Association Study of the *SLC1A2* (rs4354668), *SLC6A9* (rs2486001) and *SLC6A5* (rs2000959) Polymorphisms in Major Depressive Disorder

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Research Article

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Abstract

Background: In recent years, a growing body of evidence highlights a causal link between glutamate and depression. The membrane excitatory amino acid transporter 2 (EAAT2), encoded by *SLC1A2* is responsible for the uptake and redistribution of most of the synaptic glutamate. Glycine, an inhibitory neurotransmitter, acts as an obligatory co-agonist of N-methyl-D-aspartate (NMDA) receptors and modulates excitatory neurotransmission. The clearance of synaptic glycine is performed by glycine transporters encoded by *SLC6A9* and *SLC6A5*. Higher synaptic glycine and glutamate levels could enhance the activation of NMDA receptors, therefore counteract the hypofunction of glutamate neurotransmission described in major depressive disorder (MDD). The aim of the study was to assess whether polymorphisms of *SCL1A2*, *SCL6A5* and *SCL6A9* play a role in the development of MDD and its clinical picture in the Polish population.

Methods: The study group consisted of 161 unrelated Caucasian patients with MDD, and 462 healthy controls. Polymorphisms of *SLC1A2*, *SLC6A5* and *SLC6A9* were genotyped with PCR-RLFP and TaqMan assays. The relationship between the studied single nucleotide polymorphisms (SNPs) was assessed based on the comparison of genotype and allele distribution in the study and the control group. The research also evaluated the relationships between the studied polymorphisms and clinical variables such as age of disease onset, number of episodes, duration of depression or severity of symptoms.

Results: We found a statistically significant association between *SLC1A2* rs4354668 polymorphism and MDD development (the frequency of rs4354668 CC genotype and allele C was 2-fold higher in patients than in the control). Such associations were not detected for *SLC6A5* and *SLC6A9* polymorphisms. No statistically significant impact of the studied SNPs on clinical variables of MDD was also observed.

Conclusions: The results of the current study indicate an association of rs4354668 polymorphism in *SCL1A2* with depression development in Polish population. Further studies with larger samples should be performed in the future to clarify the current findings.

Background

Depression is a commonly occurring, serious and recurrent disorder linked to diminished role functioning and quality of life, medical morbidity, and mortality with more than 264 million people affected worldwide [1, 2]. The heritability of depression is significant with around 35% of the risk associated with genetic predisposition [3]. The understanding of its etiopathogenesis has seriously evolved over the last decades. Although numerous prominent discoveries have been made in the field of genetics and biochemistry, many remains unknown to this day. In the mid of the 20th century, mostly based on the observations of reserpine and iproniazid work of action, the monoaminergic theory of depression arose [4]. The vast majority of currently available antidepressants shares the same action mechanism, which involves the modulation of monoaminergic neurotransmission at a synaptic level by serotonin, dopamine and norepinephrine reuptake, degradation and receptor dynamics [5]. Those drugs however, though invaluable and revolutionary, have serious limitations according to several recent large clinical studies [6, 7, 8, 9]. Some researches proved that monoamines depletion in healthy volunteers does not precipitate depressive symptoms, neither worsens symptoms in depressed medication-free patients [10]. Taken together, these observations led the scientist to the notion that other neurotransmitters might play a crucial role in the pathogenesis of major depression. In recent years, a growing body of evidence highlights a causal link between glutamate and depression. Ketamine, an antagonist of glutamatergic N-methyl-D-aspartate receptor (NMDAR), demonstrated ultra-rapid efficacy for the treatment of refractory depression [11].

L-glutamic acid (glutamate) is the principal excitatory neurotransmitter in the central nervous system [12]. Almost all the glucose that enters the brain is ultimately converted to glutamate [13]. The packaging and release of glutamate is carried out through vesicular glutamate transporters (VGLUTs) [14]. After traversing the synaptic cleft, glutamate binds to cognate ionotropic receptors that are divided into three major classes: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA), as well as to metabotropic glutamate receptors, which have been demonstrated to modulate ion channels activity [15]. The extracellular level of glutamate is strictly regulated by a complex machinery. The uptake and redistribution of synaptic glutamate is performed through the membrane excitatory amino acid transporters, EAAT1 and EAAT2, encoded by *SLC1A3* and *SLC1A2* respectively [16]. SLC1A2 and SLC1A3 transporters are primarily astroglial in distribution [17]. Over 90% of glutamate in the brain is uptaken by EAAT2 [18]. A significant reduction in astroglia has been observed in MDD [19, 20].

The level of the astrocytic glutamate transporter EAAT2 is decreased in an animal model of depression [21]. Furthermore, the blockade of glutamate uptake with EAAT2 inhibitor dihydrokainic acid is sufficient to induce both anhedonia and anxiety in rats [22, 23]. Hence, an impaired glutamate uptake in glia could hold an important place in producing specific symptoms of depression.

In the post-mortem study the gene expression of the membrane transporter SCL1A2 was observed to be diminished in hippocampal tissue of MDD group compared to controls [24]. Choudary et al. documented significant downregulation of the glial high affinity glutamate transporters SLC1A2 in MDD subjects within anterior cingulate cortex and left dorsolateral prefrontal cortex (DLPFC) [25]. However, in the white matter *SLC1A2* mRNA was significantly lower in the subjects with MDD as compared to the superficial and deep gray matter of DLPFC [26]. A similar downregulation in *SCL1A2* mRNA was observed also in locus coeruleus of patients with ante-mortem diagnosis of MDD [27]. Such depletion in glutamate SCL1A2 transporter may result in a significant elevation of extracellular glutamate levels, which is potentially neurotoxic both to neurons and glia [28, 29]. In depressed patients elevated glutamate levels have been reported in the prefrontal and occipital cortex, as well as in the peripheral blood serum [30, 31, 32]. Also, an imbalance between neurotransmitters gamma-aminobutyric acid (GABA) and glutamate, resulting in cortical inhibition and excitation respectively, have been suggested to play a role in MDD [31, 33]. Mallolas et al. detected a novel *SCL1A2* gene promoter polymorphism that was an A-to-C change at 181 bp from the transcriptional start site [34]. Subjects with the mutant genotype, i.e., C allele carriers, were found to have a 30% reduction in promoter activity, which resulted in decreased expression of EAAT2 and therefore correlated with greater glutamate concentrations.

Glycine is an ubiquitous inhibitory neurotransmitter in the central nervous system (CNS), widely expressed in brainstem and spinal cord [35]. In glutamatergic synapses, glycine acts as an obligatory co-agonist of NMDA receptors, and therefore modulates excitatory neurotransmission [36]. Two members of SLC6 family of sodium/chloride-dependent neurotransmitter transporters are responsible for the clearance of synaptic glycine: glycine transporter type 1 (GlyT1) and glycine transporter type 2 (GlyT2) [37]. GlyT1 is strongly associated with glycine uptake in astrocytes and glutamatergic neurotransmission in cortex and hippocampus, while GlyT2 performs presynaptic glycine uptake on inhibitory glycinergic synapses in spinal cord, brainstem and cerebellum [38, 39]. In humans, GlyT1 and GlyT2 are encoded by *SLC6A9* and *SLC6A5* genes respectively [40, 41]. Hence, higher synaptic glycine levels could enhance the activation of NMDA receptors. Sarcosine, a GlyT1 inhibitor, was found to produce greater and quicker improvement in depressive symptoms in MDD patients, comparing to active control of citalopram [42].

Considering the findings of the above cited studies, *SLC1A2, SLC6A5* and *SLC6A9* have been chosen as the candidate genes in our case-control association study.

In the current work we focus on examining the association of the *SLC1A2* rs4354668, *SLC6A9* rs2486001 and *SLC6A5* rs2000959 polymorphisms with major depressive disorder and its clinical variables in the Polish population.

Materials And Methods

Patient and control groups profile

The study group consisted of 161 unrelated Caucasian patients matching the diagnostic criteria of DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, 5th Edition) for major depressive disorder (MDD). The severity of the depression symptoms was evaluated with the Hamilton Depression Rating Scale (HDRS). The symptoms of bipolar disorder and bipolar spectrum features were excluded based on Hirschfeld Mood Disorder Questionnaire and diagnostic criteria of bipolar disorder by Ghaemi. The study group comprised 116 females (72.1%, mean age 57 ± 12, range 19–84) and 45 males (27.9%, mean age 57 ± 9, range 29–80) recruited from inpatients treated at the Department of Psychiatry and Psychotherapy, Medical University of Silesia in Katowice, the Neuropsychiatric Hospital in Lubliniec and the State Hospital for Mental Diseases in Rybnik. Exclusion criteria involved intellectual disability, anxiety disorder, mixed anxiety and depressive disorder, schizoaffective disorder, schizophrenia, bipolar disorder, organic or substance-related psychosis, autoimmune and chronic inflammatory diseases, and lack of informed consent to participate in the study.

All patients were assessed to be capable of giving an informed consent to participate in the study. Detailed patients' profile was presented in Table 1. The control group included 462 healthy, unrelated individuals, aged from 18 to 65 [females 238 (51.5%), mean age 40 ± 8, range 28–62; males 224 (48.5%), 41 ± 9, range 23–68], blood donors of Regional Blood Donation and Blood Treatment Center in Katowice. Exclusion criteria for controls were abnormal blood tests result, contagious and autoimmune disease, psychoactive substance abuse except for nicotine, past mental illness episodes including family members declared in the questionnaire.

The study was approved by the Bioethics Committee of the Medical University of Silesia (resolution KNW/0022/KB1/34/14).

Table 1
Detailed patients' profile

Variable	Total group			Females			Males		
	Mean (± SD)	Median (Q_1-Q_3)	Range	Mean (± SD)	Median (Q_1-Q_3)	Range	Mean (± SD)	Median (Q_1-Q_3)	Range
Number of episodes	5.6 ± 6	4 (3–6)	1–50	6 ± 5	4 (3–7)	2–35	6 ± 7	4 (3–5)	1–50
Duration of the disease	12 ± 10	10 (5–17)	0.5–47	13 ± 10	10 (6–18)	1–47	10 ± 8	7 (3–15)	0,5–30
Total HDRS score	14.8 ± 9	13 (7–21)	0–42	14.1 ± 8.7	13 (7–20)	0–40	16.5 ± 9.8	15 (9–23)	2–42
Age of onset	45 ± 12	45 (36–53)	14–70	44 ± 12	43 (35–52)	14–70	47 ± 12	48 (40–55)	21–68
SD – standard deviation, Q_1 – lower quartile, Q_3 – upper quartile									

SNP choice and genotyping

We selected three SNPs (rs4354668 in the *SLC1A2*, rs2486001 in the *SLC6A9* and rs2000959 in the *SLC6A5*) with the minor allele frequency ≥ 0.10 in the European population (data available at website http://www.ncbi.nlm. nih.gov/SNP). Other selection criteria were: assay availability, the potential functional significance of SNP or the association of SNP with other related diseases while literature references were strongly taken under consideration [43].

Genomic DNA was extracted from the peripheral blood lymphocytes using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer protocol. Purity and concentration of DNA extracts was assessed using a BioPhotometer plus (Eppendorf AG, Germany). The genotyping of SNPs: rs4354668 A/C in the *SLC1A2* and rs2486001 G/A in the *SLC6A9* was performed by PCR-RFLP method as described previously [44], using the following published primers: *SLC1A2* forward 5'-GAGCGGCGGGGCCTCTTTTC-3' and reverse 5'-TGCAGCCGCTGCCACCTGTG-3' [45], *SLC6A9* forward 5'-TTCTATTCCCTGGGGTTCAGCA-3' and reverse 5'-AGCCTGGGCTGAGGCACACCAC-3' [43]. Amplified products were digested by two restriction enzymes (*Bcn*I - rs4354668 and *Ava*II - rs2486001) (Thermo Fisher Scientific), according to the protocol, and digested products were separated by electrophoresis in 2% agarose gels stained with ethidium bromide. Product sizes were: rs4354668: A allele 381 bp, C allele 262 bp/119 bp; rs2486001: G allele 210 bp, A allele 123 bp/87 bp.

Genotyping of rs2000959 polymorphism in the *SLC6A5* was performed using an allele-specific TaqMan assay (ABI/Life technologies, USA, catalog number: C__12032096_1_) on a CFX96 real-time PCR detection system (Bio-Rad), in a 96-well format. The PCR reaction was performed in a final volume of 25 µL, containing 10 ng of the DNA template, 12.5 µL TaqMan Universal PCR Master Mix (ABI/Life technologies, USA), 1.25 µl of a combined primers and probes mix (ABI/Life technologies, USA), and nuclease free water. Real-time PCR was performed with a holding stage at 95◦C for 10 min, followed by 40 cycles at 95◦C for 15 s and 60◦C for 1 min. Each analysis was performed with two no-template controls as a negative control, along with a three previously genotyped control samples representing particular rs2000959 genotypes as a positive controls.

As a quality control measure, 5% of randomly selected samples were repeatedly genotyped, and the concordance rate of these repeated samples reached 100%. Samples with missing genotypes have been removed from the further analysis.

Statistical analysis

Statistical analysis was performed using STATISTICA 13.0 PL (StatSoft, TIBCO Inc., Palo Alto, CA, U.S.) and R software (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.). All tests were two-tailed and $p < 0.05$ was considered to be statistically significant. Nominal and ordinal variables were expressed as percentages, whilst descriptive variables were expressed as mean value ± standard deviation in case of data with normal distribution or as median (lower quartile – upper quartile) in case of data with skewed distribution. Hardy–Weinberg equilibrium (HWE) was examined by the Fischer's exact test to compare the actual genotypes with the expected number. The differences in allele frequencies and genotypes distribution between groups were assessed using either the Chi-square ($\chi 2$) test or the Fisher exact test. Distribution of variables was evaluated by the Shapiro-Wilk test and quantile-quantile (Q-Q) plot, and homogeneity of variances was assessed by the Levene test. Logistic regression was applied to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). The logistic regression models (co-dominant, dominant, recessive, over-dominant, log-additive) were also used to assess potential association with schizophrenia risk and the best fitting models were determined by Bayesian information criterion (BIC). The linkage disequilibrium (LD), haplotype analysis as well as inheritance models (co-dominant, dominant, recessive, over-dominant, log-additive) were done using *SNPAssoc* package in R. The two-way ANOVA (sex, genotype) with Tukey's post-hoc test was used to examine the effect of genotypes on clinical variables (number of episodes, age of onset, duration of the disease and total HRDS score).

Results

Comparison of genotype and allele distribution of the studied polymorphisms between patients and controls.

The analysis of the *SLC1A2* rs4354668 polymorphism (Table 2) showed statistically significant differences in the genotype ($p < 0.001$) and allele ($p < 0.001$) distribution between a group of MDD patients and controls. We observed that genotype CC and allele C were more represented in the study group than in the control group with the allele C statistically significantly increased the risk of developing MDD [OR = 1.57 (95% CI: 1.25–2.11), $\chi 2 = 12.02$, $p < 0.001$]. The sex-stratified analysis showed statistically significant differences in the genotype distribution in women ($p < 0.01$). In men only a trend towards statistical significance was observed ($p = 0.09$). In both female ($p < 0.01$) and male group ($p < 0.05$), statistically significant differences in the allele distribution were found. Moreover, C allele significantly increased the risk of developing MDD in both groups: male [OR = 1.66 (95% CI: 1.14–3.04)], female [OR = 1.52 (95% CI: 1.15–2.22)].

The polymorphisms rs2486001 (*SLC6A9*) and rs2000959 (*SLC6A5*) showed no statistically significant difference in the genotype (rs2486001: $p = 0.53$; rs2000959: $p = 0.71$) and allele (rs2486001: $p = 0.91$; rs2000959: $p = 0.69$) distributions between the study and the control groups. The analyses in subgroups stratified according to gender also did not show any statistically significant differences in the genotype (rs2486001: females: $p = 0.22$, males: $p = 0.97$; rs2000959: females: $p = 0.29$, males: $p = 0.71$) and allele (rs2486001: females: $p = 0.55$, males: $p = 0.94$; rs2000959: females: $p = 0.26$, males: $p = 0.40$) distributions.

Each of the 3 analyzed SNPs did not depart from the Hardy–Weinberg equilibrium both in the study [rs4354668 ($p = 0.16$), rs2486001 ($p = 0.53$), rs2000959 ($p = 0.48$)] and in the control group [rs4354668 ($p = 0.39$), rs2486001 ($p = 0.60$), rs2000959 ($p = 1.00$)].

In the next step we explored the potential association between the risk of MDD and individual polymorphisms based on different genetic inheritance models (dominant, recessive, co-dominant and over-dominant). Genotypes of the two analyzed polymorphisms (rs2486001, rs2000959) were not significantly associated with the risk for MDD in any of the inheritance models (data not shown).

The *SCL1A2* SNP genotypes were significantly associated with MDD in the co-dominant and recessive models with the genotype CC increasing the risk of developing MDD in both models [co-dominant: OR = 2.50 (95% CI: 1.52–4.17, $p < 0.001$), recessive: OR = 2.38 (95% CI: 1.56–3.70, $p < 0.001$)]. After gender-stratification significant differences were observed only in females [co-dominant: OR = 2.50 (95% CI: 1.33–4.76, $p < 0.01$), recessive: OR = 2.56 (95% CI: 1.52–4.35, $p < 0.001$)]. In males the differences were statistically insignificant, however in the recessive model a trend towards statistical significance was observed ($p = 0.052$) (Table 3).

Table 2
Analysis of genotype and allele distributions in the entire population and stratified by gender.

SNP		Total group		χ2	p-value	Females		χ2	p-value	Males		χ2	p-value
		Patients	Controls			Patients	Controls			Patients	Controls		
Genotypes													
rs4354668	A/A	41 (25.47%)	154 (33.33%)	15.98	**< 0.001**	30 (25.86%)	73 (30.67%)	12.58	**< 0.01**	11 (24.44%)	81 (36.16%)	4.80	**0.09**
	A/C	71 (44.10%)	234 (50.65%)			51 (43.97%)	131 (55.04%)			20 (44.45%)	103 (45.98%)		
	C/C	49 (30.43%)	74 (16.02%)			35 (30.17%)	34 (14.29%)			14 (31.11%)	40 (17.86%)		
Alleles													
rs4354668	A	153 (47.52%)	542 (58.66%)	12.02	**< 0.001**	111 (47.84%)	277 (58.19%)	6.74	**< 0.01**	42 (46.67%)	265 (59.15%)	4.77	**< 0.05**
	C	169 (52.48%)	382 (41.34%)			121 (52.16%)	199 (41.81%)			48 (53.33%)	183 (40.85%)		

Nominal associations are bolded.

Table 3
Analysis of different inheritance models for the SNP rs4354668 in *SLC1A2* between study and control group.

Model	Genotype	Total group (n = 623)					Females (n = 354)					Males (n = 269)	
		Depression	**Control**	**OR (95% CI)**	**p-value**	**BIC**	**Depression**	**Control**	**OR (95% CI)**	**p-value**	**BIC**	**Depression**	**Control**
Co-dominant	A/A	41 (25.5%)	154 (33.3%)	1.00	**< 0.001**	700.9	30 (25.9%)	73 (30.7%)	1.00	**< 0.01**	453.4	11 (24.4%)	81 (36.2%)
	A/C	71 (44.1%)	234 (50.6%)	1.08 (0.69–1.67)			51 (44%)	131 (55%)	0.94 (0.56–1.61)			20 (44.4%)	103 (46%)
	C/C	49 (30.4%)	74 (16.0%)	**2.50 (1.52–4.17)**			35 (30.2%)	34 (14.3%)	**2.50 (1.33–4.76)**			14 (31.1%)	40 (17.9%)
Dominant	A/A	41 (25.2%)	154 (33.3%)	1.00	0.10	707.3	30 (25.9%)	73 (30.7%)	1.00	0.35	458.7	11 (24.4%)	81 (36.2%)
	A/C-C/C	120 (74.5%)	308 (66.7%)	1.41 (0.93–2.13)			86 (74.1%)	165 (69.3%)	1.27 (0.77–2.08)			34 (75.6%)	143 (63.8%)
Recessive	A/A-A/C	112 (69.6%)	388 (84.0%)	1.00	**< 0.001**	694.6	81 (69.8%)	204 (85.7%)	1.00	**< 0.001**	447.6	31 (68.9%)	184 (82.1%)
	C/C	49 (30.4%)	74 (16%)	**2.38 (1.56–3.70)**			35 (30.2%)	34 (14.3%)	**2.56 (1.52–4.35)**			14 (31.1%)	40 (17.9%)
Over-dominant	A/A-C/C	90 (55.9%)	228 (49.4%)	1.00	0.09	707.1	65 (56%)	107 (45%)	1.00	0.05	455.7	25 (55.6%)	121 (54%)
	A/C	71 (44.1%)	234 (50.6%)	0.72 (0.50–1.05)			51 (44%)	131 (55%)	0.64 (0.41-1.00)			20 (44.4%)	103 (46%)

OR – odds ratio; CI – confidence interval; BIC – Bayesian information criterion. Nominal associations are bolded.

The linkage disequilibrium analysis showed a weak linkage disequilibrium (LD) only between the polymorphisms rs2486001 and rs2000959 (D'=0.097, r = 0.060, p < 0.05).The polymorphisms rs4354668 and rs2000959 showed only a trend to LD (D'=0.071, r = 0.055, p = 0.053).

The haplotype analysis revealed statistically significant differences in the frequency of haplotype CGC between the study and the control groups. The patients with CGC haplotype had almost 1.75 higher risk of developing MDD in the entire group than those with the most frequent haplotype AGC [OR = 1.75 (95% CI: 1.16–2.63, p < 0.01)]. Such differences were not observed after stratification according to gender, however males with CGC haplotype showed a trend towards statistical significance (p = 0.06) (Table 4).

Correlation between gender, genotype, and clinical variables of MDD

Two-way ANOVA showed no statistically significant impact of rs4354668, rs2486001 and rs2000959 genotypes on clinical variables of the MDD (Table 5). However, for rs2486001 SNP within *SLC6A9*, a tendency towards statistically significant effect of the interaction gender x genotype on mean HDRS score was observed (p = 0.08). Females with rs2486001

G/G genotype had higher mean HDRS score than those with G/A genotype (15.2 ± 9.1 vs 11.1 ± 6.8), whereas males with rs2486001 G/A genotype had higher mean HDRS score than those with G/G genotype (18.4 ± 8.2 vs 16.2 ± 10.3). Patients with rs2486001genotype A/A were excluded from the analysis due to scarce sample number. Moreover, a statistically significant impact of gender on the duration of the disease was detected for each of the studied SNP [A/C genotype of rs4354668: F vs M median duration of the disease 11 vs 6 years ($p < 0.01$); G/G genotype of rs2486001: F vs M median duration of the disease 11 vs 8 years ($p < 0.01$); C/A genotype of rs2000959: F vs M median duration of the disease 12 vs 6 years ($p < 0.01$)].

Table 4
Haplotype analysis of the studied polymorphisms in patients with MDD and control subjects.

Haplotype			Total group (n = 623)			Females (n = 354)			Males (n = 269)		
rs4354668	rs2486001	rs2000959	Freq (%)	OR (95% CI)	p-value	Freq (%)	OR (95% CI)	p-value	Freq (%)	OR (95% CI)	p-value
A	G	C	33.89	1.00	—	31.29	1.00	—	36.65	1.00	—
C	G	C	24.27	1.75 (1.16–2.63)	**< 0.01**	26.34	0.63 (0.37–1.05)	0.12	21.97	0.53 (0.27–1.04)	**0.06**
A	G	A	13.68	1.23 (0.74–2.08)	0.41	15.19	0.66 (0.36–1.21)	0.30	12.44	1.73 (0.52–5.70)	0.41
C	G	A	12.59	1.28 (0.80–2.08)	0.30	11.78	0.75 (0.41–1.35)	0.29	13.14	0.83 (0.36–1.89)	0.65
A	A	C	5.32	0.52 (0.18–1.47)	0.22	6.30	3.06 (0.63–14.76)	0.13	4.28	1.18 (0.27–5.13)	0.95
C	A	C	4.18	1.54 (0.67–3.57)	0.31	4.01	0.44 (0.15–1.24)	0.10	4.39	1.19 (0.24–5.86)	0.80
C	A	A	3.18	2.04 (0.77–5.56)	0.15	3.07	0.34 (0.09–1.26)	0.19	3.44	0.75 (0.15–3.77)	0.67
A	A	A	2.89	1.32 (0.41–4.35)	0.64	2.02	1.05 (0.18–6.00)	0.76	3.70	0.49 (0.10–2.31)	0.47
Global haplotype association p-value < 0.05											

OR – odds ratio; CI – confidence interval. Nominal associations are bolded.

Table 5
Results from the two-way ANOVA on clinical variables of MDD.

Variable	rs4354668			rs2486001			rs2000959		
	sex	genotype	sex^{x}genotype	sex	genotype[1]	sex^{x}genotype	sex	genotype	sex^{x}genotype
Age of onset	0.10	0.27	0.33	0.57	0.23	0.85	0.33	0.46	0.82
Number of episodes	0.73	0.24	0.20	0.71	0.73	0.31	0.79	0.68	0.89
Duration of the disease	**< 0.01**	0.40	0.64	**< 0.05**	0.91	0.51	**< 0.01**	0.45	0.19
Total HDRS score	0.15	0.28	0.15	**< 0.05**	0.60	**0.08**	0.45	0.45	0.69

[1]A/A genotype was excluded due to scarce sample number. Nominally significant *p-values* are bolded.

Discussion

The results of the present study indicate an association of rs4354668 *SLC1A2* polymorphism with the risk of developing MDD in a Polish population. We found that the genotype CC and C allele were more frequent in depressed patients than in healthy ones. As far as we know, only a few studies on genetic polymorphism of *SLC1A2* in MDD were conducted. The majority of studies reported an association of *SLC1A2* SNPs with bipolar disorder [46, 47, 48, 49] and schizophrenia [50, 51, 44]. One of the polymorphisms (-181 A/C, rs4354668) evaluated in this paper was also analyzed in a Thai sample of 100 patients with MDD with and without suicide attempt [52]. In contrast to our findings, no significantly different genotype/allele distributions of *SLC1A2* rs4354668 were found between patients in the MDD group and volunteers in the control group. Various problems may validly lead to such discrepancies between the studies. A possible heterogeneity of used diagnostic methods and applied clinical criteria of inclusion and exclusion may be one of them. Contrary to the above cited study, we used slightly more strict protocol, that encompassed ruling out symptoms of bipolar disorder and bipolar spectrum features based on standardized questionnaires. In both studies no data on family history of mental disorders was collected so one population might have been more heavily genetically predisposed to develop MDD than the other one. Moreover, different gene-gene and gene-environment interaction across various populations may also contribute to the inconsistency of the results between studies. Our study population comprised significantly higher number of participants (161 vs 100) with similar female to male ratio, that might have also impacted the statistical analysis. Interestingly, the distribution of genotypes in Thai healthy controls (n=100) differs greatly from the Caucasian population analyzed in our study (n=462): 7 participants had TT genotype (7%), 47 participants had GT genotype (47%) and 46 participants had GG genotype (46%). In the current paper 154 participants (33.33%) in the control group had AA genotype, 234 participants (50.65%) had AC genotype and 74 participants (16.02%) had CC genotype. Both populations were in Hardy-Weinberg equilibrium. Therefore, the genetic differences among ethnic groups could result in the predominance of AA homozygotes and the paucity of CC homozygotes in case of our study. Further research is

needed to establish the role of rs4354668 polymorphism in MDD development across different ethnic groups. So far, no other studies of *SLC1A2* rs4354668 polymorphism in MDD have been reported.

A particular gene-by-environment interaction of early experienced stress and the functional polymorphism rs4354668 in *SLC1A2* has been shown in the hippocampal gray matter in subjects with bipolar disorder. After exposure to higher levels of adverse childhood experiences G/G homozygotes were found to be more vulnerable to stress reporting lower gray matter volume in the hippocampus. As the mutant G allele is associated with less transporter expression and a 30% reduction in promoter activity compared with the T allele, the levels of synaptic glutamate have been hypothesized to be increased that could lead to neurotoxicity and brain damage [47]. Morphometric studies of individuals with bipolar disorder showed reduced gray matter volumes in the hippocampal formation unlike individuals with unipolar depression who showed reduced gray matter volumes in the anterior cingulate gyrus [53]. Two different SNPs (rs3812778 and rs3829280) of the *SLC1A2* were associated with the level of glutamate within the anterior cingulate in both bipolar and unipolar depression, with minor allele carriers having significantly higher glutamate levels in comparison with common allele homozygotes [48]. In the study of Zhang et al. the rs4354668 SNP in the *SLC1A2* gene was significantly associated with schizophrenia and there were better executive function performances in all subjects homozygous for the T allele compared with the G allele carriers [51]. These findings are consistent with previous reports demonstrating that rs4354668 G allele has a disadvantageous effect on cognitive functions such as working memory and executive functions [54, 55]. Cognitive impairment is believed to be a core feature of depression [56]. Therefore, the lack of assessment of cognitive deficits seems an important limitation in the present study. Further work is encouraged to delineate the impact of polymorphisms in the glutamate system on cognitive performances in depressed population.

Lithium salts are a common mood stabilizer, highly effective in the treatment of bipolar disorder. The *SLC1A2* rs4354668 polymorphism was reported to influence the total episode recurrence rate and the efficacy of lithium treatment response in a sample of Italian patients with BD [46]. These findings seem to be particularly interesting in the context of lithium in the augmentation strategies of antidepressant medications. The World Federation of Societies of Biological Psychiatry Task Force recommends lithium as the first-line augmentation option for treatment-resistant depression with a recent meta-analysis supporting this approach [57, 58].

We did not analyze the potential influence of the studied SNPs on suicidal behaviors among depressed patients. Since suicide is inseparably linked to mood disorders, that is a serious limitation to this study. No research on association of *SLC1A2* rs4354668 polymorphism with suicidal behavior has been conducted to date. However, in the Irish study of Murphy et al. other *SLC1A2* variant, rs4755404, was associated with suicide attempts, but the findings are inconsistent with other researchers' results [59]. Hee-Yeon Choi et al. studied various psychological and genetic risk factors of suicidal behaviors in Korean population and did not find any significant associations between rs4755404 *SCL1A2* variant and suicidal behavior [60]. Similarly, the Italian researchers failed to replicate these results [49]. Thus, the role of *SLC1A2* polymorphisms in suicide remains to be clarified.

The results of our study indicate no association of SNPs in genes involved in the glycinergic neurotransmission, i.e., rs2486001 of *SLC6A9* and rs2000959 of *SLC6A5* with the development of MDD. To our knowledge, the associations of these two SNPs with MDD have not been reported previously. The number of published association studies regarding glycine transporters is scarce and limited mostly to schizophrenia and substance use disorder. Tsai et al. observed no significant differences in allelic frequencies or genotypic distributions of the *SCL6A9* polymorphisms (rs1766967, rs16831541, rs2248632, rs2248253) between the group of patients with schizophrenia and healthy controls in the Chinese population [61]. Also, Deng et al. who tested various SNPs in glycine transporters genes, concluded that *SLC6A9* is unlikely to be major susceptibility gene for schizophrenia in the Japanese population [62]. Morita et al. examined three SNPs of the *SLC6A9* (rs2486001, rs2248829, rs2248632) in the Japanese sample of methamphetamine-addicted subjects and found, that the TG haplotype consisting of rs2486001 and rs2248829 SNPs approximately doubled the risk of predisposition to methamphetamine-use disorder [43]. The rs2486001 polymorphism in *SLC6A9* and 4 other SNPs in *SLC6A9* and *SLC6A5* (currently not analyzed), was not associated with alcohol dependence in the group of 644 German alcohol-dependent subjects [63]. Noteworthy, the interest in glycine transporters has been growing constantly in recent years and encompasses numerous fields of medicine. Mutations in *SLC6A9* have been linked to development of essential hypertension and became causal factors of glycine encephalopathy [64, 65].

Several limitations of our study must be considered. Firstly, the sample size of the study group was relatively small, what might contribute to false-positive or false-negative results. Secondly, the study group comprised significantly higher number of females than males. In the future, we believe that assessment of cognitive functions and suicide attempts should be included in the research protocol to place the possible findings in a wider perspective. Finally, we analyzed only one SNP of each studied gene. Therefore, it cannot be excluded that other SNPs of the *SLC1A2*, *SLC6A9* and *SLC6A* might be associated with MDD or its clinical features.

Overall, we conducted our study on a relatively significant number of patients with depression of Caucasian ethnicity with the presence of the control group. Bearing in mind all the limitations of the current paper, we cautiously speculate that the mutant *SLC1A2* carriers are more susceptible to develop MDD. Further studies are needed to establish the influence of *SLC1A2*, *SLC6A9* and *SLC6A* polymorphisms on gene/protein expression and to explain their role in the pathogenesis of depressive disorders.

Conclusions

In conclusion, the current study indicates an association of rs4354668 polymorphism in *SCL1A2* with development of MDD in a Polish population. The frequency of genotype CC and allele C was twice as high in the study group as in the control group. The analysis of different genetic inheritance models showed that the genotype CC of the rs4354668 polymorphism increased the risk of developing MDD in the co-dominant and recessive models. Haplotype analysis revealed that patients with CGC haplotype had almost 1.75 higher risk of developing MDD in the entire group than those with the most frequent haplotype AGC. No associations between *SLC6A9* and *SLC6A5* polymorphisms and susceptibility to MDD were observed.

Abbreviations

AMPA
α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
CNS
central nervous system
DLPFC
dorsolateral prefrontal cortex
DSM-5
Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
EAAT
excitatory amino acid transporter
GABA
gamma-aminobutyric acid
GlyT
glycine transporter
HDRS
Hamilton Depression Rating Scale
HWE
Hardy-Weinberg equilibrium
KA
kainate
LD
linkage disequilibrium
MDD
major depressive disorder
NMDA
N-methyl-D-aspartate
PCR-RLFP
polymerase chain reaction - restriction fragment length polymorphism
SLC
solute carrier
SNP
single nucleotide polymorphism
VGLUT
vesicular glutamate transporter

Declarations

1. Ethics approval and consent to participate

The study was approved by the Bioethics Committee of the Medical University of Silesia (resolution KNW/0022/KB1/34/14). All participants have given an informed consent
to participate in the study. All methods were carried out in accordance with relevant guidelines and regulations.

2. Consent for publication (not applicable)

3. Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

4. Competing interests

The authors declare that they have no competing interests.

5. Funding

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the paper for publication.

6. Authors' contributions

PR and KK conceptualized and designed the study, provided DNA samples and clinical data, and contributed equally to drafting and revising the manuscript. AO and PCh contributed to interpreting the data and statistical analyses. MK and JK selected the SNPs, performed genotyping, and prepared the genetic analysis. All authors contributed toward data analysis, drafting, and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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