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RESEARCH ARTICLE

The methylenetetrahydrofolate reductase (*MTHFR*) 677 C>T polymorphism increases the risk of developing chronic myeloid leukemia—a case-control study

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Abstract The methylenetetrahydrofolate reductase (*MTHFR*) 677 C>T and 1298 A>C polymorphisms are associated with variations in folate levels, a phenomenon linked to the development of various malignancies. The aim of this study was to investigate the influence of the 677 C>T and 1298 A>C polymorphisms in the *MTHFR* gene on the risk of developing chronic myeloid leukemia (CML). Our study included 151 patients with CML and 305 controls. The *MTHFR* 677 C>T and 1298 A>C polymorphisms were investigated by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and allele-specific PCR techniques. The CT and TT genotypes of the *MTHFR* 677 C>T polymorphism were associated with an increased risk of developing CML (odds ratio (OR)=1.556, 95 % confidence interval (CI)=1.017–2.381, p value=0.041, and OR=1.897, 95 % CI=1.046–3.44, p value=0.035, respectively). No association was observed between the prognostic factors (blasts, basophils, additional chromosomal abnormalities, EUTOS score, Sokal and Hasford risk groups) and the *MTHFR* 677 C>T and 1298 A>C variant genotypes in CML patients. Our study shows that the *MTHFR* 677 C>T polymorphism is significantly associated with the risk of CML in Romanian patients.

Keywords MTHFR · Polymorphisms · Chronic myeloid leukemia

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Introduction

Chronic myeloid leukemia (CML), one of the most studied malignancies, is characterized by the presence of the fusion gene *BCR-ABL* [1]. This genetic alteration is considered a driver mutation in CML. However, little is known about the factors leading to the acquisition of this specific mutation.

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in folate metabolism along with DNA methylation and synthesis. Thus, we might assume that any alteration in its activity, arising from polymorphisms in the *MTHFR* gene, could cause genomic instability and modify susceptibility to cancer.

The most studied polymorphisms of the *MTHFR* gene are 677 C>T and 1298 A>C, which are responsible for a reduction in enzyme activity in variant allele carriers [2]. Thereby, individuals with variant homozygous genotype for the *MTHFR* 677 C>T polymorphism have 30 % MTHFR enzyme activity compared with those harboring the homozygous wild-type genotype, whereas the enzyme activity is 60 % in those

with heterozygous genotype. Individuals with variant homozygous genotype for the *MTHFR* 1298 A>C polymorphism have been shown to have 60 % MTHFR activity [2]. Deficiency in folate balance is probably associated with carcinogenesis. Low folate levels may lead to errors in DNA synthesis, double-strand breaks, chromosomal repair, and DNA hypomethylation [3, 4].

Previous reports have studied the association between *MTHFR* polymorphisms and various cancers. Some of them have shown a decreased cancer risk in individuals with variant *MTHFR* genotypes, in the case of colorectal cancer [5, 6]. In contrast, a high frequency of variant homozygous genotype for the *MTHFR* 677 C>T polymorphism was observed in esophageal and gastric cancer [7], pancreatic cancer [7, 8], as well as postmenopausal breast cancer [9]. The association between the risk of leukemia and the presence of the *MTHFR* 677 C>T and 1298 A>C polymorphisms has been previously described, particularly in acute lymphoblastic leukemia (ALL) [10–15]. A new meta-analysis that included 5710 cases and 10,798 controls suggested that the 677 C>T polymorphism in *MTHFR* gene is associated with decreased susceptibility to ALL and 677 C>T variant plays a protective role in pediatric patients and Caucasian subjects [15]. The same meta-analysis indicated a lack of positive relationship between 1298 A>C polymorphism in *MTHFR* gene and ALL [15].

There are only a few studies which investigated the role of *MTHFR* 677 C>T and 1298 A>C polymorphisms in acute myeloid leukemia (AML) [16–19] and CML [20–24].

Da Costa Ramos et al. reported that the CT genotype of the *MTHFR* 677 C>T polymorphism was associated with decreased risk (odds ratio (OR)=0.37, 95 % confidence interval (CI) 0.14–0.92), whereas the AC genotype of the *MTHFR* 1298 A>C genotype was linked to an increased risk (OR= 2.90, 95 % CI 1.26–6.71) of developing AML in non-white Brazilian children [25]. Skibola observed no significant difference in *MTHFR* 677 C>T and 1298 A>C genotype frequencies between 237 AML patients and 377 healthy controls [26].

The findings derived from published studies regarding the *MTHFR* polymorphisms and the risk of CML are conflicting [2, 3, 16, 17, 20–24].

The aim of the present work was to evaluate the relationship between the *MTHFR* 677 C>T and 1298 A>C polymorphisms and CML in a representative cohort of Romanian patients.

Material and methods

Patients and controls

One hundred fifty-one patients (65 women and 86 men) diagnosed with CML at the Hematology Clinic from Tirgu-Mures and Hematology Department of the "Ion Chiricuta" Cancer Institute from Cluj-Napoca, Romania, were included in the study. All our CML patients had a Philadelphia chromosome demonstrated by standard cytogenetics and/or displayed the *BCR-ABL* fusion, as assessed by reverse-transcription PCR reaction. The mean age [± standard deviation (SD)] of our CML group was 51.0±13.24 years. At the time of diagnosis, 133 patients (88.1 %) were in chronic phase, 14 patients (9.3 %) were in blast phase, while 4 of them (2.6 %) were in accelerated phase. Blood samples were collected from CML patients on admission in the Hematology Clinics, at diagnosis and prior to starting the therapy. Our CML patients originated from North-Western and Central Romania, regions that comprise a population of around 6.1 million inhabitants.

Three hundred five individuals (179 women and 126 men) with no history of malignancy were included in the control group. The mean age (±SD) in the control group was 47.0± 15.41 years.

Our study protocol was reviewed and endorsed by the Ethics Committees of the Universities of Medicine and Pharmacy from Tirgu-Mures and Cluj-Napoca. All the individuals included in the study gave their written informed consent.

We evaluated Sokal and Hasford risk scores as well as EUTOS score for the CML patients included in our study. The Sokal risk score was developed initially for chronic phase CML patients treated with hydroxyurea, while the Hasford score was initially developed for patients treated with interferon (IFN-α). The Sokal and Hasford risk scores are widely used as prognostic indicators in CML patients. Sokal score is based on the age, spleen size, platelet count, and peripheral blood blasts. Hasford score includes two additional important prognostic factors in CML patients (the percentage of the peripheral blood eosinophils and basophils), allowing the prediction of overall survival in CML patients and comparing the results of different therapeutic regimens. EUTOS score predicts complete cytogenetic remission 18 months after the start of therapy, which is considered as an important predictor for the course of disease. EUTOS score is a simple prognostic score, and in CML patients treated with imatinib, it is considered to have a greater prognostic value than Sokal and Hasford risk score systems. EUTOS score is based on the spleen size and percentage of basophils. Sokal, Hasford, and EUTOS scores were calculated based on the data collected at the time of diagnosis, prior to treatment. Online calculation formulas on the European Leukemia Net (ELN) network site (http://www.leukemia-net.org) were used to assess Sokal, Hasford, and EUTOS scores, calculating the result of the score and the respective risk group.

Genotyping procedures

Genomic DNA was extracted from peripheral blood using commercially available kits (Wizard Genomic DNA Purification Kit, Promega, USA, and Quick-gDNA MiniPrep kit, ZymoResearch, USA). The *MTHFR* 1298 A>C polymorphism was investigated by allele-specific PCR technique using the PCR conditions and primers (5′-TGTTTGTTCT TGGGAGCGG-3′, 5′-CGAAGACTTCAAAGACACTTG-3′, 5′-CGAAGACTTCAAAGACACTTT-3′) as previously described [27]. Briefly, amplification conditions were as follows: after an initial denaturation at 95 °C, 30 cycles were performed (each cycle consisted of denaturation at 94 °C for 30 s, annealing at 57 °C for 20 s, and extension at 72 °C for 20 s) followed by a final extension at 72 °C for 7 min.

The *MTHFR* 677 C>T polymorphism was genotyped using the polymerase chain reaction and the restriction fragment length polymorphism (PCR-RFLP) method, utilizing the published forward primer 5′-CATCCCTATTGGCAGGTTAC-3′ and reverse primer 5′-GACGGTGCGGTGAGAGTG-3′ [28]. The amplified products were digested by FastDigest *Hinf*I restriction enzyme (Thermo Fisher Scientific, MA, USA). The PCR protocol consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min.

Statistical analysis

Contingency table-based methods were used for studying the existence of possible associations between two nominal variables. The statistical significance of the association was tested by the Pearson chi-squared test or Fisher's exact test. If a statistically significant result was achieved, we performed the quantification of association by calculating the OR and the associated 95 % CI. The sample size was estimated a priori by power analysis applied in R environment for statistical computing and graphics (R version 3.1.0, package "pwr"). To study the significant differences of the various quantitative features that follow a normal distribution in independent groups, the ANOVA test was used, the variances being tested by the test of homogeneity of variances (Levene). In case of rejection of the null hypothesis, the post-test analysis by performing Tukey HSD tests was used.

Binary logistic regression was used to assess the simultaneous effects of patient features on the probability of attaining a CML. ORs were estimated with their 95 % CIs using unconditional univariate and multivariate logistic regressions. The final binary logistic regression model involved the following parameters: age, gender, and *MTHFR* 1298 A>C and 677 C>T polymorphisms. Any covariate with a p value <0.05 was considered significant. The model selection strategy used an all-subset regression approach based on the likelihood ratio test to build the multivariate model. For statistical processing, SPSS version 13 (SPSS, Chicago, IL, USA) was used.

Results

In our population, both *MTHFR* polymorphisms were in Hardy-Weinberg equilibrium (data not shown).

The distribution of the *MTHFR* polymorphism genotypes and the frequencies of the alleles in patients with CML and controls are presented in Table 1. The frequencies of the variant CT and TT genotypes of the *MTHFR* 677 C>T polymorphisms were higher among CML patients (45 and 16.6 %) than in controls (38 and 11.5 %). The frequency of the variant T allele in CML patients was 39.1 %, while in controls, it was 30.5 % (p value=0.011). In addition, the frequency of the variant C allele for 1298 A>C polymorphism was 33.1 % in the CML group and 31.6 % in the control group, but with no statistical significance (p value=0.652).

The variant AC and CC genotype frequencies of *MTHFR* 1298 A>C polymorphisms were 45 and 10.6 % for CML patients and 39.0 and 12.1 % for controls. Regarding the 1298 A>C polymorphism, no statistically significant difference was observed in CML patients compared to control group. In addition, we investigated the combined effect of the investigated polymorphisms. We noticed that only the 677CT/1298AC genotype was associated with the risk of CML.

We performed a comparison of several demographic, clinical, and laboratory findings among CML patients according to the *MTHFR* 677 C>T and 1298 A>C genotypes.

When analyzing the median age at diagnosis of CML patients, we noticed a significant difference of the genotypes for *MTHFR* 677 C>T polymorphism (ANOVA test, p value= 0.033), but not for *MTHFR* 1298 A>C polymorphism (ANOVA test, p value=0.435).

According to the results presented in Table 2, no significant difference was found between gender and the genotypes of the two *MTHFR* polymorphisms analyzed in CML patients.

Our findings revealed no statistically significant difference between laboratory parameters (blasts, hemoglobin, LDH), clinical phases of the disease, and MTHFR 677 C>T and 1298 A>C genotypes in CML patients.

No statistically significant differences were found regarding the number of white blood cell (WBC) count and the genotypes for *MTHFR* 677 C>T polymorphism in CML patients (Kruskall-Wallis test, p value=0.273). In contrast, we observed a statistically significant difference between the WBC count and *MTHFR* 1298 A>C polymorphism (Kruskall-Wallis test, p value=0.032).

Table 1 Analysis of *MTHFR* polymorphisms in CML patients versus controls

MTHFR genotypes/alleles	CML group, *N* (%)	Control group, *N* (%)	OR, 95 % CI	*p* value
677 C>T genotype frequency				
677CC	58 (38.4)	154 (50.5)	1.00	–
677CT	68 (45)	116 (38)	1.556 (1.017–2.381)	0.041*
677TT	25 (16.6)	35 (11.5)	1.897 (1.046–3.44)	0.035*
677CT+TT	93	151	1.635 (1.099–2.433)	0.016*
1298 A>C genotype frequency				
1298AA	67 (44.4)	149 (48.9)	1.00	–
1298AC	68 (45)	119 (39)	1.271 (0.839–1.924)	0.257
1298CC	16 (10.6)	37 (12.1)	0.962 (0.50–1.849)	0.907
1298AC+CC	84	156	1.197 (0.809–1.772)	0.372
677CC/1298AA	17 (11.1)	50 (16.4)	1.00	–
677CT/1298AA	25 (16.6)	64 (21)	1.149 (0.559–2.358)	0.720
677TT/1298AA	25 (16.6)	35 (11.5)	2.101 (0.989–4.459)	0.060
677CC/1298AC	25 (16.6)	67 (22)	1.097 (0.535–2.248)	0.856
677CT/1298AC	43 (28.5)	52 (17)	2.432 (1.229–4.814)	0.013*
677CC/1298CC	16 (10.6)	37 (12.1)	1.272 (0.569–2.843)	0.681
Total (genotypes)	151 (100)	305 (100)		
677 C>T polymorphism allele frequency				
677C allele	184 (60.9)	424 (69.5)	–	–
677T allele	118 (39.1)	186 (30.5)	1.462 (1.096–1.950)	0.011*
Total (alleles)	302 (100)	610 (100)		
1298 A>C polymorphism allele frequency				
1298A allele	202 (66.9)	417 (68.4)	–	–
1298C allele	100 (33.1)	193 (31.6)	1.070 (0.797–1.435)	0.652
Total (alleles)	302 (100)	610 (100)		

*Statistically significant

In the current study, no association was observed in the distribution of any of the *MTHFR* polymorphisms regarding the percentage of basophils in peripheral blood (Kruskall-Wallis test, p value>0.05 for all these comparisons) and clinical phases of the disease (chronic phase versus accelerated or blast phase, Perason chi-squared test, p value=0.235 for the 677 C>T polymorphism and p value=0.934 for the 1298 A>C polymorphism).

No association was seen in the distribution of the two *MTHFR* polymorphisms regarding the Sokal risk groups (p value=0.294 and p value=0.144, respectively) and Hasford risk groups (p value=0.13 and 0.588, respectively). Furthermore, no association was observed between variant genotypes for the studied *MTHFR* polymorphisms and EUTOS score.

Regarding the cytogenetic analysis performed in CML patients, additional chromosomal abnormalities (ACA) comprising of trisomy 8, duplication of Ph chromosome, isochromosome 17q, trisomy 19 and 21, loss of Y chromosome, and del(9q) were found in 20 cases. There was no significant difference between patients with ACA and variant genotypes for the *MTHFR* 677 C>T (p value=0.297) and 1298 A>C (p value=0.788) polymorphisms.

Binary logistic regression was used to assess the simultaneous effects of patient features (age, gender, and *MTHFR* 677 C>T and 1298 A>C polymorphisms) on the probability of attaining a CML. We can state that *MTHFR* 677 C>T is the most significant exposure variable, and gender is the most important covariate in developing CML. We observed that the risk to develop CML is 2.99 times higher in individuals with TT genotype than those with CC genotype (p value=0.002) and 1.63 than those with CT genotype (p value=0.04), after adjusting for *MTHFR* 1298 A>C polymorphism, age, and gender. Regarding the patients gender, we noticed that the risk to develop CML is 2.03 times higher in men than in women (OR=2.03, 95 % CI 1.36–3.06, p value=0.001).

Table 2 Comparison of demographic, clinical, and laboratory findings (at diagnosis) in CML patients, stratified after the *MTHFR* genotypes

Variable	*MTHFR* 677CC genotype	*MTHFR* 677CT genotype	*MTHFR* 677TT genotype	*p* value[a]	*MTHFR* 1298AA genotype	*MTHFR* 1298AC genotype	*MTHFR* 1298CC genotype	*p* value[a]
Mean age at diagnosis (years)	53.0±12.66	48.0±13.73	56.0±11.8	0.033*	53.0±13.51	49.5±12.14	52.0±16.37	0.435
Gender								
Female	29	23	13	0.115	31	28	6	0.432
Male	29	45	12		36	40	10	
Blasts; mean (% in peripheral blood)	7.68	12.28	10.25	0.974	8.97	12.88	3.19	0.728
LDH (median)	700.0	629.0	653.0	0.798	665.0	690.0	670.0	0.971
Basophils; median (% in peripheral blood)	4.0	5.0	4.5	0.979	4.0	4.5	5.0	0.495
Hemoglobin; mean (g/dl)	10.6	10.74	9.64	0.524	10.03	11.04	10.13	0.815
Clinical phases								
Chronic phase	53	54	21	0.235	57	56	15	0.934
Accelerated/blastic crisis phase	4	10	4		7	10	1	
EUTOS score								
High risk	20	24	7	0.766	20	25	6	0.750
Low risk	37	40	17		43	41	10	
Sokal risk groups								
Low	19	22	4	0.294	14	26	5	0.144
Intermediate	19	14	9		23	13	6	
High	18	25	11		23	26	5	
Data missing	1	4	1		6	0	0	
Hasford risk groups								
Low	19	26	7	0.13	20	26	6	0.588
Intermediate	27	16	10		24	21	8	
High	10	19	7		16	18	2	
Data missing	1	4	1		6	0	0	

*Statistically significant

[a] Variant genotype versus homozygous wild-type genotype

Discussion and conclusions

In the present work, we studied the impact of *MTHFR* 677 C>T and 1298 A>C polymorphisms on the development of adult CML in a Romanian population.

In our study, the CT and TT genotypes of the *MTHFR* 677 C>T polymorphisms were associated with an increased risk (OR=1.556, 95 % CI 1.017–2.381, and OR=1.897, 95 % CI 1.046–3.44, respectively) of developing CML. Also, the variant T allele was significantly enriched in CML cases compared to controls (OR=1.462, 95 % CI=1.096–1.950, *p* value=0.011). Therefore, we may consider the presence of the variant allele as a modifier factor in the risk of developing CML. However, in the case of 1298 A>C polymorphism, there was no significant excess neither in heterozygous genotype (AC) nor in variant homozygous genotype (CC) among patients with CML compared with controls. This indicates that the presence of the *MTHFR* 1298 A>C polymorphism is not a risk factor for developing CML.

Moon et al. demonstrated that the *MTHFR* 677CC/1298CC genotype increased the risk of CML in Korean patients (OR= 8.80, 95 % CI 2.03–22.1, *p* value=0.002) (21). We failed to reveal any association of CML with *MTHFR* 677CC/1298CC genotype. Likewise, Barbosa et al. found no association between the *MTHFR* 677 C>T and 1298 A>C polymorphisms among CML patients from Brazil [16]. Similarly, Pazhakh et al. found no statistically significant association between *MTHFR* polymorphisms and CML risk [17].

A recent study conducted on 52 patients and 53 controls from Serbia reported that the *MTHFR* 677 C>T polymorphism has no significant influence on the susceptibility to CML [22]. In contrast, we noticed a positive association between the *MTHFR* 677 C>T variant genotypes and CML (*p* value=0.016).

In contrast to our results, Lordelo et al. showed that the AA genotype of the *MTHFR* 1298 A>C polymorphism significantly increased the risk of developing CML, particularly in the case of young adult females (OR=2.052, 95 % CI=1.08–3.92, *p* value=0.028), whereas the AC genotype significantly decreased this risk (OR=0.630, 95 % CI=0.40–0.99, *p* value=0.047). In addition, no association between the *MTHFR* 677 C>T polymorphism and CML was globally observed, although a lower risk was noticed for men harboring the variant homozygous CC genotype (OR=0.495, 95 % CI=0.26–0.95, *p* value=0.032) [23].

Another recent study performed on 208 patients with myeloproliferative neoplasms negative for the *BCR-ABL* fusion (polycythemia vera, essential thrombocythemia, and primary myelofibrosis) and 245 healthy individuals from Romania did not support a significant involvement of the *MTHFR* 677 C>T and 1298 A>C polymorphisms as modifier factors in the risk of developing these diseases, except for the 677CT/1298 AC compound heterozygous genotype alone, which was seen significantly more frequently in patients than in controls [29]. Similarly, in the present work, we observed an excess of 677CT/1298 AC compound heterozygotes in patients with CML compared with controls (OR=2.432, 95 % CI=1.229–4.814, *p* value=0.013). According to our findings, we may consider that 677CT/1298 AC compound heterozygous genotype is a risk factor for developing CML. We might assume that in the presence of *MTHFR* 677 C>T polymorphism, the *BCR-ABL* fusion, and thus the CML phenotype are more likely to occur.

Both *MTHFR* variants reduce the enzymatic activity of the MTHFR enzyme. The compound heterozygous genotype (677 C>T/1298 A>C) has similar effects to the 677 C>T homozygous genotype. As both variants are frequent in the general populations, the compound heterozygous genotype is frequent as well, being worthy to analyze it in this study. Thus, when analyzing *MTHFR* 677 C>T and 1298 A>C variant combined genotypes (677 CT/1298AA+677 CT/1298AC+677 TT/1298AA), a positive association was seen between variant genotypes for the *MTHFR* 677 C>T and 1298 A>C polymorphisms and the risk of CML (OR=1.635, 95 % CI=1.099–2.433, *p*=0.016).

These discrepancies between different studies regarding the association between CML and the *MTHFR* polymorphisms might be due to the differences in sample size or the ethnic background. Another explanation of the conflicting results might be the complexity of the folate metabolic pathway as *MTHFR* is just one of the many enzymes involved in the folate pathway [19].

According to Zanrosso et al. these discrepant results may be explained by the influence of environmental factors (racial, ethnic, and nutritional) in several population groups and *MTHFR* 677 C>T and 1298 A>C polymorphisms in the relationship between leukemia and *MTHFR* polymorphisms [30]. Similar incriminating factors, as well as gene-gene and gene-environment interactions, were also reported by other authors to be the cause of the contradictory results [2, 18, 31, 32]. Our study and the other published studies that investigated the relationship between *MTHFR* gene polymorphisms and leukemia have not assessed the dietary folate intake. It is well known that the intake of B vitamins (namely, B6 and B12) can influence the folate metabolism while the chronic alcohol consumption/intake will lead to folate deficiency [2].

We evaluated the impact of *MTHFR* 677 C>T and 1298 A>C polymorphisms taking into account the clinical phases of the disease. The two polymorphisms had a similar distribution in patients with chronic phase and accelerated or blast phase. Our findings are not in agreement with those observed in a recent study performed on 97 patients with chronic myeloid leukemia from Egypt [3]. Khorshied et al. reported an association between *MTHFR* 677 TT variant genotype and the risk of disease progressing to accelerated and blast transformation phase [3]. According to Khorshied et al., *MTHFR* 677 C>T homozygous variant might be considered a molecular predictor for disease progression [3].

To our knowledge, the relationship between *MTHFR* 677 C>T and 1298 A>C gene polymorphisms and EUTOS score, Sokal and Hasford risk groups in CML patients has not been previously considered. No association was observed between the prognostic factors (blasts, basophils, additional chromosomal abnormalities, EUTOS score, Sokal and Hasford risk groups) and the *MTHFR* 677 C>T and 1298 A>C variant genotypes in patients with CML.

In conclusion, our study showed that the *MTHFR* 677 C>T polymorphism is significantly associated with the risk of CML in the Romanian patients. According to our findings 677CT/1298AC compound heterozygous genotype is a risk factor for developing CML. Our results do not support a significant involvement of the *MTHFR* 1298 A>C polymorphisms as risk factors in the development of CML.

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Conflicts of interest None

Author contributions CB designed the research, performed genetic analysis, analyzed the data, and wrote the manuscript. MI analyzed the data. APT designed the research, performed genetic analysis, and wrote the manuscript. IM and DD collected the samples and patients' data. MD designed the research and revised the manuscript.

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