess of LR and LQ haplotypes (Table 3).

**DISCUSSION**

The pooled frequency of the R allele was 0.54 in the study population. This is consistent with studies by Sanghera et al (1997), Hu et al (2003) and Zhang et al (2005) in Chinese populations (R = 0.57, 0.53, 0.54, respectively). However, this result differed significantly from the value obtained for the English (R = 0.30; Rice et al, 1997), Asian Indian (R = 0.39, Sanghera et al, 1998) and Thai (R = 0.29, Phuntuwate et al, 2005) populations which were on one end of the frequency spectrum. It also differed from the value calculated for the Korean (R = 0.666; Hong et al, 2001), Bogalusa black (R = 0.67) and the Amerindian populations (R = 0.73; Santos et al, 2005). Amerindians showed higher R allele frequency when compared to Asians. The relatively low R allele frequency in Thais was unexpected, given that their geographical neighbors, Malaysians, showed frequencies of R >0.40. Nevertheless, the frequency of the PON1-192R allele generally ranged from 0.25 in Cauca-

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>LR</th>
<th>LQ</th>
<th>MR</th>
<th>MQ</th>
<th>L</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaysians</td>
<td>285</td>
<td>0.540</td>
<td>0.371</td>
<td>0.002</td>
<td>0.085</td>
<td>0.912</td>
<td>0.540</td>
</tr>
<tr>
<td>Chinese</td>
<td>142</td>
<td>0.574</td>
<td>0.387</td>
<td>-</td>
<td>0.039</td>
<td>0.961</td>
<td>0.574</td>
</tr>
<tr>
<td>Koreans</td>
<td>191</td>
<td>0.620</td>
<td>0.325</td>
<td>-</td>
<td>0.055</td>
<td>0.945</td>
<td>0.620</td>
</tr>
<tr>
<td>Japanese</td>
<td>2,196</td>
<td>0.666</td>
<td>0.261</td>
<td>-</td>
<td>0.073</td>
<td>0.927</td>
<td>0.666</td>
</tr>
<tr>
<td>Amerindians</td>
<td>259</td>
<td>0.730</td>
<td>0.237</td>
<td>-</td>
<td>0.033</td>
<td>0.967</td>
<td>0.730</td>
</tr>
<tr>
<td>Euro-Brazilians</td>
<td>101</td>
<td>0.307</td>
<td>0.302</td>
<td>-</td>
<td>0.391</td>
<td>0.609</td>
<td>0.307</td>
</tr>
<tr>
<td>Afro-Brazilians</td>
<td>70</td>
<td>0.465</td>
<td>0.250</td>
<td>0.064</td>
<td>0.222</td>
<td>0.714</td>
<td>0.529</td>
</tr>
</tbody>
</table>

*aSanghera et al, 1997; bHong et al, 2001; cYamada et al, 2003; dSantos et al, 2005; e,fAllebrandt et al, 2002*
Serrato et al, 1995). The R allele is a risk factor for coronary heart disease in Asian Indians but not in Chinese (Sanghera et al, 1998). Such inconsistency is probably because the PON1 codon 192 polymorphism was in linkage disequilibrium with other functional mutations in a particular haplotype of the PON1 gene (Koda et al, 2004) or nearby genes.

For codon 55 polymorphism, the prevalence of the PON1-55 L allele (L = 0.912) in this investigation is similar to that found in the Japanese (L = 0.927; Yamada et al, 2003), Singaporean Chinese (L = 0.961; Sanghera et al, 1998) Han Chinese (Zhang et al, 2006), Amerindians (L = 0.967; Santos et al, 2005), and Thais (L = 0.950, Phuntuwate et al, 2005). Comparison of the M allele frequencies for Malaysian Chinese (M = 0.161) and Indians (M = 0.043) with their Singaporean counterparts showed similar frequencies (Singaporean Chinese, M = 0.202; Singaporean Indians, M = 0.036, respectively) although differing distinctly between the two ethnic groups. This emphasizes the local and ethnic differences in gene frequencies among populations. The generally low frequency of the M allele was such that in some studies the MM genotype was not observed at all (Santos et al, 2005). The prevalence of the L allele in this study was higher than that found in the western white (0.640) (Brophy et al, 2001) and Italian populations (0.63) (Arca et al, 2002).

Only seven genotypes were observed in this study, and this was probably due to the strong linkage disequilibrium between the two polymorphic sites (only 8.6 kb apart) across the populations (Santos et al, 2005). As the R allele frequency is higher in Malaysians, it is reasonable to expect a higher frequency of the LL/QR (36%) and LL/RR (32%) genotypes, compared to LLQQ (43%) and LLQR (41%) for Thais. There were no MMQR or MMRR carriers observed, suggesting that this genotype combination is very rare in Malaysians; this is similar to the low incidence observed in the Thai population. Only MMQQ genotypes were observed for the M allele in the present study. Kao et al (2002) found that patients with retinopathy had a higher frequency of the LL genotype than those without retinopathy in a diabetic population. These findings suggested that the M allele may have a protective role. Conversely, the L allele was regarded as a risk factor for diabetic complications.

The present study is the first to record the distribution of the 55LM and 192QR genotype frequencies of the PON1 gene in three major Malaysian ethnic groups. The Malays and Chinese had a higher frequency of the R allele, which is considered a risk factor for cardiovascular disease in a number of populations from previous studies.

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