Free Radical Research, January 2008; 42(1): 82–92

informa
healthcare

Insight into the role of CYBA A640G and C242T gene variants and coronary heart disease risk. A case-control study

A. MACÍAS-REYES[1], F. RODRÍGUEZ-ESPARRAGÓN[1], A. CABALLERO-HIDALGO[1,2], Y. HERNÁNDEZ-TRUJILLO[1], A. MEDINA[1,3], & J. C. RODRÍGUEZ-PÉREZ[1,2,4]

[1]*Research Unit,* [2]*Universidad Las Palmas de Gran Canaria,* [3]*Cardiology, and* [4]*Nephrology Departments, HUGC Dr. Negrín, Las Palmas de Gran Canaria, Spain*

Accepted by Professor Henrik Poulsen

(Received 17 May 2007; in revised form 8 November 2007)

Abstract
The CYBA gene variants have been inconsistently associated with coronary heart disease (CHD) risk. A case-control study was conducted genotyping 619 subjects to explore the contribution of C242T and A640G to CHD risk in the population. A significant risk was found associated with GG homozygosity (odds ratio (OR) 2.132, 95% confidence interval, 1.113–4.085). The C242T variant was associated with CHD risk in women. Bias due to population stratification was analysed. Phenotype changes linked to these polymorphisms were evaluated. Superoxide measurements revealed higher production as indicated by the presence of the G and T alleles. Differences in mRNA concentration in heterozygous A640G samples were analysed. Higher levels of G allele mRNA compared with A allele mRNA were found. NAD(P)H oxidase p22phox sub-unit expression was evaluated with Western blot. Experiments revealed a gradual relationship in p22phox protein expression according to genotypes of the analysed variants. Those GG TT double homozygous showed increased p22phox protein expressions regarding AA CC double homozygous. This study has demonstrated increased expression and activity of the NAD(P)H system components during atherogenesis and the results could help explain the relevance of the A640G variant as a CHD marker.

Keywords: *NADPH Oxidase, p22phox, CYBA, polymorphisms, lipid hydroperoxide*

Abbreviations: *CHD, Coronary Heart Disease; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; CYBA, cytochrome b-245 alpha polypeptide; p22phox, p22 phagocyte b-cytochrome; LOOH, Lipid hydroperoxide; PBMC, peripheral blood mononuclear cell; SOD, superoxide dismutase.*

Introduction

The major source of superoxide in the vascular smooth muscle cells (VSMCs), endothelial cells and myocites is the NAD(P)H oxidase system [1–3]. Increased superoxide production has been implicated in atherosclerosis, arterial hypertension, cancer and diabetes [4,5].

There is accumulated evidence of differences between vascular NAD(P)H oxidase and that which is expressed in neutrophils [6]; however; the molecular identity of the p22phox component between both systems has been confirmed [7] as p22phox constitutes a critical component of vascular NAD(P)H oxidase [7,8]. Thus, it has been shown that the complete inhibition of p22phox mRNA expression in VSMCs results in a decrease in cytochrome b content and superoxide production. Fukui et al. [9] found an increased expression of

Correspondence: Francisco Rodríguez-Esparragón, Research Unit, Hospital de Gran Canaria Dr. Negrín, 35010 Las Palmas de Gran Canaria, Spain. Tel: 34-928 449277. Fax: 34- 928 4491. E-mail: frodesp@gobiernodecanarias.org

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760701796918

p22phox and gp91phox in the necrotic sites after myocardial infarction. Azumi et al. [1,10] demonstrated the presence of p22phox–based NAD(P)H oxidase in human atherosclerotic plaques and reported that ROS production and the presence of oxidized-LDL were spatially associated with the p22phox sub-unit. Sorescu et al. [11] observed the co-localization of the Nox2 and p22phox components of the NAD(P)H system and also found an increased expression of these mRNAs concomitantly with the progression of human atherosclerotic plaques in coronary arteries.

Among the described polymorphisms in the CYBA gene that encodes p22phox the C242T transition that results in a Tyr for His substitution at residue 72 has been suggested to play a major role in p22phox function [12]. The 242T allele has been associated with differences in coronary artery risk and superoxide production [12–17]. On the contrary, the A640G gene variant, another polymorphism of the CYBA gene, has been less studied [12,18,19].

We have analysed the relationships of both CYBA gene variants with CHD risk in a case-control study. Because it is known that allele frequencies vary widely within and between populations regardless of disease status, we tested for stratification in our population. Lipid hydroperoxide concentrations according to genotypes were evaluated in the plasma of control subjects. The differences in superoxide anion production according to genotype were evaluated in cells and vascular homogenates. Also protein biosynthesis differences according to genotypes were evaluated in cells by Western blots. Based on our findings, we analysed the differences in mRNA levels according to genotype and in heterozygous individuals, which could explain the relevance of our results.

Materials and methods

Subjects

Participants were selected from a case-control trial carried out in Gran Canaria (Canary Islands, Spain). The sample design and sampling inclusion selection criteria have been described in detail elsewhere [20]. Briefly, cases were defined as an individual of Canaries/Spanish origin admitted to any hospital of Gran Canaria with a diagnosis of myocardial infarction or unstable angina and documented evidence of coronary artery disease by angiography. We minimized the effects of selection bias of subjects suffering a coronary event by acutely studying consecutive patients admitted to the coronary unit.

Age and gender-matched controls were selected from the Gran Canaria population. A two-stage stratified selection process from the Gran Canaria population census randomly identified controls. Controls underwent a structured clinical interview and a physical exam and diagnostic procedures (EKG and echocardiogram) to exclude cardiovascular disease. Subjects, interviewed by a trained nurse, completed a standard questionnaire consisting of demographic information, lifestyle and anthropometry data and history of employed medications. Participants were Canary Islanders with at least two generations of Canarian ancestors. The present study was conducted in subjects from which serum and DNA samples were available.

Blood samples were also obtained from healthy male volunteers and affected patients for peripheral blood mononuclear cell (PBMC) isolation and DNA and RNA studies. CHD patient recruitment was carried out under the inclusion criteria described above.

The study protocol was approved by the Institutional Ethics Committee. Participants provided written informed consent.

Laboratory procedures

Total cholesterol, HDL and triglycerides were measured by enzymatic-colourimetric methods. LDL cholesterol was calculated according to the Friedewald formula when triglyceride levels were ≤ 4.52 mmol/L. Lipoprotein (a) [Lp(a)] was analysed with an immunoturbidimetric method. Total plasma lipid hydroperoxide (LOOH) content was calculated by the Xylenol Orange (FOX) assay as described [21,22]. Briefly, plasma samples were analysed under basal conditions and after being mixed with copper sulphate at a final concentration of 10 μM at 37°C for 24 h. Triphenyl phosphine was used for signal authentication. The absorbance of the supernatants was monitored at 560 nm and the hydroperoxide content determined using a molar absorption coefficient of 4.3×10^4 M^{-1} cm^{-1} or by reference to an H_2O_2 standard curve.

P22Phox variant genotyping

Genotypes of the CYBA gene variants were determined as described [18].

Detection of population stratification

To address the possibility that, in our study, the observed differences in allele frequencies between analysed populations might be related to population structure, we applied Pritchard et al.'s [23] approach. The software STRUCTURE was downloaded from http://pritch.bsd.uchicago.edu. The programme was first used to infer the most probable number of groups according to Pariset et al. [24] and, secondly, it was used to estimate the natural logarithm of the probability that a given genotype X is part of a given population K. To this end, Ya5NBC5, Yb8NBC65, Yc1RG68, Ya5NBC27, Ya5NBC51, Ya5NBC109, Ya5NBC102 and ACE(I/D) polymorphic *Alu* markers

were amplified by PCR in all subjects. Primer sequences and PCR conditions were as described [25]. Implemented data was run using the admixture model assuming K = 1–5 using a burn-in period of 100 000 steps followed by 2 000 000 Markov Chain Monte Carlo replicates.

Cell isolation and culture

PBMCs were isolated from normal healthy blood donors and CHD patients by density-gradient centrifugation using Ficoll-Hypaque (Pharmacia). Isolated cells from healthy subjects were cultured in RPMI 1640 medium containing 10% (vol/vol) normal heat-inactivated calf serum and antibiotics. The cells were allowed to adhere overnight in the presence of 5% CO_2 in a humidified incubator at 37°C. Non-adherent cells were washed away twice with phosphate-buffered saline (PBS). For RNA stability studies, monocytes/macrophages of known genotypes for the CYBA A640G and C242T variants were isolated, cultured and treated with 5 μg/ml of actinomycin D or vehicle for 6 and 12 h prior to RNA isolation.

Cytochrome C test

Monocyte/macrophage cells were isolated as described above from healthy male subjects who had previously been genotyped for the A640G and C242T variants of the CYBA gene. All possible p22phox gene combinations for the two polymorphisms were assayed for superoxide production. Superoxide anion release was measured under basal and stimulated conditions according to the procedure of ferricytochrome C reduction in the presence or absence of superoxide dismutase (SOD). All the chemicals used were from Sigma Chemical Co. (St Louis, MO). Samples were assayed on an ELISA reader (EL_x 800, BIO-TEK Instruments, Inc). Each microwell contained 5×10^4 cells incubated with cytochrome C (150 μM) and SOD (400 U/ml) (SOD+) or an equal volume of Hank's balanced salt solution (HBSS) (SOD−). To activate monocytes/macrophages, 60 ng/ml of phorbol myristate acetate (PMA) was used (PMA+). Under basal conditions, an equal volume of DMSO was added (PMA−). Total final volume was adjusted with HBSS. The reduction of ferricytochrome C was measured from changes in absorbance at 550 nm. The amount of $O_2^{\cdot -}$ released was calculated by dividing the difference in absorbance with and without SOD under basal and PMA-stimulated conditions by the extinction coefficient ($E_{550} = 18.5\ mM^{-1}\ cm^{-1}$) and the results are expressed as nanomoles $O_2^{\cdot -}$ per 10^7 cells.

Chemiluminescent detection of $O_2^{\cdot -}$ production

Small distal mammal artery fragments rejected after histological examination because of surgical reasons were collected from patients undergoing coronary revascularization surgery. Fragments were cleaned from fat, homogenized on a glass homogenizer and conserved at −70°C until use. Protein content was measured using the Bio-Rad Protein Assay with serial dilutions of bovine serum albumin (BSA) as standard. Superoxide anion generation was measured in homogenate samples by using the chemiluminescent-enhanced lucigenin assay. Samples were equilibrated in PBS at pH 7.5. A total of 500 μg per well was placed in opaque 96-well microtiter plates and incubated at 37°C under 95% O_2 and 5% CO_2 for 30 min with and without substrate and inhibitor before counting in an FL_x800 Microplate Fluorescence Reader. Lucigenin was added for determining basal NAD(P)H-derived $O_2^{\cdot -}$ production. A lucigenin concentration of 50 μM avoids problems related to its own autoxidation [26]. This measure was validated by treating the homogenates with the NAD(P)H inhibitor, diphenyl iodonium (DPI, 100 μmol/L) for 10 min before adding lucigenin. To evaluate the NADH-activated $O_2^{\cdot -}$ production, a high concentration of NADH (100 μmol/l) was added to the wells containing lucigenin and homogenates. These measures were also validated by DPI treatment. Signals were integrated over 30 min and averages of the plateau phase used for calculations. Validation experiments were also conducted with intact artery rings used immediately after extraction. Superoxide anion was measured and subsequently rings were dried in an oven for 60 min at 60°C to obtain the tissue dry weight. The specific chemiluminescent signal was expressed as counts per min per milligram of dry weight (cpm/mg). Basal and activated-NAD(P)H oxidase activities were expressed as the difference of aortic $O_2^{\cdot -}$ production under each condition in the presence and in the absence of DPI.

Western Blot Analysis

PBMCs were isolated from double homozygous subjects for the A640G and C242T variants. Cells were lysed in a buffer containing 6 M urea, 25% glycerol, 0.1% SDS, 0.5% sodium deoxycholic acid, 1% Nonidet P-40 substitute (Sigma), 150 mM NaCl, 50 mM Tris/HCl (pH 7.8) and a protease inhibitor mixture (Roche) essentially as described [27]. Lysates were incubated with Laemmeli sample buffer containing 5% 2-mercaptoethanol for 30 min at room temperature and then boiled for 5 min. Approximately equal amounts of proteins were resolved by 15% SDS-PAGE and transferred to nitrocellulose membrane. Following blocking, proteins were probed with polyclonal rabbit anti-p22phox and anti-CD68 antibodies (Santa Cruz Biotechnology) diluted 1:100

in blocking solution. Visualization was carried out with SuperSignal West Pico chemiluminescent substrate (Pierce) using horseradish peroxidase-conjugated secondary antibodies against rabbit IgG (1:2000) (Dako). Blots were photographed in a Gene Genius device and analysed by the GeneTools Software from Syngene. For all of the Western blot analysis experiments, CD68 was used as an internal loading control. The ratio of p22phox to CD68 Western blot products, as pixel density in densitometric units was used as indexes of p22phox protein expression.

cDNA synthesis and polymerase chain reaction (PCR) amplification and restriction analysis

Total RNA was extracted from PBMCs isolated from CHD patients. Reverse transcription of mRNA was carried out using MMLV reverse transcriptase (Roche Applied Science) according to manufacturer's instructions.

The allele-mixing experiment was performed as described [28] with changes. Total RNA from cultured cells of an AA homozygote of the A604G polymorphism was added in increasing amounts (from 0.1–1.6 μg in 0.1-μg increments) to 1 μg of the total RNA from PBMCs of a GG homozygote. These mixtures were used for cDNA synthesis. Amplification of the p22phox cDNA fragment encompassing the A640G polymorphism was performed with 2 μl of the cDNA synthesized with the same primers used for genotyping the A640G variant except that the sense primer was 5′-labelled with 6-carboxyfluorescein (6-FAM) (Applied Biosystem UK). PCR conditions were as described [18] [Genbank accession number GI:4557504] except that the annealling temperature was 60°C and cycle number was 29. Restriction analysis of labelled PCR products was performed with *DraIII*; an excess of enzyme was used to ensure a complete digestion yielding labelled, allele-specific fragments of 258 bp (G) and 227 bp (A). The digested PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and by ethanol precipitation and separated on an ABI Prism 310 Analyser.

To analyse mRNA decay rates according to A640G genotypes, actinomycin D chase experiments were performed. Total RNA prepared from cultured, non-treated monocytes/macrophages (controls) and actinomycin D treated cells was used to measure CYBA mRNA stability. Following retrotranscription, a 5′-fluorescence-labelled fragment of the CYBA gene was amplified as described above. Normalization was performed against a 147-bp amplified fragment of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) with the forward primer 5′GGCCTCCA AGGAGTAAGACC3′ (5′-labelled with 6-FAM) and the reverse primer 5′AGGGGTCTACATGGCAA CTG3′ (spanning GAPDH 1031–1177 bp) [Genbank accession number BC020308]. Products were separated on an ABI Prism 310 Analyser.

Statistical analysis

Distortions from Hardy-Weinberg equilibrium (HWE) were estimated for single and bi-loci disequilibria by using Genetic Data Analysis (GDA) version 1.1 and Arlequin version 2.000 software, respectively [29]. Arlequin was also used to infer haplotype frequency from genetic data. SPSS statistical software, version 12.0, was used for the remaining data analysis. Quantitative variables are presented as mean ± SD and qualitative variables as percentages. Assumption of normal distribution for continuous variables was tested by Kolmogorov-Smirnov z-statistics. Normally distributed continuous variables were compared by one-way ANOVA; the Kruskal-Wallis test was used for comparisons of non-normally distributed variables. Means and pairwise comparisons were performed using unpaired Student's t-tests and the Mann-Whitney U-test when appropriate. Chi-square tests and odds ratio (OR) with 95% confidence interval (CI) analyses were carried out to estimate the risk of CHD associated with categorical variables and the analysed polymorphisms.

Logistic regression analysis was performed using the Wald stepwise method including as the dummy variable the genotypes of polymorphic variants. To compare superoxide production using fluorometric readings between unprocessed artery samples and those processed by homogenization, we also considered the following ANCOVA model: $y_{i,j} = \alpha + \gamma_i + \beta_i \times (time)_{i,j}$ (with i = vascular artery homogenates, intact artery rings and $j = 1, \ldots, 30$). Thus, $y_{i,j}$ represents the expected superoxide production in the i group in the sample j. The γ_i parameter represents the group effects (with $\gamma_{\text{artery rings}} = 0$, as the reference category) and the β_i is the variation rate for $y_{i,j}$ for each time unit variation. Therefore, when the sample processing method does not alter the superoxide production, slopes in both groups are expected to be equal ($\beta_{\text{artery homogenates}} = \beta_{\text{artery rings}}$).

Results

Distribution of A640G and C242T genotypes among cases and controls

Three hundred and four cases (mean age 56 ± 10 years, 22% females) and 315 matched community controls (mean age 54 ± 10 years, 26% females) were randomly selected. The main clinical characteristics of study subjects are listed in Table I. Genotype distribution for the analysed polymorphisms was in accordance with those frequencies expected in HWE for both gene variants in the whole study and in cases and controls. We found that genotype distribution for

Table I. Main clinical characteristics of the study subjects.

	Cases	Controls	*p*-value
Sample size, *n*	304	315	0.658
Sex (male/female), *n*	237/67	232/83	0.211
Age, years	56±10	54.5±11	0.062
MI, %	60	–	–
Smoking status, %	50	27.3	<0.001
Diabetes, %	33.9	12.1	<0.001
BMI, Kg/m^2	27.2±3.7	27.3±3.8	0.693
SBP, mm Hg	135±24	136±26	0.954
DBP, mm Hg	76±13	84±12	<0.001
Alcohol, g/day	16±27	11±19	0.011
Total cholesterol, mmol/L	5.2±1.1	6.1±1.0	<0.001
LDL cholesterol, mmol/L	3.5±1.0	4±1.0	<0.001
Triglycerides, mmol/L	1.24±0.67	1.52±0.77	<0.001
Lp (a), mg/dL	53.5±68	36.8±43	<0.001
CYBA A640G			
A allele	0.4045	0.577	
G allele	0.5955	0.423	<0.001
CYBA C242T			
C allele	0.6155	0.622	
T allele	0.3845	0.378	0.828

A640G gene polymorphisms was significantly different between groups ($\chi^2_2 = 37.4$; $p < 0.001$). Genotype distribution for cases and controls, respectively, was AA: 15% and 30.6%; AG: 50.9% and 54.2%; and GG: 34.1% and 15.2%. Bivariate analysis showed a strong cardiovascular risk associated with the GG genotype with an OR of 2.9, 95%CI 1.93–4.32. G allele carriers had an OR of 2.5, 95%CI 1.66–3.76. No gender-dependent difference was observed, although a higher cardiovascular risk associated with the GG genotype was found among the males (OR = 3.36, 95%CI 2.08–5.434) compared to females (OR = 2.04, 95%CI 0.96–4.34).

There was no difference in the genotype distribution among groups for the C242T polymorphism ($\chi^2_2 = 0.12$; $p = 0.94$). Genotype distribution for cases and controls, respectively, was CC: 39% and 39.2%; CT: 45.1% and 46%; and TT: 15.9% and 14.8%. No differences were observed among cases and controls either between C allele carriers vs AA homozygotes or A allele carriers vs CC homozygotes for the total population studied. A gender difference was observed: there was a trend towards a significant difference in the C242T genotype distribution in women ($\chi^2_2 = 5.924$; $p = 0.05$). Bivariate analysis showed that female T allele carriers had a significant cardiovascular risk with an OR of 2.23, 95%CI 1.08–4.58.

Both variants were found to be in linkage disequilibrium in controls ($\chi^2_1 = 14.75$; $p < 0.001$); the A and G alleles of the A640G variant were preferentially found with the T and C alleles of the C242T variant, respectively. Linkage disequilibrium tested among the cases was at the threshold of significance ($\chi^2_1 = 3.65$; $p = 0.056$). The overall comparison of the inferred haplotypes was significantly different between patients and controls.

The cardiovascular risks associated with classic cardiovascular risk factors have been previously analysed using multivariate model approaches in our population [20,22]. Among others, those models included a detailed lipid profile, hypertensive status, blood pressure values, diabetes, glycemia, familial antecedents of diabetes and coronary diseases. Also, we have interpreted the associated risk obtained when considering the fact that some of the analysed variables were clearly modified because of the pharmacological treatment. Since our present interest remains estimative rather than predictive, the selected model depicted in Table II was the best obtained resultant of the inclusion of variables that differed significantly in bivariate analysis. Thus, in this model we obtained an OR of 2.132, 95%CI 1.113–4.085 for the GG genotype of the A640G variant and an OR of 3.85, 95%CI 1.748–8.481 for the interaction term 'T allele carrier of the C242T variant and gender (women)'.

Population admixture analysis

We explored population characteristics by the model-based inference framework used in the Structure software. Genotype distributions were initially tested for significant deviations from HWE. Using an approach similar to described [24], the likelihood values and the variance of the bootstrap samples obtained with the Structure software were plotted against K for choosing the optimal K value. We found that the likelihood reaches a maximum around K = 2. A similar result was obtained when the recommendations of Pritchard et al. [23] were applied. Thus, the smallest probability was associated with a prior K of 2 (Table III). Results from STRUCTURE for K = 2 showed that the distribution of members was approximately similar between inferred clusters. The same conclusion was reached by examining global probabilities regardless of the value of K between the ranges of 2–5 (Table III). This symmetry was strongly

Table II. Multivariate model.

	OR	IC 95%
Diabetes	2,926	1.550–5.523
HTA	1,939	1.127–3.335
Lp(a)	1,009	1.003–1.015
Tobacco	3,506	1.996–6.160
Triglycerides	0,988	0.984–0.999
HDL-cholesterol	0,854	0.825–0.884
Total colesterol	0,992	0.984–0.999
Alcohol	1,019	1.006–1.015
GG genotype (A640G)	2,132	1.113–4.085
T allele carrier (C242T)*Sex (women)	3,850	1.748–8.481
Global adjustment		84.4%

HTA: hypertension; T allele carrier (C242T)*Sex (women) refers to the interaction term when these two variables were considered.

Table III. (a) Estimates of log probability of data under the admixture model and (b) Proportion of membership of the whole studied population under the admixture model.

(a)		Ln P (X/D)			P (K/X)	
	K = 2	−3164.9			1	
	K = 3	−3797.6			≅0	
	K = 4	−3232.3			5.35×10^{-30}	
	K = 5	−3290.7			2.32×10^{-55}	
				Inferred groups		
(b)		1	2	3	4	5
	K = 2	0.428	0.572			
	K = 3	0.330	0.341	0.329		
	K = 4	0.277	0.223	0.277	0.223	
	K = 5	0.193	0.193	0.211	0.211	0.192

suggestive of the absence of population structure in our study sample.

Genotype-phenotype associations: Lipid hydroperoxides

Total lipid hydroperoxide (LOOH) content was evaluated in the plasma of control subjects. We found a gradual but not significant relationship in LOOH content according p22phox genotypes. Those G and T allele carriers possessed higher plasma LOOH levels than those A and C allele carriers. For simplicity, the subjects were grouped as double homozygotes of the p22phox analysed variants; therefore, only those subjects with truly informative haplotype were considered. As depicted in Figure 1, we found a significant difference in LOOH levels in the oxidized plasma samples of those subjects with G T haplotype regarding those subjects with the A C haplotype ($p = 0.038$).

Genotype-phenotype associations: Superoxide generation from cultured monocytes/macrophages

A graded but not statistically significant relationship in basal superoxide production in cultured cells

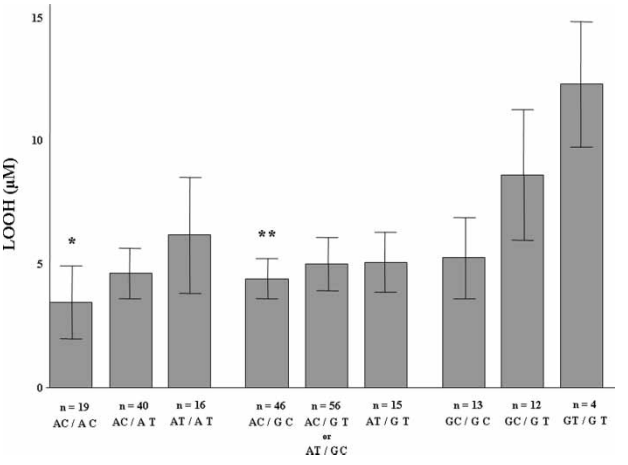

Figure 1. Lipid hydroperoxides concentration values according to p22phox haplotypes. Values are means ± mean standard error. *$p = 0.038$ and **$p = 0.032$ for the Mann-Whitney U-test comparing LOOH concentrations of the indicated haplotype vs G T haplotype.

($n = 41$) according to A640G and C242T genotypes was observed. Analysis revealed that GG and TT homozygous samples produced more superoxide anion than did AA and CC homozygotes. These graded relationships based on genotype were lost when cells were analysed after PMA-treatment. Because of linkage disequilibrium, we analysed superoxide production according to genotype combinations in healthy controls. A graded but not significant relationship according to A640G variant was observed under basal conditions in the sub-set of TT homozygotes of the C242T. This relationship was lost under stimulated conditions. Similarly, we found that under basal and stimulated conditions, a graded but not significant relationship was observed according to C242T genotypes only in the sub-set of GG homozygotes of the A640G variant.

Genotype-phenotype associations: Superoxide production from mammal artery fragments

Chemiluminescent detection of NAD(P)H-derived superoxide production was analysed in vascular homogenates obtained from 15 patients as described in the Methods section. Previously performed validation experiments using intact artery rings and vascular homogenates showed no differences in slopes ($p = 0.34$). We observed a higher but not statistically significant NADP(H)-derived superoxide anion production was found in GG and TT double homozygotes of the A640G and C242T variants.

Allele mixing experiment

We used a non-radioactive approach that was sensitive enough to detect changes in the relative concentrations of A- and G-type mRNA. Figure 2A shows the ratios of the fluorescence signals corresponding to the labelled fragments of the A and G alleles (219/258). There was a decrease in the A:G signal ratio according to the increase in the amount of the A allele digestion product. Five experiments with three replicates were performed. Data from Figure 2A indicate the mean ratios of representative experiments.

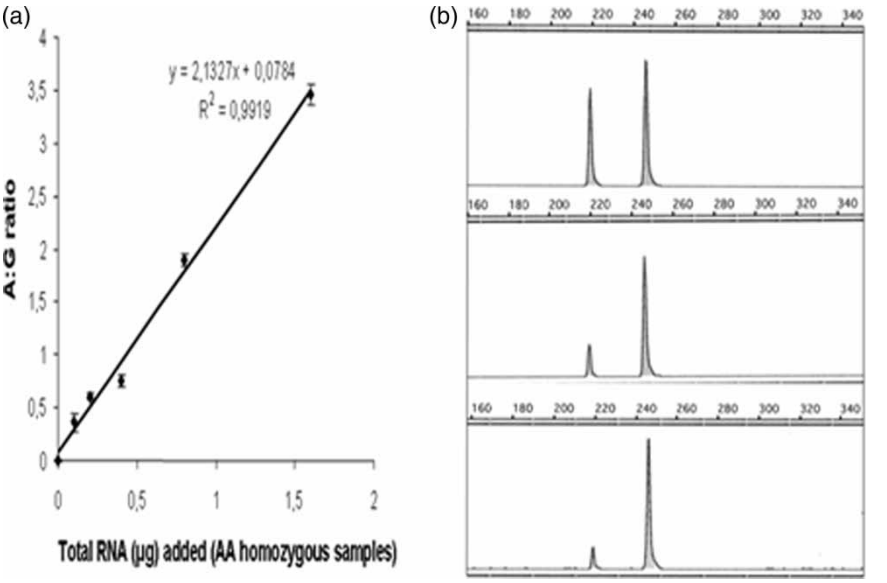

Figure 2. (A) Graphic representation of the A allele:G allele signal ratios obtained in the allele-mixing experiments. Data are means ± SD of three different mixed samples replicated twice. (B) Selected examples of the AG heterozygotic samples analysed.

Analysis of heterozygotic A640G monocytes/macrophages

Total RNA samples from PBMCs of previously genotyped heterozygous subject for the A640G variant were obtained and analysed as described above. To avoid C242T-dependent effects, all analysed A640G heterozygous samples were genotyped and confirmed to be CC homozygous for the C242T variant. Twenty-one A640G heterozygous samples were analysed. Results demonstrate significantly higher levels of G allele mRNA compared with A allele mRNA with a mean value of 2.58 ± 1.75. Selected examples are depicted in Figure 2B.

Analysis of mRNA degradation rates

We compared the mRNA degradation rates of the CYBA gene transcript in cultured cells by actinomycin D chase experiments. Cells were isolated from previously genotyped homozygous subjects (GG and AA) for the A640G gene variant. Results are depicted in Figure 3A. At the indicated time points there were no statistically significant differences among the CYBA gene transcripts according to A640G genotypes.

Effects of genotypes on p22phox protein expression

To analyse the influence of A640G and C242T variants of the CYBA gene on p22phox expression, we evaluated the expression of the NAD(P)H oxidase subunit p22phox using Western blot. Figure 3B showed a clear effect on p22phox expression according to genotypes of the analysed variants. Moreover in accord with mRNA analysis we found that p22phox protein expression was significantly higher in cell lysates from GG TT double homozygous subjects than those obtained from AACC double homozygous subjects.

Discussion

We found that the A640G gene variant of p22phox constitutes a strong genetic risk marker of CHD in the whole study population and is even more significant among males.

As has been shown, the most important source of superoxide anion is the enzyme NADP(H) oxidase [30]. Increased expression of the NAD(P)H components in infarcted areas [1,31] has been demonstrated, as has its relevance to atherosclerotic disease progression [10,11]. Polymorphic variants described in the CYBA gene have been controversially associated with CHD risk [12,13,15,16,19]. Gardemann et al. [18] found that the A640G gene variant constituted an independent coronary risk factor for males and suggested that the A-to-G transition might influence CYBA mRNA processing and stability or alternatively act as a neutral marker. We have analysed CYBA gene mRNA production in A640G heterozygous samples testing for the sensitivity of the method by allele-mixing experiments. Our results revealed higher levels of G allele mRNA compared with A allele mRNA. We did, however, identify a great variability, but this could suggest that the precise combination of other gene variants or gene–gene interactions with the analysed polymorphism determines *in vivo* transcriptional gene regulation. Nevertheless, we used only one set of primers, and the possibility that the A640G polymorphism could be associated with conformational changes of mRNA should not be ruled out. Data from Western blots are also consistent with mRNA assays showing a

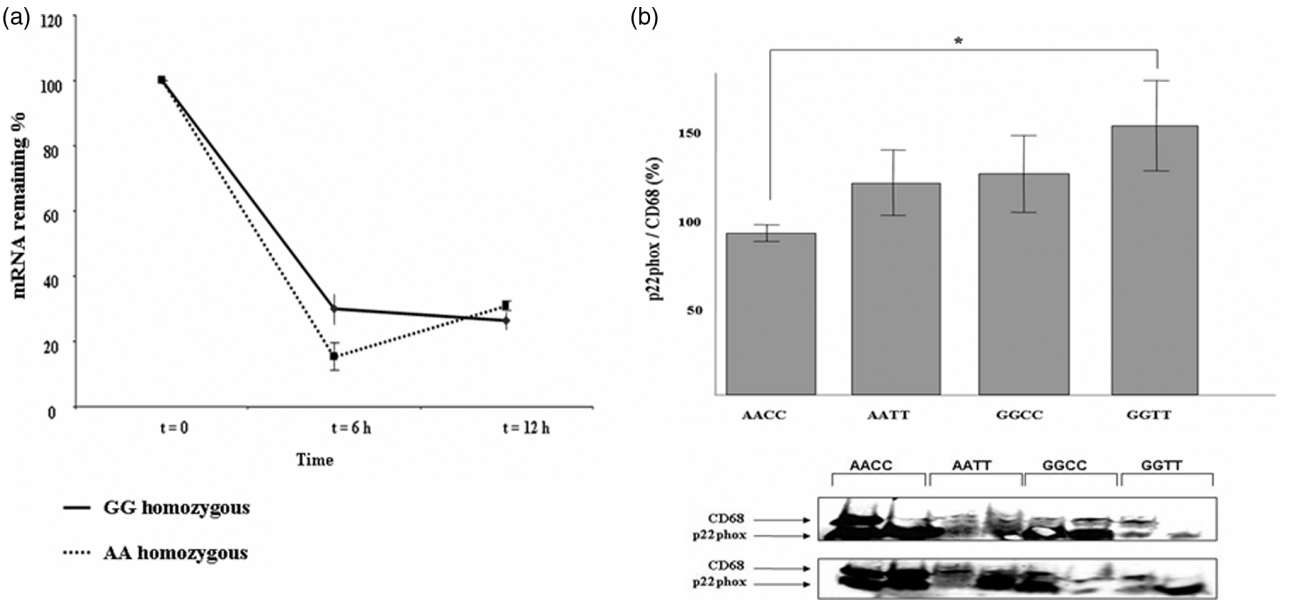

Figure 3. (A) Actinomycin D (Act D) chase experiments. Monocyte/macrophage cells were isolated from previously genotyped GG and AA homozygous subjects for the A640G variant. Cells were treated with Act D and vehicle and harvested at the indicated time points. mRNA remaining at the indicated time point is expressed as a percentage of the initial value, after being normalized to GAPDH. Signal intensity at time 0 was defined as 100%. Results are shown as means ± SD of three (GG) and three (AA) samples replicated five times. (B) Bars plot of protein data of Western blotting expressed as percentages of p22phox signal compared to CD68 signal. Bars represent Mean ± SEM values of four lysates from subjects belonging to each type of double homozygosis at least three times repeated. $*p = 0.011$.

gradual relationship according to genotypes of the analysed variants. Thus, the NAD(P)H oxidase subunit p22phox protein level normalized to CD68 protein level was found to be higher when passing from AA to GG genotype of the A640G variant but also when passing from CC to TT genotype of the C242T gene variant.

Lipid hydroperoxide (LOOH) can be considered a reliable marker of oxidative stress [32]. Measurements in plasma of control subjects showed a gradual relationship according to p22phox genotypes and haplotypes. Moreover when LOOH levels were analysed according to haplotypes we found a significantly higher level in subjects with the G T haplotype than in subjects of the A C haplotype.

Superoxide anion production was measured in monocyte/macrophages and internal mammal artery homogenates. Data from experiments showed a trend toward a difference in NADP(H) system activity in both A640G and C242T variants. Therefore, although these experiments did not reach statistical significance, it seems that our phenotypic data reinforce genetic findings. Superoxide production derived from the NADP(H) oxidase system was analysed in monocytes and vascular homogenates by two reference methods whose sensitivities are known to differ [26,33]. However, data obtained from healthy subjects tended in the same direction as data obtained using artery fragments from affected patients where, as reported [17], it is difficult to investigate the effect of a polymorphism free of other factors known to increase superoxide results. It is known that under certain physiological situations, increased ROS production is associated with compensatory pathways leading to an up-regulation in expression levels of antioxidant enzymes [34]; however, these compensatory pathways are lost in disease situations. It is conceivable that these mechanisms could help explain the relevance of the A640G gene variant that we have identified. Moreover, basal differences in mRNA levels according to the A640G variant could be relevant when considered in conjunction with the up-regulation of all NAD(P)H components as well as enzyme activity that has been described during atherogenesis [11].

There are conflicting reports in the literature [18] with our findings. Authors studied a large male population composed of individuals that underwent coronary angiography for diagnostic purposes. Cases and controls were then selected according to the presence/absence of 50% of vessels stenosis. Therefore, studies comparisons differ according to control selection criteria; sample size; and gender selection criteria, mainly because they found higher cardiovascular risk in AA homozygous and we found higher cardiovascular risk in GG homozygous subjects. Regarding the differences in control selection criteria, some reports argue that in case-control studies patient-based controls vs population controls constitute study strengths rather than a limitation [18,35]. Others suggest caution in the interpretation of results [36]. We have analysed a well-characterized selected population. Our controls were selected from the same population as cases and their risk-factor information

was collected. Diagnostic procedures were used to exclude coronary heart disease. Moreover, it is known that cases should be the same individuals who would have been considered cases in a hypothetical cohort study. To this end, incident cases were recruited in our study period to minimize survival bias. We found that the strength of the association was statistically high therefore, although we have analysed a smaller than Gardemann et al. [18] population, our study seems not to be under-powered because of limited sample size. Another important issue is that population admixture and ethnic heterogeneity that has been recognized qualitatively as one major potential error cause in association studies [37,38] was tested in our study. Also, we explored some genetic and phenotypic changes linked to the presence of the analysed variants. Moreover, studies have reported associations between CYBA-p22phox gene variants and some clinical phenotypes [39–41]. Some of these works argue in the same direction as our study while others show inverse relationships. Thus, a significant interaction between systemic oxidative stress level response to exercise training and A640G polymorphism has been found [40]. Authors found that subjects with one or two copies of A allele at the A640G locus showed a greater reduction in thiobarbituric acid reactive substances (TBARS) level after exercise training than those without an A allele. Furthermore, their diplotype analysis showed that TBARS was decreased to a greater extent in the C242/A640 haplotype carriers compared with the non-carriers [40]. Similarly, a genotype-phenotype association has been recently reported between the NADPH oxidase p22phox genotypes and plasma nitrotyrosine level [41]. On the contrary, a study that analysed among others the A640G and C242T CYBA gene variants identified the C242/A640 haplotype as a risk haplotype for end stage renal disease [39]. It turns out paradoxical to find this result in a sub-group of non-diabetic patients taking into account the largely documented impact of oxidative stress in diabetes [4,5]. It might be possible that interactions between unadjusted confounders at least partly explained the results obtained in this work.

Therefore, some but not all case-controls studies confounders were avoided in our study. There are other possible but not analysed sources of heterogeneity including gene–gene or gene–environment interactions; locus heterogeneity; variable effects of linkage disequlibrium; variable disease expression or differential disease susceptibilities; variable phenotypes and different cut-off points and genotyping error [42]. To this end our analysis does not solve the problem of a false positive; but it does a spurious association unlikely to be responsible for the observed link. Interestingly, new functional variants have been described in the CYBA gene [43]. It is possible that these variants could be linked to the A640G polymorphism. Alternatively, the A640G variant could be linked to other not studied variants either in the same gene or elsewhere.

According to some [16,17] but not all studies [14], we found a higher but not statistically significant increased superoxide production linked to the T allele of the C242T variant. The C242T genotype distribution was also statistically different in women in our population. Most of the association reports that evaluated the C242T on CHD risk provided conflicting results [13,14,18,44,45]. Notably, the C242T polymorphism is clearly influenced by ethnicity. It is known that genotype frequencies differ between Japanese and other populations [12,16]. Peculiarly, the association between C242T and ischemic cerobrovascular disease (CVD) has been found in the Japanese [46], whereas studies carried out in other populations failed to find such association [47]. As has been suggested [46], polymorphism of the putative heme-binding site in the p22-phox gene may have different effects on NADPH oxidase in the central nervous system and the cardiovascular system and even have opposite effects in CVD and CHD.

Although we cannot speculate on the functional properties of the C242T variant since this aspect was insufficiently explored in our study, it is possible that the C242T could be linked to other functional variants either in the same gene or elsewhere. Moreover, there are numerous indications that steroid hormone levels affect NAD(P)H-mediated superoxide production [48,49]. This is suggestive that there may be differential regulation of p22phox gene expression by oestrogen by genotype as described [49]. Additional studies of the mechanisms of p22phox regulation are needed.

Acknowledgements

This work was supported by grants/04 from the Fundación Canaria de Investigación y Salud (FUNCIS) and Fundación Mapfre-Guanarteme. Authors gratefully acknowledge the technical assistance Lidia Estupiñán-Quintana, Erika Hernández Velazquez, Ramón Saavedra, Nuri Purswani Ramchandi and Clara Martel. We are in debt to B. López-Valcarcel of the University of Las Palmas (ULPGC) for contributions in helping us with the study design.

References

[1] Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. Expression of NADH/NADPH oxidase p22phox in human coronary arteries. Circulation 1999;100:1494–1498.

[2] Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase

activity in cultured vascular smooth muscle cells. Circ Res 1994;74:1141–1148.

[3] Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci USA 1997;94:14483–14488.

[4] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–844.

[5] Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM. NADPH oxidases in cardiovascular health and disease. Antioxid Redox Signal 2006;8:691–728.

[6] Griendling KK, Ushio-Fukai M. Redox control of vascular smooth muscle proliferation. J Lab Clin Med 1998;132:9–15.

[7] Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK. p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. J Biol Chem 1996;271:23317–23321.

[8] Foubert TR, Bleazard JB, Burritt JB, Gripentrog JM, Baniulis D, Taylor RM, Jesaitis AJ. Identification of a spectrally stable proteolytic fragment of human neutrophil flavocytochrome b composed of the NH2-terminal regions of gp91(phox) and p22(phox). J Biol Chem 2001;276:38852–38861.

[9] Fukui T, Yoshiyama M, Hanatani A, Omura T, Yoshikawa J, Abe Y. Expression of p22-phox and gp91-phox, essential components of NADPH oxidase, increases after myocardial infarction. Biochem Biophys Res Commun 2001;281:1200–1206.

[10] Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. Arterioscler Thromb Vasc Biol 2002;22:1838–1844.

[11] Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429–1435.

[12] Inoue N, Kawashima S, Kanazawa K, Yamada S, Akita H, Yokoyama M. Polymorphism of the NADH/NADPH oxidase p22 phox gene in patients with coronary artery disease. Circulation 1998;97:135–137.

[13] Cahilly C, Ballantyne CM, Lim DS, Gotto A, Marian AJ. A variant of p22(phox), involved in generation of reactive oxygen species in the vessel wall, is associated with progression of coronary atherosclerosis. Circ Res 2000;86:391–395.

[14] Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Functional effect of the C242T polymorphism in the NAD(P)H oxidase p22phox gene on vascular superoxide production in atherosclerosis. Circulation 2000;102:1744–1747.

[15] Schachinger V, Britten MB, Dimmeler S, Zeiher AM. NADH/NADPH oxidase p22 phox gene polymorphism is associated with improved coronary endothelial vasodilator function. Eur Heart J 2001;22:96–101.

[16] Shimo-Nakanishi Y, Hasebe T, Suzuki A, Mochizuki H, Nomiyama T, Tanaka Y, Nagaoka I, Mizuno Y, Urabe T. Functional effects of NAD(P)H oxidase p22(phox) C242T mutation in human leukocytes and association with thrombotic cerebral infarction. Atherosclerosis 2004;175:109–115.

[17] Wyche KE, Wang SS, Griendling KK, Dikalov SI, Austin H, Rao S, Fink B, Harrison DG, Zafari AM. C242T CYBA polymorphism of the NADPH oxidase is associated with reduced respiratory burst in human neutrophils. Hypertension 2004;43:1246–1251.

[18] Gardemann A, Mages P, Katz N, Tillmanns H, Haberbosch W. The p22 phox A640G gene polymorphism but not the C242T gene variation is associated with coronary heart disease in younger individuals. Atherosclerosis 1999;145:315–323.

[19] Zafari AM, Davidoff MN, Austin H, Valppu L, Cotsonis G, Lassegue B, Griendling KK. The A640G and C242T p22(phox) polymorphisms in patients with coronary artery disease. Antioxid Redox Signal 2002;4:675–680.

[20] Rodriguez-Perez JC, Rodriguez-Esparragon F, Hernandez-Perera O, Anabitarte A, Losada A, Medina A, Hernandez E, Fiuza D, Avalos O, Yunis C, Ferrario CM. Association of angiotensinogen M235T and A(-6)G gene polymorphisms with coronary heart disease with independence of essential hypertension: the PROCAGENE study. Prospective Cardiac Gene. J Am Coll Cardiol 2001;37:1536–1542.

[21] Nourooz-Zadeh J, Tajaddini-Sarmadi J, Ling KL, Wolff SP. Low-density lipoprotein is the major carrier of lipid hydroperoxides in plasma. Relevance to determination of total plasma lipid hydroperoxide concentrations. Biochem J 1996;313:781–786.

[22] Rodriguez-Esparragon F, Rodriguez-Perez JC, Hernandez-Trujillo Y, Macias-Reyes A, Medina A, Caballero A, Ferrario CM. Allelic variants of the human scavenger receptor class B type 1 and paraoxonase 1 on coronary heart disease: genotype-phenotype correlations. Arterioscler Thromb Vasc Biol 2005;25:854–860.

[23] Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics 2000;155:945–959.

[24] Pariset L, Cappuccio I, Ajmone MP, Dunner S, Luikart G, England PR, Obexer-Ruff G, Peter C, Marletta D, Pilla F, Valentini A. Assessment of population structure by single nucleotide polymorphisms (SNPs) in goat breeds. J Chromatogr B Analyt Technol Biomed Life Sci 2006;833:117–120.

[25] Ray DA, Walker JA, Hall A, Llewellyn B, Ballantyne J, Christian AT, Turteltaub K, Batzer MA. Inference of human geographic origins using Alu insertion polymorphisms. Forensic Sci Int 2005;153:117–124.

[26] Munzel T, Afanas'ev IB, Kleschyov AL, Harrison DG. Detection of superoxide in vascular tissue. Arterioscler Thromb Vasc Biol 2002;22:1761–1768.

[27] Kawahara T, Ritsick D, Cheng G, Lambeth JD. Point mutations in the proline-rich region of p22phox are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation. J Biol Chem 2005;280:31859–31869.

[28] Leviev I, Negro F, James RW. Two alleles of the human paraoxonase gene produce different amounts of mRNA. An explanation for differences in serum concentrations of paraoxonase associated with the (Leu-Met54) polymorphism. Arterioscler Thromb Vasc Biol 1997;17:2935–2939.

[29] Scheneider L, Roessli D, Excoffier L. A software for population genetics data analysis. Switzerland: Genetics and Biometry Laboratory, University of Geneva; 2000.

[30] Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 2000;86:494–501.

[31] Yokoyama M, Inoue N, Kawashima S. Role of the vascular NADH/NADPH oxidase system in atherosclerosis. Ann NY Acad Sci 2000;902:241–247.

[32] Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 1998;39:1529–1542.

[33] Tarpey MM, White CR, Suarez E, Richardson G, Radi R, Freeman BA. Chemiluminescent detection of oxidants in vascular tissue. Lucigenin but not coelenterazine enhances superoxide formation. Circ Res 1999;84:1203–1211.

[34] Laude K, Cai H, Fink B, Hoch N, Weber DS, McCann L, Kojda G, Fukai T, Schmidt HH, Dikalov S, Ramasamy S,

Gamez G, Griendling KK, Harrison DG. Hemodynamic and biochemical adaptations to vascular smooth muscle overexpression of p22phox in mice. Am J Physiol Heart Circ Physiol 2005;288:H7–H12.

[35] Winkelmann BR, Russ AP, Nauck M, Klein B, Bohm BO, Maier V, Zotz R, Matheis G, Wolf A, Wieland H, Gross W, Galton DJ, Marz W. Angiotensinogen M235T polymorphism is associated with plasma angiotensinogen and cardiovascular disease. Am Heart J 1999;137:698–705.

[36] Andreotti F, Burzotta F, De SV, Maseri A, Iacoviello L. The G20210A prothrombin mutation and the Physicians' Health Study. Circulation 2000;101:E207–E208.

[37] Chakraborty R, Smouse PE. Recombination of haplotypes leads to biased estimates of admixture proportions in human populations. Proc Natl Acad Sci USA 1988;85:3071–3074.

[38] Lander ES, Schork NJ. Genetic dissection of complex traits. Science 1994;265:2037–2048.

[39] Doi K, Noiri E, Nakao A, Fujita T, Kobayashi S, Tokunaga K. Haplotype analysis of NAD(P)H oxidase p22 phox polymorphisms in end-stage renal disease. J Hum Genet 2005;50:641–647.

[40] Park JY, Ferrell RE, Park JJ, Hagberg JM, Phares DA, Jones JM, Brown MD. NADPH oxidase p22phox gene variants are associated with systemic oxidative stress biomarker responses to exercise training. J Appl Physiol 2005;99:1905–1911.

[41] Perianayagam MC, Liangos O, Kolyada AY, Wald R, MacKinnon RW, Li L, Rao M, Balakrishnan VS, Bonventre JV, Pereira BJ, Jaber BL. NADPH oxidase p22phox and catalase gene variants are associated with biomarkers of oxidative stress and adverse outcomes in acute renal failure. J Am Soc Nephrol 2007;18:255–263.

[42] Cardon LR, Palmer LJ. Population stratification and spurious allelic association. Lancet 2003;361:598–604.

[43] Moreno MU, San Jose G, Orbe J, Paramo JA, Beloqui O, Diez J, Zalba G. Preliminary characterisation of the promoter of the human p22(phox) gene: identification of a new polymorphism associated with hypertension. FEBS Lett 2003; 542:27–31.

[44] Cai H, Duarte N, Wilcken DE, Wang XL. NADH/NADPH oxidase p22 phox C242T polymorphism and coronary artery disease in the Australian population. Eur J Clin Invest 1999;29:744–748.

[45] Li A, Prasad A, Mincemoyer R, Satorius C, Epstein N, Finkel T, Quyyumi AA. Relationship of the C242T p22phox gene polymorphism to angiographic coronary artery disease and endothelial function. Am J Med Genet 1999;86:57–61.

[46] Ito D, Murata M, Watanabe K, Yoshida T, Saito I, Tanahashi N, Fukuuchi Y. C242T polymorphism of NADPH oxidase p22 PHOX gene and ischemic cerebrovascular disease in the Japanese population. Stroke 2000;31:936–939.

[47] Krex D, Ziegler A, Konig IR, Schackert HK, Schackert G. Polymorphisms of the NADPH oxidase P22PHOX gene in a Caucasian population with intracranial aneurysms. Cerebrovasc Dis 2003;16:363–368.

[48] Imanishi T, Hano T, Nishio I. Estrogen reduces angiotensin II-induced acceleration of senescence in endothelial progenitor cells. Hypertens Res 2005;28:263–271.

[49] Wassmann K, Wassmann S, Nickenig G. Progesterone antagonizes the vasoprotective effect of estrogen on antioxidant enzyme expression and function. Circ Res 2005;97:1046–1054.