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Research Article

Implications of *MTHFR* polymorphisms in the development of treatment-resistant schizophrenia

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Abstract. Objectives: The aim of the current study is to investigate whether the C677T and A1298C *MTHFR* polymorphisms are more frequent in patients with treatment-resistant schizophrenia compared to treatment-responsive patients, and if these mutations are associated with a higher severity of the disease and a poorer functionality of the affected persons. Material and method: The study included 60 patients diagnosed with schizophrenia divided into two subgroups: patients treatment-resistant schizophrenia, treated with clozapine (36 patients), and patients with non-resistant, partially responsive schizophrenia, treated with non-clozapine antipsychotics (24 patients). The intensity of negative, positive and general symptoms was quantified using the PANSS scale (Positive and Negative Syndrome Scale), the severity of the disease was assessed using the CGI-S (Clinical Global Impression) score, and global functionality was evaluated using the GAF (Global Assessment Functioning) scale. The *MTHFR* 677C>T and 1298A>C gene polymorphisms were determined by Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP-PCR). Results: We found a greater frequency of the compound heterozygous genotypes in the treatment resistant group ($p=0.04$). There was no significant association between the *MTHFR* C677T and A1298C polymorphisms, respectively, in the group of treatment-resistant patients compared to non-resistant patients, although a greater number of patients with an altered genotype was observed in the treatment-resistant group for both positions compared to non-resistant patients ($p=0.3$; $p=0.1$, respectively). Regarding the implication of *MTHFR* gene variants in the severity of the disease and patient functionality, no significant associations were found. Conclusions: There was an association between the C677T/A1298C compound heterozygous genotypes and treatment resistant schizophrenia. No significant association was found between *MTHFR* C677T and A1298C and therapy resistance, the severity of the disease or global functionality in patients with schizophrenia.

Key Words: *MTHFR*, polymorphism, treatment-resistant schizophrenia.

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Introduction

Mental diseases affect 2-3.5% of the population with schizophrenia affecting about 0.3-0.7% up to 1% of the world's population regardless of race, ethnicity or geographic area (McGrath et al 2008). The age of onset is normally around the age of 21-25 years, 3-4 years earlier in men, compared to women, in whom onset is around the age of 25-30 years (Falkenburg et al 2014). Current research focuses on the impact that genetic changes have on treatment response, which might lead in the future to a therapeutic approach taking into consideration individual genetic mutations (Ozbek et al 2008). The treatment of schizophrenia still represents an enormous challenge both for research and for the practicing psychiatrist. Previous research has shown that the initiation of an early treatment increases the chances of remission and reduces the severity of symptoms as well as the decline in the patient's social and global functioning (Marshal et al 2005). However, despite the entry on the market in recent years of a series of atypical antipsychotics, about 30% of patients do not have an adequate response to usual antipsychotic medication and are classified as treatment-resistant. These patients have a much poorer prognosis (Schennach et al 2012).

The classification of schizophrenia depending on resistance or response to treatment is made based on clinical criteria allowing to differentiate three subtypes of schizophrenia: treatment-responsive, treatment-resistant schizophrenia and ultraresistant (clozapine-resistant) (Lee et al 2015).

Methylenetetrahydrofolate reductase (MTHFR) has a central role in the folate cycle by irreversibly converting 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-MTHF, the predominant circulating form of folic acid. 5-MTHF plays an important role in one-carbon metabolism, as well as in DNA methylation (Bottiglieri et al 1994), being involved in the recycling of homocysteine to methionine, which later leads to the generation of S-adenosylmethionine (SAM). SAM represents the major source of methyl groups in the brain (Gilbody et al 2007) and a major donor of methyl groups for the synthesis of DNA, proteins, neurotransmitters, hormones and phospholipids (Peerbooms et al 2011; Sugden 2006; Krebs et al 2009). Moreover, SAM serves as a cofactor and methyl group donor for the epigenetic regulation process of gene expression. Since recent studies have already highlighted the involvement of epigenetics in limiting the therapeutic effects of atypical antipsychotic medication and considering the role of MTHFR in the cycle responsible for providing methyl groups needed for histone methylation in the silencing gene process, we sought to explore the implications

of *MTHFR* functional polymorphisms in treatment resistant schizophrenia (Kurita et al 2012).

The two common polymorphisms namely C677T and A1298C, are associated with a decrease of the activity of the enzyme. The *MTHFR* C677T variant reduces MTHFR activity and raises the homocysteine level, resulting in a reduction of DNA methylation, thus representing risk factors for schizophrenia and other neuropsychic disorders (Yoshimi et al 2010). Homocysteine and its metabolites can have a excitotoxic effect on brain glutamate receptors, N-methyl-D-aspartate (NMDA) and may inhibit central nervous system methylation (Cohen-Woods et al 2010).

The main aim of this study was to evaluate the association between the presence of *MTHFR* C677T and A1298C gene polymorphisms and treatment-resistant schizophrenia. We also attempted to establish a correlation between the presence of these polymorphisms and a higher severity of the disease, as well as a poorer functionality of these patients.

Material and method

This was a prospective, observational, transversal, analytical case-control study.

Seventy-eight patients diagnosed with schizophrenia according to ICD-10 criteria (World Health Organization Classification of Mental and Behavioural Disorders: Clinical Descriptions and Diagnostic Guidelines) and DSM-IV-TR criteria (American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th-TR), who were admitted to the Clinic of Psychiatry I and the Mental Health Center of the Cluj County Clinical Emergency Hospital in the period 1.04.2011-30.10.2011, were invited to participate in the study. From these, 60 patients with a mean age of 40.9±8.9 years, 51 males (85%) and 9 females (15%), signed the informed consent. The study protocol was approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy.

According to Kane, treatment-resistant schizophrenia involves the administration of three atypical antipsychotics (with different action mechanisms) at a dose higher or equal to 1000 mg/day chlorpromazine equivalents for 6 weeks, with a BPRS scale higher or equal to 45 points, a CGI-S higher or equal to 4 and persistent socio-professional dysfunction for 5 years, without at least a 20% reduction of symptoms (Kane et al 1988). Since 1988, these criteria have been known as "the Kane criteria" for treatment-resistant schizophrenia (Kane et al 2013; Farooq et al 2013). The only evidence-based treatment for these patients is clozapine (Leucht et al 2013; Essali et al 2009), an atypical antipsychotic with a relatively weak dopamine antagonism.

The selection criteria in the study were: patients with primary diagnosis of schizophrenia divided into two subgroups of patients diagnosed with resistant schizophrenia, treated with clozapine (36 patients – 60%) and non-resistant schizophrenia patients, responding to non-clozapine antipsychotics (partial response rate of 24 patients -40%) according to criteria that define schizophrenia resistance (duration of disease, medication switches, GAF poor functioning and severity of disease at the time of study-CGI-S). Patients included in both subgroups are patients able to understand study related information.

The exclusion criteria were: (1) treatment resistance defined after administration of a single antipsychotic in the appropriate dose over a suitable time period without obtaining the therapeutic outcome, (2) addiction to alcohol or illicit drugs, (3) drug treatments that can influence the clinical picture, (4) patients with associated organic pathologies, (5) pathologies of psychiatric development or other types of psychotic disorders. (6) Patients who were unable to understand the instructions or signing informed consent and subjects under 18 years of age, were not included in the study.

Patients underwent a complete psychiatric examination. The presence and intensity of negative, positive and general symptoms was evaluated using the PANSS scale (Positive and Negative Syndrome Scale - Kay et al 1987), the severity of the disease at the inclusion in the study was assessed using the CGI-S score (Clinical Global Impression - Guy 1976), and global functionality was quantified using the GAF scale (Aas 2011). The following data were recorded: age and sex, the age of onset of the disease, the clinical form of schizophrenia, antipsychotic medication and response to treatment.

All participants in the study were Romanians aged over 18 years. Each patient was examined by at least two experienced psychiatrists, who assigned a diagnosis of paranoid schizophrenia to 48 patients (80%) and undifferentiated schizophrenia to 12 patients (20%) depending on criteria defined by the Diagnostic and Statistical Manual of Mental Disorders, fourth revised edition (DSM-IVTR), and the Classification of Mental and Behavioural Disorders (ICD-10). Diagnosis was made according to clinical diagnostic criteria of treatment-resistant schizophrenia (disease duration, drug switches, deficient functioning – GAF, and disease severity at the inclusion in the study – CGI-S). No patients had a special diet that might have altered drug absorption. From each patient, 2 ml of venous blood was drawn on EDTA tubes, and the blood samples were transported for processing to the Medical Biochemistry Department of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca.

DNA was isolated using a Zymo Research kit (ZR Genomic DNA II kitTM).

The *MTHFR* 677C>T and 1298A>C polymorphisms were determined by Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP-PCR) as previously described by Frosst et al (1995) and Van Der Put et al (1998) with some modifications according to our laboratory. Briefly, for the 677C>T polymorphism we amplified a 198bp fragment using the following primers: forward 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′, reverse 5′-AGGACGGTGCGGTGAGAGTG-3′. For the 1298A>C we used the following primers: forward 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′, reverse 5′-CACTTTGTGACCATTCCGGTTTG-3′ to produce a 163bp amplicon.

The PCR reaction occurred in an iCycler (Bio-Rad Laboratories, Inc, Life Science Group USA). Enzymatic digestion was done in 10µl and we used 5U restriction enzymes (HinfI for 677C>T and MboII for 1298A>C).

All the reagents were purchased form Fermentas (Thermo Fisher Scientific) except the primers which were from Eurogentec (Liege, Belgium).

Statistical data analysis was performed using the Statistical Pack for Social Sciences version 22 (SPSS IBM, USA). For data characterization, absolute and relative frequencies or means and standard deviations were calculated, depending on the type of variables. The distribution of nominal data between the groups

was tested using the chi-square test. The normal distribution of quantitative variables was tested with the Kolmogorov-Smirnov test. Differences of quantitative variables between two groups were tested using the t-Student test. Differences of quantitative variables between more than two groups were tested using the ANOVA test. A p-value less than 0.05 was considered statistically significant.

Results

The mean age of onset was 22.17 years in patients with paranoid schizophrenia (80%), and 21.75 years in those with undifferentiated schizophrenia (20%).

Patients with treatment-resistant schizophrenia were treated with clozapine (60%) and those with partially responsive schizophrenia (40%) received treatment with non-clozapine antipsychotics: risperidone (16.7%), quetiapine (15%), others: olanzapine, aripiprazole, amisulpride, paliperidone (8.3%).

Among patients with treatment-resistant schizophrenia, the severity of the disease was high in 55.5% of patients and moderate in 44.5%; in contrast, all patients with partially responsive schizophrenia presented a moderate severity (CGI-S score).

The functionality of patients with treatment-resistant schizophrenia was severely affected in a 52.78% proportion compared to patients with partially responsive schizophrenia, who had only moderately affected functionality (GAF scale).

The evaluation of the *MTHFR* C677T gene polymorphism in the group of treatment-resistant patients evidenced no difference in the genotype distribution frequency. A greater number of patients with an altered genotype was observed in the group of patients with therapy resistance compared to non-resistant patients. Both *MTHFR* C677T and A1298C genotypes and alleles were similarly distributed between the two groups of patients (p=0.3 and p=0.1 respectively) (Table 1). When analyzing the compound heterozygous genotypes, we observed a higher frequency in the treatment resistant group compared to the non-resistant one (p=0.04).

No significant differences were observed when analyzing the distribution of the genotypes in regards of the severity of the disease (Table 2). There was also no significant difference between patients when analyzing the genotypes in relationship with the patients functionality score (GAF scale), as shown in Table 3.

Discussions

In our study, no significant association was found between the *MTHFR* C677T and A1298C polymorphisms when analyzing the patients' resistance to treatment, although a greater number of patients with therapy resistance had the risk alleles T for the C677T and C for the A1298C, respectively, compared to the group of non-resistant patients. These data cannot be correlated with the literature data, as there are no studies that have made similar comparisons. The analysis of *MTHFR* C677T and the severity of the disease (CGI-S) showed no significant differences in severity between the heterozygous genotype and the normal homozygous genotype; similarly, for the A1298C polymorphism, no significant differences were found between the heterozygous genotype and the normal homozygous genotype.

The evaluation of the relationship between *MTHFR* C677T polymorphism and patient functionality (GAF score) showed

Table 1. *MTHFR* C677T and A1298C genotypes analysis in relationship with treatment resistance

***MTHFR* polymorphism**		**Resistant**	**Non-resistant**	**p**
		N(%)	**N(%)**	
C677T	**CC**	13(36.1)	12(50.0)	
(rs1801133)	**CT**	19(52.8)	8(33.3)	0.3
	TT	4(11.1)	4(16.7)	
Alleles frequency	**C**	45(62.5)	32(66.7)	0.6
	T	27(37.5)	16(33.3)	
A1298C	**AA**	14(38.9)	14(58.3)	0.1
(rs1801131)	**AC**	22(61.1)	10(41.7)	
Alleles frequency	**A**	50(69.4)	38(79.2)	0.2
	C	22(30.6)	10(20.8)	
C677T/A1298C compound heterozygous	**TT/AC and CT/AC**	13(36.1)	3(12.5)	0.04

Table 2. *MTHFR* C677T and A1298C genotypes analysis in relationship with the severity of the disease (CGIS score)

***MTHFR* polymorphism**		**Moderate severity (CGIS 3-4)**	**High severity (CGIS 5-6)**	**p**
		N(%)	**N(%)**	
C677T	**CC**	18(45.0%)	7(35.0%)	
	CT	17(42.5%)	10(50.0%)	0.7
	TT	5(12.5%)	3(15.0%)	
A1298C	**AC**	20(50%)	12(60%)	
	AA	20(50%)	8(40.0%)	0.6
C677T/A1298C compound heterozygous	**TT/AC and CT/AC**	9(22.5%)	7(35%)	0.3

Table 3. The relationship between *MTHFR* C677T, A1298C polymorphisms and the GAF score

MTHFR polymorphism		DPA	DPM	p
		N(%)	N(%)	
C677T	**CC**	7(36.8)	18(43.9)	0.7
	CT	9(47.4)	18(43.9)	
	TT	3(15.8)	5(12.2)	
A1298C	**AA**	8(42.1)	20(48.8)	0.4
	AC	11(57.9)	21(51.2)	
C677T/A1298C compound heterozygous	**TT/AC and CT/AC**	7(35%)	9(22.5%)	0.3

no statistical significance between the altered genotype in patients with severely affected functionality compared to those with moderately affected functionality. Similarly, no association was found in the care of the A1298C polymorphism.

Regarding the implications of the *MTHFR* gene polymorphisms in treatment-resistant schizophrenia, to our knowledge there are no representative studies in literature. A study performed in men with treatment-resistant schizophrenia demonstrated that the *MTHFR* C677T polymorphism influences one-carbon metabolism. This study consisted of supplementing dietary choline intake for 12 weeks, and showed the fact that men with the *MTHFR* 677TT genotype and low folate levels use choline as a methyl donor (Yan et al 2011).

An extensive study was conducted using PubMed, EMBASE, PsycInfo, CINAHL and OpenGrey databases, in order to identify all current studies comparing treatment-resistant schizophrenia (defined either as a lack of response to two antipsychotics administered for an adequate time period or clozapine recommendations) with treatment-responsive schizophrenia (defined as a response to non-clozapine antipsychotics) (Gillespie et al 2017).

It is known that genetic factors play an important role in schizophrenia because it has been observed that the disease has a high heritability (80%) (Sullivan et al 2003). The genetics of schizophrenia is complex, being determined by the interaction between multiple genes and only to a small extent by individual genes (Joober et al 2002; Allen et al 2008). There are very few studies on genetic mutations involved in the development of treatment-resistant schizophrenia.

Methylentetrahydrofolate reductase is the central enzyme in the folate cycle and contributes to the metabolism of the amino acid homocysteine, the deficiency resulting in hyperhomocysteinaemia and homocysteinuria. Severe deficiency of MTHFR with enzymatic activity below 20-30% can cause developmental delays, seizures, motor dysfunction and even schizophrenic disorders (Botto 2000; Peerbooms et al 2011).

The two genetic polymorphisms associated with decreasing MTHFR activity, 667C/T and 1298 A/C were extensively studied, the first being relatively frequent in the world, about 12% of the population having the TT genotype, with a different distribution among different ethnic groups (Foldinger et al 1999). The 667C/T polymorphism was the most studied, its clinical consequences being dependent on the state of the folate, while the second polymorphism is less clearly outlined. Most studies refer to patients with schizophrenia and high homocysteine levels (Regland et al 1997) and conclude that homozygosity for thermolabile MTHFR may be a risk factor for schizophrenia-like psychosis, folate administration diminishing this risk. Arinami et al (1997) found the T677 variant both in schizophrenia and depression, which contradicts the findings of Kunugi et al (1998) that does not find a causal relationship between C677T polymorphism and the occurrence of schizophrenia or affective disorders. A series of studies in the literature show that the T allele is commonly associated with schizophrenia (Peerbooms et 2011, meta-analysis by Mohamed et al 2014; Zintzaras 2006; and Cohen-Woods et al 2010; Kempisty et al 2006; Lewis et al 2005; Muntjewerff et al 2006; Lajin et al 2012).

An association of the *MTHFR* 1298A>C polymorphism with schizophrenia was not found in most studies, including the meta-analysis of Peerbooms et al (2001). The A1298C variant cannot fundamentally affect the risk of the disease unless it is present in homozygous form. This variant may not have such a profound effect on homocysteine levels alone, but may contribute to hyperhomocysteinemia if present with the C677T allele and low folate levels (Nazki et al 2014).

The association between *MTHFR* polymorphisms and resistance to treatment has not been studied so far in Romania, which is in agreement with the absence of studies regarding this association in other European countries. It is known that about one-third of patients are treatment-resistant, although according to some estimates, the proportion is 40-60% (Juarez-Reyes et al 1995; Essock et al 1996). Among mental diseases, treatment-resistant schizophrenia is associated with some of the highest levels of dysfunction (Iasevoli et al 2016) and rates of hospitalization (Meltzer et al 2001), and high costs for society. While in Romania, the costs generated by treatment-resistant schizophrenia are not estimated, in USA, direct health care costs for patients with treatment-resistant schizophrenia require a supplementation of 34 billion USD (Kennedy et al 2014).

The main limitation of this study is the relatively small size of the samples used, although a strong effect should have been detected at this size. Nevertheless, our results must be replicated in larger size samples to exclude small effects, like those estimated for most of the mutations involved in the etiology of schizophrenia in general.

Conclusions

This study shows the absence of a significant association between *MTHFR* C677T and A1298C polymorphisms and therapy resistance, although there was a positive association between the C677T/A1298C compound heterozygous genotypes and treatment resistance. With regards to the severity of the disease or the global functionality of the affected persons, no relationship between the two *MTHFR* variants could be demonstrated.

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