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Association of rs780094 and rs1260326 glucokinase regulatory protein gene polymorphisms with dyslipidemia in a group of Serbian acute ischemic stroke patients

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Abstract: Although genetic variations rs780094 and rs1260326 of the glucokinase regulatory protein gene (*GCKR*) could be associated with lipid profile imbalance, their influence on acute ischemic stroke (AIS) risk has not yet been established. The aim of this study was to investigate the influence of *GCKR* single nucleotide polymorphisms (SNPs) rs780094 and rs1260326 on lipid profile parameters in patients with AIS, and to evaluate the association of these SNPs with the risk of AIS. In a case-control study, a total of 148 subjects were screened for *GCKR* rs780094 and rs1260326 SNPs using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The lipid profile was determined based on serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triacylglycerol (TG) concentrations. The frequencies of the minor rs780094T allele and the minor rs1260326T allele were significantly lower in AIS patients compared to controls. The rs780094TT genotype and the rs1260326TT genotype were associated with decreased risk of AIS compared to wildtype carriers. In conclusion, this is the first study implying that decreased risk of AIS in rs780094 and rs1260326 homozygous minor allele carriers is not caused by dyslipidemia, but possibly by the lack of coagulation factor glycosylation.

Keywords: acute ischemic stroke; glucokinase regulatory protein; rs780094; rs1260326; lipid profile

INTRODUCTION

Acute ischemic stroke (AIS) is an episode of neurological dysfunction caused by focal cerebral, spinal or retinal infarction [1]. The three leading causes of AIS are large-artery atherosclerosis, thromboembolism and lacunar infarction due to diseases of small blood vessels in the brain [2]. Stroke is the third leading cause of death, after cardiovascular and malignant diseases, and the first leading cause of disability in developed countries. Among patients older than 65 years, 26% of patients need help performing daily activities and 46% have some form of cognitive impairment [3].

Atherogenic dyslipidemia, which is characterized by low levels of high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A1, and high levels of total cholesterol (TC), triacylglycerol (TG) and low-density lipoprotein cholesterol (LDL-C), is associated with an increased incidence of AIS [4]. It is known that single nucleotide polymorphisms (SNPs) in different genes associated with lipid metabolism can affect serum lipid levels and increase the risk of AIS [5-7]. Among the genes whose genetic variations can influence dyslipidemia is the glucokinase-regulatory protein (GKRP) gene (*GCKR*). GKRP regulates glucokinase, an enzyme that catalyzes the phosphorylation of glucose in the first glycolysis reaction, producing glucose-6-phosphate. Glucokinase activity is not directly inhibited by glucose 6-phosphate as are the other hexokinases. Instead, it is indirectly inhibited by fructose 6-phosphate. In the presence of fructose 6-phosphate, glucokinase binds tightly to GKRP,

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forming an inactive heterodimer, and is translocated to the hepatocyte nuclei. On the other hand, when glucose levels in the blood increase, glucokinase is released from GKRP and the enzyme reenters the cytosol reverted to its active form. As a result, glucose is metabolized, dihydroxyacetone phosphate, glycerol 3-phosphate, acetyl-CoA and malonyl-CoA concentrations are increased, which can result in increased triacylglycerol and very-low-density lipoprotein (VLDL) synthesis. GKRP thus regulates both glucose and lipid metabolism pathways [8].

The *GCKR* gene is located on chromosome 2p23 and is comprised of 19 exons and encodes a 68 kDa protein. The rs780094 SNP is located at intron 16, while the rs1260326 SNP is located at exon 15. Both SNPs are characterized by the replacement of cytosine (C) by thymine (T). In rs1260326 SNP, this replacement results in the substitution of proline by leucine at position 446 (Pro446Leu) [9]. Previous studies have shown an association between these two SNPs and the risk of developing type 2 diabetes mellitus, obesity, metabolic syndrome and dyslipidemia [7,8,10], but their functional significance in AIS is still not sufficiently known. The aim of this study was to investigate the influence of *GCKR* rs780094 and rs1260326 SNPs on the lipid profile in patients with AIS, as well as to evaluate the association between these SNPs and the risk of AIS.

MATERIALS AND METHODS

Ethics statement

The study was performed in compliance with the Declaration of Helsinki; the Ethical Committee of the Medical Faculty University of Niš approved the study protocol. All participants agreed to participate in the study, and informed consent was signed by healthy subjects and by the patients or their legal guardians.

Participants

The study was performed on 148 subjects, including 68 patients with a diagnosis of AIS in the acute phase of the disease, diagnosed and treated at the Clinic of Neurology, University Clinical Center Niš, Serbia between 2019 and 2020. The control group consisted of 80 healthy subjects, matched with the patients by age and sex, without a previous history of AIS or other diseases. Ischemic stroke was diagnosed using computerized tomography and/or magnetic resonance imaging of the brain. Patients with diabetes mellitus, obesity (body mass index >30 kg/m^2), cholestatic liver disease, thyroid disease, Cushing syndrome, nephrotic syndrome, chronic kidney disease, cancer, as well as patients with atrial fibrillation and other sources of cardioembolism were excluded from the study.

Blood sample preparation and genotyping

Blood samples were taken in the morning after fasting, within seven days after the ischemic attack, during hospitalization. From the blood samples (with EDTA as an anticoagulant), we used 200 µL of blood for DNA isolation. A second test tube (without the anticoagulant) was centrifuged at 2000 × g for 10 min at 4°C, after which the serum was separated and used for lipid profile assessment. The isolation of DNA was performed using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen GmbH, Hilden, Germany). *GCKR* rs780094 and rs1260326 SNPs were determined by PCR-RFLP.

Fragments of 427 base pairs (bp) (rs780094) and 231 bp (rs1260326) were amplified using the appropriate primers (F: 5'-GATTGTCTCAGGCAAACCTGGTAG-3' and R: 5'- CAGGTCTATGCCACCACTCCTAG-3' for the rs780094; F: 5'-TGCAGACTATAGTGGAGCCG-3' and R: 5'-CATCACATGGCCACTGCTTT-3' for the rs1260326). The 25-µL PCR reaction mixture contained: 12.5 µL KAPA 2G Fast HS Ready-Mix PCR solution kit (KAPA Biosystems, Germany), 0.5 µL of primer (10 pmol/µL) each (Fermentas, GmbH, St. Leon-Roth, Germany) and 20 ng of DNA. Gene amplification was performed under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 15 s, elongation at 72°C for 15 s and termination at 72°C for 30 s. PCR products were checked by agarose gel electrophoresis (2%) and visualized under UV light. After confirmed amplification on an agarose gel, PCR products were digested with PscI (rs780094) and HpaII (rs1260326) restriction enzymes (Fermentas) at 37°C overnight and analyzed by vertical electrophoresis on an 8% polyacrylamide gel under UV light.

Genotyping of rs780094

Genotype CC (wildtype) was detected on a polyacrylamide gel as three fragments (188, 177 and 62 bp), while the TT genotype is detected as two fragments (365 and 62 bp). A heterozygous genotype (CT) was confirmed by the presence of four fragments on the gel (365, 188, 177 and 62 bp).

Genotyping of rs1260326

Genotype CC (wildtype) was detected on a polyacrylamide gel in the form of three fragments of 150, 63 and 18 bp, while the TT genotype was presented as two fragments (213 and 18 bp). Heterozygous genotype (CT) was confirmed by the presence of four fragments on the gel (213, 150, 63 and 18 bp).

Lipid profile assessment

Sera from early morning blood samples were used for the assessment of the lipid profile. Total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels were determined using standard biochemical analyses on an Olympus AU400 device. The research was conducted in the Laboratory for Functional Genomics and Proteomics at the Faculty of Medicine, University of Niš, Serbia.

Statistical analysis

The frequencies of alleles and genotypes in patients and controls were analyzed and compared using the $\chi 2$ test; we also determined possible deviation from the expected values of the Hardy-Weinberg equilibrium. Univariate logistic regression analysis was used to analyze the association between SNPs and AIS and genetic risk was assessed by the odds ratio (OR) with a 95% confidence interval (CI). For testing the normality of parameter distribution, the Kolmogorov-Smirnov test was used. There was a normality assumption for all studied parameters. The lipid profile parameter levels were expressed as mean (M)±standard deviation (SD). Statistically significant differences in values between patients and controls, as well as between different genotypes in a group of patients, were determined by the student t-test for two independent samples. A P-value of <0.05 was considered statistically significant. The statistical analysis was conducted using the SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Among the patients were 40 women and 28 men, with a mean age of 63.17±2.43 years. Out of 80 healthy subjects, there were 52 females and 28 males, with a mean age of 61.85±3.28 years. No significant differences were found between age (P=0.095) and gender (P=0.775) of patients and controls.

Genotype and allele frequencies of the rs780094 *GCKR* gene polymorphism

Genotype frequencies of *GCKR* rs780094 and rs1260326 SNPs did not deviate from the normal distribution of the Hardy-Weinberg equilibrium in the patient and control groups (P>0.05). The distribution of *GCKR* rs780094 genotypes did not show a statistically significant difference between patients with AIS and the group of healthy subjects (x^2=5.322, df=2, P=0.07). AIS patients had a significantly lower frequency of the minor rs780094T allele compared to the control group (x^2=4.488, df=1, P=0.034). Univariate logistic regression showed that the rs780094 TT genotype was associated with a lower risk of having AIS as compared to the CC genotype (OR=0.333, 95% CI: 0.124-0.895, P=0.029), while the presence of the T allele was not associated with a lower risk of AIS compared to the C allele (P=0.186; Table 1).

Genotype and allele frequencies of the rs1260326 *GCKR* gene polymorphism

The distribution of the rs1260326 SNP genotypes in AIS patients showed a statistically significant difference compared to the control group (x^2=8.931, df=2, P=0.011). Patients with AIS had a significantly lower frequency of the minor rs1260326 T allele compared to the group of healthy subjects (x^2=5.623, df=1, P=0.018). Univariate logistic regression showed that the rs1260326 TT genotype was associated with a lower likelihood of having AIS in comparison to the CC genotype (OR=0.277, 95% CI: 0.100-0.765, P=0.013),

Table 1. Genotype and allele frequencies of the *GCKR* rs780094 SNP in the studied groups.

Genotype (rs780094)	Control N=80	AIS N=68	P-value (χ2 test)	OR (95% CI)	P-value
CC	16 (20.0%)	20 (29.4%)	0.070	1	
CT	40 (50.0%)	38 (55.9%)		0.760 (0.344-1.680)	0.498
TT	24 (30.0%)	10 (14.7%)		0.333 (0.124-0.895)	0.029
Allele					
C	72 (45.0%)	78 (57.4%)	0.034	1	
T	88 (55.0%)	58 (42.6%)		0.600 (0.282-1.278)	0.186

AIS – acute ischemic stroke, N – number of subjects, OR – odds ratio, 95% CI – 95% confidence interval

Table 2. Genotype and allele frequencies of the *GCKR* rs1260326 SNP in the studied groups.

Genotype (rs1260326)	Control N=80	AIS N=68	P-value (χ2 test)	OR (95% CI)	P-value
CC	18 (22.5%)	20 (29.4%)	0.011	1	
CT	36 (45.0%)	40 (58.8%)		1.000 (0.458-2.181)	1.000
TT	26 (32.5%)	8 (11.8%)		0.277 (0.100-0.765)	0.013
Allele					
C	72 (45.0%)	80 (58.8%)	0.018	1	
T	88 (55.0%)	56 (41.2%)		0.697 (0.332-1.460)	0.339

AIS – acute ischemic stroke, N – number of subjects, OR – odds ratio, 95% CI – 95% confidence interval

Table 3. Lipid profile in the studied groups

Lipid profile	Control M (SD)	AIS M (SD)	P
TC (mmol/L)	4.56 (0.48)	5.71 (0.65)	<0.001
LDL-C (mmol/L)	2.68 (0.57)	3.79 (0.84)	<0.001
HDL-C (mmol/L)	1.30 (0.29)	0.74 (0.31)	<0.001
TG (mmol/L)	1.28 (0.29)	2.59 (0.55)	<0.001

AIS – acute ischemic stroke, TC – total cholesterol, LDL-C – low-density lipoprotein cholesterol, HDL-C – high-density lipoprotein cholesterol, TG – triglyceride

while the presence of the T allele was not associated with a reduced risk of AIS compared to the C allele (P=0.339; Table 2).

Lipid profile in the studied groups

The mean values of TC, LDL-C and TG in AIS patients were significantly higher in comparison to healthy subjects (P<0.001, P<0.001, P<0.001, respectively). On the other hand, HDL-C values were significantly lower in patients compared to the matching control (P<0.001; Table 3). There were no statistically significant differences in the values of lipid status parameters (TC, LDL-C, HDL-C and TG) in either the AIS patients with the rs780094 CC genotype compared to patients carrying the rs780094 CT/TT genotypes (P>0.05), or between carriers of different genotypes (CC vs CT/TT) of rs1260326 SNP (P>0.05; Table 4).

DISCUSSION

Although rs780094 and rs1260326 are the most frequently examined *GCKR* SNPs, their association with ischemic stroke is not completely clear, nor is their influence on lipid profile in this disease. The results of this study showed that there were no statistically significant differences in the distribution of *GCKR* rs780094 genotypes between AIS patients and control,

Table 4. The influence of *GCKR* rs780094 and rs1230326 SNPs on lipid profile in AIS patients.

Lipid profile	Genotype (rs780094)		P	Genotype (rs1230326)		P
	CC	CT/TT		CC	CT/TT	
TC (mmol/L)	5.64 (0.70)	5.73 (0.64)	0.602	5.48 (0.68)	5.80 (0.63)	0.069
LDL-C (mmol/L)	3.89 (0.81)	3.75 (0.86)	0.531	3.54 (0.85)	3.89 (0.83)	0.118
HDL-C (mmol/L)	0.64 (0.24)	0.78 (0.33)	0.081	0.80 (0.31)	0.72 (0.31)	0.293
TG (mmol/L)	2.45 (0.61)	2.64 (0.52)	0.190	2.51 (0.53)	2.62 (0.56)	0.438

TC – total cholesterol, LDL-C – low-density lipoprotein cholesterol, HDL-C – high-density lipoprotein cholesterol, TG – triglyceride

while patients had a significantly lower frequency of the minor rs780094T allele compared to healthy subjects. Regression analysis showed that homozygous carriers of the minor allele (TT genotype) have a significantly lower risk of AIS. To our knowledge, based on our literature search, there is only one other study that has examined the association of this SNP and the risk of AIS so far. Our results are not consistent with the results of Bi et al. [11], who did not find an association between the rs780094 SNP and the occurrence of stroke in an African American population.

The distribution of *GCKR* rs1260326 genotypes in our study showed a significant difference between patients with AIS and healthy subjects. The frequency of the minor T allele in patients was significantly lower compared to the control. Regression analysis showed that homozygous carriers of the minor allele (TT genotype) have a significantly lower risk of AIS. Data from the literature on the association between this SNP and AIS are contradictory. Zhou et al. [5] did not find a significant difference between genotype and allele distribution in AIS patients and controls but found a reduced risk for AIS in carriers of the *GCKR* rs1260326 TT genotype in a female Chinese population. On the other hand, in a Hungarian population an association between rs1260326 and the occurrence of stroke was not reported [12]. Differences in results could be due to patient selection, sample size, subject population and ethnicity. To our knowledge, this is the first study performed in a Serbian population.

The replacement of proline with leucine at position 446 (which occurs in carriers of the minor allele of rs1260326 SNP) has been shown to result in impaired interaction of GKRP and glucokinase, which indirectly results in increased glucokinase activity in hepatocytes, mobilizes "glycolytic machinery" leading to lipogenesis stimulation [13-15]. Similar effects were shown by López Rodríguez et al. [16], who found increased expression of the *GCKR* gene in hepatocytes in CGGC haplotype carriers by examining the effects of four SNPs (rs780094, rs780095, rs780096 and rs1260326) with a stronger inhibitory effect on glucokinase in comparison to carriers of a haplotype consisting of minor alleles.

In our study, the values of lipid profile parameters (TC, LDL-C and TG) were significantly higher and HDL-C values were significantly lower in AIS patients compared to the control. However, our results did not show a statistically significant difference in lipid profile between carriers of minor alleles and carriers of wildtype genotypes of both examined SNPs in the group of AIS patients. To date, based on our literature search, there are two studies on the influence of rs1260326 SNP on lipid profile in AIS patients [5,12]; our results are consistent with these.

It was suggested that homozygous carriers of the minor allele of rs780094 and rs1260326 SNPs are at decreased risk of type 2 diabetes mellitus, despite higher TG levels compared to wildtype genotype carriers, indicating that other potential molecular mechanisms may influence disease onset [17,18]. The mechanism that could explain the association of AIS and the two examined SNPs is the role of *GKRP* in the activation of coagulation factors, especially factor XI, whose increased concentration is associated with thromboembolism and AIS. The results of a genome-wide association study showed the association of *GCKR* rs780094 with the regulation of this factor, indicating the influence of rs780094 SNP on the level of factor XI, as well as the association of rs780094 and *GCKR* expression in the liver [19]. Factor XI is known to have several N-glycosylated sites at key sites of the glycoprotein to which ligands that participate in the coagulation cascade bind. It is possible that minor allele carriers of the examined SNPs in our study lack this protein-inhibitory effect on glucokinase due to genetic variations and a reduced expression of *GCKR*. Glucokinase, a regulatory protein, thus "enables" the breakdown of glucose, rendering it less available for glycosylation and activation of coagulation factors, which reduces the risk of AIS.

Research on a large number of patients is needed to investigate the molecular mechanisms and potential protective effects of rs780094 and rs1260326 SNPs in AIS patients, the possible effects on the coagulation cascade and especially the effects of SNP-SNP interactions on AIS risk, as we have undertaken for different SNPs previously [20-24].

The limitations of our study are related to the relatively small sample size. Additionally, it has been shown that inadequate physical activity and diet could impair endothelial function and influence

dyslipidemia, involving nitric oxide and oxidative stress as potent modulators of the inflammatory response [25-27]. As we did not evaluate the potential influences of daily physical activity and diet on the lipid profile, this could be considered a study limitation.

CONCLUSIONS

The results of this study showed that homozygous minor allele carriers of *GCKR* rs780094 and rs1260326 SNPs were at a decreased risk of AIS compared to carriers of the wildtype genotype. Although the values of TC, LDL-C and TG were significantly higher and the values of HDL-C significantly lower in patients with AIS compared to the control, no significant influence of the examined SNPs on the lipid profile in patients was shown. However, future research on a large sample should focus on testing the underlying mechanisms of the potential protective effects of these SNPs in AIS.

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Conflict of interest disclosure: The authors declare that there is no conflict of interest**.**

Data availability: The Data Report is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Basic%20et%20al_Data%20Report.pdf

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