Pathol. Oncol. Res. (2015) 21:743–749
DOI 10.1007/s12253-014-9884-5

RESEARCH

Functional Variants of Lipid Level Modifier MLXIPL, GCKR, GALNT2, CILP2, ANGPTL3 and TRIB1 Genes in Healthy Roma and Hungarian Populations

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Received: 14 March 2014 / Accepted: 22 December 2014 / Published online: 9 January 2015

Abstract The role of triglyceride metabolism in different diseases, such as cardiovascular or cerebrovascular diseases is still under extensive investigations. In genome-wide studies several polymorphisms have been reported, which are highly associated with plasma lipid level changes. Our goal was to examine eight variants: rs12130333 at the ANGPTL3, rs16996148 at the CILP2, rs17321515 at the TRIB1, rs17145738 and rs3812316 of the MLXIPL, rs4846914 at GALNT2, rs1260326 and rs780094 residing at the GCKR loci. A total of 399 Roma (Gypsy) and 404 Hungarian population samples were genotyped using PCR-RFLP method. Significant differences were found between Roma and Hungarian population samples in both MLXIPL variants (C allele frequency of rs17145738: 94.1% vs. 85.6%, C allele frequency of rs3812316: 94.2% vs. 86.8% in Romas vs. in Hungarians, $p<0.05$), in ANGPTL3 (T allele frequency of rs1213033: 12.2% vs. 18.5% in Romas vs. Hungarians, $p<0.05$) and GALNT2 (G allele frequency of rs4846914: 46.6% vs. 54.5% Romas vs. in Hungarians, $p<0.05$), while no differences over SNPs could be verified and the known minor alleles showed no correlation with triglyceride levels in any population samples. The current study revealed fundamental differences of known triglyceride modifying SNPs in Roma population. Failure of finding evidence for affected triglyceride metabolism shows that these susceptibility genes are much less effective compared for example to the apolipoprotein A5 gene.

Keywords Polymorphisms · Triglyceride · Roma · Hungarian

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Introduction

The recent genome-wide association studies (GWAS) revealed genetic polymorphisms associated with blood lipid level changes. Nowadays, special attention gained on

metabolic consequences, including triglyceride level increases, confirming risk for cardiovascular diseases, metabolic syndrome or for cerebrovascular diseases, especially stroke events [1–19]. The National Cholesterol Education Program (NCEP) in 2001 ascertained several markers which are in strong association with coronary risk, stratified as risk factors related to lifestyle, such as physical inactivity, obesity, atherogenic diet; and emerging risk factors, as lipoprotein profile, homocysteine level, changed fasting glycaemia and evidence of subclinical atherosclerosis. Approach to lipoprotein management in 2001 National Cholesterol Guidelines [20].

As a prominent example, the functional role of *APOA5* polymorphisms had already been widely investigated [1–7]. Several of them are associated with elevated triglyceride levels and higher risks for ischemic stroke and cardio- or cerebrovascular diseases or for metabolic syndrome [4, 5, 8–11, 21, 22]. Recently, other triglyceride modifying polymorphisms came into focus, which may also have role in development of different diseases [2, 12, 15, 16, 19, 23–25]. Some variants of these are mentioned in connection with increased, while others with decreased triglyceride levels [16, 23, 24, 26, 27]. The elevated levels of certain triglycerides may have a higher risk for several vascular diseases, moreover significant associations between triglyceride level-elevating and polymorphisms were confirmed [1, 2, 4, 5, 12–15, 17, 28, 26, 29–32, 25].

Romani people, who are often neglected, strongly differ from other nations [33]. In this work, our goal was to investigate the possible relationship of functional polymorphisms of *GCKR*, *MLXIPL*, *ANGPTL3*, *CILP2*, *GALNT2* and *TRIB1* gene loci with altering triglyceride levels in Roma and in Hungarian population samples.

Materials and Methods

Patients

The DNA samples were from the central Biobank managed by the University of Pecs, belonging to the National Biobank Network of Hungary (http://www.biobanks.hu); clinical features are shown in Table 1. The molecular investigations were carried out on genomic DNA, which was isolated from peripheral EDTA-anticoagulated blood leukocytes, by a standard desalting method [34]. The maintenance, management and governance principles of the Biobank had been endorsed by the national Scientific Research Ethics Committee, Budapest (ETT TUKEB). During the collection and use of DNA samples and the consorting clinical and personal data were in complete compliance with the guidelines of the 1975 Helsinki Declaration and the currently operative national laws and regulations.

Table 1 Major clinical and laboratory data of Roma and Hungarian population samples

	Roma (399)	Hungarians (404)
Males/females	179/221	141/263
Age (years)	55.7±0.94	61.5±0.79
Plasma triglyceride (mmol/l)	1.61±0.04	1.44±0.02
Total cholesterol (mmol/l)	4.70±0.06	5.58±0.06

Here we studied eight polymorphisms reportedly associated with triglyceride-level changes: rs17145738 and rs3812316 of the *MLXIPL* locus, rs1260326 and rs780094 of the *GCKR* gene, rs4846914 variant of *GALNT2* gene, rs1699614 of *CILP2*, rs1213033 of *ANGPTL3* gene locus and rs17321515 of *TRIB1* gene locus in biobanked samples in Roma and Hungarian populations. Sample size determination was based on our preliminary analyses of the prevalence of these SNPs. Based on the important significant difference in frequencies of the genetic alterations between Roma and Hungarian samples; we calculated how many samples we would need per group to be adequately small and large enough to detect a statistically significant difference and to exclude Type I and Type II errors (alpha=0.05 and beta<0.03, two tailed). Thus, a total of 399 Roma samples compared with 404 Hungarians, rs4846914 variant of *GALNT2* gene, rs1699614 of *CILP2*, rs1213033 of *ANGPTL3*, rs17321515 of *TRIB1*, rs17145738 and rs3812316 of the *MLXIPL* locus, rs1260326 and rs780094 of the *GCKR* gene were enrolled in this study.

Molecular Biology Methods

The allele specific amplification was performed by synthetic oligonucleotide primers, using standard polymerase chain reaction technique. After the PCR reaction, restriction fragment length polymorphisms procedures were used to get the genetic pattern. All the methods were designed to involve an obligate cleavage site on the amplicon in the amplified DNA sequence thus enabling us to verify the efficacy of the digestion. The position of the analyzed gene loci, the sequences of the primers, the restriction enzymes and cleavage sites and patterns were shown in Table 2.

3Statistical Analysis

All clinical data were represented as means±SEM where appropriate. For continuous variables the Mann–Whitney *U* test and for discrete variables the Chi-square tests were applied to compute the differences between the clinical parameters in Roma population and in Hungarian participants. The value of $p<0.05$ was considered as statistically significant. SPSS 20.0 package for Windows (SPSS Inc., Chicago, IL, USA) was employed for all statistical analyses.

Table 2 Primer sequences and PCR-RFLP conditions

Gene	Functional variants	Forward primer	Reverse primer	Melting temperature (°C)	PCR product size (bp)	Restriction endonuclease	Ancestral genotype fragments (bp)	Heterozygous genotype fragments (bp)	Minor allele genotype fragments (bp)
GCKR	C1337T rs1260326	TGCAGACTATAGTGGAGCCG	CATCACATGGCCACTGCTTT	60	231	*HpaII*	18, 63, 150	18, 63, 150, 213	18, 213
	rs780094	ATTGTCTCAGGCAAACCTGGTA	CCCGGCCTCAACAAATGTAT	60	273	*PscI*	63, 210	33, 63, 177, 210	33, 63, 177
MLXIPL	rs17145738	ATGGTCCAGGAGTCTGCCC	AGCCATCGTGCCTAGCTAAA	60	615	*TaaI*	49, 113, 253	49, 113, 253, 366	49, 366
	rs3812316	CCATCCCCAGCCATCCCT	TTCTCCAGTGTGGTCCCCGT	60	239	*BspLI*	16, 17, 203	16, 17, 26, 177, 203	16, 17, 26, 177
ANGPTL3	rs12130333	TTTCTAAACCTTGGTATCTT CATTTG	CATTTTCATGGTTGCTTTGT AATTT	58	372	*DraI*	79, 294	26, 79, 268, 294	26, 79, 268
CILP2	rs16996148	TCTCATCATTCACCCATCCA	AATGTGTGTTCTCCCAAGCC	58	466	*Hin1II*	184, 283	47, 184, 235, 283	47, 184, 235
GALNT2	rs4846914	CTGTGCCTTCTGGGACTGCTA	AGTGAGGAAGGACTATGA GATGATG	57	200	*HpyF3I*	19, 74, 107	19, 74, 107, 126	74, 126
TRIB1	rs17321515	AAGGAAGGGTTAGGTAGACC AATTA	GACACCAGCTGTAGAGAA CCAAATA	57	596	*FspBI*	56, 90, 450	56, 90, 450, 541	56, 541

Results

All allele distribution and allele frequencies of polymorphisms summarized in Table 3 and Table 4 were in Hardy–Weinberg equilibrium both in Roma and in Hungarian individuals. In allele frequencies significant differences were found for *MLXIPL* both variants, *GALNT2* rs4846914 and *ANGPTL3* rs1213033 polymorphisms comparing Roma participants to the Hungarians. The C alleles in rs17145738 and rs3812316 variants of *MLXIPL* occurred more frequently in Roma population than in Hungarians. Contrary to this, variants rs1213033 of *ANGPTL3* and rs4846914 of GALNT2 genes exhibited a significantly lower allele frequency in Romas than in Hungarians.

Serum triglyceride and total cholesterol levels in two examined populations with different genotypes are summarized in Table 5 and Table 6. We found no association between serum triglyceride levels and carrying minor alleles analyzed compared with the non-carriers in Roma and Hungarian population samples.

Discussion

Worldwide, the role of serum triglycerides and total cholesterol in relation to development of several diseases, especially cardio-and cerebrovascular diseases, metabolic syndrome and diabetes mellitus is extensively investigated [4, 10, 11, 30, 35–38]. In the past few years, several studies described new genetic polymorphisms which have an effect on triglyceride level alteration, like *GCKR* and *APOA5* variants [11, 30, 35–38]. The mechanism of glucokinase enzyme of the liver is under control by glucokinase regulatory protein (GCKR), which enzyme has a dominant glucose phosphorylase role of

Table 3 Allele distribution of polymorphisms of GCKR and MLXIPL gene loci

	Roma (399)		Hungarians (404)	
GCKR rs1260326	CC (n=119)	CT+TT (n=205+75)	CC (n=102)	CT+TT (n=208+94)
T allele frequency	44.5%		49.0%	
GCKR rs780094	GG (n=119)	GA+AA (n=180+100)	GG (n=99)	GA+AA (n=218+87)
A allele frequency	47.6%		48.5%	
MLXIPL rs17145738	TT (n=2)	TC+CC (n=43+354)	TT (n=9)	TC+CC (n=98+297)
C allele frequency	94.1%*		85.6%	
MLXIPL rs3812316	GG (n=5)	GC+CC (n=36+358)	GG (n=9)	GC+CC (n=89+306)
C allele frequency	94.2%*		86.8%	

*p<0.025 vs. Hungarians

Table 4 Allele distribution of polymorphisms of CILP2, GALNT2, ANGPTL3 and TRIB1 gene loci

	Roma (394)		Hungarians (400)	
CILP2 rs1699614	GG	GT+TT	GG	GT+TT
	(*n*=333)	(*n*=60+1)	(*n*=342)	(*n*=56+2)
T allele frequency	7.86%		7.50%	
GALNT2 rs4846914	AA	AG+GG	AA	AG+GG
	(*n*=90)	(*n*=243+63)	(*n*=91)	(*n*=182+127)
G allele frequency	46.6%*		54.5%	
ANGPTL3 rs1213033	CC	CT+TT	CC	CT+TT
	(*n*=309)	(*n*=81+8)	(*n*=270)	(*n*=112+18)
T allele frequency	12.2%*		18.5%	
TRIB1 rs17321515	AA	GA+GG	AA	GA+GG
	(*n*=103)	(*n*=203+93)	(*n*=107)	(*n*=186+107)
G allele frequency	48.8%		50.0%	

*$p<0.025$ vs. Hungarians

the liver and of the pancreatic β-cells in the glucose homeostasis of the blood [39–41]. In genome-wide association studies the possible effect of functional variants in *GCKR* gene in association with hypertriglyceridemia was analyzed [15, 42]. The intronic rs780094 and the exonic rs1260326 variants are the most investigated, the last variant causes a Leu/Pro change at 446 amino acid position, which indirectly affects triglyceride levels alteration, has a role in impaired fasting glycaemia, and is a possible risk for type II diabetes mellitus, as Veiga-da Cunha observed [43]. Santoro et al. examined 455 obese children and adolescents (181 Caucasians, 139 African Americans, and 135 Hispanics) for rs1260326 of *GCKR* gene. The variant showed an association with hepatic fat accumulation along with large VLDL and triglyceride levels. Two genes, as *GCKR* and *PNPLA3* act together and have a susceptible effect for manifestation of fatty liver in obese young people [44].

Several studies confirmed the fact, that Angiopoietin-like protein 3 (ANGPTL3) has an effect on lipid metabolism; the protein indirectly inhibits the activity of lipoprotein and other endothelial lipases. The loss-of-function mutations of *ANGPTL3* gene causes total ANGPTL3 absence, which shows a high association rate with recessive hypolipidemia. This type of hypolipidemia is characteristic for decrease of apolipoprotein B and apolipoprotein A-I-enclosing lipoproteins, which leads to altering levels of high-density lipoprotein. By contrast, the incomplete scarcity of ANGPTL3 is related to attenuation of low-density lipoprotein. Pisciotta et al. investigated *ANGPTL3* gene in 4 persons with low levels of LDL cholesterol and HDL cholesterol, and they found homozygous, compound heterozygous for *ANGPTL3* loss-of-function mutations (p.I19LfsX22/p.N147X, p.G400VfsX52) associated with the deficiency of ANGPTL3 in plasma. Decreased plasma levels of triglyceride-containing lipoproteins and of HDL particles were observed, moreover, the heterozygous carriers showed normal level of plasma high-density lipoprotein cholesterol, but low plasma level of ANGPTL3 and attenuated level of low-density lipoprotein cholesterol [45].

A Max-like-interacting-protein-like (*MLXIPL*; or carbohydrate response element binding protein, *ChREBP*) gene is located in the WBSCR14 deletion region at chromosome 7q11.23. Recently, in genome-wide association studies between the plasma triglyceride-level alterations and *MLXIPL*

Table 5 The effect on lipid parameters of polymorphisms of GCKR and MLXIPL gene loci

	Roma (399)		Hungarians (404)	
GCKR rs1260326	CC	CT+TT	CC	CT+TT
	(*n*=119)	(*n*=205+75)	(*n*=102)	(*n*=208+94)
Plasma triglyceride (mmol/l)	1.47±0.06	1.66±0.05	1.58±0.06	1.52±0.03
Serum cholesterol (mmol/l)	4.57±0.11	4.76±0.07	5.66±0.10	5.54±0.07
GCKR rs780094	GG	GA+AA	GG	GA+AA
	(*n*=119)	(*n*=180+100)	(*n*=99)	(*n*=218+87)
Plasma triglyceride (mmol/l)	1.50±0.06	1.65±0.05	1.51±0.05	1.54±0.03
Serum cholesterol (mmol/l)	4.57±0.10	4.76±0.07	5.69±0.10	5.54±0.07
MLXIPL rs17145738	TT	TC+CC	TT	TC+CC
	(*n*=2)	(*n*=43+354)	(*n*=9)	(*n*=98+297)
Plasma triglyceride (mmol/l)	1.22±0.12	1.61±0.04	1.44±0.09	1.54±0.03
Serum cholesterol (mmol/l)	4.75±0.15	4.70±0.06	6.23±0.53	5.56±0.06
MLXIPL rs3812316	GG	GC+CC	GG	GC+CC
	(*n*=5)	(*n*=36+358)	(*n*=9)	(*n*=89+306)
Plasma triglyceride (mmol/l)	1.51±0.25	1.61±0.04	1.41±0.09	1.54±0.03
Serum cholesterol (mmol/l)	4.88±0.30	4.7±0.06	5.90±0.52	5.57±0.06

Values are means ± SEM. Triglycerides and serum total cholesterol levels are mmol/l

Table 6 The effect on lipid parameters of polymorphisms of CILP2, GALNT2, ANGPTL3 and TRIB1 gene loci

	Roma (394)		Hungarians (400)	
CILP2 rs1699614	GG	GT+TT	GG	GT+TT
	(*n*=333)	(*n*=60+1)	(*n*=342)	(*n*=56+2)
Plasma triglyceride (mmol/l)	1.60±0.04	1.62±0.10	1.54±0.03	1.52±0.05
Serum cholesterol (mmol/l)	4.71±0.06	4.69±0.14	5.59±0.06	5.53±0.16
GALNT2 rs4846914	AA	AG+GG	AA	AG+GG
	(*n*=90)	(*n*=243+63)	(*n*=91)	(*n*=182+127)
Plasma triglyceride (mmol/l)	1.65±0.09	1.59±0.04	1.57±0.05	1.53±0.03
Serum cholesterol (mmol/l)	4.72±0.11	4.70±0.07	5.66±0.11	5.56±0.07
ANGPTL3 rs1213033	CC	CT+TT	CC	CT+TT
	(*n*=309)	(*n*=81+8)	(*n*=270)	(*n*=112+18)
Plasma triglyceride (mmol/l)	1.61±0.04	1.61±0.08	1.52±0.03	1.58±0.04
Serum cholesterol (mmol/l)	4.67±0.06	4.81±0.12	5.57±0.07	5.60±0.10
TRIB1 rs17321515	AA	GA+GG	AA	GA+GG
	(*n*=103)	(*n*=203+93)	(*n*=107)	(*n*=186+107)
Plasma triglyceride (mmol/l)	1.71±0.07	1.57±0.04	1.51±0.04	1.55±0.03
Serum cholesterol (mmol/l)	4.76±0.11	4.69±0.06	5.59±0.11	5.57±0.07

Values are means ± SEM. Triglycerides and serum total cholesterol levels are mmol/l

locus correlations were found. Moreover, the influence of triglyceride level increase of the major alleles of the rs17145738 and rs3812316 variants in *MLXIPL* locus were observed [15, 16].

In recent GWAS studies, the minor G-allele of the rs4846914 intronic variant of the *GALNT2* (UDP-N-acetyl-alpha-D-galactosamine: polypeptide-N-acetyl–galactose-aminotransferase 2) gene associated with increased triglyceride concentrations of the plasma. In a case–control study, which was performed on Han Chinese population analyzing 4192 individuals for type 2 diabetes, the association between elevated triglyceride levels and genotypes for *MLXIPL* rs17145738 variant and for *GCKR* rs780094 was confirmed, but not for *GALNT2* rs4846914 polymorphism [46].

In the last years an association has been detected between dyslipidemia and the rs16996148 (near *CILP2*), rs17321515 (near *TRIB1*), rs12130333 (near *ANGPTL3*) variants [16]. Moreover, these loci were correlated with the manifestation of cardiovascular diseases [15].

The *CILP2* gene the proteins' relation to lipid metabolism is not well studied yet. In a genome-wide association study, a triglyceride level reducing role of the rs16996148 variant was confirmed analyzing Caucasian individuals [23]. Vrablík et al. investigated 895 Czech patients with primary dyslipidemia comparing with 672 healthy controls. There was no significant effect of the polymorphisms *CILP2* on lipid levels after receiving statin treatment [47].

The human tribbles–1 (TRIB1) facilitates the proteosome-dependent protein degradation. In an Asian Malay population, the variant adjacent to the *TRIB1* locus (rs17321515) showed a significant correlation with increased total cholesterol and LDL-cholesterol, moreover higher risk for coronary heart disease and cardiovascular disease was described [48].

Conclusion

Our findings could verify earlier results [15], in allele frequencies showed significant differences in both variants of *MLXIPL*, *GALNT2* rs4846914 and *ANGPTL3* rs1213033 polymorphisms comparing Roma individuals to Hungarians. Analyzing Roma and Hungarian population samples we could not confirm any associations between altering levels of triglycerides and minor allele carriers compared with the non-carriers. This shows a much weaker triglyceride influencing activity for all these genotypes compared with the apolipoprotein A5 gene, which had been showed to influence the triglycerides using almost the same biobanks and populations as we used the current study [4, 5, 49].

Acknowledgments Study was supported by the grant of Hungarian Science Foundation OTKA K103983 and T73430.

Conflict of Interests The authors declare that they have no conflict of interests related to this manuscript.

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