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Original Article

Genomewide DNA Methylation Responses in Patients with β-Thalassemia Treated with Yisui Shengxue Granules (益髓生血颗粒)*

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ABSTRACT **Objective**: To examine the clinical effects of Yisui Shengxue Granules (益髓生血颗粒) in the treatment of β-thalassemia and explore its mechanism on DNA methylation levels. **Methods**: A randomized placebo-controlled double-blinded trial was conducted. Forty patients with β-thalassemia were recruited and distributed randomly by envelope method into an experimental group and a control group, 20 patients in each group. The patients were given Yisui Shengxue Granules in the experimental group and placebo in the control group (12 g/bag, 3 times a day) during a 3-month intervention. Before and after 1, 2, and 3 months of treatment, peripheral intravenous blood was sampled, and blood parameters such as hemoglobin (Hb), red blood cells (RBCs), reticulocytes (Ret), and fetal hemoglobin (HbF) were analyzed. Mononuclear cells from 5 patients, who showed an obvious treatment effect, were isolated by density gradient centrifugation. DNA methylation was analyzed using an Affymetrix USA GeneChip Human Promoter 1.0 Array and Input-promoter 1.0. **Results**: Compared with pre-treatment, there was an obvious increase in Hb and RBCs counts after 1, 2, and 3 months in the experiment group ($P<0.01$ or $P<0.05$). Meanwhile, HbF increased from the 2nd to the 3rd month ($P<0.05$). In the control group, Hb and RBCs showed no obvioas change. After 3-month treatment, DNA methylation results from 5 patients revealed that there were 24 hypomethylated genes and 3,685 hypermethylated genes compared with pre-treatment. Genes of insulin-like growth factor 1 receptor (IGF1R) and Janus kinase 3 (JAK3) revealed the most relations with other genes (degree: 21) and genes of 1-phosphatidylinositol-4, 5-bisphosphate phosphodiesterase gamma 2 (PLCG2) and mitogen-activated protein kinase 10 (MAPK10) showed a stronger intermediary role (betweenness centrality=0.04). **Conclusions**: JAK3 and MAPK10 are two key genes in bone marrow and the lymphatic system, and JAK3 is likely to be related to hematopoietic cytokines in the process of early hematopoiesis. (Registration No. NCT01549080)

KEYWORDS β-thalassemia, Yisui Shengxue Granule, Chinese medicine, DNA-methylation

β-thalassemia is known as a group of monogenic hereditary hematological diseases and results from deficient or zero synthesis of β-globin controlled by genes on chromosome 11.[1,2] The areas with high prevalence of β-thalassemia are the Mediterranean, Africa, Southeast Asia, and South China.[3] In China, β-thalassemia is the most prevalent in the Southeast, e.g., Guangdong and Guangxi, Hainan, Taiwan, and Hong Kong. The total annual incidence of symptomatic β-thalassemia individuals is estimated at 1 per 100,000 throughout the world. For β-thalassemia, the most common are gene mutations CD41-42(-CTTT), CD17(AAG-TAG), -28(A>G), and IVS-Ⅱ-654(C>T).[4,5]

Blood transfusion and iron chelation are traditional treatments of thalassemia, and others such as gene therapy and a bone marrow transplant are

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still not popular among the patients in China.[6-8] In recent years, scientists attempted to find the drugs reactivating γ-globin genes,[9] such as 5-azacytidine and hydroxyurea, which can reactivate the γ-globin gene, thus replacing the β-globin gene.

Yisui Shengxue Granules (YSSXG, 益髓生血颗粒), a traditional Chinese herbal medicine, has been used as an alternative therapy for thalassemias for 30 years. Our previous case studies have revealed that YSSXG activated γ-globin gene, formed HbF(α2γ2) and thus replace HbA(α2β2).[10-12] YSSXG can improve the anemia symptoms of thalassemia patients not by changing the genotype, but via modification of gene expression.

In this study, a randomized, double-blinded and placebo-controlled clinical trial was designed to examine the clinical effects, and a global DNA methylation assay was performed to analyze DNA methylation in 5 patients with a good response to treatment. We aimed to find the possible mechanism of action of YSSXG on DNA methylation levels, especially on chromosomes 11 and 16.

METHODS

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: (1) Patients received a diagnosis according to "Diagnosis and curative effect evaluation standard of hematopathy"[13] and "The principles of usage of the new Chinese herbs"[14] for Chinese medicine syndrome of Gan (Liver) and Shen (Kidney) yin deficiency, and insufficiency of blood and essence, (2) 3–40 years old, (3) no blood transfusion or use of anti-anemia medication in the past 6 months, (4) have signed consent to the treatment plan (minors signed the consent under supervision of their guardians).

Exclusion criteria included: (1) the presence of other primary diseases, (2) upper respiratory tract infection, (3) allergy to this experimental substance, (4) blood transfusion within 45 days, and (5) psychiatric diagnosis or pregnancy.

Study Design

A randomized double-blinded, placebo-controlled clinical trial (registration No. NCT01549080) was conducted with the approval of the Research Ethics Committee of Guang'anmen Hospital, China (2011, No.078) in accordance with the principles outlined in the Declaration of Helsinki. Sample size was estimated a total of 40 β-thalassemia patients assuming a relative risk of sample loss (no more than 20%) in the whole trial, an accrual period of 3 months treatment. Forty patients with β-thalassemia were allocated randomized by envelope method into 2 groups receiving either YSSXG or the placebo, and the allocation ratio was 1:1. Blinding design was as follows: all drugs must be repackaged and reallocated according to regulations of standard double-blind clinical trial. The encoded blinding data will be kept in Guang'anmen Hospital. When all patients had fulfilled the procedures of the trial, the data were record into electronic Case Report Forms (eCRFs) and locked, and then the blind was disclosed.

Drug and Reagents

YSSXG was produced by Guang'anmen Hospital according to the protocol described in a patent (No. CN1872182, batch No. 121171618), including 11 Chinese herbal medicinal components: *Fructus Corni*, *Prepared Radix polygoni multiflori*, *Radix Rehmanniae preparata*, *Radix Astragali*, *Radix Codonopsis*, *Radix Angelicae sinensis*, *Fructus Psoraleae*, *Collacorii Corii Asini*, *Caulis Spatholobi*, *Carapax Trionycis* and *Fructus Amomi*. An ultra-high-performance liquid chromatography analysis of YSSXG was carried out as previously reported[15-17] to guarantee good quality. Placebo contained dextrin and starch and was made by Guang'anmen Hospital. YSSXG and the placebo were packed in completely similar packages.

Grouping and Intervention

Forty β-thalassemia patients were randomized into either the experimental group or control group by envelope method, 20 patients in each group. Patients in the experimental group took YSSXG (No.20110602), 12 g/bag, granules were dissolved in warm water and taken orally following the instructions. Patients in the control group took the placebo(No. 20110519), and the use of the placebo was the same as that for YSSXG. The intervention period was 3 months.

Evaluation of Clinical Efficacy

Before treatment (baseline) and after 1 month (1st), 2 month (2nd), and 3 month (3rd) treatment, the parameters hemoglobin (Hb), red blood cells (RBCs), reticulocytes (Ret), and fetal hemoglobin (HbF) in peripheral blood were analyzed using a Cell Dyn 1711

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Table 1. Baseline Clinical Characteristics of the Two Groups

Group	Case	Age (Year, $\bar{x} \pm s$)	Male/female	Nationality (Han/Zhuang)	Hb (g/L)	RBC ($\times 10^{12}$/L)	Ret (%)	HbF (%)
Experiment	17	13.76 ± 9.05	12/5	8/9	64.24 ± 15.20	3.24 ± 0.73	5.31 ± 4.86	44.96 ± 23.26
Control	18	16.28 ± 9.63	11/7	7/11	70.06 ± 12.47	3.36 ± 0.54	5.19 ± 3.22	57.43 ± 25.23

automatic blood analyzer (USA). An increase in Hb concentration to more than 5 g/L was regarded as effectiveness of treatment. Increase in Ret alone was not regarded as a good response to treatment.[13]

DNA Methylation

This parameter was analyzed in 10 blood samples before and after treatment in 5 patients. Venous blood samples (5 mL) were collected into heparinized tubes before and after treatment. Then blood mononuclear cells were isolated by density gradient centrifugation (500 × *g*, 10 min).

DNA methylation was analyzed using an Affymetrix USA GeneChip Human Promoter 1.0 Array and Input-promoter 1.0 according to the manufacturer's protocol (http://www.affymetrix.com/). Briefly, peripheral blood mononuclear cells from β-thalassemia patients were collected before and after 3-month treatment. A DNA target was polymerase chain reaction-amplified, fragmented, and end-labeled. After hybridization, washing, and staining, the chip was scanned according to the instructions. Gene Ontology (GO) analysis from GO database, Pathway analysis from the Kyoto encyclopedia of genes and genomes (KEGG) database, and signal-net analysis from the KEGG database were used to analyze the DNA methylation data.[18]

Data Analysis

Data on the blood parameters of Hb, RBC, Ret, and HbF were analyzed in SPSS 17.0, and descriptive data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Comparisons before and after treatment were conducted by the paired *t* test. Differences with a probability value of less than 0.05 were considered statistically significant. Statistical significance of the differences between values of methylation levels for different samples/groups was assessed by Fisher's test and multiple-comparison test with $P<0.05$, and false discovery rate (FDR) <0.05 as a filtering cutoff point.[19-21]

RESULTS

Patient's Characteristics

There were 25 males and 15 females, ages of the patients ranged from 4 to 34 years old (14.65 ± 8.99). The baseline clinical characteristics of the 2 groups, including gender as well as Hb, RBC, Ret, and HbF levels are presented in Table 1.

Consort flow diagram is presented in Figure 1. After the whole course of treatment (or placebo) was finished, there were 2 patients who had transfusion during the trial and 1 patient did not took the granules in the experiment group (17 cases); 1 patient had transfusion and 1 patient discontinued the intervention in the control group (18 cases).

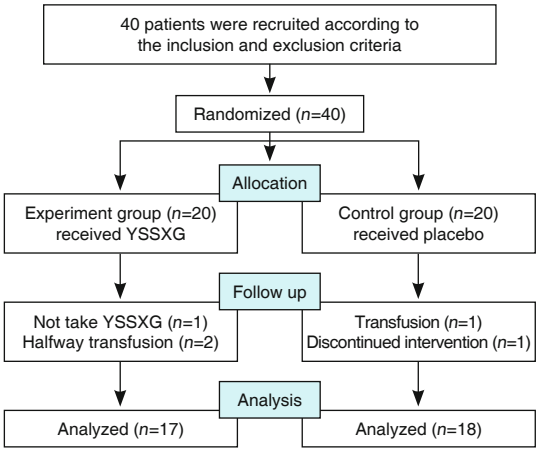

Figure 1. Consort Flow Diagram of YSSXG for Treatment of β-Thalassemia Patients

Analysis of Clinical Blood Parameters

The changes of blood parameters are presented in Table 2. In the experimental group, since the 1st month, the parameters Hb and RBC increased, and HbF started rising from the 2nd month compared with pre-treatment ($P<0.05$ or $P<0.01$). In the control group, HbF showed an increase in the 2nd month ($P<0.05$). There were no statistically significant differences between the 2 groups ($P>0.05$).

Clinically Related Parameters of 5 Patients Subjected to DNA Methylation Analysis

Basic Information of 5 patients with β-thalassemia is presented in Table 3. Peripheral blood parameters Hb, RBC, Ret, and HbF of the 5 patients subjected to DNA methylation analysis were

Table 2. Comparison of Hb, RBC, Ret and HbF Levels before and after Treatment ($\bar{x} \pm s$)

Group	Case	Time (month)	Hb (g/L)	RBC ($\times 10^{12}$/L)	Ret (%)	HbF (%)
Experiment	17	0	64.24 ± 15.20	3.24 ± 0.73	5.31 ± 4.86	44.96 ± 23.26
		1st	70.53 ± 15.47**	3.58 ± 0.82**	4.65 ± 2.93	45.35 ± 21.27
		2nd	70.82 ± 17.18**	3.57 ± 0.92*	5.22 ± 3.41	51.81 ± 22.63**
		3rd	71.35 ± 17.04**	3.80 ± 0.72**	5.39 ± 3.17	52.01 ± 20.01*
Control	18	0	70.06 ± 12.47	3.36 ± 0.54	5.19 ± 3.22	57.43 ± 25.23
		1st	70.22 ± 12.80	3.44 ± 0.57	4.79 ± 2.90	54.64 ± 22.30
		2nd	67.61 ± 14.85	3.35 ± 0.60	4.83 ± 3.41	61.73 ± 24.96*
		3rd	68.50 ± 13.97	3.50 ± 0.45	4.84 ± 3.88	57.93 ± 21.51

Note: *P<0.05, **P<0.01, compared with pre-treatment

Table 3. Basic Information of 5 Patients with β-Thalassemia

No.	Gender	Age (Year)	Nationality	Genotype	Hb (g/L)	RBC ($\times 10^{12}$/L)	Ret (%)	HbF (%)
1	Male	23	Han	β 41-41/β IVS-I-1	79	3.98	2.2	86.0
2	Female	6	Yao	β 41-41/β 17	41	1.92	5.2	20.1
3	Male	8	Han	β -28/β -28	70	3.41	4.1	53.1
4	Female	25	Zhuang	β 43/β E	70	3.8	1.6	31.5
5	Male	6	Han	β 654/β E	60	3.42	1.8	48.1

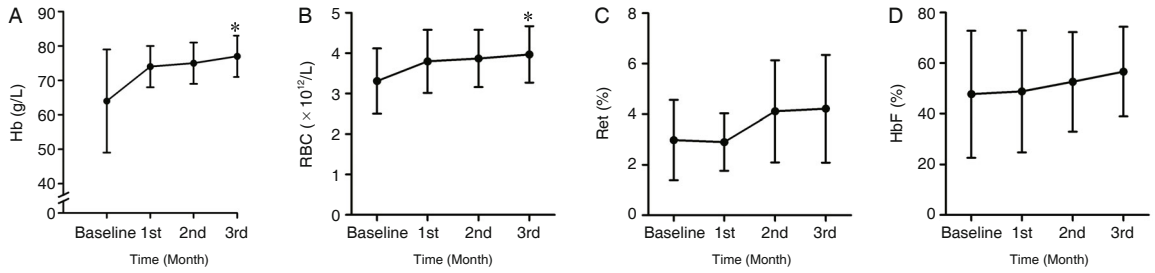

Figure 2. Related Blood Parameters of the 5 Patients

Note: *P<0.05, compared with pretreatment

studied in detail (Figure 2). Hb level after 3 months treatment showed an obvious improvement (P<0.05). HbF showed a rising tendency but no statistical significance (P=0.35).

Genomewide DNA Methylation

After 3-month treatment, there were 24 hypomethylated genes and 3,685 hypermethylated genes as compared with pre-treatment. The hypermethylated genes were XPA, ASL, AC0685337, MRPS5, and SCGN, and the hypomethylated genes were PDGFB, PPP1R1B, NAA38, CHAMP1, SORBS2, and SLC16A1.

According to GO, functions of the genes with hypermethylation include transcription, DNA-dependent and small-molecule metabolic process, whereas functions of the genes showing hypomethylation include substructure-dependent cell migration and blood coagulation Ⅰ (Table 4). Through the pathway analysis, we found that the pathways affected by hypermethylation included metabolic pathways and endocytosis, whereas pathways affected by hypomethylation after treatment were cancer, transcriptional misregulation in cancer, and others (Table 5).

Besides, after the GO analysis and pathway analysis, we conducted a signal-net analysis, and found that 2 genes, IGF1R and JAK3, had the most numerous functional connections with other genes (degree: 21). PLCG2 and MAPK10 genes showed a stronger intermediary role (Table 6).

DISCUSSION

β-Thalassemia is caused by the absent or reduced synthesis of beta globin chains. β globin gene maps in the short arm of chromosome 11, a region also containing fetal A-gamma and G-gamma genes. Fetal hemoglobin genes expressed during fetal period, and replaced by beta hemoglobin gene after birth. Now reactivation of fetal γ globin is appealing as a therapeutic

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Table 4. Parts of Genes Functions with Hypermethylation and Hypomethylation after Treatment

GO Id	GO name	GO diffgene count	*P* value	FDR
Hypermethylation				
GO:0006351	Transcription, DNA-dependent	340	3.05791E-47	1.50296E-43
GO:0044281	Small molecule metabolic process	253	1.21724E-34	2.99138E-31
GO:0006355	Regulation of transcription, DNA-dependent	230	3.95402E-28	6.47800E-25
GO:0045944	Positive regulation of transcription from RNA polymerase II Promoter	148	9.08687E-26	1.11655E-22
GO:0010467	Gene expression	135	3.62516E-22	2.78637E-19
GO:0007165	Signal transduction	181	3.96838E-22	2.78637E-19
GO:0006915	Apoptotic process	132	1.22632E-21	7.53417E-19
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	112	1.46458E-21	7.99823E-19
GO:0015031	Protein transport	92	3.68698E-20	1.64741E-17
GO:0055085	Transmembrane transport	109	5.23725E-18	1.98008E-15
Hypomethylation				
GO:0006929	Substrate-dependent cell migration	2	1.051E-05	0.0013025
GO:0007596	Blood coagulation	5	1.506E-05	0.0013025
GO:0031954	Positive regulation of protein autophosphorylation	2	6.919E-05	0.0039901
GO:0043536	Positive regulation of blood vessel endothelial cell migration	2	0.0001424	0.0061567
GO:0030168	Platelet activation	3	0.0006553	0.0161965
GO:0051781	Positive regulation of cell division	2	0.0010734	0.0213416
GO:0016049	Cell growth	2	0.0013202	0.0213416
GO:2000194	Regulation of female gonad development	1	0.0020972	0.0213416
GO:0006468	Protein phosphorylation	3	0.0028642	0.0250149
GO:0002576	Platelet degranulation	2	0.0033228	0.0250149

Notes: GO Id: gene ID number in Gene Ontology database; GO name: category of function of gene; diffgene count: number of gene methylated; *P* value: $P<0.01$ indicating significant methylation

Table 5. Pathways with Hypermethylation and Hypomethylation after Treatment

Path Id	Path name	Path diffgene count	*P* value	FDR
Hypermethylation				
1100	Metabolic pathways	214	1.692E-27	4.400E-25
4144	Endocytosis	58	7.342E-17	9.544E-15
5200	Pathways in cancer	69	1.107E-12	9.594E-11
4080	Neuroactive ligand-receptor interaction	57	2.605E-10	1.354E-08
4510	Focal adhesion	47	4.055E-10	1.576E-08
4514	Cell adhesion molecules	38	4.243E-10	1.576E-08
4010	MAPK signaling pathway	54	7.545E-10	2.452E-08
4151	PI3K-Akt signaling pathway	65	1.411E-09	4.077E-08
4020	Calcium signaling pathway	41	1.087E-08	2.356E-07
4060	Cytokine-cytokine receptor interaction	52	2.813E-08	5.627E-07
Hypomethylation				
5200	Pathways in cancer	3	0.0026025	0.0754736
5202	Transcriptional misregulation in cancer	2	0.0160094	0.2011683
4510	Focal adhesion	2	0.0208105	0.2011683
4060	Cytokine-cytokine receptor interaction	2	0.0343277	0.2488759

Notes: Path Id: gene ID number in KEGG database

Table 6. IGF1R/JAK3/PLCG2/MAPK10 in Signal-Net Analysis

Gene symbol	Betweenness centrality	Degree	Indegree	Outdegree	Style
PLCG2	0.0442117	11	8	6	up
MAPK10	0.0402092	14	10	5	up
IGF1R	0.0382059	21	14	11	up
JAK3	0.0251484	21	18	3	up

Notes: Gene symbol: gene name; betweenness centrality: the mediation of signal transmission; degree: number of genes to one specific gene in signal net; indegree: gene number in upstream of one gene; outdegree: gene number downstream of one gene; style: state of genes

approach to β-thalassemias. DNA methylation is an epigenetic modification and changes at the β globin locus and has a switch from fetal to adult hemoglobin. Lessard, et al[22] found that DNA methylation may play a important role on synthesis of HbF through BCL11A. Chu, et al[23] indicated that YSSXG had an inhibition influence on activity of DNA methyltransferase. Based on the β-globin locus has no CpG islands, we conducted the genomewide DNA methylation analysis to identify the mechanism of action of YSSXG.

Blood parameters from the clinical trial showed that

YSSXG increased Hb, RBC, and HbF of β-thalassemia patients in experimental group. Other studies have shown that YSSXG could prolong the time interval between transfusions for β-thalassemia patients in a clinical trial.[24,25] But our study showed that there was no similar rising tendency of Hb, RBC and HbF among different individuals. And this may be related with the different genotypes as well as content of Hb before treatment. DNA methylation results found that compared with pre-treatment, genes LGR4 and PP11-159H22.2 on chromosome 11 as well as genes CCDC101, NUPR1, and RP11-666O2.1 on chromosome 16 were hypomethylated. Using genomewide DNA methylation, we could find the relevant targets and pathways more easily. JAK3 and MAPK10 were found to be the two key genes during the treatment, and in another study, Rane, et al[26] has shown that JAK3 might perform an important function in the early hematopoietic process, especially in the expression of some hematopoiesis-related cell factors (e.g., SCF and G-SCF). Goren, et al[27] found that demethylation might be an obligatory step in the molecular mechanisms that bring about the abnormal expression of γ globin seen in hereditary persistence of fetal hemoglobin and may explain the HbF rising from the 2nd month with a statistical significance compared with pre-treatment.

As for the possible mechanism of action, in our previous work we studied this topic at different levels, such as genes, proteins, and cells.[28-31] DNA methylation is an important parameter in epigenetics and plays an important role in regulation of gene expression.[32,33] YSSXG does not change the patients' genotype, and studies have shown that DNA methylation was important for globin gene expression.[34,35] This is the first attempt to study the mechanism of action of YSSXG on thalassemia at the epigenetic level, and we obtained preliminary data on the possible relevant genes. Further in-depth research and validation studies will be needed in the future.

There were no statistically significant differences in clinical blood parameters between the experimental and control groups, and we chose 5 β-thalassemia patients to conduct DNA methylation analysis, and a big sample size should be used in the future. We studied only β-thalassemia, and it remains unclear whether YSSXG has any influence on DNA methylation of α-thalassemia. Epigenetics change has been considered the most promising new strategy for disease control and prevention. Searching for new drugs from Chinese medicine can be a new thinking for the treatment of β-thalassemia.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

Cheng YL and Wu ZK conceived and designed the experiments; Cheng YL and Zhang XH performed the experiments, and contributed significantly to analysis and manuscript preparation; Wang WJ, Huang J, Chu NL and Fang SP collected the blood samples; Sun YW perform the quality control on experimental drugs.

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