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Original article

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

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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.

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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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