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Original Research

Expression and variability of lipid metabolism genes in intracranial aneurysm

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Abstract: The objective of this study was to investigate the association between mRNA expression and single nucleotide polymorphisms (SNPs) of the ATP-binding cassette transporter (*ABCA1*) gene, apolipoprotein A1 (*APOA1*) gene, low-density lipoprotein (*LDLR*) gene and RNA gene located in the CDKN2B-CDKN2A cluster (*CDKN2B-AS1*) involved in lipid metabolism and the occurrence of intracranial aneurysm (IA). Fifty three IA patients, and 27 controls (IA-free) were enrolled in this study and were genotyped for seven single nucleotide polymorphisms. Increased expression of the *LDLR* gene in IA patients was observed. The A/G genotype and the A allele of the c. –113G>A polymorphism of the *APOA1* gene were associated with increased occurrence of IA (ORs 12.36 and 14.14, respectively), while the G/G genotype and G allele showed the opposite tendency (ORs 0.06 and 0.07, respectively). We also detected that the A/A–G/A combined genotype of the c. –113G>A – *APOA1* and g.46859A>G – *LDLR* SNPs was associated with a decreased occurrence of IA. Moreover, the A/G–G/G combined genotype of the c.656G>A – *ABCA1* and c. –113G>A – *APOA1* was associated with a decreased occurrence of IA. The results of our study suggest the association between expression and variability of lipid metabolism genes and occurrence of IA.

Key words: Intracranial aneurysm, cholesterol metabolism, lipid metabolism, gene expression, polymorphism, subarachnoid hemorrhage.

Introduction

Intracranial aneurysm (IA) is a regional bulge in cerebral vessels, usually located at arterial bifurcations. Its prevalence is estimated to be 0.2–9% in the general population (1). Most of the aneurysms are asymptomatic and clinically silent, but larger malformations may manifest by headache, nausea, vision impairment, and loss of consciousness (2, 3). Rupture of IAs is a major cause of subarachnoid hemorrhage (SAH) (4). Despite medical advances, the mortality rate of patients with SAH still reaches 50% (5-7). Survivors often have long-term neurological and neuropsychological deficits.

Development of modern imaging techniques contributes to a higher detection of unruptured IAs (8). However, the current state of knowledge does not allow predicting the risk of rupture of IA. The life threatening and other catastrophic consequences of SAH may be prevented by surgical or endovascular treatment of aneurysm, but both are associated with a relatively high risk for patients, including death and permanent disability. Thus, the choice of treatment strategies, if any, for IA patients raises many concerns (2, 9). The management of IA patients should consider individual predispositions to IA rupture, which should be included in the IA treatment.

A variety of factors influence the risk of SAH, including age, hypertension, size, location and morphology of IA (4, 10, 11). Social risk factors, such as smoking, alcohol abuse and diet are also implicated in the formation and rupture of IAs (12, 13). Moreover, the high family aggregation suggests a genetic basis of IAs and SAH (14). Genome-wide association studies (GWAS) also revealed linkages to several *loci*, including 2q33.1, 4q31.23, 8q11.23–q12.1, 9p21.3, 10q24.32, 12q22, 13q13.1, 18q11.2 and 20p12.1 (15-18). In addition, several case-control studies have confirmed the association between single nucleotide polymorphisms (SNPs)/mutations and IA and SAH (15, 19-22).

Although many aspects of the pathobiology of formation and rupture of IA remain unknown, a growing body of evidence suggests that lipid metabolism may play an essential role in these processes (23). Interestingly, GWAS identified the 13q13.1 locus, encompassing the *STARD13* gene encoding a protein containing a C-terminal STAR-related lipid transfer (START) domain (15). It was shown that the accumulation of lipids and their oxidation products in IA was associated with an increased tendency of the aneurism wall to rupture (23). How do lipids, such as low density lipoproteins (LDL), accumulate in the aneurism wall is still unclear. It was demonstrated that exposure of the endothelium to increased hemodynamic stress, including hypertension, induced disturbances in lipid metabolism of the arterial wall and then led to the intimal accumulation of chemically modified lipids. Aneurysm wall is constantly exposed to non-physiologic hemodynamic shear stress, suggesting that altered lipid metabolism in endothelium of the aneurism wall or complete or partial loss of endothelium and its barrier function, may be a key

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mechanism that leads to accumulation of lipids in the aneurism wall (24, 25).

Because atherosclerotic changes are present in IA, their pathogenesis may therefore be closely linked with atherosclerosis (25, 26). LDL have significant pro-atherogenic effects and may activate NADPH to produce $O_2^{\bullet-}$ and H_2O_2 (27). Thus, by inducing peroxidation of lipoproteins, free radicals promote atherosclerosis and possibly also aneurysmal changes in cerebral vessels. Several studies also reported high total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), triglycerides, low high-density lipoprotein-cholesterol (HDL-C) levels as risk factors for abdominal aortic aneurysms (28-31). However, the exact role of cholesterols in IAs formation and rupture is unclear. Some results suggest that hypercholesterolemia independently decreased the risk of rupture, whereas others found no such effect (32-35). Congenital or inherited defects weakening the arterial wall are considered a main risk factor for IA formation and rupture. Because the concentration of cholesterol in blood is correlated with arterial stiffness, changes of its levels may affect the development and rupture of IAs (36-37).

As mentioned, recent observations have highlighted the important role of lipid metabolism and genetic factors in development of IA. We therefore hypothesized that changes in gene expression and genetic variations in lipid metabolism genes can modify the risk of IA. The aim of this study was to determine the role of genes involved in lipid metabolism in the pathogenesis of IA. We investigated the association between ATP-binding cassette transporter, also known as the cholesterol efflux regulatory gene (*ABCA1*), *APOA1*, *LDLR* and *CDKN2B-AS1* mRNA expression levels and the following polymorphisms in these genes: c.–327C>T (rs1800977), c.656G>A (rs2230806) in the *ABCA1* gene, c.–113G>A (rs670), g.5085C>T (rs5069) in the *APOA1* gene, c.177C>T (rs688), g.46859A>G (rs6413504) in the *LDLR* gene and g.22125348A>C (rs1333048) in the *CDKN2B-AS1* gene, and occurrence of IA. We selected the common, potentially functional SNPs, which are mainly associated with the lipid levels (38-44).

Materials and Methods

Study subjects

Fifty three IA patients and 27 subjects with the exclusion of IA (controls) were recruited from inpatients and outpatients facilities of the Department of Neurosurgery and Oncology of the Central Nervous System, Medical University of Lodz, (Lodz, Poland). This work was conducted under the approval of the Bioethics Committee of University of Lodz (Approval no. KBBN-UŁ-I/6/2013), and each participant enrolled in this study provided informed consent in writing. All clinical and genetic investigations were conducted according to the principle expressed in the Declaration of Helsinki. Venous blood (2 × 3 ml) was taken from each individual into EDTA-containing tubes for genotyping and Tempus™ Blood RNA Tubes for analysis of gene expression.

A standard questionnaire was used to collect information about age, family history among 1st degree relatives for IA and drug treatment (aspirin and statins). All participants also underwent routine fasting laboratory tests, such as measurement of cholesterol level, lipid profile and clinical blood pressure. The demographic and baseline characteristics of the study population are provided in Table 1.

Table 1. Demographic and baseline characteristics of patients with intracranial aneurysm (IA) and controls enrolled in this study.

Characteristics	Controls (n = 27)		IA (n = 53)		*p*	OR(95% CI)	p^{OR}
	number	frequency	number	frequency			
Sex							
females	20	0.74	36	0.68	0.757	0.87 (0.26 – 2.84)	0.813
males	7	0.26	17	0.32		1.15 (0.35 – 3.78)	0.813
Age							
Mean ± SD	43.8±13.7		53.8±11.3		**<0.001**	**1.10 (1.04 – 1.15)**	**0.001**
Range	21–65		30–74				
IA in family							
yes	0	0.00	6	0.11	0.171	–	–
no	27	1.00	47	0.89		–	–
Hypertension							
yes	10	0.37	23	0.43	0.756	1.40 (0.47 – 4.13)	0.542
no	17	0.63	30	0.57		0.71 (0.24 – 2.11)	0.542
Hypercholesterolaemia							
yes	8	0.30	27	0.51	0.114	2.28 (0.75 – 6.92)	0.147
no	19	0.70	26	0.49		0.43 (0.14 – 1.33)	0.147
Lipids profile							
incorrect	3	0.11	7	0.13	0.929	1.91 (0.42 – 8.61)	0.398
correct	24	0.89	46	0.87		0.52 (0.12 – 2.35)	0.398
Statins							
yes	5	0.19	12	0.23	0.891	1.80 (0.51 – 6.32)	0.359
no	22	0.81	41	0.77		0.55 (0.16 – 1.95)	0.359
Aspirin							
yes	3	0.11	12	0.23	0.344	4.07 (0.97 – 17.07)	0.055
no	24	0.89	41	0.77		0.24 (0.06 – 1.03)	0.055

p values for a two-sided χ^2-test; ∗*p* values for t-test. $p < 0.05$ were considered statistically significant and are in bold; OR – odds ratio; 95% CI – 95% confidence interval; p^{OR} values < 0.05 along with corresponding ORs are in bold.

Diagnosing of patients

Diagnosis of the aneurysm was based on CTA (computerized tomographic angiography) or DSA (digital subtraction angiography), examinations performed in Department of Neurosurgery and Oncology of the Central Nervous System on inpatient and outpatient basis. In some patients the incidence of subarachnoid hemorrhage (SAH) from ruptured cerebral aneurysm occurred in the past and was followed by the microsurgical clipping or intravascular coiling. In others the aneurysm was found incidentally during diagnostics for other pathologies and never bled (unruptured intracranial aneurysm, UIA). Patients with UIA fulfilling treatment criteria were qualified for the clipping or coiling and underwent an appropriate procedure. The control subject were selected from group of patients who underwent vascular diagnostics CTA or DSA which confirmed the absence of aneurysm or other vascular pathology in cerebral circulation.

Study group also underwent routine neurological examination and ultrasonography evaluation of hemodynamic parameters of blood flow, also mechanical features of vascular wall with stiffness evaluation of large body arteries. The ultrasonography evaluation of large vessels was performed in study patients by means of competent USG device (My Lab™ 25 GOLD, Esaote, Florence, Italy and Vivid™ 7, GE Healthcare Medical, USA). The hemodynamic parameters of vessel blood flow and mechanical features of vascular wall was estimated. These data was provided based on performing of chosen and established special ultrasonography tests (examinations), including brachial artery endothelium-dependent and -independent flow-mediated dilatation (FMD [%]), carotid arterial stiffness index-β (SI), intima-media thickness (IMT [mm]). Also, pulse β-wave velocity (PWV [m/s], aortic stiffness) was assessed. The PWV are currently regarded as the reference method for noninvasive assessment of arterial compliance. The examination was performed by means of a non-invasive device (Complior, Artech Medical, Pantin, France) with the TY-306 Fukuda pressure sensitive transducers (Fukuda, Tokyo, Japan) with the patients in the supine position. The transducers were placed above the area with the best palpable pulse on the carotid and femoral arteries. The distance between the sensors was measured. The system calculates PWV on the basis of about 12-16 records as the distance between the sensors divided by the time in which the pulse wave arrived along the aorta from the carotid artery to the femoral artery.

RNA isolation

Total RNA was extracted from whole blood with the commercially available Tempus™ Spin RNA Isolation Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA concentration was quantified using a Bio-Tek Synergy HT Microplate Reader with absorbance measurements performed at 280 nm, and its purity was analyzed using the 260/280 absorbance ratio. The purified RNA samples were stored in Nucleic Acids Purification Elution Solution at –20°C until further analysis.

Gene expression and data analysis

Gene expression was analyzed by TaqMan probe-based real-time PCR (RT-PCR) assay and the reaction was performed with KAPA PROBE FAST Universal qPCR Kit (KAPA Biosystems, Wilmington, MA, USA). The thermocycling RT-PCR conditions used were as follows: 42 ºC for 5 min (cDNA synthesis), 95 ºC for 5 min (inactivate RT), followed by 40 cycles at 95 ºC for 3 s (denature) and 60 ºC for 30 s (anneal/extend) with plate reading at this step. The final reaction volume was 10 µl, consisting of 1 µl RNA template, 5 µl KAPA PROBE FAST qPCR Master Mix (2×), 0.2 µL KAPA RT Mix (50×), and 0.5 µl TaqMan probe (20×). The TaqMan probes used for amplification are presented in Table 2. The reactions were carried out in a thermal cycler CFX96 ™ Real-Time PCR Detection System (BIO-RAD Laboratories, Hercules, CA, USA). Data acquisition and the analysis of the RT-PCR assays were performed using CFX Manager ™ Software (version 3.0). The expression level was calculated using the $2^{-\Delta Ct}$ model, with the Ct values normalized using 18S rRNA as internal (endogenous) control.

Polymorphism genotyping

Polymorphisms were chosen on the basis of their potential functions in lipid metabolism using public domain of the National Center for Biotechnology Information the Single Nucleotide Polymorphisms database (NCBI dbSNP) at http://www.ncbi.nlm.nih.gov/snp. We selected SNPs with a minor allele frequency 5% or greater in the European population. Primers for each polymorphism were designed using the Primer3 software (http://frodo.wi.mit.edu/) and published nucleotide sequence found at ENSEMBL database. DNA was isolated from peripheral blood leukocytes using Extractme DNA blood kit (Blirt, Gdansk, Poland). Polymorphisms were analyzed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. All PCR reaction mixtures contained 25 ng of genomic DNA, 0.25 U HotStarTaq Plus DNA Polymerase (Qiagen, Venlo, The Netherlands), 1 µL of 10× PCR buffer, 0.25 µM each primer and 200 µM dNTPs. The primers and thermal cycling conditions for amplifying PCR products are presented in Table 3. The amplification conditions were as follows: initial denaturation step at 95°C for 5 min, 39 cycles at 95°C for 30 s, 30 s annealing (temperature in Table 3), extension 60 s at 72°C, and the final extension at 72°C for 5 min. All amplifications were performed in a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were

Table 2. TaqMan probes used to quantifying mRNA level with RT-PCR assays.

Gene name and symbol	Assay ID
Eukaryotic 18S rRNA – endogenous control (*18S rRNA*)	X03205.1
ATP-binding cassette, sub-family A, member 1 (*ABC1*)	Hs01059118_m1
Apolipoprotein A-I (*APOA1*)	Hs00985000_g1
Low density lipoprotein receptor (*LDLR*)	Hs01092524_m1
CDKN2B antisense RNA 1 (*CDKN2B-AS1*)	Hs04259476_m1

Table 3. Forward (F) and reverse (R) PCR primers, annealing temperatures, and PCR-RFLP product sizes in polymorphisms analysis of the *ABCA1*, *APOA1*, *LDLR* and *CDKN2B-AS1* genes.

Gene	Polymorphism	Location	Primer sequences (5'→3')	Annealing temperature [°C]	Restriction enzymes	Genotypes - fragment size after digestion (bp)
ABCA1	c.-327C>T rs1800977	5′ UTR	F: CGTGCTTTCTGCTGAGTGAC R: TGCGTCTCTTTCTCCTACCC	57	*Bsm*AI	C/C – 333, 13 C/T – 333, 199, 134, 13 T/T – 199, 134, 13
	c.656G>A (p.Arg219Lys) rs2230806	exon 7	F: AAAGACTTCAAGGACCCAGCTT R: CCTCACATTCCGAAAGCATTA	64	*Eco*NI	A/A – 184, 125 A/G – 309, 184, 125 G/G - 309
APOA1	c.-113G>A rs670	5′ near gene	F: AGGGACAGAGCTGATCCTTGAACTCTTAAG R: TTAGGGGACACCTACCCGTCAGGAAGAGCA	63	*Msp*I	G/G-C/C – 209, 114, 66, 46 G/G-C/T – 255, 209, 114, 66, 46 G/G-T/T – 255, 114, 66 A/A-C/C – 209, 180, 46 A/A-C/T – 255, 209, 180, 46 A/A-T/T – 255, 180 A/G-C/C – 209, 180, 114, 66, 46 A/G-C/T – 255, 209, 180, 114, 66, 46 A/G-T/T – 255, 180, 114, 46
	g.5085C>T rs5069	5′ UTR				
LDLR	c.1773C>T (p.Asn591) rs688	exon 12	F: TCTCCTTATCCACTTGTGTGT R: CTTGCATCTCGTACGTAAGC	60.5	*Hinc*II	C/C – 100, 54, 36 C/T – 136, 100, 54, 36 T/T – 136, 54
	g.46859A>G rs6413504	intron	F: GAGGGGTAAAAAGGCAGGAG R: CCATCTGTCTCTGCCAACAA	63	*Bsm*I	A/A – 227, 47 A/G – 274, 227, 47 G/G – 274
CDKN2B-AS1	g.22125348A>C rs1333048	antisense RNA	F: ACCCGAAGTAGAGCTGCAAA R: CACAAGTTGGAATATGAAGCAGA	64	*Dra*I	C/C – 152 C/A – 152, 84, 68 A/A – 152

digested with 1.5 U of allele-specific restriction enzyme (Table 3). Digested fragments were visualized on 8% polyacrylamide gel with ethidium bromide staining (0.5 µg/ml). Marker DNA M100-500 (DNA Gdansk, Gdansk, Poland) was used as a molecular mass marker. For quality control, 10% of samples were genotyped again and 100% compliance was achieved.

Statistical analysis

Statistical analysis was performed with Prism 5 (Graph-Pad, San Diego, CA, USA), SigmaPlot v11.0 software (Systat Software, Inc., San Jose, CA, USA) and

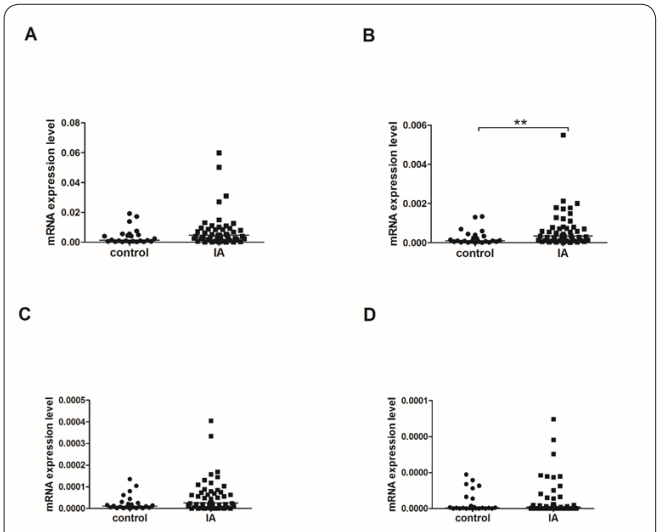

Figure 1. Basal expression of *ABCA1* (A)*, LDLR* (B), *APOA1* (C) and *CDKN2B-AS1* (D) mRNA, in patients with intracranial aneurysm (IA; n = 48) as compared to controls (n = 24). The expression of mRNA was calculated by the $2^{-\Delta Ct}$ method (where ΔCt is the value obtained by subtracting C_t of 18S rRNA from C_t of *ABCA1*, *LDLR*, *APOA1* and *CDKN2B-AS1* genes, respectively). The data are plotted as individual values and the median is indicated by the horizontal bars in each graph, * – $p < 0.05$.

MedCalc Version 12.3.0 (MedCalc Software, Mariakerke, Belgium). The magnitude of the association of the genotypes of polymorphisms and combined genotypes with IA was estimated using odds ratio (OR) tests with 95% confidence interval (CI) by unconditional multiple logistic regression model, both with and without adjustment for age. Demographic and baseline characteristics of patients were compared by unpaired Student's t-test or χ^2 test, as appropriate and mRNA expressions were compared by the Mann-Whitney test.

Results

Patients

53 patients with IA and 27 IA-free controls were recruited in our study (Table 1). The number of females was higher than males in both groups. The lipid profile, cholesterol level, blood pressures values did not differ between patients and controls. There were no significant differences between distributions of subpopulations of individuals taking aspirin or statins in IA cases and controls.

ABCA1, APOA1, LDLR and CDKN2B-AS1 mRNA levels analysis

Analysis of the expression of the *LDLR* gene showed a significant increase ($p < 0.01$) mRNA level in IA patients group compared to the controls (Fig. 1). Moreover, there were no differences in *ABCA1*, *APOA1* and *CDKN2B* mRNAs expression levels in IA group when compared to the control group (Fig. 1).

Polymorphism of lipid metabolism genes

The genotype and allele frequencies of seven polymorphisms are presented in Table 4. Evaluation of genotype and allele distributions showed that the G/G genotype and the G allele of the c. –113G>A polymorphism in *APOA1* were associated with a lower occurrence of IA. Our results also show a significant association between the A/G genotype and the A allele of this polymorphism and an increased occurrence of IA. Moreover, the A/A genotype was only detected in IA group (Table 4).

Gene-gene interaction

In this study we investigated the association between the occurrence of IA and combined genotypes of c.–327C>T, c.656G>A, c.–113G>A, g. 5085C>T, c.177C>T, g.46859A>G and g.22125348A>C polymorphisms. We detected that the A/A–G/A genotype of the c. –113G>A – *APOA1* and g.46859A>G – *LDLR* SNPs was associated with a decreased occurrence of IA (Table 5). Moreover, the A/G–G/G genotype of the c.656G>A – *ABCA1* and c. –113G>A – *APOA1* was associated with a decreased occurrence of IA (Table 6).

Discussion

The present study carried out on blood from 80 individuals, including 53 patients with IA and 27 controls (IA-free). We performed our research on rather small group of patients, but these patients were thoroughly interviewed and investigated. In particular, the group consisted of individuals, in whom the presence of IA was excluded on the basis of highly specific examination. In the first part of this study, we examined the relationship between potential risk factors for IA and the occurrence of this disease. There were no significant differences between distribution of sex, hypertension, hypercholesterolemia, lipid profile, statins and aspirin usage and family history for IA among patients and control cohorts. One of the cause such result may be disproportion between numbers of samples, therefore further studies on a larger population are necessary.

Although the pathogenesis of IA remain largely unknown, results of several studies have shown that lipid metabolism and genetic factors may be involved (23-25). The identification of genes associated with IA is important to understand better the development of this disease.

In the present work we did not found any differences in the level of ABCA1 mRNA gene expression between IA patients and controls. The ATP-binding cassette transporter A1 (ABCA1) encoded by the *ABCA1* gene is one of the important proteins involved in lipid metabolism. ABCA1 mediates the initial step of the reverse cholesterol transport as it facilitates the efflux of cholesterol and phospholipids from peripheral cells to lipid-poor apolipoprotein A1 creating nascent high-density lipoprotein (HDL) particles (45, 46). *ABCA1* gene is highly expressed in macrophages and leucocytes as well as in many tissues, such as liver, lung, placenta, adrenal gland, intestine, brain and fetal tissues (47, 48). It was shown that macrophages play a critical role in the formation of IA (49, 50). Moreover, it was documented that normal macrophages displayed a blunted increase in ABCA1 expression and were more likely to differentiate into foam cells – a process which is recognized as pro-atherosclerotic (51, 52).

Our results also show that the c.656G>A-*ABCA1* SNP (rs2230806) was not significantly associated with IA. This SNP results in a single amino acid change in

Table 4. Distribution of genotypes and alleles of the c.–327C>T, c.656G>A of the *ABCA1* gene, c.–113G>A, g.5085C>T of the *APOA1* gene, c.177C>T, g.46859A>G of the *LDLR* gene and g.22125348A>C in IA patients and controls.

Genotypes /alleles	Controls (n = 27) number	IA (n = 53) number	Crude OR (95% CI)	*p*	Adjusted OR[a] (95% CI)	*p*
c.-327C>T						
C/C	10	18	0.87 (0.33 – 2.30)	0.785	1.08 (0.36 – 3.28)	0.886
C/T	11	28	1.63 (0.64 – 4.16)	0.308	1.31 (0.45 – 3.73)	0.617
T/T	6	7	0.53 (0.16 – 1.78)	0.306	0.54 (0.13 – 1.11)	0.385
$\chi^2 = 1.478$; $p = 0.477$						
C	31	64	1.13 (0.58 – 2.12)	0.716	1.25 (0.58 – 2.70)	0.573
T	23	42	0.88 (0.45 – 1.72)	0.716	0.80 (0.37 – 1.73	0.573
c.656G>A						
AA	1	3	1.56 (0.15 – 15.75)	0.706	1.35 (0.10 – 18.85)	0.821
AG	16	20	0.42 (0.16 – 1.07)	0.070	0.54 (0.19 – 1.55)	0.252
GG	10	30	2.22 (0.86 – 5.74)	0.101	1.76 (0.61 – 5.11)	0.295
$\chi^2 = 3.348$; $p = 0.187$						
A	18	26	0.60 (0.27 – 1.33)	0.211	0.69 (0.28 – 1.68)	0.413
G	36	80	1.65 (0.75 – 3.63)	0.211	1.45 (0.59 – 3.55)	0.413
c.-113G>A						
GG	25	32	**0.12 (0.02 – 0.57)**	**0.007**	**0.06 (0.01 – 0.39)**	**0.003**
AG	2	16	**5.40 (1.14 – 25.60)**	**0.033**	**12.36(1.95 – 78.25)**	**0.008**
AA	0	5	-	-	-	-
$\chi^2 = 9.279$; $p = 0.010$						
G	52	80	**0.14 (0.03 – 0.63)**	**0.010**	**0.07 (0.01 – 0.44)**	**0.005**
A	2	26	**6.91 (1.58 – 30.25)**	**0.010**	**14.14 (2.27 – 88.22)**	**0.005**
g.5085C>T						
CC	24	50	2.08 (0.39 – 11.10)	0.390	1.68 (0.26 – 10.72)	0.585
CT	3	0	-	-	-	-
TT	0	3	-	-	-	-
$\chi^2 = 7.475$; $p = 0.024$						
C	51	100	0.99 (0.32 – 3.00)	0.983	0.90 (0.26 – 3.10)	0.864
T	3	6	1.01 (0.33 – 3.08)	0.983	1.11 (0.32 – 3.86)	0.864
c.1773C>T						
TT	3	9	1.64 (0.40 – 6.62)	0.490	2.26 (0.39 – 13.01)	0.360
CT	13	22	0.76 (0.30 - 1.94)	0.572	0.96 (0.33 – 2.80)	0.944
CC	11	22	1.03 (0.40 – 2.65)	0.947	0.73 (0.25 – 2.14)	0.567
$\chi^2 = 0.594$; $p = 0.743$						
T	19	40	1.11 (0.57 – 2.15)	0.759	1.42 (0.64 – 3.15)	0.387
C	35	66	0.90 (0.46 – 1.75)	0.759	0.70 (0.32 – 1.56)	0.387
g.46859A>G						
G/G	6	14	1.26 (0.42 – 3.75)	0.682	1.09 (0.32 – 3.66)	0.887
A/G	15	26	0.77 (0.30 – 1.95)	0.583	0.86 (0.30 – 2.46)	0.779
A/A	6	13	1.14 (0.38 – 3.42)	0.819	1.13 (0.32 – 4.00)	0.854
$\chi^2 = 0.313$; $p = 0.855$						
G	27	54	1.04 (0.53 – 2.02)	0.909	0.99 (0.46 – 2.11)	0.981
A	27	52	0.96 (0.49 – 1.87)	0.909	1.01 (0.47 – 2.15)	0.981
g.22125348A>C						
CC	7	11	0.75 (0.25 – 2.22)	0.601	0.99 (0.28 – 3.61)	0.999
AC	13	27	1.12 (0.44 – 2.83)	0.813	1.24 (0.43 – 3.53)	0.686
AA	7	15	1.13 (0.40 – 3.22)	0.822	0.76 (0.23 – 2.45)	0.650
$\chi^2 = 0.277$; $p = 0.870$						
C	27	49	0.86 (0.44 – 1.66)	0.651	1.12 (0.52 – 2.40)	0.771
A	27	57	1.16 (0.60 – 2.25)	0.651	0.89 (0.42 – 1.91)	0.771

p values < 0.05 along with corresponding ORs are in bold; OR[a] adjusted for age.

Table 5. Distribution of combined genotypes of the c.-113G>A– *APOA1* and g.46859A>G– *LDLR* polymorphisms and odds ratio (OR) with 95% confidence interval (95% CI) in patients with intracranial aneurysm (IA) and controls.

Combined Genotypes	Controls (*n* = 27) Number	Frequency	IA (*n* = 53) Number	Frequency	Crude OR (95% CI)	*p*	Adjusted OR[a] (95% CI)	*p*
A/A–G/G	5	0.19	8	0.15	0.78 (0.23 – 2.67)	0.695	0.73 (0.19 – 2.86)	0.655
A/A–G/A	15	0.56	16	0.30	**0.35 (0.13 – 0.90)**	**0.030**	**0.28 (0.09 – 0.86)**	**0.026**
A/A–A/A	5	0.19	8	0.15	0.78 (0.23 – 2.67)	0.695	0.62 (0.15 – 2.57)	0.508
A/G–G/G	1	0.04	5	0.09	2.71 (0.30 – 24.43)	0.375	2.35 (0.22 – 25.08)	0.481
A/G–G/A	0	0.00	8	0.15	-	-	-	-
A/G–A/A	1	0.04	3	0.06	156 (0.15 – 15.75)	0.706	3.64 (0.29 – 46.16)	0.319
G/G–G/G	0	0.00	1	0.02	-	-	-	-
G/G–G/A	0	0.00	2	0.04	-	-	-	-
G/G–A/A	0	0.00	2	0.04	-	-	-	-

p values < 0.05 along with corresponding ORs are in bold; OR[a] adjusted for age.

Table 6. Distribution of combined genotypes of the c.656G>A – *ABCA1* and c.-113G>A – *APOA1* polymorphisms and odds ratio (OR) with 95% confidence interval (95% CI) in patients with intracranial aneurysm (IA) and controls.

Combined Genotypes	Controls (n = 27)		IA (n = 53)		Crude OR (95% CI)	p	Adjusted OR[a] (95% CI)	p
	Number	Frequency	Number	Frequency				
A/A–G/G	1	0.04	2	0.04	1.02 (0.09 – 11.77)	0.988	1.16 (0.07 – 18.30)	0.916
A/A–G/A	0	0.00	1	0.02	-	-	-	-
A/A–A/A	0	0.00	0	0.00	-	-	-	-
A/G–G/G	14	0.52	9	0.17	**0.19 (0.06 – 0.54)**	**0.002**	**0.20 (0.06 – 0.66)**	**0.008**
A/G–G/A	2	0.07	9	0.17	2.56 (0.51 – 12.78)	0.253	4.93 (0.77 – 31.72)	0.093
A/G–A/A	0	0.00	2	0.04	-	-	-	-
G/G–G/G	10	0.37	21	0.40	1.12 (0.43 – 2.90)	0.822	0.72 (0.24 – 2.19)	0.570
G/G–G/A	0	0.00	6	0.11	-	-	-	-
G/G–A/A	0	0.00	3	0.06	-	-	-	-

p values < 0.05 along with corresponding ORs are in bold; OR[a] adjusted for age.

codon 219 from arginine to lysine. It was shown that the A allele has been related to low risk of coronary artery disease, and to lower triglycerides concentration, but the results on the level of HDL-cholesterol among patients carrying this allele are controversial (39, 53).

We did not found any association between second potentially functional polymorphism c.-327C>T in the *ABCA1* gene (rs1800977) and occurrence of IA. Our findings are similar to studies conducted on a Japanese population (54). It was shown that this SNP causes the loss of binding motif for the Sp2 transcription factor and generates a new binding motif for the Smad4 transcription factors (41). This SNP was previously shown to be associated with the plasma concentration of HDL-cholesterol (38). Case-control studies showed that the subjects carrying TT genotype had a higher risk of developing of Alzheimer's disease than subjects without that genotype (41).

We also found, that the expression of the *LDLR* gene was higher in IA patients group as compared to control. The *LDLR* gene encodes the low density lipoprotein receptor (LDLR) – a cell surface glycoprotein that mediates the uptake of low-density lipoprotein (LDL) particles from the circulation via receptor-mediated endocytosis and plays an essential role in maintaining cellular cholesterol homeostasis (55). It was shown that pathogenic changes in LDLR resulted in an impaired uptake or processing of LDL, leading to elevated serum cholesterol and LDL levels, promoted the formation of atherosclerosis and increased the risk of coronary heart disease (56, 57). LDLR is highly expressed in liver, vascular cells and macrophages. Studies have also shown that increases in LDLR expression significantly increase cholesterol delivery into macrophages in culture (58). According to our knowledge there is no information on *LDLR* mRNA expression in lymphocytes of patients with IA.

Recent GWAS identified several common SNPs at the *LDLR* locus that altered its expression and could contribute to inter-individual differences in lipoprotein levels (42). The c.1773C>T (p.Asn591) – a common synonymous SNP in exon 12 of this gene (rs688), associated with increased plasma total and LDL cholesterol in several independent populations (40, 42, 59). Some synonymous SNPs can alter protein conformation by transforming frequent codons into rare ones, thus reducing translational efficiency and disrupting the process of co-translational protein folding (60-62). Lee *et al*. (63) demonstrated that the T/T genotype of this polymorphism was associated with a 73% decreased risk of primary intracerebral hemorrhage. We also investigated the g.46859A>G SNP located in intron 18 of the *LDLR* gene. In the multiethnic SHARE study significant relationship was identified between the G allele and plasma levels of TG, HDL, and LDL (64). In our study no association between the c.1773C>T and g.46859A>G SNPs and IA was found.

We did not observe any differences in the level of *APOA1* mRNA gene expression between IA patients and controls. The apolipoprotein A1 (APOA1) serves as a cofactor for cholesterol esterification and promotes reverse cholesterol transport from peripheral tissues to the liver (65). APOA1 plays a critical role in the lipid metabolism and several studies showed its protective effect against atherosclerosis and coronary heart disease (66, 67).

Several polymorphisms in the *APOA1* gene were associated with HDL-cholesterol and APOA1 levels, including polymorphisms studied in the present work – the c.–113G>A (rs670) and g.5085C>T (rs5069) SNPs located within promoter region of the *APOA1* gene (43, 68). To our knowledge, these polymorphisms have not been studied in IA patients so far. It was shown that the c.–113G>A SNP enhanced promoter function – increased transcriptional efficiency was observed in A allele carriers in comparison with G allele (69, 70). The minor allele (A) was also associated with higher APOA1 and HDL levels in several populations (71-74). This SNP has recently been linked to many disease, such as hyperlipidemia (72), myocardial infarction (75), hypertension (76), Alzheimer's disease (77) and coronary atherosclerosis disease (78). Our results reveal an association of the A allele with increased risk of IA. Several studies showed that the minor allele g.5085T is associated with increased HDL-cholesterol (79-81) and decreased TG levels (74). Additionally, the T allele was also associated with a higher transcription rate of *APOA1* (68). Moreover, it was shown that the c.–113G>A and the g.5085C>T SNPs were in linkage disequilibrium and subjects who carried the rare alleles presented higher levels of HDL (82). In our study, we did not detect any association between this SNP and IA risk.

In recent years, two GWAS have shown a strong association between the chromosomal locus 9p21.3 and IA (15, 83). These observations have been further confirmed in large-scale case-control studies (84, 85). This locus is also associated with other lipid as-

sociated diseases, such as coronary artery disease, myocardial infarction, aortic aneurysms and peripheral artery disease (44, 86). The 9p21.3 locus contains only a sequence for an antisense RNA. The antisense noncoding RNA *CDKN2B-AS1* gene is located within the CDKN2B-CDKN2A gene cluster, overlaps with the tumour suppressor gene p15 coding sequence. The gene product is a functional RNA molecule that interacts with polycomb repressive complex-1 (PRC1) and -2 (PRC2), leading to epigenetic silencing of other genes in this cluster. The *CDKN2B-AS1* gene was shown to transcriptionally silence p15 directly as well as through induction of heterochromatin formation. Furthermore, *CDKN2B-AS1* gene expression was increased in human leukemias with an inverse correlation with p15 expression (87). In this work, we did not found any difference in the level of *CDKN2B-AS1* mRNA gene expression between the compared groups. Several functional SNPs of the 9p21.3 locus have been described, including rs10757278, rs1333049 and rs1333048 (44). In this work, we searched for the association between the occurrence of IA and the g.22125348A>C (rs1333048). Our findings showed that this SNP is not associated with this disease.

The present study has some limitations. First, it was carried out on peripheral blood and not the target brain tissue. Second, the sample size was relatively small, which does not allow for analysis of the *ABCA1*, *APOA1*, *LDLR* and *CDKN2B-AS1* mRNA expression levels according to genotypes among IA patients. These two limitations follows from the specificity of the target material and its limited accessibility. Third, it could be interesting to determine other SNPs, especially these that are located in *APOA1*/*C3*/*A4*/*A5* gene cluster which is strongly associated with plasma lipids and lipoproteins (88).

To conclude, our study showed a significant association between lipid metabolism genes and occurrence of IA. This knowledge might help to identify specific molecular markers of IA and develop new therapeutic strategies.

In conclusion, data reported here suggest that high *LDLR* mRNA expression levels and the c.-113G>A–*APOA1* (rs670), c.656G>A–*ABCA1* (rs2230806), g.46859A>G– *LDLR* (rs6413504) polymorphisms are associated with IA. Therefore, lipid metabolism genes can play a role in the pathogenesis of intracranial aneurysm.

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