Study of global DNA methylation in monozygotic twins with cerebral palsy

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Abstract: The objective of this paper is to study the global DNA Methylation in monozygotic (MZ) twins with cerebral palsy. Two pairs of twins (a cerebral palsy children, a normal child) admitted to the First Affiliated Hospital of Zhengzhou University were selected as subjects. The phenol-chloroform method was used to extract DNA from venous blood and micro satellite DNA genotyping technique was used to identify the eggs of the twins. DNA methylation fragments were enriched by MBD affinity column chromatography, followed by Solexa sequencing and bioinformatics analysis. In this study, we found that there were different DNA hypermethylation regions between each pair of twins and normal children through global DNA methylation analysis technique by analyzing the blood cells of two pairs of monozygotic twins with cerebral palsy and normal infants. The results revealed the region of DNA methylation and the protein coding genes of promoter region of common methylation of cerebral palsy were both higher than normal children. These common promoter hypermethylation genes in cerebral palsy are involved in a variety of biological processes such as membrane protein transport, neuronal development, apoptosis, and metabolism. Moreover, DNA methylation plays an important role in gene expression. We hypothesized that the onset of cerebral palsy in twins is associated with hypermethylation of the promoter which inhibiting the expression of hypermethylation genes in children with cerebral palsy. The current research provided a basis for further study of the large sample of twins and sporadic cerebral palsy.

Keywords: DNA Methylation; CpG island; MBD2b protein.

INTRODUCTION

Although monozygotic (MZ) twins have the same DNA sequence and growth environment, but sometimes there will be a huge phenotypic difference, but the mechanism of its generation is still unclear. It was found that epigenetic modification was the main reason for the genetic differences of MZ twins with consistent genetic materials (Kaminsky *et al*, 2009; Petronis *et al*, 2003; Kaminsky *et al*, 2009; Yamazawa *et al*, 2008). The study of Fraga *et al* (Fraga *et al*, 2005) on the possible mechanism of MZ phenotypic differences found that there was a significant difference between MZ twins in DNA methylation level, X chromosome inactivation and site-specific histone acetylation, and the difference was significantly increased with age increase. Kaminsky *et al*'s (Kaminsky *et al*, 2009) study on the DNA methylation difference between 114 pairs of MZ twins and 80 pairs of monozygotic twins by CpG Island chip showed that there was a difference in the degree of methylation between monozygotic twins and fraternal twins, and the degree of methylation in some CpG island regions of MZ twins was even greater than that of unrelated individuals. Petronisx (Kaminsky *et al*, 2009) pointed out that the molecular mechanism of phenotypic differences between MZ should be focused on its epigenetic differences. Some subtle differences in epigenetic modifications may lead to significant phenotypic differences between genetically identical MZ twins, which are the individual differences brought by epigenetic information variability under same genetic coding information(Souren and Tierling *et al*, 2011; Kegley and Sellers *et al*, 2010).

Epigenetics is mainly concerned with the cell differentiation process of non-DNA sequencing mechanisms and its consequences. The operation of the epigenetic information interface associates the genotype with environmental influences to determine the memory of the gene transcription model, and then determining the phenotype of the cell. This regulation is essential to the life activities of higher eukaryotes. Its abnormality will directly or indirectly affect the integrity of the genome and intracellular gene expression patterns, thereby leading to several major diseases, including cancer.

Compared with the genetic variation at the sequence level, DNA methylation is more likely to be affected by environmental factors. Thus, differences at the level of DNA methylation may be important factors in the different between twin-cerebral palsy and normal individuals (Ollikainen *et al*, 2015). Based on the above understanding, global DNA methylation analysis method (MBD affinity column chromatography enrichment of DNA methylation fragments, followed by Solexa sequencing/bioinformatics analysis) was used to analyze the blood cells of the two pairs of cerebral palsy and normal individuals, to obtain the protein coding genes with distinct DNA methylation status in the promoter region of cerebral palsy state.

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MATERIALS AND METHODS

Research objects

Two pairs of twins (a cerebral palsy children, a normal child) admitted to the First Affiliated Hospital of Zhengzhou University from May 2013 to October 2014 were selected randomly as subjects. Twin pair I: male, 14-month years old. No.1 was spastic diplegia children, and No.2 normal children; Gestational age: 37 months; Birth weight: 3.8kg; Imaging findings: Leucomalacia; Clinic symptom: Language barrie. Twin pair II: male, 31-month years old. No.5 was normal children, and No.6 spastic quadriplegia children. Gestational age: 30 months; Birth weight: 3.2kg; Imaging findings: Cerebral agenesis; Clinic symptom: Language barrie. In order to screen these children, the child who had Inherited metabolic diseases, neurological sequelae and the progressive neuromuscular disease has beened ruled out. Diagnosis and classification were performed according to the diagnostic and classification criteria of cerebral palsy established by the National Symposium on Cerebral Palsy Children (2004, Kunming). The present study has been approved by the ethic committee of the First Affiliated Hospital of Zhengzhou University. Informed consent was obtained from all parents involved.

Blood sample collection and DNA extraction

Blood sample collection: The peripheral venous blood was extracted and mixed with glucose, citric acid and sodium citrate or heparin, and stored in a refrigerator at -20°C. Phenol chloroform method was used for DNA extraction:

1) Freeze-thawed anti-coagulated blood sample 500 μl was fetched and placed in a 2 ml eppendorf tube.
2) Two times the volume of sterile saline was added and gently shaken to wash the blood cells and centrifuged for 2-3 min at 4000-6000rpm removing the supernatant carefully;
3) Step 2 was repeated 2-3 times until the supernatant was essentially colorless or slightly red.
4) After discarding the supernatant, 300 μl of buffer containing 120 μg/ml proteinase K (10 mmol/L of HCI pH = 8.0, 0.5% SDS) was added and put in a water-bath for 3-5 h in 50°C. After the enzyme digestion, the solution should be clear and transparent.
5) Equal volume (about 500μl) of Phenol was added (pH=8.0), shaking about 20 times until mixed evenly.
6) Centrifugation for 5 min with a high-speed centrifuge (13,000 rpm) was performed.
7) The supernatant was transferred to another 2 ml eppendorf tube;
8) 250 μl phenol and 250 μl chloroform were added in sequence. Repeat steps 5-7.
9) 500 μl chloroform were added in sequence. Repeat steps 5-7.
10) The supernatant was transferred to a 1.5 ml tip tube, and 1ml of anhydrous ethanol was added and mixed, and then flocculent precipitation of DNA could be seen.
11) Centrifugation was performed for 8 min at 13,000 rpm, discarding the supernatant.
12) Pouring the tip pipe on the absorbent paper and drying at room temperature for about 10-15 min; After discarding the supernatant, the remaining sediment in the tip pipe is tipped on the absorbent paper and dried at room temperature for about 10 minutes.
13) 100 μl of TE buffer solution (Tris-EDTA, pH = 7.4) was added and incubated at 37°C for 3 hor overnight at room temperature to facilitate DNA lysis;
14) Dissolved DNA was packed and store at -20°C.
15) After that, the concentration of DNA was determined by fluorescence quantitative method and UV Spectrophotometry. The former was used to estimate the content of double-stranded DNA through fluorescent dyes to bind specifically to macromolecules; the latter mainly uses DNA, RNA and proteins with different absorption spectra to identify their content. Each DNA would undergo three separate measurements. If the error is within the allowable range, the average concentration would be taken as the final concentration, and then diluted and stored at -20°C.
16) The volume of water and DNA added to each well in the dilution process was calculated until the same of final concentration of each well, volume between 100-200μl.
17) A number of sterilized 96-well plate were taken according to the needs;
18) DNA saved in -20°C was fetched out and melted at room temperature, and then centrifuged for 1 min at 1000 rpm;
19) Based on the pre-calculated values, the corresponding volume of sterile water and DNA was disassembled in different wells of a 96-well plate;
20) Gentle shaking, and centrifuging for 1 min at 1000rpm, so that there was no water attached to the inner hole of the 96-well plate;
21) The well-separated 96-well plate was sealed with sealing paper and allowed to stand for 3 h at room temperature;

Zygosity identification

Microsatellite DNA typing was used to identify the zygosity of the twins. A short tandem repeat sequence with high degree of heterozygosity in the Chinese population (short tandemrepeat, STR) (D16S3068, D13S159, D18S474, D19S220, D19S210, D21S266, D14S258, D14S63, D12S1682) was selected on 9 different chromosomes to synthesize the primers and labeled with the fluorescent dyes.

PCR reaction system	5μl
DNA	20ng
$MgCl_2$	3.0mmol/L
Primer	0.2pmol/L

dNTP	200mmol/L
10 ×buffer	0.5μl
Hotstart Tag	2U

1) 95°C 15min
2) 94°C 30s
3) 60°C 30s (-0.5°C/cycle)
4) 72°C 30s to 2 10 times
5) 94°C 30s
6) 55°C 30s

0.5μl PCR products were mixed with 0.15μl internal standard GneScan350 and 0.75μl form amide and then put on the ice after 95°C for 30 min. Gel electrophoresis was performed on the sequencer. Then Gne Scan and Genotyper software were used for genotyping analysis.

Enrichment of methylated DNA

Genomic DNA was broken into 150-1000bp fragment by ultrasound. Ultrasound 5 s, stop 5 s, last for 4 cycles;

4μg of each DNA sample after sonication was added to 50μl Sepharose 4B gel of the GST-MBD2b protein in which the *Escherichia coli* DNA was removed. Binding buffer containing 0.1M KC1 was used to balance the gel for twice (180μl/per time, centrifuging for 1 min at 0.5rcg). 10μl Sss 1 methylase was added to the binding buffer containing 0.1M KCl until the volume was 180μl, and then combined for 2 h in the oscillator under 4°C. 1.5 ml supernatant was absorbed to a new centrifuge tube after the combination was centrifuged for 1min. After that, the supernatant was combined with 180μl of binding buffer containing 0.5M KCl for 2min. The supernatant was fetched out to a new tube after centrifugation, and the DNA was eluted with an elution buffer containing 1.0 M KCl.

1μl glycogen as the carrier in each tube, adding the same amount of isopropanol precipitation subsided for 30 min, and centrifuged for 10 min with the maximum speed under 4°C. After washing with 75% ethanol, the supernatant was abandoned and the centrifugal concentrator was used to dry the liquid, adding 30μl of sterile water dissolved.

Subsequent treatment of enriched DNA
DNA end-filling:
(1) The obtained product was transferred into PCR tube, with 60μl in each tube; The 60μl of obtained product was transferred into each PCR tube
(2) Compounding a portion of MIX 1
ddH2O 38.7μl
10×NEB buffer 2 11μl
10mg/ml BSA (NEB) 0.5μl
T4polymerase (NEB) 0.2μl
(3) 50μl of the above mixture was added to each sample, and incubated at 37°C for 30 min;
(4) Recovery: 11μl 3M NaAC?, 1μl Glycogen (50ng/μl), 120μl pre-cooled phenol were added to each tube and mixed completely;
(5) Centrifuging for 10 min at 13200rpm;
(6) 110μl supernatant was absorbed?? to a new tube, a 230μl absolute ethyl alcohol was added, and was placed for 30 min at -20°C after mixing;
(7) Centrifuged for 10 min at 4°C 13200rpm;
(8) After discarding the supernatant, 500μl 70% ethanol was added to the precipitate and centrifuged for 5min at 4°C 13200rpm;
(9) Discarded the supernatant and dried at room temperature;
(10) 25μl of sterile water was added to each tube and was placed on ice:

Connectors 1 part??

ddH2O 13μl
10×ligase buffer NEB 5μl
Linker (15P) 6.7μl
400U/μl T4DNA ligase (NEB) 0.4μl
Samples were mixed with 25μl of the above mixture at 16°C for 16 h; The QIA quick kit was used to recover the ligation product, and 25μl sterile water was used to dissolve DNA;

PCR amplification of enriched DNA

MIX A 1part??
ddH2O 2μl
10×buffer 4μl
dNTP (10mM) 1.25μl
oligo JW 102(40uM) 1.25μl
betaine (5M) 6.5μl
MIX B 1 part
ddH2O 8.5μl
10×buffer 1μl
rTaq 0.5μl

15μl of MIX A was added to each sample and proceeded to the following procedure, and pause at the beginning of the first step; 10μl of MIX B was added to each tube and continue with the next procedure.

Step 1 60°C 4 min
Step 2 72°C 3 min
Step 3 95°C 2 min
Step 4 95°C 30 s
Step 5 60°C 30 s
Step 6 72°C 1 min
Step 7 go to Step 4 14 cycles
Step 8 72°C 1 min
Step 94°C Hold

Finally , the amplified product was transferred to a 1.5 ml sterile tube and the sample was diluted ten-fold to serve as a template for the second round PCR reaction.

Second rounds of PCR: 1 parts reaction system

ddH20 30.5p1
10xbuffer 5p1
dNTP (10mM) 1.25p1
oligo JW102 (40uM) 1.25p1

Betaine (5M) 6.5p1
template 5p1
rTaq 0.5pl

Reaction condition
Step 1 95°C 2 min
Step 2 95°C 30 s
Step 3 67°C 30 s
Step 4 72°C 1 min
Step 5 go to Step2 24cycles
Step 6 72°C 5 min
Step 7 40°C Hold
The QI Aquick kit was used to recover the PCR product, and Nanodrop was used to quantify.

Solexa sequencing and analysis
Solexa sequencing was used for the amplification of PCR product. The fragment was located on the human genome, and the distribution map of the DNA fragment was established, which took P value>103th as the methylation state (peaks). Software eland provided by the Illumina Company was used to convert the sequencing information into a suitable format and locate it on the genome. Then, macs was used to find peaks for the comparison.

RESULTS

Zygosity identification: The 9 STR genotypes between the 2 pairs of twins were completely consistent with each other (fig. 1, fig. 2), so that the two pairs of twins were determines as identical twins.

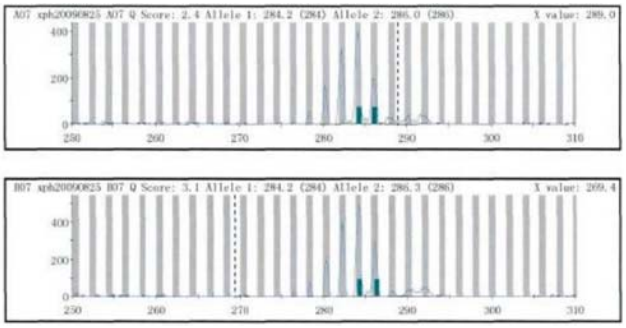

Fig. 1: Micro satellite DNA genotype: This is a pair of MZ at the same locus --- D19S220 genotype

Results of global DNA methylation pattern analysis of two pairs of twins (table 1)

Specific hypermethylation regions of cerebral palsy and normal individuals in twins
By comparing the DNA methylation status between each pair of cerebral palsy and normal children, it was found that the hypermethylation regions of cerebral palsy were: No.1: 25440, No.6: 103885; the normal hypermethylation regions were: No.2: 38772; No.5: 3622. By comparing the hypermethylation regions of two cases of cerebral palsy and normal children, we found that the region numbers of common methylation of cerebral palsy children were 10679, and the numbers of common methylation region of normal children were 626 (table 2).

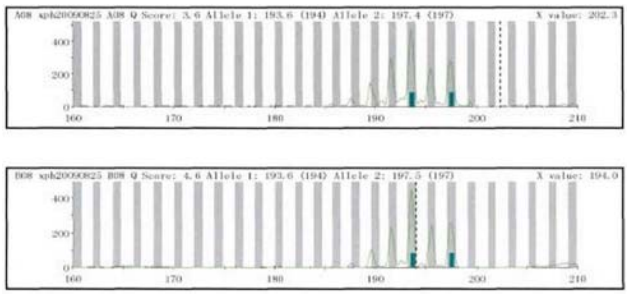

Fig. 2: Micro satellite DNA genotype: This is a pair of MZ at the same locus --- D14S63 genotype

The promoters of the protein-coding gene in the promoter region of the common methylation of the cerebral palsy (10679) and the promoters of the protein-coding gene in the region of the normal co-methylated region (626) were selected and found that former had 661, the latter 41. The promoter regions of the differentially methylated genes were selected and listed in table 3 and table 4.

DISCUSSION

A pair of MZ fetuses is born with the same DNA structure, but different cell environment, so each individual gene expression is not the same. Because MZ has the same genetic background, it can maximize the exclusion of genetic heterogeneity and can be concentrated in the cell environment. Therefore, the MZ model has its unique advantages in exploring the biological mechanism of cerebral palsy (Bonellie *et al*, 2005; Kohsaka *et al*, 1996; Poulsen *et al*, 2003; Reuss *et al*, 2002).

Based on the analysis of globe DNA methylation of two pairs of MZ, we found the region of DNA methylation difference between every pair of twins with cerebral palsy and normal individuals, and further found the common DNA hypermethylation region in two cerebral palsy or normal individuals in MZ. We further identified the methylation status of these candidate regions in these two pairs of twins' blood cells by bisulfite sequencing, and 661 promoters of protein coding genes were found in the region of common methylation of two pairs of MZ cerebral palsy individuals, and 41 protein coding genes in common methylation regions of normal individuals.

These genes are involved in many biological processes, such as membrane protein transport (TOMMT,VPS53, CRB2, GPRINI), neural development (GPRINI), apoptosis (PHLDA3 ,VDAC2, HUS1) and metabolism (ATP1B4, CETP, VDAC2), in which VDAC2 is one of the three subtypes of voltage-dependent anion-selective channels (VDAC). The main function of VDAC is to

Table 1: Basic data of DNA methylation pattern

Twins	Sample name	Reads	Peaks (p value>1e-3)
I	1 (cerebral palsy)	9069341	274140
	2 (normal children)	10016810	300963
II	6 (cerebral palsy)	9796832	305692
	5 (normal children)	8622768	152271

Note: Reads: Sequencing fragments sequenced to Solexa on the genome; peaks: DNA region above background

Table 2: Specific hypermethylation regions of cerebral palsy and normal individuals in twins

	1 vs 2		6 vs 5		1 vs 6 methylation	2 vs 5 methylation
Hypermethylation region	1methylation	2 methylation	6 methylation	5 methylation	Co-methylation	Co-methylation
Total number (pvalue>1e-3)	39181	66889	165219	6461	16258	1172
Total number of specific methylation regions*	25440	38772	103885	3622	10679	626

Note: The identity of the sample was shown in table 1; * Hypermethylation in the labeled samples in this column (p value>1e-3), which there was no peak (p value<1e-3) in the corresponding samples.

Table 3: There was hypermethylation in the gene promoter of cerebral palsy (p value>1e-3), but not in normal samples (p value<1e-3)

Gene ID	Gene Symbol	Gene ID	Gene Symbol
NM-001146069	MFSD10	NM-001005498	RHBDF2
NM-152678	FAM116A	NM-004570	HUS1
NM-173689	CRB2	NM-001128159	VPS53
NM-152463	EME1	NM-000078	CETP
NM-004668	MGAM	NM-153824	PYCR1
NM-019059	TOMM7	NM-004140	LLGL1
NM-001142447	ATP1B4	NM-012396	PLDA3
NM-006618	KDM5B	NM-001707	BCL7B
NM-199047	TBPL2	NM-003375	VDAC2
NM-052899	GPRIN1		

maintain the permeability of the mitochondrial outer membrane, which is related to the trans membrane transport of various metabolites (Massa *et al*, 2000). At present, it is believed that the main role of VDAC2 is related to energy metabolism and cell apoptosis. Recently, it is reported that VDAC is the main component of the death pathway (Madesh and Hajnóczky, 2001; (Shoshan-Barmatz and Israelson *et al*, 2006). On the one hand, the binding of VDAC2 to outer dense fibers (ODF) influences the opening of mtPTP and releases cytochrome C (Cytc) from the mitochondria into the cytoplasm. In cytoplasm, Cytc binds to apoptotic protease activating factor-1 (Apaf-1) and activates caspase-dependent apoptosis pathway (Klaus-Dieter *et al*, 2004). On the other hand, VDAC2 directly bind with Bcl-pro-apoptotic Bcl2 protein to enhance the activity of VDAC, mt PTP open, and promote the release of Cytc(Gross *et al*, 1999; Shimizu *et al*, 1999; Kelekar *et al*, 1998). GPRIN1 mRNA (GProtein-Regulated Inducer of Neurite Outgrowth I, GRIN 1) is expressed extensively throughout the embryonic developmental stage and postnatal in the differentiation region of the nervous system, and the expression of GPRIN1 mRNA is localized in the neural differentiation region. Human GRIM gene is located in 9q34.3, a total of 22 exons, and a total length of about 31 kb. Its gene expression regulates the formation of early brain development synapses, plasticity, neurons and the number of their connections. The spatio-temporal expression pattern of GRIM is closely related to the peak of neuronal differentiation, suggesting that GPRIN 1 plays an important role in neural cell differentiation and distribution (Chen *et al*, 1999; Masuho *et al*, 2008). Studies have confirmed that the 1001t3/C polymorphism in the GR1N1 gene is associated with schizophrenia. The CETP gene is the coding gene of Cholesteryl ester transfer protein (CETP) and plays an important role in cholesterol transport. Recent studies have found that CETPC-629A mutations are associated with vascular dementia, and may lead to a reduction in white matter (Herman, 1998).

The effect of DNA methylation on gene expression has been studied extensively (Qureischie *et al*, 2009). CpG island methylation can inhibit the activity of promoter and

Table 4: There was hypermethylation in the gene promoter of normal children (pvalue>1e-3), but not in cerebral palsy children (pvalue<1e-3)

Gene ID	Gene Symbol	Gene ID	Gene Symbol
NM-004146	NDUFB7	NM-001001520	HDGF2
NM-003373	VCL	NM-005596	NFIB
NM-012384	GMEB2	NR-0244%	LOC442421
NM-172348	IL4	NR-015410	FLJ22536
NM-002596	RFPL3S	NM-203424	IQCF2
NM-030789	HM13	NM-182513	SPC24
NM-198839	ACACA	NM-001164313	ZBTB24
NM-001007176	C8orf22	NM-012317	LDOC1
NM-152666	PLD5	NM-174897	BPIL3
NM-006183	NTS	NM-021706	LAIR1
NM-020169	LXN		

inhibit gene expression. Vitro gene transfection experiments showed that the hypermethylation of Ha-ras-1 gene promoter of CAT (chloramphenicolacetyl transferase) gene significantly reduced the expression of the gene (Esteller *et al*, 1999). The whole methylation of CpG island of β-globin genes in adult chickens will result in complete inhibition of transcription. The results show that DNA methylation can inhibit gene expression. The mechanisms of inhibition of promoter activity by CpG island methylation are: (1) Methylation of CpG islands directly interferes with the binding of transcription factors with regulatory region DNA. Some methylated DNA binding proteins (MDBP 1, MDB2) bind specifically to methylated DNA, thereby inhibiting the binding of transcription factors to the promoter. (2) Methylation can alter chromatin structure and indirectly inhibit gene transcription.

It is not clear whether the high methylation genes of cerebral palsy can inhibit the expression of genes due to hypermethylation of the promoter, or whether it is associated with the onset of cerebral palsy in twins. The discovery of these hypermethylated promoters common to cerebral palsy is only a preliminary result of our series of studies. We will conduct further studies on the sporadic cerebral palsy cases/normal individuals in twins (both cerebral palsy and normal individuals) by MSP to identify genes associated with the pathogenesis of cerebral palsy and to conduct more in-depth studies.

CONCLUSION

Our study found the region of DNA methylation differences between twins with cerebral palsy and normal individuals, as well as a common DNA hypermethylation region in two pairs of twins with cerebral palsy or normal individuals. The protein coding genes of promoter region of common methylation region of cerebral palsy and normal individuals were selected, providing a basis for the further study of large sample of twins and sporadic cerebral palsy.

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