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Research paper

Identification of gene variants related to the nitric oxide pathway in patients with acute coronary syndrome

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ABSTRACT

Dysfunction of vascular endothelium is known to have an essential role in the atherosclerotic process by releasing mediators including nitric oxide (NO). Nitric oxide maintains endothelial balance by controlling cellular processes of vascular smooth muscle cells. Evidence suggests that variations in the NO pathway could include atherosclerotic events. The objective of this study was to determine the possible effects of genes on the nitric oxide pathway in the development of acute coronary syndrome (ACS). The blood samples of 100 patients with ACS and 100 controls were collected at Istanbul University, Department of Cardiology. DNA samples were genotyped by using Illumina Cyto-SNP-12 BeadChip. The additive model and Correlation/Trend Test were selected for association analysis. Afterwards, a Q–Q graphic was drawn to compare expected and obtained values. A Manhattan plot was produced to display p-values that were generated by $-\log_{10}(P)$ function for each SNP. The p-values under 1×10^{-4} were selected as statistically significant SNPs while p-values under 5×10^{-2} were considered as suspicious biomarker candidates. Nitric oxide pathway analysis was then used to find the single nucleotide polymorphisms (SNPs) related to ACS. As a result, death-associated protein kinase 3 (DAPK) (rs10426955) was found to be most statistically significant SNP. The most suspicious biomarker candidates associated with the nitric oxide pathway analysis were vascular endothelial growth factor A (VEGFA), methionine sulfoxide reductase A (MSRA), nitric oxide synthase 1 (NOS1), and GTP cyclohydrolase I (GCH-1). Further studies with large sample groups are necessary to clarify the exact role of nitric oxide in the development of disease.

1. Introduction

Acute coronary syndrome (ACS) arises from the formation of thrombus at damaged atherosclerotic plaques in coronary arteries (White and Chew, 2008; Birnbaum et al., 2014a, 2014b; Achar et al., 2005; Kumar and Cannon, 2009; Santos-Gallego et al., 2014; Libby, 2013; Cimmino et al., 2012; Burke and Virmani, 2007; Abbate et al., 2012; Massberg et al., 2003). Acute coronary syndrome is a complex disease determined by both genetic and environmental issues. Almost 80% of ACS occurs because of a thrombus occlusion, which leads to an oxygen imbalance and causes atherosclerotic conditions (Burke and Virmani, 2007; Abbate et al., 2012; Massberg et al., 2003). The dysfunction of vascular endothelium is known to perform an essential function in the atherosclerotic process by releasing mediators such as nitric oxide (NO) (Kawashima and Yokoyama, 2004; Davignon and Ganz, 2004; Matsuzawa and Lerman, 2014 Dec; Napoli et al., 2006). Nitric oxide maintains endothelial balance by controlling cellular processes of vascular smooth muscle cells (Kawashima and Yokoyama, 2004; Davignon and Ganz, 2004; Matsuzawa and Lerman, 2014 Dec; Napoli et al., 2006). The release of NO starts to reduce at the same time that vascular endothelial dysfunction develops (Oemar et al., 1998; George and Johnson, 2010). The level of NO regulated by both neuronal nitric oxide synthase (NOS 1) and endothelial nitric oxide synthase (NOS 3) signals through changes in expression levels or enzyme activities (Park et al., 2004; Dosenko et al., 2006; Casas et al., 2006; Napoli and Ignarro, 2007; Jaramillo et al., 2010; da Costa Escobar Piccoli et al., 2012; Yang et al., 2014). Previous studies revealed that genetic alternations that occur in nitric oxide biosynthesis signaling pathways could promote atherosclerosis and the development of coronary syndromes [18-38]. However, the relationship between genetic changes in up- and down-regulators of nitric oxide pathway and ACS has yet to be elucidated. The goal of this study was to identify SNPs in genes that are regulated by nitric oxide in patients with acute coronary syndrome by using genome-wide association

Abbreviations: ACS, acute coronary syndrome; CHB, Han Chinese in Beijing; DAPK3, death-associated protein kinase 3; GCH1, GTP cyclohydrolase I; JPT, Japanese from Tokyo; MSRA methionine, sulfoxide reductase A; NO, nitric oxide; NOS1, nitric oxide synthase 1 neuronal; VEGFA, vascular endothelial growth factor A; YRI, Yoruba in Ibadan.

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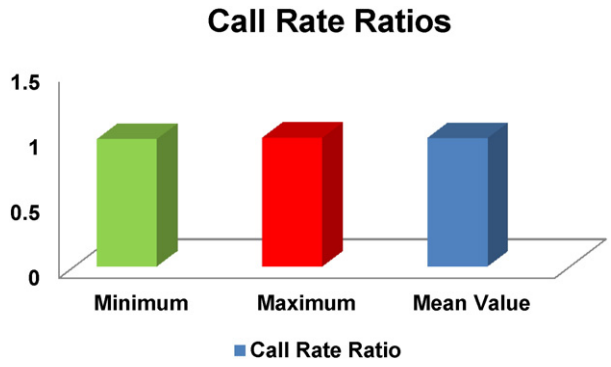

Fig. 1. Call rate values of samples. Distribution of samples. All samples has call rate values >95%.

microarray technology. With the obtained results, the aim was to gain new insight into the pathophysiologic mechanism of acute coronary syndrome and find new biomarkers for novel therapeutic strategies.

2. Material & methods

2.1. Study population

In all, 300,000 SNPs were genotyped from blood samples of patients with ACS (n = 100) and controls (n = 100). All blood samples of patients were collected 1–3 h after a heart attack. Patients who had a history of myocardial infarction (MI) were excluded from the study. Controls were selected from patients attending the Department of Cardiology who had proven to have no acute myocardial or metabolic disease history. Local Ethical Protocol approval consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects) was obtained before the study.

2.2. DNA extraction

The DNA samples were extracted using blood samples from the patients and controls in accordance with the manufacturer's instructions (Invitrogen, Life Technologies). The quality and quantity of DNA samples was measured using Nanodrop (Thermo Scientific, USA). DNA samples that possessed of 1.8–2.0 OD260/OD280 ratios and 200 ng yield were chosen for the genotyping process.

2.3. Genotyping

The Illumina CytoSNP-12 BeadChip 300 K Array was used to genotype approximately 300,000 SNPs. Hybridization procedures were performed using methods as described by Illumina. Arrays were scanned on iScan System (Illumina). Genotyping data was visualized, normalized and clustered using the Illumina Genome Studio Genotyping Module.

2.4. Genotyping quality control

At the first step, the sex of samples was predicted with sample sex identifier module. After comparing the predicted and observed sex of individuals, the mismatched samples were elected from the study. Samples that had call rate under <95% and an MAF < 0.01 elected from SNP sets. Fisher's exact test was used to calculate the Hardy-Weinberg equilibrium (HWE), and SNPs that had p value under 10^{-5} were filtered. Furthermore, we used linkage disequilibrium (LD) to select SNPs that had LD\0.5 before undertaking the principal component analysis (PCA). All of these analyses were performed using the Golden Helix SNP & Variation Suite version 7 software package. After SNP filtering, the obtained genetic data were compared against different ethnic populations: Han Chinese in Beijing (CHB), Japanese from Tokyo (JPT), Yoruba in Ibadan (YRI), and Caucasian.

2.5. Statistical analysis

For statistical analysis, the additive model [dd, Dd, DD] was used for analysis and the Correlation/Trend test was selected as the statistical process. A p-value was calculated for each SNP and scores were generated with $-\log_{10}(P)$. SNPs that belonged to genes involved in the nitric oxide pathway were selected and matched against SNP values calculated after the association study using $-\log_{10}(P)$ function. SNPs with p-values under $1x10^{-4}$ were listed as the most statistically significant markers.

3. Results

After defining minimum (0.976379) and maximum (0.9865547) call rate values based on the genotyping markers, the mean value was calculated (0.984657). Distribution of all samples that had call rate values >95% is shown in Fig. 1. Afterwards, the sex of samples was identified using heterozygosity properties of the X chromosome. In total, 299,671 markers were used to analyze the 181 samples because SNPs are found on the Y chromosome. The study design is summarized in Table 1.

3.1. SNP filtering

SNPs were excluded from the analysis if their call rate was <95% (7825 SNPs) and with MAF < 0.01 (29,943 SNPs). Fisher's exact test was used to calculate the HWE, which identified 9783 SNPs that had p-value under 10^{-5}. In all, 47,530 SNPs were filtered from the study.

Table 1
Study Design. The GWAS contained five stages: genotyping, quality control, principal component analysis, whole genome association analysis and pathway analysis.

Stage	Samples
Stage 1 (Genotyping)	• 300,000 SNPs genotyped in blood samples of ACS cases (n = 100) and controls (n = 100)
Stage 2 (Quality control)	• SNPs showing a calling rate < 95% (7825 SNPs) and an MAF\0.01 (29,943 SNPs) excluded from the analysis. • Fisher's exact test used for HWE (Hardy-Weinberg Equilibrium) and determined 9,783 SNPs that had p- value under 10^{-5}. • Totally 47,530 SNPs filtered from the study.
Stage 3 (Principal component analysis)	• Before PCA analysis, linkage disequilibrium (LD) used to select SNPs that had LD < 0.5. We filtered 98,327 SNPs that had LD < 0.5 from SNP pool
Stage 4 (Whole genome association analysis)	• A whole genome association study performed with 151,133 SNPs in 181 samples (91 patients and 90 controls). • Additive model [dd, Dd, DD] selected for a case/control analysis and Correlation/Trend Test as the statistical method. • p-Value calculated for each SNP and score made with $-\log10(P)$ was shown in Manhattan Plot as genomewide
Stage 5 (Pathway analysis)	• SNPs belonged to genes involved in nitric oxide pathway selected and matched each SNP value calculated after association study with $-\log(10)$ function. SNPs that p-value under 1×10^{-4} listed as the most statistically significant markers