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Original article

Associations between genetic variation in one-carbon metabolism and leukocyte DNA methylation in valproate-treated patients with epilepsy

Guanzhong Ni [a, 1], Jiaming Qin [a, 1], Ziyi Chen [a], Hongliang Li [b], Jueqian Zhou [a], Min Huang [b], Liemin Zhou [a, *]

[a] Department of Neurology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, Guangdong Province, China
[b] Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, Guangdong Province, China

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SUMMARY

Background: Valproate (VPA) as a first-line antiepileptic drug is useful for the most types of epileptic seizure treatment. Previous studies observed that VPA influenced one-carbon metabolism (OCM), consequently, DNA methylation. However, other individual genetic variations, as well as VPA, modify DNA methylation.

Objective: In this study, we investigated associations between genetic variations in OCM and leukocyte DNA methylation in VPA-treated patients with epilepsy.

Methods: This was a cross-sectional study of 101 epileptic patients who underwent VPA monotherapy and 68 healthy controls. All subjects were measured OCM-related nutrients (folate, homocysteine and vitamin B12), and DNA methylation of specific regions were analyzed. Furthermore, we examined the associations between genetic variations in OCM and DNA methylation levels in epileptic patients.

Results: VPA-treated patients with epilepsy exhibited both higher serum homocysteine and vitaminB12 levels and lower folate levels relative to controls ($P = 0.018$, $P = 0.003$, $P < 0.001$ respectively), the methylation level of the MTHFR amplicon was significantly lower in the VPA group compared with those in the controls ($P = 0.043$). VPA-treated epileptic patients carrying the T-allele of methylenetetrahydrofolate reductase (MTHFR) c.677C>T showed higher serum Hcy levels than those observed in the 677CC group ($P < 0.01$). Epileptic patients who carried G-allele of methionine synthase (MTR) c.2756A>G showed significantly lower MTHFR amplicon methylation levels compared to carriers of the wild-type MTR 2756AA genotype ($P = 0.028$).

Conclusion: Our study provided evidence that the MTR c.2756A>G polymorphism is associated with MTHFR amplicon hypomethylation in VPA-treated patients with epilepsy.

1. Introduction

DNA methylation is the most extensively studied mechanism that contribute to the regulation of gene expression and maintenance of genome stability [1,2]. It is widely recognized that aberrant genomic DNA methylation, not only in the genome overall, but also in specific genes is associated with congenital malformations [3]. For example, previous studies suggested that hypomethylation of methylenetetrahydrofolate reductase (MTHFR) gene and long interspersed nucleotide element-1 (LINE-1) were associated with increased risk of neural tube defects (NTDs) [4,5]. According to the results of several epilepsy pregnancy registers over the last 15–20 years, exposure to valproate (VPA) during pregnancy increased the

Abbreviations: AED, antiepileptic drug; ANOVA, one-way analysis of variance; BHMT, betaine-homocysteine methyltransferas; FA, folate; Hcy, homocysteine; HWE, hardy–weinberg equilibrium; LINE-1, long interspersed nucleotide element-1; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; NTDs, neural tube defects; OCM, one-carbon metabolism; RFC-1, reduced folate carrier-1; SNP, Single nucleotide polymorphisms.

* Corresponding author. Department of Neurology, The First Affiliated Hospital, Sun Yat-Sen University, No. 58 Zhongshan 2nd Road, Guangzhou 510080, Guangdong Province, China. Fax: +86 20 37620001.
E-mail address: lmzhou56@163.com (L. Zhou).
[1] These authors contributed equally to this work.

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risk of NTDs from a background risk of 1‰ to between 1% and 2% [6–8]. In animal research, Alonso-Aperte et al. found that VPA through altered methionine synthase activity, induced DNA hypomethylation, which may be involved in VPA-induced teratogenesis [9]. Although there have been tissue-level investigation of aberrant DNA methylation under VPA treatment, the epigenetic markers in peripheral or cord blood still have great interest. Recent studies found hypomethylation of DNA from cord or peripheral blood mononuclear cells in antiepileptic drug (AED)-treated patients with epilepsy [10,11].

Nutrients (homocysteine, folate and vitamin B12) in one-carbon metabolism (OCM) are cosubstrates and cofactors associated with methylation and also function as regulatory molecules. The abnormal status of these nutrients may cause disturbances in methylation reactions [12]. Patients with epilepsy and involved in long-term VPA therapy are more susceptible than the general population to abnormal status of nutrients in OCM [13]. We previously demonstrated that VPA, through its known effects on OCM, affects MTHFR amplicon methylation levels [14]. Additionally, observational studies revealed associations between genetic variation in OCM and global methylation levels in leukocyte DNA from healthy individuals [15,16].

To the best of our knowledge, no study has yet investigated the interaction between genetic variation in OCM and DNA methylation levels in VPA-treated patients with epilepsy. Therefore, we examined the associations between genetic variation in OCM and DNA methylation status in VPA-treated patients with epilepsy.

2. Methods

2.1. Subjects

This study is designed to investigate the effects of genetic variants in OCM on DNA methylation in epileptic patients. Epileptic patients (aged between 16 and 55 years) who were treated with VPA as monotherapy for at least 6 months were included in this study. Patients who had discontinued medication or had been treated with other AEDs were excluded from this study. Epilepsy caused by ischemic stroke, history of cardiac and peripheral vascular disease, diabetes mellitus, tobacco use, hematologic diseases, endocrine disorders, tumors, pregnancy, liver or renal diseases constituted criteria resulting in exclusion from the study. All subjects using folate (FA) antagonists and vitamins, as well as vegetarians, were excluded. Healthy volunteers who received annual physical checkups were recruited as controls. Both patients and controls were from the same geographic area and were matched for age, sex and ethnic background. The current study was approved by the human ethics committee of the first affiliated hospital, Sun Yat-Sen university, and a written informed consent was obtained from each participant.

2.2. Laboratory tests and serum concentration assay

Blood samples were collected from subjects for laboratory evaluations between 08:00 and 08:30 AM after overnight fasting. The levels of serum FA, homocysteine (Hcy) and vitamin B12 were measured using an autoanalyzer Immulite 2000 and suitable kits, (DPC Diagnostic Products Corporation, Los Angeles, USA) according to manufacturer's instructions.

The serum VPA concentration was assayed using a high-performance liquid chromatographic technique with an ultraviolet detector (Chromsystems, Waters Company, Milford Massachusetts, USA).

2.3. DNA extraction and genotyping

Whole-blood genomic DNA was extracted from the peripheral blood samples using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were determined by absorbance at 260 nm and 280 nm using a NanoDropTM 1000 Spectrophotometer (Thermo Scientific, Wilmington,USA). The polymorphisms within OCM genes that include MTHFR c.677C>T, methionine synthase (MTR) c.2756A>G, methionine synthase reductase (MTRR) c.66A>G, betaine-homocysteine methyltransferas (BHMT) c.716G>A, reduced folate carrier-1 (RFC-1) c.80G>A were genotyped using the Sequenom MassARRAY technology platform with the iPLEX gold chemistry (Sequenom, CA, USA) in the conditions recommended by the manufacturer (primers details are listed in additional file 1: Table S1). The MassARRAY Typer 4.0 software was used for proper data acquisition and analysis. A manual review was carried out to further clarify uncertain genotype calls. Assays with less than 80% call rate within the same SpectroCHIP was considered as having failed.

2.4. DNA bisulfite conversion and quantitative methylation analysis

Extracted whole-blood genomic DNA was treated with sodium bisulfite using the EZ 96 DNA-methylation kit (Zymo Research, Irvine, CA, USA). The bisulfite converted DNA was resuspended in 10 μl of elution buffer and stored at −80 °C until the samples were ready for analysis. The Sequenom MassARRAY platform was used to perform quantitative methylation analysis of the LINE-1 element and MTHFR amplicon. Bisulfite converted DNA was amplified by PCR using primers designed by Epidesigner online (primers details are listed in additional file 2: Table S2), followed by fragmentation after transcription and analysis on a mass spectrometer (Sequenom, Inc, San Diego,USA). This generated mass signal patterns were translated into quantitative DNA-methylation levels of different CpG sites in the earlier mentioned genes by MassARRAY EpiTYPER Analyzer software (version 1.0, Sequenom, Inc, San Diego,USA). Measurements were done in triplicate on DNA from the same bisulfite-treatment batch on different PCR plates. The methylation level was expressed as the percentage of methylated cytosines over the total number of methylated and unmethylated cytosines. Prior to analysis, strict quality control was carried out to remove potentially unreliable measurements, such as low mass, high mass and silent peak overlap CpG units. The CpG units that failed to produce data for more than 30% of samples (unreliable CpG units) and samples lacking more than 30% of their data points (unreliable samples) were discarded [17].

2.5. Statistical analysis

Statistical analyses of the results were conducted using SPSS version 21.0. Mean differences of continuous variables between the two groups were compared using the Student's t-test for normally-distributed variables or the Mann–Whitney U-test for non-normally-distributed variables. Analysis of parametric variables among the three subgroups were performed using one-way analysis of variance (ANOVA) with a post hoc bonferroni's test. For analysis of non-parametric variables, a Kruskal–Wallis test with a post hoc Mann–Whitney U-test was employed. Deviation from Hardy–Weinberg equilibrium (HWE) was tested for all genotypes studied using Haploview 4.2. A P-value <0.05 was considered to be statistically significant.

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3. Results

VPA-treated patients with epilepsy (101) and sixty-eight healthy controls of matched age and sex were studied. Demographic features are shown in Table 1.

VPA-treated patients with epilepsy exhibited both higher serum Hcy and vitamin B12 levels and lower FA levels relative to controls ($P = 0.018$, $P = 0.003$, $P < 0.001$ respectively), and the methylation level of the MTHFR amplicon was significantly lower in the VPA group compared with those in the controls ($P = 0.043$); however LINE-1 amplicon methylation level was indifferent from the control group ($P = 0.593$). (Table 2).

The associations between genetic polymorphisms of OCM enzymes and serum levels of Hcy in VPA-treated epileptic patients were analyzed. In this study, we detect that the allelic distributions of all SNPs were consistent with Hardy–Weinberg equilibrium. We observed MTHFR (rs1801133) was significantly associated with increased serum levels of Hcy in VPA-treated epileptic patients. VPA-treated epileptic patients carrying the T-allele of MTHFR c.677C>T showed serum Hcy levels higher than those observed in the 677CC group ($P = 0.008$) (Table 3). Furthermore, we compared the levels of serum Hcy and FA between the VPA group and controls for the different MTHFR genotypes. The serum FA levels in VPA-treated epileptic patients were lower than those in controls; however, the serum Hcy levels were higher relative to those in controls and were only observed in T-allele of MTHFR genotypes (Table 4).

Table 5 lists the genotypes investigated and their associations with MTHFR amplicon methylation in VPA-treated patients with epilepsy. Epileptic patients who carried G-allele of MTR c.2756A>G showed significantly lower MTHFR amplicon methylation levels compared with carriers of the wild-type MTR 2756AA genotype ($P = 0.028$).

4. Discussion

The present study confirmed our previously research that VPA-treated epileptic patients were more susceptible to developing hyperhomocysteinemia and hypomethylation of MTHFR amplicon relative to the general population [14]. Here we showed that MTHFR c.677C>T contributed to increased serum Hcy levels in epileptic patients receiving VPA treatment. Furthermore, we revealed that the G-allele of the MTR c.2756A>G variant was associated with reduced MTHFR amplicon methylation in VPA-treated epileptic patients. To our knowledge, our study represents the first published findings showing associations between genetic variations in OCM and DNA methylation in VPA-treated epileptic patients.

Hcy is an intermediate product of OCM. Our results are consistent with previous reports reporting high levels of Hcy in VPA-treated epileptic patients [13,18]. Elevated Hcy concentrations may be resulted from a deficiency in cofactors indispensible for OCM. As an important cofactor, FA participates in OCM by donating a methyl group for vitaminB12 dependent remethylation of Hcy to methionine. Our research indicated that FA deficiency occurs in VPA-treated patients with epilepsy. Although the mechanisms by which VPA induce FA deficiency are unclear, a proposed mechanism is that VPA may interfere with the intestinal absorption of FA [19].

Recent research suggested that Hcy may reflect a better indicator of methyl group supplement and utilization in genome DNA methylation [20]. To better understand the status of DNA methylation in VPA-treated epileptic patients, the levels of LINE-1 and MTHFR amplicon methylation were also determined. We found that the levels of MTHFR amplicon methylation in VPA-treated patients with epilepsy were significantly lower than those of the controls. MTHFR is a key enzyme in OCM that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl donor required for methylation of Hcy to methionine. Lower MTHFR amplicon

Table 1
Demographic details of valproate and control groups.

	Controls (n = 68)	VPA (n = 101)
Gender M/F (n)	35/33	53/48
Age (years)	26.8 ± 5.4	26.8 ± 10.6
Duration of treatment (months)		24 (6.5,60)
Dose of treatment (mg/d)		750 (500,1000)
AEDs concentration (μg/ml)		59.06 ± 25.64

VPA = valproate, AEDs = antiepileptic drugs.

Table 2
Levels of one-carbon metabolism nutrients and DNA methylation.

	Controls (n = 68)	VPA (n = 101)	*p*-value
OCM nutrients			
Hcy (μmol/l)	9.91 ± 1.97	11.38 ± 5.58	0.018
FA (μg/l)	11.54 ± 4.02	7.94 ± 2.93	<0.001
VitB12 (ng/l)	555.94 ± 217.46	670.72 ± 275.28	0.003
DNA methylation (%)			
LINE-1 amplicon	56.55 ± 4.56	56.99 ± 4.81	0.593
MTHFR amplicon	6.11 ± 3.28	5.05 ± 2.99	0.043

VPA = valproate, OCM = one-carbon metabolism, Hcy = homocysteine, FA = folate, VitB12 = vitamin B12, LINE-1 = long interspersed nucleotide element-1, MTHFR = methylenetetrahydrofolate reductase.

Table 3
The associations between one-carbon metabolism related genotypes and serum homocysteine levels in valproate-treated patients with epilepsy.

Gene	SNP	Serum Hcy (μmol/l) (n)	*p*-value
MTHFR	rs1801133	CC 9.94 ± 2.41 (47)	<0.001[a]
		CT 12.19 ± 5.92 (40)	
		TT 19.41 ± 13.04 (6)	
		CT + TT 13.13 ± 7.43 (46)	0.008[b]
MTR	rs1805087	AA 10.83 ± 4.58 (73)	0.179
		AG + GG 12.67 ± 7.98 (21)	
MTRR	rs1801394	AA 10.73 ± 5.26 (52)	0.664
		AG 11.81 ± 6.17 (37)	
		GG 11.30 ± 3.32 (6)	
		AG + GG 11.74 ± 5.83 (43)	0.228
BHMT	rs3733890	GG 11.28 ± 6.21 (41)	0.798
		GA 11.72 ± 5.48 (41)	
		AA 10.30 ± 2.04 (8)	
		GA + AA 11.49 ± 5.09 (49)	0.48
RFC-1	rs1051266	AA 10.75 ± 3.36 (34)	0.905
		AG 10.83 ± 3.90 (33)	
		GG 11.27 ± 6.40 (24)	
		AG + GG 11.02 ± 5.06 (57)	0.542

SNP = single nucleotide polymorphism, MTHFR = methylenetetrahydrofolate reductase, MTR = methionine synthase, MTRR = methionine synthase reductase, BHMT = betaine-homocysteine methyltransferas, RFC-1 = reduced folate carrier-1. [a,b]The MTHFR variant c.677C>T was significantly associated with serum Hcy levels in VPA treated patients with epilepsy.

Table 4
Levels of homocysteine and folate, according to methylenetetrahydrofolate reductase genetic polymorphism.

	Hcy (μmol/l)	*p*-value	FA (μg/l)	*p*-value
Controls CC	9.81 ± 2.12 (36)		12.02 ± 3.77 (36)	
VPA CC	9.94 ± 2.41 (47)	0.793	8.78 ± 2.99 (47)	<0.001
Controls CT/TT	10.07 ± 1.80 (30)		10.88 ± 4.05 (30)	
VPA CT/TT	13.13 ± 7.43 (46)	0.010	7.20 ± 2.69 (46)	<0.001

Hcy = homocysteine, FA = folate, VPA = valproate.

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Table 5
The associations between one-carbon metabolism related variants and methylenetetrahydrofolate reductase methylation status in valproate-treated patients with epilepsy.

Gene	SNP	MTHFR methylation (%) (n)	*p*-value
MTHFR	rs1801133	CC 4.82 ± 2.31 (42) CT 5.59 ± 3.86 (37) TT 5.04 ± 1.68 (5)	0.54
MTR	rs1805087	AA 5.41 ± 3.27 (63) AG + GG 4.04 ± 1.96 (19)	0.028[a]
MTRR	rs1801394	AA 5.14 ± 2.73 (45) AG 5.24 ± 3.45 (33) GG 4.79 ± 3.51 (6)	0.946
BHMT	rs3733890	GG 5.49 ± 3.35 (37) GA + AA 4.62 ± 2.36 (44)	0.174
RFC-1	rs1051266	AA 4.59 ± 3.04 (29) AG 5.93 ± 3.45 (30) GG 4.82 ± 2.54 (22)	0.213

SNP= Single nucleotide polymorphism, MTHFR = methylenetetrahydrofolate reductase, MTR = methionine synthase, MTRR = methionine synthase reductase, BHMT = betaine-homocysteine methyltransferas, RFC-1 = reduced folate carrier-1. The MTR variant c.2756A>G was significantly associated with MTHFR amplicon methylation status in VPA treated -patients with epilepsy.

methylation levels could indicate increased MTHFR enzyme activity, thereby promoting Hcy remethylation in VPA-treated epileptic patients.

Hyperhomocysteinemia in epileptic patients is caused by not only exogenous factors (VPA related FA deficiency) but also endogenous factors (genetic polymorphisms of OCM enzymes) [21]. To determine the effects of endogenous factors on Hcy metabolism in epileptic patients, we investigated the relationship between five genetic variants of OCM enzymes and plasma Hcy levels. Our results indicated that VPA-treated epileptic patients carrying the T-allele of MTHFR c.677C>T showed serum Hcy levels higher than those observed in the 677CC group. MTHFR c.677C>T is known to be associated with reduced enzyme activity, in the homozygous state, implies a 50−60% decrease in the enzymatic activity [22]. One possible explanation for results differing from those previously report is the difference in the study population. Yoo et al. observed that epileptic patients with homozygous (MTHFR TT) exhibited higher serum Hcy levels in carbamazepine and phenytoin treated groups, but not in the VPA-treated group [23]. However, the number of VPA-treated patients enrolled in that study was 33, which may have been too few to constitute a statistically significant conclusion. In another study, Semmler et al. [24] found that MTHFR c.677C>T did not contribute to the risk of hyperhomocysteinemia during AED treatment; however, the patients in that study were treated with both monotherapy and combined therapy.

Mutations in the genes which encode one-carbon unit metabolizing enzymes can lead to aberrant DNA methylation by affecting the synthesis of methyl donor. At present the most widely studied common mutation in general population is the MTHFR c.677C>T. Our result is not consistent with previous studies reporting that the MTHFR c.677C>T mutation possess a lower degree of DNA methylation [25,26]. Our study differed in that the subjects recruited in our study were VPA-treated patients with epilepsy. The MTHFR c.677C>T affects genomic DNA methylation through alterations of FA status, and VPA interferes with intestinal absorption of FA and induce DNA hypomethylation in epileptic patients. Therefore, we suggest that the effect of VPA on DNA methylation may overshadow the effect of the MTHFR c.677C>T mutation in epileptic patients.

A novel finding in our study was that there was a relation between the G-allele of MTR c.2756A>G and the reduced MTHFR amplicon methylation in VPA-treated epileptic patients. MTR is an important enzyme involved in methionine biosynthesis and methionine is an essential methyl donor for DNA methylation reactions. Previous associations between MTR c.2756A>G and DNA methylation levels were controversial [16,27]. Bleich et al. reported that the G-allele of MTR c.2756A>G had an association with reduced global DNA methylation. The mechanism associated with MTR c.2756A>G reduced MTHFR amplicon methylation remains unclear. Here, not association was detected between MTR c.2756A>G and serum Hcy levels. Therefore, our results suggest that the MTR c.2756A>G may have an effect on DNA methylation independent of effects on Hcy metabolism.

VPA as a first-line AED is useful for the most types of epileptic seizure treatment. However, prenatal exposure to VPA is associated with increased risk of NTDs [28]. Vajda et al. reported that pregnant women for whom VPA treatment resulted in malformed fetuses had substantially increased risk of having other malformed fetuses in future pregnancies [29]. This finding suggested the existence of genetic polymorphisms that determined individual susceptibility to fetal malformation. Our study found an association between MTR c.2756A>G and MTHFR amplicon hypomethylation in VPA-treated patients with epilepsy. Because MTHFR amplicon hypomethylation is a modifiable risk factor for NTDs [4], we suggest that genetic variations in OCM maybe predictors of individual susceptibility to the teratogenic effect of VPA.

Limitations of this research should be considered. First, our study did not contemplate a possible interaction caused by each AED between the one-carbon metabolic changes and epilepsy syndromic category. Second, because of the well known sensitivity level (approximately 5%) of the MassARRAY technique, the method we used to detect DNA methylation levels had an insufficient sensitivity when some of our results of MTHFR methylation were below the sensitivity level. Third, other environmental factors that might affect the results were not excluded in this cross-sectional study. Finally, the small sample size in this work was also a limitation.

5. Conclusions

In summary, we investigated associations between variations involved in OCM and DNA methylation in VPA-treated patients with epilepsy. Our results indicated that the T-allele of MTHFR c.677C>T was associated with increased Hcy levels and that the G-allele of MTR c.2756A>G was associated with reduced MTHFR amplicon methylation.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

GN and LZ conceived and designed the experiments. GN, JQ and HL collected blood samples and patients' data. GN, JQ, HL performed the experiments. GN, JQ and ZC analyzed the data. GN, ZC, and JZ contributed reagents/materials/analysis tools. GN, MH and LZ contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2017.01.004.

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