C242T polymorphism of the NADPH oxidase p22PHOX gene and its association with endothelial dysfunction in asymptomatic individuals with essential systemic hypertension

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Received August 7, 2012; Accepted November 22, 2012

DOI: 10.3892/mmr.2014.1992

Abstract. Vascular oxidative stress is an important factor in hypertension-associated vascular damage and is mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. The C242T polymorphism at the p22PHOX gene affects binding of p22PHOX to heme, leading to variants of NADPH oxidase that produce different levels of reactive oxygen species (ROS). Specific variations in ROS are associated with an altered risk of developing cardiovascular disease. In the present study, 140 permanent Kashmiri-resident individuals were recruited (75 with essential systemic hypertension and 65 normotensive controls). Endothelial function was assessed non-invasively using high-resolution ultrasonography of the brachial artery. Endothelium-dependent vasoreactivity was expressed in terms of flow-mediated dilation. The TT genotype was identified in 2% of hypertensive and 7% of normotensive individuals. Frequency of the T-allele was not observed as significantly different between hypertensive and normotensive individuals. Blood pressure or the prevalence of hypertension did not vary between C242T p22PHOX genotypes or in the presence or absence of the T-allele.

Introduction

Endothelial dysfunction was one of the first vascular abnormalities of atherosclerosis to be identified (1). Abnormalities in endothelial function resulting from non-denuding injuries caused to the endothelium by conditions or risk factors that predispose to atherosclerotic cardiovascular (CV) disease may be present prior to development of detectable arterial wall lesions (2-4). Oxidative processes in the vessel wall result in low-grade inflammation with attachment and subsequent migration of monocyte-macrophages, leading to a number of humoral and cellular events characteristic of endothelial dysfunction (5-7). Endothelial dysfunction of risk-factor-exposed or atherosclerotic vessels appears to be a consequence of excessive production of reactive oxygen species (ROS) within the vascular wall (8,9). Moreover, ROS scavenging by superoxide dismutase has been demonstrated to improve coronary endothelial vasodilatation (10).

The most important source of superoxide in the vasculature is the membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (11,12). NADPH oxidase is an important contributor to impaired endothelium-dependant vasodilatation in animal (12) and human (13,14) vascular tissue. NADPH oxidase is a multi-subunit enzyme complex comprising p47PHOX, p67PHOX, gp91PHOX, p22PHOX and rac-2 protein. The membrane-bound p22PHOX protein, expressed in vascular smooth muscle and endothelial cells, functions as the final electron transporter from NADPH to molecular oxygen and is essential for the assembly and activation of NADPH oxidase (15). Therefore, the expression and activity of this component may be a key determinant of superoxide generation by vascular NADPH oxidase (16). Expression of p22PHOX has been identified at higher levels in human atherosclerotic coronary compared with non-atherosclerotic arteries, indicating that p22PHOX may be involved in the pathophysiology and pathogenesis of atherosclerotic CV disease (17). Several genetic variations in the p22PHOX gene have been identified, a number of which correlate with CV diseases (18). The C242T variation is a functional single nucleotide polymorphism, located on exon 4 and encodes a non-conservative C→T replacement that results in the substitution of histidine 72 for tyrosine. By affecting p22PHOX binding to heme, the C242T polymorphism may produce variants of NADPH oxidase associated with different levels of ROS production and an altered risk of developing CV diseases (19,20).

Although the pathogenesis of hypertension is complex and multifactorial, a role for increased ROS generation has been hypothesized in a number of studies, particularly with respect to AngII-dependent hypertension (21). For example, vascular NADPH oxidase activity is increased in rats made hypertensive by chronic AngII infusion (12), together with increases in the expression of Nox1, 2 and 4 (22) and p22PHOX mRNA (23).

Vascular oxidative stress is involved in hypertension-associated vascular damage (24-28) and appears to be mediated...
by NADPH oxidase activation, through rennin-angiotensin system (RAS)-dependent (12,21) and RAS-independent (29) mechanisms. Previous studies demonstrated that an increased expression of p22PHOX is associated with enhanced NADPH oxidase activity in hypertension (23,30,31). Furthermore, elevated NADPH oxidase activity (32) and an increased expression of p22PHOX (33) have been identified to correlate with impaired bioavailability of nitric oxide (NO) in hypertension. Since the C242T polymorphism at the p22PHOX gene modulates production of ROS in vasculature by affecting NADPH oxidase enzyme activity (20,34,35), existence of this genetic polymorphism may be an important determinant of vascular dysfunction in patients with essential hypertension. In an additional study, hypertensive individuals carrying the CC genotype of this polymorphism exhibited features of NADPH oxidase-mediated oxidative stress and endothelial damage as indicated by enhanced plasma levels of von-Willebrand factor (36).

Ethnicity is an important determinant of genetic polymorphisms. While distribution of allelic variations in the gene for p22PHOX have been established for several ethnic groups worldwide (34,35,37-47), to date, no studies have been performed on individuals living on the Indian subcontinent. Therefore, the aim of the present study was to analyze the distribution of p22PHOX C242T genotypes and alleles in a community-based sample drawn from the Kashmiri population. In view of the potential significance of the p22PHOX gene C242T polymorphism for NO bioavailability, the functional relevance of this genetic variation to vascular function is of interest.

Materials and methods

Patient population. The present cross-sectional study was conducted at the Sher-i-Kashmir Institute of Medical Sciences (Srinagar, India). Local permanent residents were invited to take part in the study. A total of 140 independent, apparently healthy individuals, aged 30-65 years were recruited (75 with essential systemic hypertension and 65 normotensive controls).

The ethics committee of the Sher-i-Kashmir Institute of Medical Sciences approved the study, and written informed consent was obtained from the patient/ the patient’s family.

General evaluation. Peripheral venous blood samples for routine laboratory tests were obtained from individuals instructed to fast overnight. Estimated glomerular filtration rate (eGFR) was calculated according to the modification of diet in renal disease formula (48). Plasma lipid parameters were measured using standard enzymatic methods on a fully automatic multichannel chemistry analyzer (Hitachi 912, Roche Diagnostics, Tokyo, Japan). Commercially available kits from Randox Laboratories, Ltd. (Crumblin, UK) were used for estimation of total cholesterol (T-C), triglyceride (TG) and direct low-density lipoprotein cholesterol (LDL). High-density lipoprotein-cholesterol (HDL) was estimated by the second generation enzymatic assay using a kit obtained from Roche Diagnostics. High-resolution ultrasonography of the brachial artery was performed to determine flow-mediated dilatation (FMD) as a measure of endothelium-dependent vasodilatation.

Genotyping analysis. Total genomic DNA was extracted from whole blood using the phenol-chloroform method. The 353-bp target region of the p22PHOX gene was amplified by polymerase chain reaction using the following primers: sense 5’-TGCTTGTGGGTAAACCAAGG-3’ and antisense 5’-GGAAACACACTGAGGTAAAGTG-3’ in a 25-µl reaction volume. The reaction consisted of an initial denaturation step of 2 min at 95˚C, followed by 35 cycles of 3 min at 95˚C (denaturation), 1 min at 56˚C (annealing) and 7 min at 72˚C (extension) and a final extension of 7 min at 72˚C. The 353-bp PCR product was cleaved with 1 µl RsaI restriction enzyme for 18-24 h at 37˚C. Restriction fragments were separated on a 2.5% agarose gel. Digestion of the amplicon produced a 353-bp band in CC homozygous, 193- and 160-bp bands in TT homozygous and all three bands in CT heterozygous individuals.
Statistical analysis. P<0.05 was used to indicate statistically significant differences.

Results

CV risk factors. CV risk factors in the study population are presented in Table I. Greater prevalence of low HDL concentration was observed in hypertensive compared with normotensive individuals. Smoking status was markedly correlated with prevalence of hypertension (P<0.0001). Overall CV risk factor score (Framingham score) and the calculated 10-year coronary heart disease (CHD) risk were identified as significantly higher in hypertensive compared with normotensive individuals. A 10-year CHD risk of >20% was calculated in ~1/4 of hypertensive individuals.

p22PHOX gene polymorphisms. The overall study population was in Hardy-Weinberg equilibrium. Half of the individuals were identified with the CC genotype. The TT homozygous genotype was observed in only 5% of study participants (Table II).

The clinical and laboratory characteristics of individuals with various C242T genotypes are presented in Table III. Anthropometric measurements, blood pressure (BP), serum creatinine and uric acid levels, eGFR and plasma lipid parameters were comparable among the three genotypes. However, individuals with the TT genotype were observed to have significantly lower fasting blood glucose (BG) levels compared with other genotypes. Serum T-C and TGs demonstrated a linear association among the three genotypes.

Correlation between the C242T polymorphism in the p22PHOX gene and brachial artery reactivity. Results of brachial artery reactivity testing are presented in Table IV. Blood flow through the brachial artery prior to occlusion and during the hyperemic phase was comparable between hypertensive and normotensive individuals, as was the degree of reactive hyperemia. Impaired FMD response correlated with endothelial dysfunction (Table V). A higher Framingham risk score was associated with lower FMD (Fig. 1). In the overall population, brachial artery reactivity did not vary across the p22PHOX genotypes. Baseline and hyperemic brachial artery diameter were comparable and no significant difference was detected in % FMD between the three genotypes. However, on subgroup analysis, FMD was observed to be significantly higher in hypertensive individuals with the T-allele compared with those without the T-allele (P=0.046).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Variables</th>
<th>Genotype</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Variant allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>C242T</td>
<td>Total (n=140)</td>
<td></td>
<td>70</td>
<td>63</td>
<td>7</td>
<td>0.275</td>
</tr>
<tr>
<td></td>
<td>Hypertensive (n=75)</td>
<td></td>
<td>35</td>
<td>38</td>
<td>2</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>Normotensive (n=65)</td>
<td></td>
<td>35</td>
<td>25</td>
<td>5</td>
<td>0.271</td>
</tr>
</tbody>
</table>

Table II. Genotype frequencies, ORs and CIs of p22PHOX-associated genotypes.

A, Genotype frequencies of the p22PHOX polymorphism in hypertensive and normotensive individuals

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Normotensive vs. hypertensive, OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C242T</td>
<td>CC</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>1.52 (0.76-3.0)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>0.40 (0.07-2.2)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>CT+TT</td>
<td>1.33 (0.68-2.5)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*BWild-type allele is denoted by C and the polymorphic allele by T. OR, odds ratio; CI, confidence interval.
Table IV. Results of brachial artery reactivity testing.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hypertensive (n=75)</th>
<th>Normotensive (n=65)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAD baseline (mm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.26±0.78</td>
<td>4.95±0.66</td>
<td>0.01</td>
</tr>
<tr>
<td>Range</td>
<td>3.40-7.20</td>
<td>3.90-6.00</td>
<td></td>
</tr>
<tr>
<td><strong>BAD hyperemic (mm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.53±0.83</td>
<td>5.79±0.70</td>
<td>0.04</td>
</tr>
<tr>
<td>Range</td>
<td>3.70-7.90</td>
<td>4.70-7.13</td>
<td></td>
</tr>
<tr>
<td><strong>FMD (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.22±4.58</td>
<td>17.34±7.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Range</td>
<td>-4.26-18.14</td>
<td>0-33.33</td>
<td></td>
</tr>
<tr>
<td><strong>Flow baseline (ml/min)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>116±61</td>
<td>106±49</td>
<td>0.30</td>
</tr>
<tr>
<td>Range</td>
<td>36-295</td>
<td>53-232</td>
<td></td>
</tr>
<tr>
<td><strong>Flow hyperemic (ml/min)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>792±314</td>
<td>714±297</td>
<td>0.13</td>
</tr>
<tr>
<td>Range</td>
<td>271-1498</td>
<td>207-1489</td>
<td></td>
</tr>
<tr>
<td><strong>Hyperemia (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>700±367</td>
<td>638±315</td>
<td>0.28</td>
</tr>
<tr>
<td>Range</td>
<td>114-2005</td>
<td>46-1437</td>
<td></td>
</tr>
</tbody>
</table>

BAD, brachial artery diameter; FMD, flow-mediated dilation.
Table V. Prevalence of endothelial dysfunction at baseline.

<table>
<thead>
<tr>
<th>FMD response</th>
<th>Hypertensive (n=75)</th>
<th>Normotensive (n=65)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, n (%)</td>
<td>4 (5.30)</td>
<td>54 (83.0)</td>
</tr>
<tr>
<td>Borderline, n (%)</td>
<td>14 (18.60)</td>
<td>4 (6.1)</td>
</tr>
<tr>
<td>Moderately impaired, n (%)</td>
<td>32 (42.60)</td>
<td>2 (3.0)</td>
</tr>
<tr>
<td>Severely impaired, n (%)</td>
<td>25 (33.33)</td>
<td>5 (7.6)</td>
</tr>
</tbody>
</table>

Pearson $\chi^2$=88.2, $P<0.0001$. FMD, flow-mediated dilation.

Discussion

To the best of our knowledge, the present study is the first report of allelic variation in the gene of the p22PHOX component of the superoxide-generating enzyme, NADPH oxidase, in a community-based sample from Kashmir (India). We investigated the genotype and allele frequencies of the C242T polymorphism at the p22PHOX gene in 80 asymptomatic individuals from the community. Results indicate that in the selected population of hypertensive and normotensive Kashmiri individuals, the p22PHOX gene exhibits C242T polymorphism, with allele frequencies in Hardy-Weinberg equilibrium. In this study, 50, 45 and 5% of individuals had the CC, CT and TT genotype, respectively. The frequency of the T-allele was 27.5% of the overall population. Genotype and gene frequencies were comparable between the hypertensive and the normotensive individuals. Additionally, the condition of Hardy-Weinberg equilibrium was satisfied in the subject groups and the overall study population, indicating the absence of genetic mutation, drift or selection for the locus in consideration. These data are also consistent with absence of gene flow (migration) as well as with the randomly-mating population characteristics of the Kashmir valley from where all the participants for the present study were drawn.

The distribution of C242T genotypes in this study is similar to that reported in several Caucasian populations. The T-allele frequency in our population (27%) is slightly lower than that in Caucasians (median, 33%) but higher than that in African-Americans (17%), Han-Chinese living in Beijing (6.6%) and Japanese living in Tokyo (7-13%). The frequency of T-allele in non-Caucasian populations is generally lower. A study on a mixed US population (37) reported a T-allele frequency of 35% among Caucasians in a sample of 90 healthy adults. The frequency of T-allele in non-Caucasian populations is generally lower. A study on a mixed US population (37) reported a T-allele frequency of 35% among Caucasians in a sample of 90 healthy adults.

An important observation in the present study was the significantly lower fasting BG levels in individuals with the T-allele compared with individuals without this allele. In addition, the association between the T-allele and BG appeared to be dose-dependent with glucose levels lowest in TT, intermediate in CT and highest in the CC genotype ($P=0.018$). Oxidative stress may be a factor in insulin resistance and it is possible that the p22PHOX C242T polymorphism effects plasma insulin levels by altering ROS production.

In the selected population of hypertensive and normotensive Kashmiri individuals, the p22PHOX gene was identified to exhibit a C242T single nucleotide polymorphism, with allele frequencies in Hardy-Weinberg equilibrium. The genotype and allele distribution did not differ between hypertensive and normotensive individuals. There was no differential CV risk factor burden between different genotypes or alleles with the exception of significantly lower fasting BG level in individuals with the T-allele compared with those without the allele.

References


