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Different EPHX1 methylation levels in promoter area between carbamazepine-resistant epilepsy group and carbamazepine-sensitive epilepsy group in Chinese Population

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Research Article

Keywords:

Posted Date: November 30th, 2018

DOI: https://doi.org/10.21203/rs.2.44/v1

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Version of Record: A version of this preprint was published on June 4th, 2019. See the published version at https://doi.org/10.1186/s12883-019-1308-4.

Abstract

Background: Epigenetics underlying refractory epilepsy is poorly understood. DNA methylation may affect gene expression in epilepsy patients without affecting DNA sequences. Herein, we investigated the association between Carbamazepine-resistant (CBZ-resistant) epilepsy and EPHX1 methylation in a northern Han Chinese population, and conducted an analysis of clinical risk factors for CBZ-resistant epilepsy.

Methods: 75 northern Han Chinese patients participated in this research. 25 cases were CBZ-resistant epilepsy, 25 cases were CBZ-sensitive epilepsy and the remaining 25 cases were controls. Using a CpG searcher was to make a prediction of CpG islands; bisulfite sequencing PCR (BSP) was applied to test the methylation of EPHX1. We then did statistical analysis between clinical parameters and EPHX1 methylation.

Results: There was no difference between CBZ-resistant patients,CBZ-sensitive patients and healthy controls in matched age and gender. However, a significant difference of methylation levels located in NC_000001.11 (225806929.....225807108) of the EPHX1 promoter was found in CBZ-resistant patients, which was much higher than CBZ-sensitive and controls. Additionally, there was a significant positive correlation between seizure frequency, disease course and EPHX1 methylation in CBZ-resistant group.

Conclusion: Methylation levels in EPHX1 promoter associated with CBZ-resistant epilepsy significantly. EPHX1 methylation may be the potential marker for CBZ resistance prior to the CBZ therapy and potential target for treatments.

Background

According to international League Against Epilepsy (ILAE), about 65 millions of epilepsy people have been reported in the world [1], and approximately 36% of epilepsy patients have poor or no drug-response[2]. Epigenetic modification means genomic reprogramming without affecting DNA sequence, such as microRNA expression, histone modification, and especially DNA methylation [3-4]. The most common DNA methylation usually happens at 5’carbon of cytosine in CpG islands within the promoters [5].Additionally, in recent study, DNA methylation has been suspected as one of the main epigenetic mechanisms in epilepsy and DNA methylation may affect gene expression in epilepsy without affecting DNA sequences [6-7].

Carbamazepine (CBZ) known as a first-line anticonvulsants, was commonly used for the treatment of partial seizures. However, about 30-40% of epilepsy patients were CBZ-resistant according to the recent studies [8-9]. The CYP3A4 or CYP2C8 palys an important role in the the metabolization of CBZ or the formation of carbamazepine 10-11 epoxide (CBZ-E), and CBZ-E has been known as an active CBZ metabolite equipotent [10-11]. In addition to CYP3A4 and CYP2C8, EPHX1 and PXR also participated in

the biotransformation of CBZ as major drug-metabolizing enzymes. EPHX1 is responsible for the conversion of the inactive water-soluble metabolite CBZ 10,11-diol from CBZ 10,11-epoxide [12]. ABCB1 and ABCC2 have been implicated in transport of CBZ 10-11 epoxide [13-14].

Herein, we investigated the association between CBZ-resistant epilepsy and EPHX1,PXR methylation in a northern Han Chinese population, and conducted an analysis of clinical risk factors for CBZ-resistant epilepsy. The goal of the study was to determine differential methylation profiles of 2 candidate genes in CBZ-resistant epilepsy.

Methods

Patients

All of the enrolled subjects (patients and normal controls) were of Northern Han Chinese ethnicity. Patients were from five towns in Jilin Province (Hua Dian, Pan Shi, Tao Nan, Jiao He, and Shu Lan). The patients have provided written informed consent and this research has been approved by the First Hospital of Jilin University's Research Ethics Board.

During the years 2014-2016, 25 cases that fulfilled the diagnostic criteria for CBZ-resistant epilepsy were identified at the Department of Neurology, First Hospital of Jilin University, Changchun, China. CBZ-resistant epilepsy was defined as: 1 CBZ-monotheraphy for epilepsy; 2 total dose per day was more than 1.0g or plasma concentration more than 10μg/ml; 3 invalid theraphy without any change in seizure frequency. CBZ-sensitive epilepsy was defined as: 1 CBZ-monotheraphy for epilepsy; 2 total dose per day was less than 0.8g or plasma concentration less than 10μg/ml; 3 significant decrease in seizure frequency (≥70%). In addition, normal control group involved 25 persons.

Data collected from the patients' records included: age, gender, final dose of CBZ, seizure frequency and disease course.

Methylation prediction

Using a CpG island searcher (http://www.uscnorris.com/cpgislands2/cpg.aspx), we found that EPHX1 possess obvious CpG islands in their promoter regions.

DNA extraction

DNA was extracted from peripheral blood using a SK8224 blood mini kit (Sangon Biotech, Shang Hai, China).We use methprimer to make the forward and reverse primers.The sequences for EPHX1 were M247-F (5'-TGTGGTGGAATGATATTAGTTAAGGT-3') and M247-R (5'-ACCACATTCCCTAACTTCAACTACA-3'). And primer sequences for PXR were M247-F (5'-ATTTCTTCCTCCCTCTTACC-3') and M247-R (5'-GCCTTGTCCCCACATACA-3').

Bisulfite Sequencing PCR (BSP)

To verify the methylation level of genomic DNA from the peripheral blood, BSP was used. DNA was first modified by treatment with sodium bisulfite to convert all 'C's to uracil residues except 5 mCs. Then bisulfite-modified DNA were amplified by PCR, which performed in a RT-PCR instrument (Verity 96well, ABI, USA) using 2×Power Taq PCR MasterMix (Sangon Biotech, Shang Hai, China) under the Touch-down program: 98 °C for 4 mins, followed by 9 cycles of 94 °C for 45s, 66°C for 45s (decrease 1 °C per cycle), 72 °C for 1 min, then followed by 40 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min, and final extension 72 °C for 8 mins. Moreover, the PCR product was recovered by TIANgel Midi Purification Kit (Sangon Biotech, Shang Hai, China) after verification in a 2 % agarose gel. Then the purified DNA was ligated into the vector pUC18-T by pUC18-T Cloning Kit with Competent Cell (Sangon Biotech, Shang Hai, China) and transformed into E. coli strain TOP10. Sequence determinations were carried out at SANGON (Shanghai, China) [15].

Statistical analysis

BSP sequences data are analyzed using software from BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de/) to calculate the level of DNA methylation (methylation level=methylated CpG dinucleotides/total CpG dinucleotides). Statistical analysis was carried out by Prism7.0 using Student's t-test to determine statistical significance in EPHX1 methylation (the criteria for significance were defined as p <0.05) and using Pearson's correlation to determine the association between EPHX1 methylation and seizure frequency, disease course (the criteria for significance were defined as P =0.01 or P =0.05).

Results

Clinical Parameter Evaluation

The clinical characteristics, EPHX1 methylation distribution between different groups were shown in Table 1 and Table 2 respectively. Clinical Parameters included the age, gender, final dose of CBZ, seizure frequency and disease course.

There was no significant difference between CBZ-resistant epilepsy patients, CBZ-sensitive epilepsy patients and healthy controls in terms of age (42.12±1.132, 42.44± 1.112 versus 42.68 ± 1.727, *P=0.3938，P=0.4538*), gender (male/female: 11/14, 13/12 versus 12/13). The final dose of CBZ in resistant-group was 1000mg/day or 1200mg/day, and the final dose in sensitive-group was changed from 200mg/day to 800mg/day. CBZ-resistant epilepsy patients has longer disease course than CBZ-sensitive epilepsy patients (6.720±0.6965 versus 3.480±0.259, *P＜0.01*).

Methylation in CBZ-resistant epilepsy group and CBZ-sensitive epilepsy group

For the EPHX1, we used CpG island searcher to predict the CpG clusters and found that there was a large CpG clusters located in the reference sequence NC_000001.11 (225806929.....225807108), which contained 10 CpG sites and located in promoter before extron1 (Figure 1). As shown in Figure 2, among the 10 sequence fragments, the maximium methylation level of the CpG sites was 12% in controls, 57% in

CBZ-resistant patients, and 32% in CBZ-sensitive patients. Two healthy controls had no EPHX1 methylation. In addition, methylation levels depend on CpG sites in the EPHX1 promoter. And different distribution of CG-base density among CBZ-resistant group, CBZ-sensitive group and controls have been shown in Figure 3. Besides this, the methylation level in CBZ-resistant group was much higher than CBZ-sensitive group or controls (*P<0.001*), and different methylation levels has been presented in Figure 4.

Association between EPHX1 methylation and seizure frequency, disease course

As shown in Table1, the clinical characteristics and EPHX1 methylation of the CBZ-resistant epilepsy patients have been described. We used Pearson's correlation to make an analysis of the association between EPHX1 methylation and age, final dose, seizure frequency, disease course. And as shown in Table 3, in CBZ-resistant group, there was a significant correlation found between final dose, seizure frequecy and EPHX1 methylation (*r*=0.4588/*P*=0.0211,0.4921/0.0125, respectively), and no significant correlation was found between age, disease course and EPHX1 methylation (*r*=0.2382/*P*=0.2515, *r*=0.06833/*P*=0.7455, respectively).

Additionally, in CBZ-sensitive group as shown in Table 3，there is a correlation was found between seizure frequecy and EPHX1 methylation (*r*= -0.3498/*P*=0.0865), but no statistical significance.

Discussion

We found a significant high EPHX1 methylation level in CBZ-resistant group in northern Han Chinese epilepsy patients. Previous studies focused on the EPHX1 polymorphisms in refractory epilepsy patients, few discussed the EPHX1 methylation.

As we known, about one third of the epilepsy population have poor response or no response to the antiepileptic therapy [16-17]. And studies show that the variability in drug-resistance not only comes from the environmental factors, but also associates with genetic background and heterogeneity[18]. Genetic background such as genetic polymorphisms or genetic methylation can predict drug individually and efficiently. Makmor-Bakry et al demonstrated that the maintenance dose of CBZ associated with EPHX1 416A.G and 337C.T polymorphisms. Moreover, a higher diol/epoxide ratio associated with 377C.T, and a lower ratio associated with 416A.G polymorphism [19]. But, there was conflicting reports in the literature, such as that Antonietta caruso demonstrated that EPHX1 and CYP3A4 polymorphisms have no effect on CBZ 10,11-epoxide levels [20].So, under such situation, it was necessary to evaluate effects of EPHX1 methylation on CBZ-metabolism.

Additionally, EPHX1 has been known as a biotransformation enzyme localized mainly in the endoplasmic reticulum of eukaryotic cells. It has two types, namely microsomal EPHX1 (OMIM: 132810) and soluble EPHX2 (reviewed by Harris and Hammock [21]). EPHX1 consists of nine exons and encodes three transcription variants, which was first shown to convert epoxides such as styrene oxide, 1-methyl-1-phenyloxirane, indene 1,2-oxide, and cyclohexene oxide into the respective diols [22]. As described above, the EPHX1 seems to show a detoxifying function. Besides this, in many areas of the brain, such as frontal

or occipital lobe, pons, red nucleus, and cerebellum, the EPHX1 transcripts have been found. Due to its biochemical effect on the carbamazepine-diol/carbamazepine-epoxide ratios, EPHX1 has been studied as a targed pharmacological point to increase the effective theraphy of anti-epileptic drugs and decrease the adverse effects. However, there are conflicting results have been found between the EPHX1 gene variability and anti-epileptic drugs concentration. Subsequently, studies on the EPHX1 methylation may be another choice to explore the potential association.

By sequence analysis, we found that much more CG bases in promoter area in carbamazepine-resistant epilepsy patients or carbamazepine-sensitive epilepsy patients. However, in the following study, our results demonstrated that the methylation levels of such CpG sites in CBZ-resistant group were much higher than carbamazepine-sensitive group or healthy controls (P<0.001). As the result was shown above, it means that high methylation levels in CBZ-resistant group may predict poor antiepileptic therapy. Additionally, the EPHX1 methylation positively correlated with seizure frequency and final doses in CBZ-resistant group,

which also indicated that EPHX1 methylation plays an important role in the CBZ resistance response.

In this study, we investigated EPHX1 methylation in the peripheral blood DNA. Methylation levels in peripheral blood DNA have been widely investigated in various diseases, including diabetes [23], obesity [24], major depression [25], and various cancers [26-28]. Here we provided its association with CBZ-resistant epilepsy. Glossop et al demonstrated that DNA methylation pattern vary dramatically between blood cell types and tissues [29]. In our study, tissues in the CBZ-resistant epilepsy patients were not available, but we may obtain the brain tissues from the drug-refractory patients after the epilepsy surgery in future and test our results furthermore. On the other hand, high EPHX1 methylation levels in peripheral blood DNA in CBZ-resistant epilepsy group was also meaningful equally.

Conclusions

In summary, although CBZ is the first choice drug for several epilepsy types, genetic variation in CBZ metabolic pathway like EPHX1 methylation contributed, in part, to the differences in patients' response. We found a significant association between EPHX1 methylation and CBZ-resistant epilepsy patients in the northern Han Chinese patients, and EPHX1 methylation may be the potential marker for CBZ resistance prior to the CBZ therapy. It should be noted that our results need to be further confirmed by a future study with a larger sample size or tissues samples.

Declarations

Ethics approval and consent to participate The patients have provided written informed consent and this research has been approved by the First Hospital of Jilin University's Research Ethics Board.

Consent for publication

The patients have provided written informed consent for publication, including publication of potentially identifying information.

Availability of data and material

The data and material are available freely.

Competing interests

The author(s) declared no conflicts of interest with respect to the research, authorship, funding, and/or publication of this article.

Funding

Supported by Jilin province science-technology project (Grant no.20170520021JH)

The funding supported data analysis, data interpretation, and manuscript writing.

Authors' contributions

YDL participated in the conception and drafting the manuscript. XYZ collected the clinical data and revising the manuscript. MCS participated in interpretation of data and revising the manuscript. ZW participated in design and revising the manuscript. LC was accountable for the conception, design and intellectual revising. All authors have given final approval of the version to be published and agreed to be accountable for all aspects of the work.

Acknowledgements

The authors thank Dr Jun Lu who was involved in revising the manuscript critically for important intellectual content.

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Table 1. Clinical Characteristics And Ehpx1 Methylation In The 25 Patients With Cbz-resistant Epilepsy

ID No.	Gender	Age	Final-dose (mg)	Seizure-frequency	Course of disease (year)	EHPX1 methylation
1	M	40	1000	2/week（96/year）	7	25%
2	F	43	1000	3/week（144/year）	11	29%
3	F	47	1000	3.5/week（168/year）	4	31%
4	M	38	1000	3/week（144/year）	8	30%
5	F	40	1000	2/week（96/year）	7	22%
6	M	39	1000	1.5/week（72/year）	4	21%
7	F	43	1200	5/week（240/year）	3	54%
8	M	51	1000	4/week（192/year）	5	33%
9	F	55	1200	3.5/week（168/year）	10	53%
10	F	38	1000	3/week（144/year）	6	26%
11	F	40	1000	4/week（192/year）	3	31%
12	F	42	1000	2.5/week（120/year）	4	24%
13	M	51	1200	4.5/week（216/year）	7	48%
14	F	37	1000	3/week（144/year）	6	41%
15	M	45	1000	2.5/week（120/year）	4	51%
16	M	41	1000	3/week（144/year）	6	30%
17	M	37	1200	3.5/week（168/year）	5	50%
18	F	40	1000	3/week（144/year）	2	30%
19	F	35	1000	4/week（192/year）	3	26%
20	F	36	1000	4/week（192/year）	6	38%
21	F	40	1000	3/week（144/year）	9	24%
22	M	47	1200	4.5/week（216/year）	17	26%
23	M	50	1000	3/week（144/year）	12	46%
24	F	46	1000	4/week（192/year）	11	48%
25	M	32	1000	4/week（192/year）	8	57%

Table 2. Clinical Characteristics And Ehpx1 Methylation In The 25 Patients With Cbz-sensitive Epilepsy

ID No.	Gender	Age	Final-dose (mg)	Seizure-frequency	Course of disease (year)	EHPX1 methylation
1	M	45	600	1-2/year	4	32%
2	F	38	400	2-3/year	5	6%
3	F	46	700	1/year	3	28%
4	M	41	600	-	4	25%
5	M	35	800	-	4	27%
6	M	37	200	2-3/year	5	24%
7	F	40	600	1-2/year	6	23%
8	F	43	600	-	4	16%
9	M	48	800	1-2/year	5	12%
10	M	39	600	-	2	27%
11	F	43	600	-	2	32%
12	F	50	700	1-2/year	3	24%
13	F	47	600	1-2/year	1	11%
14	M	52	600	-	3	13%
15	F	33	800	3-4/year	4	17%
16	M	54	700	2-3/year	2	20%
17	M	36	600	-	3	24%
18	M	38	600	-	2	28%
19	F	40	600	1-2/year	6	16%
20	M	42	400	-	4	30%
21	F	44	600	2-3/year	3	26%
22	M	48	400	-	4	22%
23	F	39	400	-	3	17%
24	M	36	700	1-2/year	3	20%
25	F	47	600	1-2/year	2	14%

Table 3. Correlation Between Age, Final Dose,frequency, Disease Course And Ehpx1 Methylation In Different Groups

	r		P	
	EHPX1methylation In CBZ-resistant group	EHPX1methylation In CBZ-sensitive group	EHPX1methylation In CBZ-resistant group	EHPX1methylation In CBZ-sensitive group
Age	0.2382	-0.1429	0.2515	0.4955
Final dose	0.4588*	0.00305	0.0211*	0.9885
Frequency	0.4921*	-0.3498	0.0125*	0.0865
Disease course	0.06833	-0.1354	0.7455	0.5188

There is a significant correlation between final dose, frequency and EHPX1-methylation in CBZ-resistant group (P*0.01)

There is a significant correlation between final dose, frequency and EHPX1-methylation in CBZ-sensitive group (P*0.01)

Figures

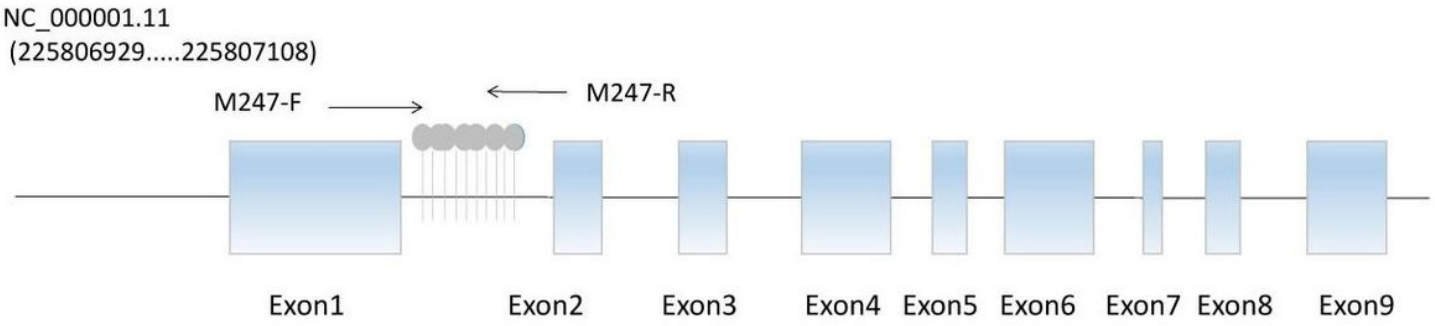

Figure 1

The schematic diagram of the CpG sites in the EPHX1 promoter. CpG sites are depicted by lollipop markers.forward and reverse primers are shown as arrows.

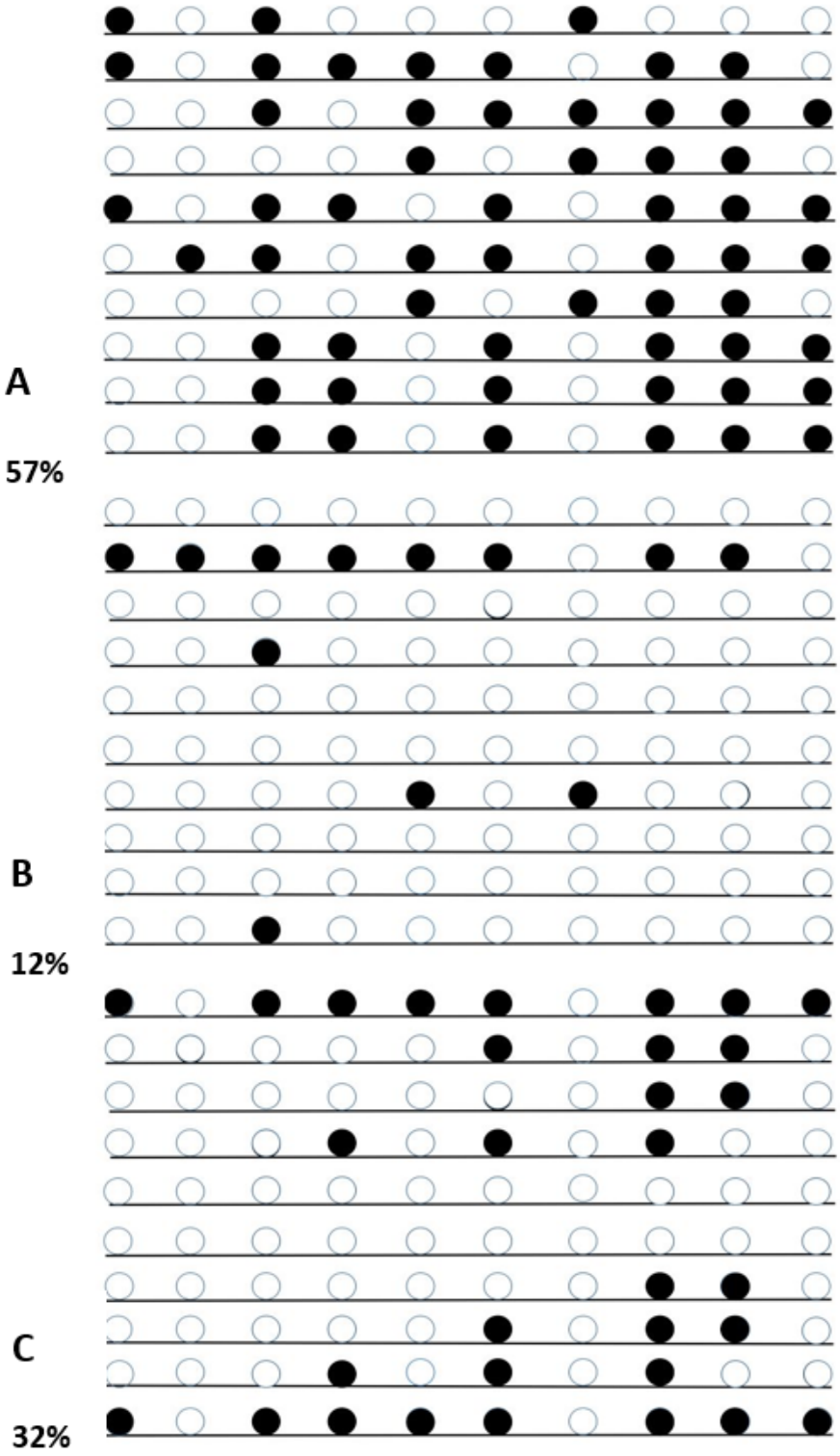

Figure 2

EPHX1 methylation density in one control and one CBZ-resistant epilepsy patient. (A) Control at the cutoff point. (B) patient with highest methylation level. White cycle: unmethylated CpG dinucleotide; Black cycle: methylated CpG dinucleotid.

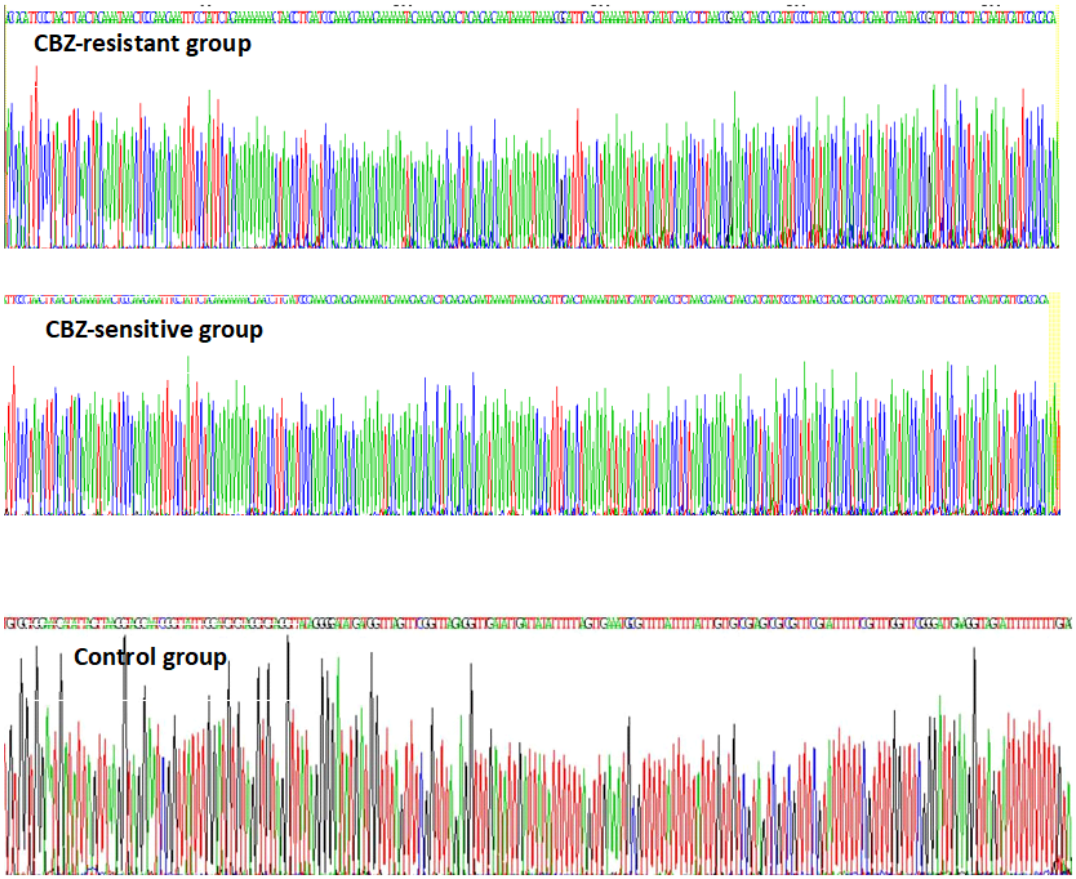

Figure 3

EPHX1 methylation density in promoter between CBZ-resistant epilepsy patient and controls (marked in blue).

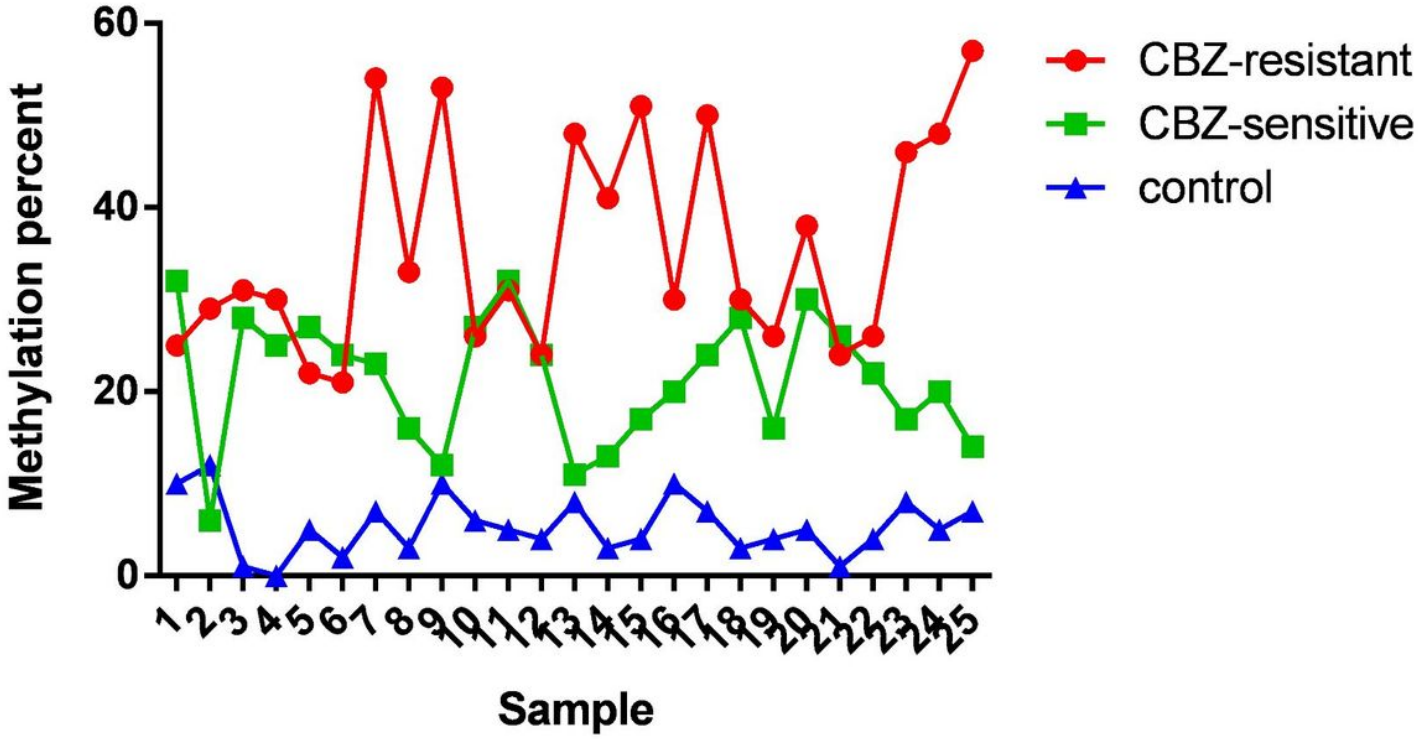

Figure 4

Different methylation levels in CBZ-resistant group, CBZ-sensitive group and controls.