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Analysis of mitochondrial DNA heteroplasmic mutations A1555G, C3256T, T3336C, C5178A, G12315A, G13513A, G14459A, G14846A and G15059A in CHD patients with the history of myocardial infarction

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ABSTRACT

The present study was undertaken in order to advance our earlier studies directed to define genetic risk of atherosclerotic vascular lesion development on a base on the analysis of sets of mutational load relevant to the mitochondrial genome mutations. A comparative evaluation of the two study participants' populations (that included coronary heart disease (CHD) patients who underwent myocardial infarction and apparently healthy donors with no clinical manifestations of coronary heart disease) on heteroplasmy levels of nine mutations of the mitochondrial genome (A1555G, C3256T, T3336C, C5178A, G12315A, G13513A, G14459A, G14846A and G15059A) that were shown previously to be associated with risk factors for atherosclerosis was performed. Close associations with the risk of cardiovascular disease were confirmed for mutation C3256T (gene MT-TL1), G12315A (gene MT-TL2), G13513A (gene MT-ND5) and G15059A (gene MT-CYB) by RT-PCR.

1. Introduction

In 97–98% of cases, atherosclerosis is the main cause of coronary heart disease (CHD). Arteries are narrowed by developing plaques which can bloc more than 50% of the lumen space without any symptoms of the disease (McCullagh et al., 1980; Turner et al., 1999; Chinnery et al., 2000; Kaufmann et al., 1996; Ingman et al., 2000; Lightowlers et al., 1997). It is established that if cardiac ischemia lasts more than 20–30 min, a serious complication of CHD, namely myocardial infarction, occurs (Turner et al., 1999). Subclinical (asymptomatic) atherosclerosis is the most widespread atherosclerotic disease (McCullagh et al., 1980; Turner et al., 1999; Chinnery et al., 2000; Kaufmann et al., 1996; Ingman et al., 2000; Lightowlers et al., 1997). Atherosclerotic lesions occur even in young people, being steadily progressing for decades, before clinical manifestations become evident (McCullagh et al., 1980; Turner et al., 1999; Chinnery et al., 2000; Kaufmann et al., 1996; Ingman et al., 2000; Lightowlers et al., 1997). In individuals of the middle age without clinical manifestations of atherosclerosis the incidence of atherosclerotic vascular lesions is about 100% (Robinson, 1998; Sukernik et al., 2002).

Despite the remarkable advances in medicine and biology, morbidity and mortality from atherosclerotic disease remain unacceptably high, while the arsenal of effective anti-atherosclerotic therapy remains limited. Modern algorithms for the evaluation of cardiovascular risk that take into account the combined interaction of several risk factors explain about 70% of the variability of acute myocardial infarction (Bruckmeier, 2003). Haplotype analysis of susceptibility genes for cardiovascular disease might therefore represent an important component of assessment for the identification of the predisposition to atherosclerotic disease. In various human pathologies the association of the pathogenesis of various diseases (including coronary artery stenosis, diabetes, deafness, myocardial infarction and cardiomyopathy) with mutations of the mitochondrial genome, localized in the coding regions of genes (and possibly occurring during ontogenesis) has been revealed (Sobenin et al., 2011; Tintinalli et al., 2001; Mitrofanov & Sazonova, 2011; Singh, 2002; Hodis et al., 1996; Mehrazin et al., 2009; Assmann et al., 2002; Benjamin et al., 1998). These mutations cause defects in the protein chains of some respiratory enzymes and transfer RNA (tRNA) in the mitochondria (Mitrofanov & Sazonova, 2011; Singh, 2002; Hodis et al., 1996; Mehrazin et al., 2009; Assmann et al., 2002; Benjamin et al., 1998), leading to a reduced concentration

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of respiratory enzymes and to mitochondrial dysfunction (Bruckmeier, 2003; Mitrofanov & Sazonova, 2011; Singh, 2002; Hodis et al., 1996; Mehrazin et al., 2009; Assmann et al., 2002; Benjamin et al., 1998).

During ontogeny the distribution of clones of mutant mtDNA in the tissues of the human body is random. But, as defective mitochondria are chronically intoxicated by free radicals, the mitochondria proliferate faster than normal, and the proportion of mutant mtDNA, on average, in an organ or tissue increases progressively (http://www.basic.northwestern.edu/biotools/OligoCalc.html/, n.d.). Mutations of the mitochondrial genome are characterized by the phenomenon of heteroplasmy (http://mfold.rna.albany.edu/?q=dinamelt/, n.d.). Accumulated data is indicative of that that 17 mutations of the mitochondrial genome are associated with coronary artery disease (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, n.d.; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch/, n.d.). Mutations in mitochondrial genes found in a number of pathologies including non-family forms of dilated (DCM) and hypertrophic cardiomyopathy (HCM), MELAS, stroke, mitochondrial myopathy, encephalopathy, MIDD (Maternally Inherited Diabetes and Deafness), type 2 diabetes and MERRF (Myoclonic epilepsy with ragged-red fibers) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, n.d.; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch/, n.d.).

The present study was undertaken in order to advance our earlier studies by evaluation of possible significance of nine mitochondrial mutations (A1555G, C3256T, T3336C, C5178A, G12315A, G13513A, G14459A, G14846A and G15059A) in coronary heart disease patients who underwent myocardial infarction.

2. Materials and methods

2.1. Materials

The material studies were DNA samples isolated from blood cells collected from 325 study participants, including 225 CHD patients who underwent myocardial infarction and 100 apparently healthy donors with no clinical manifestations of coronary heart disease. Clinical parameters of CHD patients who underwent myocardial infarction were assessed at the Cardiology Research and Production Complex and Moscow State University clinic. The study was approved by the Ethics committees of the Cardiology Research and Production Complex, Moscow, and the Institute of Atherosclerosis, Moscow. Written consent has been obtained from each study participant.

2.2. Methods

2.2.1. DNA extraction

Blood samples stored after sampling at −20 °C. DNA extraction was performed with phenol-chloroform method, according to the protocol for isolation of DNA. DNA concentration in the resultant sample was determined spectrophotometrically by NanoPhotometer Pearl UV/Vis c SDRAM P-34 (IMPLEN, Germany). For working with a collection of DNA, the samples were diluted in TE buffer to a concentration of 0.03 μg/μl and then the diluted samples were placed in separate tubes. After measuring DNA concentration, the samples were stored at −20 °C.

2.2.2. Real-time PCR

The isolated DNA was amplified as previously used (Sobenin et al., 2011). Heteroplasmy level of the 9 human mitochondrial genome mutations was analyzed by the method of excision of 5′-terminal tag (Assay TaqMan). For each of the 9 mutations, TaqMan probes were designed (the characteristics are given in Table 1: Probes and primers for Real-Time PCR PB TAQMAN).

Checking of the specificity of the primers and probes was performed on Real Time PCR System 7500 Fast (Applied Biosystems, USA). Also, for each of the species-specific probes, fluorescent dyes whose fluorescence spectra do not overlap have been selected. Signal recording Real-Time PCR was performed on channels corresponding FAM fluorophores and ROX, detecting the amplification product of the normal and mutant allele, respectively.

When choosing the oligonucleotide primers for Real-Time PCR and fluorescently labeled probes, full genome nucleotide sequence of mtDNA obtained from the GenBank database was used. To align the oligonucleotide sequences we used the program Primer 3, Oligocalculator, DinaMelt (http://www.basic.northwestern.edu/biotools/OligoCalc.html/, n.d.; http://mfold.rna.albany.edu/?q=dinamelt/, n.d.; http://www.ncbi.nlm.nih.gov/tools/primer-blast/, n.d.). The sequences of the primer pairs and estimated oligonucleotide probes were analyzed by using the program Oligocalculator, DinaMelt, PrimerBlast, NCBI nucleotide blast and checked for homology with nucleotide sequences of the nuclear DNA with a program DinaMelt, PrimerBlast (http://www.basic.northwestern.edu/biotools/OligoCalc.html/, n.d.; http://mfold.rna.albany.edu/?q=dinamelt/, n.d.; http://www.ncbi.nlm.nih.gov/tools/primer-blast/, n.d.; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch/, n.d.; Sobenin et al., 2012a). Also, a check for the absence mutual homology between pairs of primers and corresponding probes was carried out. All oligonucleotides were synthesized on an automatic synthesizer ABI 3400 DNA/RNA Synthesizer (Syntol, Moscow).

PCR analysis excision 5`- terminal tag (TaqMan system) was performed using the Real Time PCR System 7500 Fast (Applied Biosystems, USA). 25 μl reaction contained 1× TaqMan Buffer, 3 mM $MgCl_2$, 250 μM each dNTP, 300 nM primers, hybridization probes 300 nM, 0.5 units. Taq-polymerase ("Helicon", Moscow), 4 μl DNA analyzed. PCR, fluorescence intensity measurement was performed by the following algorithm:

1) Total cycle of denaturation, 2 min at a temperature 94 °C;

2) Stage amplification with fluorescence detection. It included denaturation, 10 s at 94 °C and annealing at a temperature specific for each mutation investigated (61–67 °C) for 15 s. The probes and primers for Real-Time PCR, used in the present study, are provided in Table 1.

2.2.3. Statistical analysis

The obtained data were processed using the software packages SPSS 22.0 (SPSS Inc., Chicago, IL, USA), SigmaPlot 12 (SSI, San Jose, California, USA) and Statistica 8.0 (StatSoft Inc., Tulsa, USA). Variability percent heteroplasmy was assessed using descriptive statistics and T-test. To assess homogeneity of variance, F-Test was used. Mutational load was calculated using logit regression and probit regression analysis test by Mann–Whitney.

3. Results

In this study we conducted a comparative evaluation of samples of patients with diagnosed coronary heart disease and study participants without who were considered to be healthy.

3.1. Estimation of the variability of the presence of coronary artery disease

In order to assess the significance of differences of samples generated by the criterion of having the diagnosis of coronary artery disease, a comparison of media data samples for each mutation was made (Fig. 1) and we conducted an analysis of the criterion of homogeneity of variances (F-Test for homogeneity) (the results are shown in Table 2). The graph in Fig. 1 shows the median values of differences in the two samples (without a diagnosis of ischemic heart disease and coronary artery disease) on each of the 9 analyzed mutations. Significant differences were found for mutation C3256T, A1555G, G13513A, G14846A, G15059A; for comparison, we used medians U-test by Mann–Whitney method. Table 2 shows that the F-Test for homogeneity