Hypermethylation of IGSF4 gene for noninvasive prenatal diagnosis of thalassemia

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Summary

Background: For patients with pregnancy-induced thalassemia, fetal cord blood or amniotic fluid is invasively collected in the traditional diagnosis and prediction of thalassemia. However, there is no specific molecular target in the diagnosis of thalassemia using fetal DNA from the plasma of pregnant women.

Material/Methods: The promoter of cell surface adhesion molecule (IGSF4) gene was found to be down-regulated in patients with homozygous thalassemia, and the expression of IGSF4 was closely associated with the methylation of its promoter. In the present study, mass spectrometric sequencing of methylation was performed using MassARRAY to detect the 12 CpG sites in the promoter of IGSF4 gene.

Results: The methylation degree of these 12 CpG sites was significantly higher than that in healthy subjects (P<0.05). Hierarchical clustering was done in 23 patients with thalassemia and 5 healthy individuals. Results revealed the promoter of IGSF4 gene was highly methylated in thalassemia patients, which was dramatically different from that in healthy subjects (P<0.05). Methylation-specific PCR (MSP) was employed to confirm the methylation of the promoter of IGSF4 gene and results were consistent with those obtained in sequencing with MassARRAY. Real-time PCR showed, when compared with heterozygous subjects, the expression of IGSF4 was significantly down-regulated in thalassemia patients (ratio=0.18).

Conclusions: The expression of IGSF4 was closely related to the methylation of its promoter, suggesting the methylation of IGSF4 gene is tissue-specific for thalassemia. These findings provide evidence for the non-invasive prenatal diagnosis of thalassemia in terms of epigenetics.

key words: thalassemia • DNA methylation • IGSF4 • MassARRAY • noninvasive prenatal diagnosis

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**BACKGROUND**

To date, no effective treatment has been available for thalassemia. Therefore, genetic counseling and premarital as well as prenatal screening for thalassemia are recommended. For couples carrying thalassemia genes, prenatal screening of thalassemia is critical to prevent the delivery of thalassemia major infants and to decrease the number of heterozygous thalassemia infants born. With the development and improvement of PCR technique, the testing technology in genetic diagnosis of thalassemia becomes increasingly mature. In recent years, gene chips technology has been an increasingly attractive molecular diagnostic technique for this highly heterogeneous genetic disease. However, invasive collection of fetal cord blood and amniotic fluid is needed for prenatal screening of thalassemia, and no specific molecular target has been identified for the non-invasive prenatal diagnosis using fetal DNA in the plasma of pregnant women. The design of markers is mainly based on the gene polymorphism between fetus and parents, which is relatively complex and thus limits the wide application of diagnosis with plasma DNA of pregnant women. Markers of tissue-specific methylation have become alternative targets in its diagnosis. With the development and improvement of DNA methylation analysis techniques, searching for a novel locus regulated by methylation has become one measure for the early molecular diagnosis and treatment of cancers and genetic diseases [1,2]. The methylation of β globin gene in the non-red blood cells could re-activate fetal genes at stable status and exhibit therapeutic effect on some genetic diseases (sickle cell disease and β thalassemia) [3].

The MassARRAY time-of-flight mass spectrometry (TOF-MS) biochip system was developed by the Sequenom Company (USA) which focuses on the development of biochips for the detection of genetic mutation and DNA methylation. The MassARRAY system is a unique system aiming to detect DNA methylation using spectrometry. This system can rapidly recognize different genotypes with extremely high accuracy, and identify the target DNA carrying highly methylated CpG locus. The MassARRAY system is hybridization independent, which avoids the potential mismatch in hybridization. In addition, using the high-density SpectroCHIP arrays, this system can perform more than 3840 multiple identifications within 4 h, and automatic detection of each spot takes only 3~5 sec. Therefore, this system provides a large-scale and high-throughput way to detect DNA methylation, which meets the trend of investigating diseases using genetic tools [4]. The accumulation of changes in the genetics and epigenetics may finally result in the occurrence of genetic diseases in which epigenetic changes occur at an early stage, and methylation frequently leads to inactivation of abnormal genes [5]. Thus, DNA methylation plays an important role in the molecular diagnosis of genetic diseases. In the amplification with PCR, methylation is frequently lost. Thereafter, fixation of methylation is necessary and treatment with bisulfite is widely applied [6]. After treatment with bisulfite, the methylated cytosine remains stable in PCR, and the unmethylated cytosine is changed into thymine. Thus, methylation analysis is used to detect the C/T CpG. In mass spectrometry, the DNA methylation can be qualified. Tist et al. first treated genomic DNA with bisulfite and the single methylation was effectively determined [7]. In addition, this method was also employed to analyze multiple methylations in parallel [8]. IGSF4 is a new member of the immunoglobulin superfamily. IGSF4 gene methylation can lead to the inactivation of the IGSF4 gene, which is closely related to the occurrence and prognosis of lung cancer and pancreatic cancer [9]. In normal bone marrow cells, the IGSF4 gene is normally expressed and its promoter is not methylated. Methylation of the IGSF4 gene has been found in several leukemia cell lines [9]. Our group focused on the application of microarray technology to investigate the pathogenesis of thalassemia in terms of epigenetics. In our previous study, microarray gene expression profiling and bioinformatic technique were employed to screen the thalassemia-related genes, and several differentially expressed genes were identified, including the previously reported genes IGSF4, PTPRC (CD45), CD3D, CSF1 and CSF2 [10]. Among these genes, the expression of cell surface adhesion molecule-IGSF4 is dramatically down-regulated. However, the role of the IGSF4 gene in thalassemia remains still unknown. We speculate that the IGSF4 gene may be a marker of thalassemia and can be used as a target in the screening and non-invasive diagnosis of thalassemia. In the present study, DNA methylation quantification using the MassARRAY Epityper was employed to differentially analyze the methylation of the IGSF4 gene in thalassemia, which may become a non-invasive tool for thalassemia diagnosis. Methylation of the IGSF4 gene may be a new plasma-specific fetal marker. Our study provides the basis for good prenatal and post-natal care and enhancement of population health quality.

**MATERIAL AND METHODS**

**Clinical materials**

This study included ascertained cases of thalassaemia from June 2008 to October 2010. A total of 23 cases of thalassaemia and 5 cases control groups, as described previously [11], were identified at the Third Military Medical University during the study period. None of them received radiotherapy, chemotherapy, or immunotherapy before their blood samples were collected. Prior to sample collection, appropriate permission was given from the research ethics committee. Maternal blood was collected from each patient donor. gDNA was successfully isolated from 28 blood samples.

**Bisulfite treatment**

The methylation status of a number of CpG islands were analyzed by direct sequencing of sodium bisulphate-modified gDNA [12]. Bisulfite treatment of genomic DNA was carried out with the CpGenome DNA modification kit (Qiagen). gDNA samples were subjected to bisulfite modification using a standard protocol (Qiagen Blood and Cell DNA Midi columns) [13]. Briefly, in this protocol, 2 µg of genomic DNA was added to ddH2O for a total volume 20 µl, then 85 µl Bisulfite Mix and 35 µl Protect Buffer were added, bringing the total volume to 140 µl, and then 560 µl of Buffer BL was added to 140 µl of the reaction system. After vortexing, the mixture was transferred to an EpiTect spin column, and centrifuged at maximum speed for 1 min. Buffer BW 500 µl was added to the EpiTect spin column, and the wash step was repeated. Then 500 µl of Buffer BD was added to the EpiTect spin column, incubated in a room-temperature bath 15 min, and centrifuged for 1 min at maximum speed. After removing the waste liquid, the column was centrifuged at 13 000 × g centrifugation for 1–5 min.
Table 1. Primers of IGSF4 gene.

| Gene   | Primer sequence (5'-3')          | Anticipated size (bp) | Number of CpGs | Temperature of annealing (°C) |
|--------|---------------------------------|-----------------------|----------------|-----------------------------|
| IGSF4  | TTGTAGTTTTGGAGTTAAGGAGG         | 390                   | 34             | 74.40                       |
|        | AACAATCTTTAAAAATTATCCAAAAA       |                       |                |                             |

Table 2. Primers of IGSF4 gene and β-actin gene in Real-time PCR.

| Gene   | Primer sequence (5'-3')          | Anticipated size (bp) | Temperature of annealing (°C) |
|--------|---------------------------------|-----------------------|-----------------------------|
| IGSF4  | GGTAGTCGGGTTCGTTGTTGCCTA       | 357                   | 61                          |
|        | CTACTCGACCGTCCGATTCG            |                       |                             |
| β-actin| CTTCATCTTGGGCTCAGGTGTAAATATCCCCCATC       | 238                   | 60                          |
|        | AATATCCCCCATCAGGAA              |                       |                             |

Next, 20 µl of Buffer EB was added to the spin column, followed by centrifugation at 12 000 × g for 1 min. This reaction mixture was repeatedly heated between 65°C for 15 min and 95°C for 30 sec in a PCR machine for 20 cycles. Finally, DNA purification and cleaning was done. The sulfite-modified genomic DNA was put into a 4°C refrigerator for 24 h, or –20°C storage for 2 months.

Extraction of total RNA

Total RNA was extracted from the cord blood using Trizol (Beijing Biotek, China) and concentrated by isopropanol precipitation. Purification of total RNA was carried out with the NucleoSpin RNA clean-up kit (MACHEREY-NAGEL, Germany), and the concentration and purity of RNA were determined with Nanodrop. The integrity of RNA was measured by electrophoresis. In the spectrophotometry of RNA concentration, the D260/D280 was 1.8~2.0, and the ratio of 28S rRNA to 18S rRNA was about 2.0 following formamide-gel electrophoresis of total RNA.

Sample processing and primers

After treatment with sulfite, the methylated cytosine (C) in DNA was changed into uracil (U), which results in changes in the methylation-specific sequences in the DNA template. Then, PCR was performed with primers carrying T7 promoter at the 5' end. The products were subjected to shrimp alkaline phosphatase (SAP) treatment and then base-specific cleavage. The size and molecular weight of DNA fragments following cleavage depend on the changes in the bases after sulfite treatment and can be detected by flight mass spectrometry. The designing of primers was performed according to the sequenom methylated primers (www.urogene.org/methprimer/), and the size of primers was about 200~600 bp.

Following sulfite treatment, genomic DNA was subjected to PCR amplification, cleavage and SAP by using the Complete PCR Reagent Set and MassCLEAVE Kit according to manufacturer’s instructions. PCR conditions included pre-denaturation at 95°C for 15 min, 45 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 30 sec and a final extension at 72°C for 3 min. Mixture (210 µl) in single digestion reaction included distilled water, HME buffer and SAP. The conditions were at 37°C for 30 min and at 85°C for 5 min. The products were stored at 4°C (3). In the extension reaction the mixture included distilled water, dNTP, primers and Taq polymerase. The conditions for PCR included pre-denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 5 sec, annealing at 52°C for 5 sec and a final extension at 72°C for 5 sec. Products were stored at 4°C (Kit was purchased from Takala, Japan) (4). Desalination was performed with resin (5). The CpG locus was measured with the Sequenom system (Bruker Autoflex spectrometer, Sequenom). The primers are shown in Table 1.

Methylation-specific PCR (MSP)

The bisulfite-treated DNA template (150 ng) can be used to detect gene-specific promoter hypermethylation in β-thalassaemia. Two sets of IGSF4 PCR primers were designed: the methylated primers are 5’ TTGTAGTTTTGGAGTTAAGGAGG 3’ (forward) and 5’ AACAATCTTTAAAAATTATCCAAAAA 3’ (reverse). Touchdown PCR uses a cycling program with varying annealing temperatures. It is a useful method to increase the specificity of PCR. The annealing temperature in the initial cycle should be 5°C below the Tm of the primers. In subsequent cycles, the annealing temperature is decreased in steps of 1–2°C/cycle until a temperature is reached that is equal to, or 2–5°C below, the Tm of the primers. Touchdown PCR enhances the specificity of the initial primer-template duplex formation, and thus the specificity of the final PCR product (Qiagen). The PCR conditions included initial incubation at 95°C for 12 min, the first step was incubation at 95°C for 45 sec, annealing 52–62°C, 45 sec and 1–2°C/cycle, extension 72°C, 45 sec, and 10 cycles; the second round was incubation at 95°C for 45 sec, annealing 56°C, 45 sec, extension 72°C, 45 sec, and 35 cycles, 4°C, preservation.

Real-Time PCR

To perform semi-quantitative analysis, 2 µg of RNA from each sample were used; the β-actin bands were used as internal loading controls and a minimum number of cycles were performed to maintain the linearity of the reaction. The sequences and annealing temperatures for primers were derived from an extensive literature search and are listed.
in Table 2. The cDNA synthesis conditions included incubation at 65°C for 5 min, ice bath for 5 min, and incubation at 50°C for 30 min. The PCR conditions included initial denaturing at 95°C for 3 min, 95°C for 30 sec, 60°C for 1 min, and finally 40 cycles at 72°C for 45 sec.

Data analysis

Data were analyzed with EpiTYPER™ software, which consists of a plate editor and analysis. The reaction defined by the editor program and the layout of samples and installation file were stored in the MassARRAY® database. The analyzer displayed the results from spectroscopic analysis and the detection of methylation as figures and text format. The EpiTYPER of MassARRAY EpiTYPER™1.0 was started and the MassARRAY® employed to store data. After establishing the module, the SpectroACQUIRE and MassARRAY® were used to generate and process the data. The processing and analysis using MassARRAY® RT Workstation 3.4 were performed according to manufacturer’s instructions. Unsupervised clustering analysis of the expression pattern was performed using Cluster 3.0. Treeview software was used to visualize the unsupervised clustering results.

RESULTS

DNA sequencing by mass spectrometry

The methylation of IGSF4 gene was analyzed in thalassemia patients and the scatter plot of CpG locus and its peak were obtained. Results were composed of peaks, scatters and the selected gene sequences. The results of DNA sequencing by mass spectrometry in 1 sample are shown in Figure 1. A total of 12 CpG loci were compared by DNA sequencing by mass spectrometry. Results indicated the degree of methylation of the promoter of IGSF4 gene at these 12 CpG loci in thalassemia patients was significantly higher than that in healthy controls.

Statistical analysis of IGSF4 gene methylation

Cluster analysis

The cluster analysis could completely differentiate the 23 thalassemia patients from the 5 healthy controls. Red represents a high degree of methylation and green represents a low degree (Figure 2). Results from cluster analysis showed that the degree of the promoter of IGSF4 gene successfully distinguished the thalassemia patients and healthy individuals. As shown in Figure 2, 1–23 represent samples from thalassemia patients and C1–C5 are the samples from controls. The degrees of methylation at the 12 CpG loci among patients and healthy subjects were consistent with those in DNA sequencing. These findings suggest the promoter of the IGSF4 gene is highly methylated in thalassemia patients.

Student’s T test of 12 CpG loci in patients and healthy controls

The 12 CpG loci were then subjected to T testing in sequence. Results showed there were marked differences in these 12 CpG loci.
Samples | CpG_1 | CpG_2 | CpG_3 | CpG_4 | CpG_5 | CpG_6 | CpG_7 | CpG_8 | CpG_9 | CpG_10 | CpG_11 | CpG_12
--- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | ---
1 | 0.25 | 0.11 | 0.25 | 0.12 | 0.18 | 0.09 | 0.15 | 0.15 | 0.25 | 0.11 | 0.11 | 0.11
2 | 0.21 | 0.04 | 0.18 | 0.14 | 0.11 | 0.15 | 0.18 | 0.1 | 0.25 | 0.04 | 0.14 | 0.29
3 | 0.24 | 0.08 | 0.29 | 0.18 | 0.27 | 0.17 | 0.14 | 0.13 | 0.21 | 0.08 | 0.18 | 0.11
4 | 0.22 | 0.38 | 0.32 | 0.09 | 0.2 | 0.2 | 0.16 | 0.19 | 0.66 | 0.38 | 0.38 | 0.2
5 | 0.24 | 0.08 | 0.25 | 0.14 | 0.17 | 0.09 | 0.17 | 0.12 | 0.25 | 0.08 | 0.18 | 0.14
6 | 0.18 | 0.05 | 0.16 | 0.17 | 0.15 | 0.15 | 0.1 | 0.17 | 0.15 | 0.05 | 0.25 | 0.18
7 | 0.16 | 0.08 | 0.23 | 0.18 | 0.08 | 0.25 | 0.13 | 0.19 | 0.16 | 0.08 | 0.18 | 0.12
8 | 0.23 | 0.09 | 0.24 | 0.2 | 0.15 | 0.17 | 0.13 | 0.18 | 0.13 | 0.09 | 0.19 | 0.1
9 | 0.2 | 0.11 | 0.26 | 0.12 | 0.11 | 0.08 | 0.19 | 0.14 | 0.19 | 0.11 | 0.11 | 0.12
10 | 0.2 | 0.09 | 0.25 | 0.15 | 0.26 | 0.09 | 0.16 | 0.11 | 0.17 | 0.09 | 0.19 | 0.11
11 | 0.2 | 0.06 | 0.26 | 0.11 | 0.16 | 0.15 | 0.15 | 0.27 | 0.16 | 0.06 | 0.26 | 0.19
12 | 0.21 | 0.07 | 0.2 | 0.16 | 0.14 | 0.11 | 0.18 | 0.26 | 0.24 | 0.07 | 0.17 | 0.12
13 | 0.16 | 0.06 | 0.27 | 0.1 | 0.12 | 0.08 | 0.13 | 0.17 | 0.21 | 0.06 | 0.16 | 0.12
14 | 0.13 | 0.05 | 0.16 | 0.17 | 0.17 | 0.07 | 0.13 | 0.27 | 0.22 | 0.05 | 0.1 | 0.16
15 | 0.1 | 0.05 | 0.09 | 0.11 | 0.3 | 0.15 | 0.13 | 0.15 | 0.29 | 0.05 | 0.15 | 0.16
16 | 0.25 | 0.07 | 0.3 | 0.18 | 0.1 | 0.13 | 0.13 | 0.28 | 0.21 | 0.07 | 0.13 | 0.14
17 | 0.18 | 0.06 | 0.3 | 0.17 | 0.18 | 0.13 | 0.11 | 0.18 | 0.16 | 0.06 | 0.16 | 0.17
18 | 0.26 | 0.06 | 0.32 | 0.21 | 0.1 | 0.16 | 0.11 | 0.16 | 0.37 | 0.06 | 0.26 | 0.14
19 | 0.24 | 0.12 | 0.26 | 0.24 | 0.2 | 0.2 | 0.19 | 0.16 | 0.12 | 0.13 | 0.1 | 0.21
20 | 0.16 | 0.09 | 0.17 | 0.08 | 0.17 | 0.08 | 0.13 | 0.19 | 0.2 | 0.09 | 0.09 | 0.11
21 | 0.15 | 0.13 | 0.13 | 0.19 | 0.09 | 0.17 | 0.12 | 0.1 | 0.13 | 0.1 | 0.12 | 0.19
22 | 0.18 | 0.08 | 0.2 | 0.14 | 0.06 | 0.07 | 0.18 | 0.12 | 0.19 | 0.08 | 0.18 | 0.12
23 | 0.2 | 0.1 | 0.24 | 0.08 | 0.15 | 0.1 | 0.17 | 0.15 | 0.15 | 0.1 | 0.1 | 0.13
24 | 0.02 | 0.06 | 0.09 | 0.05 | 0.01 | 0.03 | 0.09 | 0.09 | 0.09 | 0.01 | 0.06 | 0.06
25 | 0.06 | 0.08 | 0.12 | 0.05 | 0.01 | 0.02 | 0.01 | 0.06 | 0.02 | 0 | 0.08 | 0.09
26 | 0.09 | 0.01 | 0.1 | 0 | 0.03 | 0.04 | 0.05 | 0.03 | 0.09 | 0.01 | 0.01 | 0.07
27 | 0.03 | 0.05 | 0.08 | 0.06 | 0.06 | 0.06 | 0.01 | 0.09 | 0.01 | 0.04 | 0.02 | 0.05 | 0.01
28 | 0.07 | 0.02 | 0.16 | 0.04 | 0.04 | 0.03 | 0.08 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02

p-value | 0.000013 | 0.023999 | 0.000038 | 7.47E-06 | 1.22E-07 | 1.01E-09 | 0.003672 | 0.000047 | 6.00E-06 | 0.0000123 | 8.00E-06 | 0.000669

1–23 – samples from thalassemia patients; 24–28 – samples from healthy controls. Data were from DNA sequencing of 12 CpG loci by mass spectrometry; t testing was performed for each locus and results showed statistically significant differences between patients and healthy controls (P<0.05).

Table 3: t test of 12 CpG loci in patients and healthy controls.

loci between patients and healthy subjects (P<0.05). These results further confirmed the findings in cluster analysis (Table 3).

Methylation-specific PCR (MSP)

The genomic DNA was extracted from the peripheral blood of 23 thalassemia patients and 5 healthy controls, followed by sulfite treatment. Methylation-specific PCR of the IGSF4 gene was performed to validate the results above. Our results revealed that the IGSF4 gene was highly methylated in thalassemia patients as compared with controls (Figure 3).

Real time PCR of IGSF4 gene

Real-time PCR was performed to amplify the IGSF4 gene. Our results showed the expression of IGSF4 gene was
markedly down-regulated in the peripheral blood of thalassemia patients when compared with that in normal cord blood and normal peripheral blood (ratio=0.18 and ratio<0.50, respectively) (Figures 4–6). This result suggests the expression of IGSF4 gene is significantly decreased in thalassemia patients as compared with healthy controls.

**Discussion**

Epigenetics refers to the study of phenotype (appearance) and characteristics of organisms in the absence of heritable changes in DNA sequence [14,15]. Thus, a genome contains 2 types of genetic information. One is the traditional genetic information that is derived from DNA and the other is epigenetic information which provides commands on when, how and where to apply the genetic information. There are many genes at the activated state and at the inactivated state at any one time, and the transcription of different genes occurs at different stages of development [16].

The MassARRAY®EpiTYPER™ DNA methylation assay integrates the base-specific cleavage reaction and MALDI-TOF to quantitate the DNA methylation. This method can be used to analyze the CpG at multiple layers, and is a preferable method for the quantitation of DNA methylation and identification of DNA methylation at any region of the genome or at any candidate gene. It has been confirmed that the methylation of IGSF4 gene may cause its inactivation, which is closely related to the occurrence and prognosis of lung cancer and pancreatic cancer. In normal bone marrow cells, the IGSF4 expression remains normal and its promoter is not methylated. However, the methylation of IGSF4 is consistently methylated in several leukemia cell lines [17]. Our group employed gene expression profiling to investigate the pathogenesis of thalassemia in terms of epigenetics; a total of 68 genes had changes at the mRNA level in thalassemia patients. In the present study, bioinformatics was applied. The GO and pathway of the molecule annotation system were used to screen differentially expressed genes. These genes are related to hematopoietic cells, signal transduction-associated cell surface receptor, apoptosis, and proliferation. These differentially expressed genes included previously reported genes such as IGSF4, PTPRC (CD45), CD3D, CSF1 and CSF2 [18,19], which are related to abnormal globin chain synthesis (Figure 7).
The mass spectrometric sequencing of DNA methylation was employed to compare the 12 CpG loci at the promoter of IGSF4 gene between thalassemia patients and healthy controls. Results showed the incidence of methylation of these 12 CpG loci in thalassemia patients was significantly higher than that in controls. Cluster analysis revealed the methylation degree of the promoter of the IGSF4 gene could effectively distinguish thalassemia patients from healthy controls. T testing was performed to analyze these 12 CpG loci in sequence, and results indicated there were marked differences in these 12 CpG loci between thalassemia patients and healthy subjects (P<0.05). These findings were consistent with those in cluster analysis. Therefore, we speculate the promoter of the IGSF4 gene is highly methylated in thalassemia patients, and the expression of the IGSF4 gene is dramatically down-regulated in these patients when compared with healthy controls. Evidence shows CD45 can regulate the expression of ε, δ and γ globins, but not directly modulate the mature α- and β-globins [20]. CD45 may act on the CSF through the T cell surface receptor, affecting the expressions of mature globins [21]. Our study showed the mRNA expressions of CSF1 and CSF2 were decreased but those of CD45 and CD3D increased.

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The regulation network of globin-related genes is shown in Figure 8.

**Conclusions**

Taken together, we speculate there is methylation of the promoter of the IGSF4 gene in thalassemia patients, which plays an important role in the occurrence of thalassemia. Thus, the IGSF gene is closely related to the occurrence and development of thalassemia and can be used as a molecular target in the clinical classification of thalassemia. In terms of epigenetics, our study provides evidence for the genotyping of thalassemia and provides a novel non-invasive method for the prenatal diagnosis and prediction of thalassemia using fetal DNA in the plasma of pregnant women.

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Conflict of interest

The authors declare no conflicts of interests.

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