GENETIC VARIANTS THAT PARTICIPATE IN OXIDATION PROCESSES AND/OR OXIDATIVE STRESS AND ARE ASSOCIATED WITH ATHEROSCLEROSIS

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ABSTRACT

Motivation. Our previous study showed differences in the atherosclerosis phenotype between Lithuanian and Swedish men that could be influenced by complementary factors, namely oxidation processes and/or oxidative stress. The goal of this study was to evaluate the mainstream biological pathways inducing and maintaining the atherosclerotic process by analyzing genetic biomarkers particularly in inflammatory and metabolic pathways where the main focus is laid on the oxidation process.

Methods. There were 32 families recruited for the study and clinical as well as biochemical analyses were performed. For genetic analysis 150 SNPs in 89 genes were selected in order to construct a micro-array based on Arrayed Primer Extension (APEX) genotyping technology. Genotyping was carried out in 28 families and transmission disequilibrium test (TDT), sibling-TDT (S-TDT), and combined analysis were performed.

Results. Clinical and biochemical analysis revealed that probands with premature CAD were more likely to have diabetes mellitus, arterial hypertension, dyslipidemia and were male with high body mass index. Genetic analysis showed six SNPs statistically significantly associated with the atherosclerosis phenotype in the candidate genes ITGA2, IL1B, ALOX5A, OR13G1, MMP9 and NFKB1. These genes belong to different biological pathways: trombocyte adhesion and vessel damage, inflammation response, cholesterol and lypoxygenase metabolic pathway and nutrition.

Conclusions. Generalized clinical, biochemical, bioinformatical and candidate genes' association results support our hypothesis and indicate that the oxidation process may be of key importance in the formation of atherosclerosis.

UDC CODE & KEYWORDS

■ UDC: 616.1 ■ APEX genotyping technology ■ Association analysis ■ Oxidation process ■ Premature CAD ■ Transmission disequilibrium test ■

INTRODUCTION

Atherosclerosis is a complex condition that is caused by the interaction of genetic and environmental factors. A number of factors involved are: unbalanced blood cholesterol levels, high blood pressure, insulin resistance, diabetes, overweight and obesity, lack of physical activity, inappropriate diet, smoking, heavy alcohol usage, stress, older age (in men the risk increases after 45, and in women - after 55 years of age), family history of early heart disease and other. These factors participate in the etiopathogenesis and clinical manifestation of atherosclerosis, which eventually leads to many other progressive clinical conditions such as coronary artery disease (CAD) and its complications (e.g. myocardial infarction, stroke and other) [1]. These states of health constitute the most common cause of death in developing countries for men and women [2]. There are many intrinsic biological pathways contributing to the formation of atherosclerosis. Four major pathways have been identified and include lipoprotein metabolism, endothelial integrity, inflammatory response, and thrombosis process [3]. Their impact on the cardiovascular system is therefore multifactorious and different pathways give different effects as well as their reciprocity and interaction with environmental factors. Appropriate prevention and management measures should thus be taken for atherosclerosis and studies of biological pathways determining atherosclerosis seem to be of great importance.

The background of our research arises from the findings of our previous LiVicordia study (Linkoping-Vilnius coronary disease risk assessment study), which showed that Lithuanian men had more sub-clinical atherosclerosis in peripheral arteries than Swedish men [see 4-11]. Observed heterogeneity of atherosclerosis may depend not only on traditional risk factors but also on population specific genetic factors (e.g. common polymorphisms) playing role in the above mentioned four main pathways as well as in oxidative stress which might affect atherogenesis. Thus, the hypothesis for the present study evolved - whether the oxidation process might be one of the most significant factors causing atherogenesis among other known essential biological pathways involved in the formation of atherosclerosis.

The main goal of this study was to evaluate via SNP analysis prevalent biological pathways acting on the initiation and progression of atherosclerosis, represented by inflammation and disturbed metabolism. A set of candidate genes related to lipid metabolism, oxidative stress, inflammatory, metabolic processes, connective tissue formation and functioning were selected to investigate the association between the formation of atherosclerosis and nucleotide sequence variants. To achieve the aim of the study, there were three stages of accomplishments: bioinformatic analysis, genotyping, and data analysis. The Lithuanian Bioethics Committee approved the study project and the written informed consent of all tested individuals was obtained.

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Methods

Bioinformatics

The bioinformatic stage using data mining technique dealt with selecting the most promising (according to our hypothesis) candidate genes and picking out SNPs within them [12].

Ascertainment of study group

In total 32 families were recruited for the study. Probands were those with premature CAD or myocardial infarction (MI) recruited through the Clinic of Cardiology and Angiology. “Premature CAD” was defined as any previous or current evidence of significant atherosclerotic CAD, defined as MI, percutaneous coronary angioplasty (PTCA), coronary artery bypass graft (CABG), or coronary angiography with hemodinamically significant stenosis, occurring in males at the age of 55 years or in females at the age of 65 years. The diagnosis was verified by evaluating the measurements of average and maximal carotid and femoral intima-media thickness (IMT) and endothelial function.

IMT measurement. Carotid and femoral IMT was assessed by high resolution B-mode carotid ultrasound (GE, 13MHz probe) according to the standardized scanning and reading protocols. The carotid and femoral IMT scanning protocol consisted of the measurement near wall, 15-20mm proximal to the tip of the flow divider into the common carotid or femoral artery. Measurements of IMT were performed online with caliper for three times in three frames.

Anthropometrical measurements. Height and weight were determined by the use of calibrated scales. Waist measurement was performed using standard protocols. Body mass index (BMI) was calculated as weight in kilograms divided by height squared in meters.

Other risk factors for cardiovascular disease were also assessed including cigarette smoking (current, past, never), hypertension (systolic blood pressure ≥ 140 mm Hg, or taking antihypertensive medication), height, weight, waist circumference. Metabolic syndrome was diagnosed when an individual had ≥ 3 of the following: waist circumference in men >102 cm, in women >88 cm, triglycerides ≥1.7 mmol/L, high density lipoprotein cholesterol (HDL-C) <1.0 in men and <1.2 mmol/L in women, blood pressure ≥130/85 mmHg, fasting glucose ≥5,6 mmol/L.

Biochemical analysis

Contribution of metabolic and inflammatory processes on atherogenesis was based on biochemical phenotype markers in blood samples of the study population: lipid profile, concentrations of interleukin-6 (IL-6), C reactive protein (CRP), homocysteine, glucose, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), lipoprotein (a) (Lp(a)) and oxidized low density lipoproteins (ox-LDL). Total cholesterol, high- (HDL) and low density lipoprotein (LDL) cholesterol, serum triglycerides were measured by enzymatic methods (Dimension system). CRP, Lp(a), ApoA1 and ApoB were measured by immunochemical techniques (Dade Behring, BN system). IL-6 and homocysteine were evaluated by two-site sequential chemiluminescent immunometric assay (DPC, Immulite system). A monoclonal antibody 4E6 (Mercodia) based competitive ELISA was used for measuring plasma levels of ox-LDL.

Genetic analysis

Twenty-eight families for genetic analysis were selected after genotyping analysis quality control. DNA for genetic analysis was extracted from peripheral venous blood leucocytes using phenol-chloroform extraction method.

The genotyping stage was fulfilled by the Arrayed Primer Extension reaction (APEX) genotyping technique (Asper Biotech, Estonia) using the specially constructed 150 SNP genomic micro-array. Reaction is based on two steps: DNA hybridization to the complementary oligoprimers (embedded on micro-array glass slides) and single base extension of these primers with appropriate dye labeled dideoxynucleotides that match the nucleotide on polymorphic site by DNA polymerase [13]. Following the genotyping stage, the TDT, S-TDT, and combined TDT with S-TDT were carried out to identify SNPs significantly associated with atherosclerosis. Chi square analysis was carried out to test deviations in genotype frequencies from the Hardy-Weinberg equilibrium.

Results

Detailed bioinformatic analysis eventually resulted in selection of 150 SNPs in 89 candidate genes, mostly involved in oxidative stress regulation and oxidative homeostasis.

The clinical characteristics of the 32 premature CAD patients (probands) and 28 study participants - family members are summarized in Table 1.

Table 1. Clinical characteristics of study population

Variable	Probands, N=32	Family members, N=28	p-value
Age, years	45.41±6.20	44.60±7.10	0.520
Males, %	97.00	50.00	*0.001*
Body mass index	28.19±3.20	31.69±6.10	*0.030*
Current smokers, %	35.00	18.00	0.540
Arterial hypertension, %	69.00	25.00	*0.017*
Diabetes, %	5.30	0	*0.001*
MS, %	38.70	39.20	0.130
IMT, %	84.00	69.00	*0.001*
Dyslipidaemia, %	96.87	71.43	*0.006*

Source: Authors. Data are presented as mean value ± SD or (%) of subjects. Significant p-values ($p<0.05$) are in italics. MS - metabolic syndrome; IMT - elevated intima media thickness, N - sample size.

Probands were more likely to have diabetes mellitus, arterial hypertension, dyslipidaemia and were males with high body mass index. Advanced sub-clinical atherosclerosis was present in 69% family members, but prevalence of elevated IMT was higher in probands.

Laboratory and ultrasonographic measurements of study population are presented in Table 2. Probands had elevated values of Lp(a) and lower values of total cholesterol, HDL-C, ApoA1, IL-6, ox-LDL.

Table 2. Laboratory results of study population.

Variable	Probands, N=32	Family members, N=28	p-value
Total cholesterol, mmol/L	5.18±1.30	5.52±0.95	*0.190*
LDL-cholesterol, mmol/L	3.19±1.14	3.44±0.81	0.020
HDL-cholesterol, mmol/L	1.18±0.41	1.24±0.48	*0.090*
Triglycerides, mmol/L	2.10±2.01	1.79±1.13	0.890
CRP, g/L	2.28±3.51	2.57±2.56	0.220
Homocysteine, µmol/L	9.36±2.66	10.29±5.58	0.015
Lp(a), g/L	0.33±0.38	0.22±0.22	*0.020*
ApoA1, g/L	1.41 ±0.27	1.60±0.29	*0.070*
ApoB, g/L	1.08±0.35	1.06±0.24	0.490
ApoB/ApoA1, g/L	0.80±0.33	0.69±0.23	*0.049*
IL-6, pg/mL	2.59±1.06	3.70±7.40	*0.035*
Ox-LDL, U/L	58.24±40.20	72.49±33.35	*0.010*
IMT mean, cm	0.08 ±0.0031	0.08±0.0031	*0.790*
IMT max, cm	0.16±0.18	0.10±0.0037	0.79

Source: Authors. Data are presented as mean value ± SD or (%) of subjects. Significant p-values ($p < 0.05$) are in italics. N - sample size.

There were 28 families selected for genetic analysis. A micro-array for the APEX genotyping was developed. Of the 150 SNPs analyzed, 116 were polymorphic and 34 were not. Association analysis using TDT and S-TDT results are shown in Table 3. Statistically significant association with atherosclerosis was found of rs1126643 in *ITGA2* (MIM: 192974). Five other SNPs were close to statistically significant association: rs1143627, rs9551963, rs1151640, rs3918242 and rs28573147 in candidate genes *IL1B* (MIM: 146931), *ALOX5AP* (MIM: 603700), *OR13G1* (MIM: 611677), *MMP9* (MIM: 120361) and *NFKB1* (MIM: 164011) respectively (Table 3). TDT analysis showed only *ITGA2* rs1126643 statistically significantly associated with the atherosclerosis phenotype, but after combined TDT and S-TDT analysis all aforementioned SNPs showed significant results.

Table 3. SNPs statistically significantly associated with atherosclerosis

Candidate gene	SNP ID	Informative triads (for TDT)	TDT		Informative triads (for TDT and S-TDT)	Combined result (TDT and S-TDT)	
			χ^2-value	p-value		z' value	p-value
ITGA2	rs1126643	19	6.000	0.0497	19+16=35	2.245	0.0124
IL1B	rs1143627	11	5.400	0.0672	11+15=26	1.672	0.0473
ALOX5AP	rs9551963	18	5.261	0.0720	18+9=27	2.085	0.0185
OR13G1	rs1151640	16	4.765	0.0923	16+18=34	1.650	0.095
MMP9	rs3918242	15	4.765	0.0923	15+12=27	2.121	0.0170
NFKB1	rs28573147	20	3.903	0.1420	20+14=34	1.653	0.0492
Threshold values			5.991	0.05	Threshold values	1.645	0.05

Source: Authors

Discussion

The study showed that probands had more conventional risk factors than family members, and 69% family members had increased IMT (> 0.09 cm), which means that they also have advanced sub-clinical atherosclerosis. The present findings are in accordance with one of the earliest reports of the potential familial nature of carotid artery atherosclerosis from ARIC study, which found a close correlation between common carotid IMT and parental history of myocardial infarction (14). Our study reinforces the need for a greater attention to clinical examination and importance of atherosclerosis prevention in all premature CAD family members.

An unexpected was the finding of the lower values of total cholesterol, IL-6 and ox-LDL-C in probands. Possible explanation for this discrepancy includes differences in subject's treatment. In comparison with the family members, probands were treated for dyslipidaemia from the time of diagnosis. However, most of the studies have not found differences between these two groups (15).

Lp(a) levels are significantly higher in probands than in the family members. Data suggest that plasma Lp(a) concentration is a better discriminator of subclinical atherosclerosis.

Genetic analysis revealed genes associated with analysed phenotype of the study group. There are several studies supporting findings that *ITGA2* may be a genetic factor of atherosclerosis-derived and other health states [16]. We did not

find any publications specifically concerning *ITGA2* rs1126643 as associated with atherosclerosis or studies showing the association of *IL1B* rs1143627 and *NFKB1* rs28573147 with atherosclerosis or any other condition related to it. This makes our findings unique. There are several studies analyzing the specific SNPs rs9551963, rs1151640 and rs3918242 in *ALOX5AP, OR13G1* and *MMP9* respectively [17-20], which we found significant, but observations between studies differ. There were no studies revealing an association of these SNPs with atherosclerosis. According to KEGG (*Kyoto Encyclopedia of Genes and Genomes)* database six aforementioned candidate genes associated with atherosclerosis are involved in biological pathways closely related to the inflammation process and thereby indirectly or even directly to oxidation processes and/or oxidative stress: trombocyte adhesion and vessel damage (*ITGA2*, *MMP9*), inflammation response (*IL1B, NFKB1*), cholesterol and lypoxygenase metabolic pathway (*ALOX5AP*) and nutrition (*OR13G1*). Our findings reveal statistically significant association of abovementioned six genes that participate in biological oxidation processes related pathways that might potentially influence atherogenesis, with atherosclerosis. This supports the hypothesis concerning the role of oxidation processes and oxidative stress in the initiation of the development of atherosclerosis.

The main limitation in this study could be the number of samples analysed. We decided to perform the family based study design according to a smaller sample size needed for significant results compared to the case-control study design. Our results show statistically significant association but nevertheless further SNP association analysis with a larger sample size would improve these results.

Conclusion

To conclude we suggest that *ITGA2* rs1126643*, IL1B* rs1143627*, ALOX5AP* rs9551963*, OR13G1* rs1151640*, MMP9* rs3918242 and *NFKB1* rs28573147 may confer both individual and population-specific features of sensitivity to oxidation processes and oxidative stress, which are related to specific metabolomics, and in turn leads to the complex phenotype of atherosclerosis and consequent health conditions.

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