Research Article

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Effect of He Qi San on DNA Methylation in Type 2 Diabetes Mellitus Patients with Phlegm-blood Stasis Syndrome

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Abstract: This study was performed to elucidate the potential influence of He Qi San (HQS) on glucose and lipid metabolism in type 2 diabetes mellitus (T2DM) patients with phlegm-blood stasis syndrome (PBSS), and to determine DNA methylation changes. Sixty T2DM patients with PBSS were randomly divided into control and HQS groups. The control group received conventional treatments, and the HQS group received conventional treatments plus HQS. Glucose metabolism (FPG, 2hPG, FINS, and HbA1c) and lipid metabolism indexes (TG, TC and LDL-C) were determined. Genes with differential DNA methylation were subjected to GO and KEGG analyses. Glucose and lipid metabolism indexes in both groups were reduced, but were much more pronounced in the HQS group. Differential promoter CpG methylation regions were identified in 682 genes, including 426 genes with high-CpG promoters, 150 genes with intermediate CpG promoters, and 106 genes with low CpG promoters. Genes with differential DNA methylation were mainly enriched in the AMPK and insulin signaling pathways, terpenoid backbone biosynthesis, and renin secretion. We concluded that HQS remarkably improved indexes of glucose and lipid metabolism in T2DM patients with PBSS through regulating the DNA methylation of genes in

the AMPK and insulin signaling pathways and terpenoid backbone biosynthesis.

Keywords: He Qi San; T2DM; DNA methylation

1 Introduction

Diabetes is a metabolic disease characterized by persistent elevation of the blood glucose level. Thus far, diabetes is the chronic non-infectious disease that exerts the greatest impact on human health [1]. The International Diabetes Federation Diabetes Atlas, eighth edition in 2017, suggested that China had the largest number, with 114 million diabetes patients [2]. Thus, there is an urgent need for effective prevention and treatment of diabetes.

Traditional Chinese medicine (TCM) presents great efficacy and distinctive treatment characteristics in the long-term prevention and treatment of diabetes [3]. He Qi San (HQS) is an efficacious prescription that is used by the Endocrinology Department, Shenzhen TCM Hospital on the basis of years of clinical experience [4]. Previous studies in our laboratory have indicated that HQS is capable of reducing body weight and decreasing the appetite of type 2 diabetes mellitus (T2DM) patients accompanied by obesity [5, 6]. It also alleviates insulin and leptin resistance in patients with metabolic syndrome, corrects chronic inflammatory states, and decreases peripheral insulin sensitivity [6, 7]. HQS also has a strong effect on pre-diabetes, but its specific molecular mechanism remains unclear.

The level of DNA methylation directly influences the occurrence and progression of diabetes [8, 9]. Abnormal methylation of insulin resistance-related genes occurs in the visceral adipose tissues of obese patients with T2DM, which is closely related to the pathogenesis of morbid obesity in T2DM [10]. DNA methylation modification in important gene expression in the respiratory chain affects

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mitochondrial oxidative metabolism and ATP production, thus influencing islet β -cells to secrete insulin [11].

With the deepening understanding of chromatin modification in complex diseases, explorations of epigenetic information hiding in genomes and proteins are the frontier of life sciences. Methylation array analysis is currently the most cost-effective method to generate a comprehensive genome-wide profiling of human DNA methylation in the entire genome. It is used for finding and analyzing unknown methylation sites, and detecting the level of DNA methylation by calculating the probe signaling intensity of methylated and unmethylated sites.

In this study, we aimed to analyze the therapeutic effect of HQS and determine its mechanism for improving glucose metabolism and lipid metabolism in T2DM patients with phlegm-blood stasis syndrome. Methylation array analysis was then used to provide novel directions for uncovering the therapeutic targets and potential mechanism of HQS for the treatment of T2DM.

2 Methods

2.1 Subjects

A total of 60 eligible T2DM patients with phlegm-blood stasis syndrome were admitted to the Department of Endocrinology, Shenzhen Traditional Chinese Medicine Hospital from March 2017 to September 2017. Patients were randomly divided into a control group ($n=30$) or HQS group ($n=30$). Patients in control group were administrated by suitable therapeutic strategy to control the blood glucose and lipid levels. While patients in HQS group were added by HQS basis on the treatment of control group. There were 18 male patients and 12 female patients enrolled in the control group, with the age of 57.37 ± 5.20 years and BMI of $23.31 \pm 1.75 \text{ kg} \cdot \text{m}^{-2}$. In the HQS group, 16 male patients and 14 female patients were included with the age of 55.67 ± 5.43 years and BMI of $24.09 \pm 1.87 \text{ kg} \cdot \text{m}^{-2}$. The details are shown in Supplemental Materials Table S1. No significant differences in gender, age, BMI, subtypes of diabetes and other baseline characteristics were observed in T2DM patients between the two groups ($P > 0.05$).

The diagnostic criteria for T2DM patients with phlegm-blood stasis syndrome were as follows: 1) patients were diagnosed with T2DM according to a previously published reference [12]; 2) T2DM patients were diagnosed with phlegm-blood stasis syndrome according to Guidelines for the Clinical Research of Chinese Medicine New Drugs, 2002 and Phase Dialectics of Traditional Chinese Medicine

and Evaluation Criteria for Diabetes Mellitus, 1992: symptoms of fatigue and lack of strength, chest tightness and blockage of the stomach duct, sharp pain in the chest, numbness and pain of limbs, tiredness and obesity, loose stool. Tongue diagnosis revealed that the tongue was dark red and purple with ecchymosis, sublingual venous enlargement, imprints of the teeth at the edge of the tongue, thick and greasy furrow of the tongue, and wiry and rolling pulse.

Inclusive criteria of subjects were applied: 1) 30-60 years old; 2) patients were diagnosed with T2DM with phlegm-blood stasis syndrome by two associate chief physicians; and 3) patients volunteered to participate in this experiment and gave their informed consent.

Exclusive criteria of subjects were applied: 1) patients had acute complications of diabetes mellitus; 2) patients were accompanied by severe cardiovascular, cerebrovascular, respiratory, or blood system diseases; tumors; or severe damage to liver and kidney function; 3) pregnancy or lactating women; 4) increased blood glucose caused by other primary diseases; and 5) patients with poor adherence, disturbance of consciousness, or refusal of informed consent.

Elimination from the study and termination criteria were applied for: 1) patients who failed to complete the prescribed course of treatment; 2) allergies, abnormal safety indicators, or adverse reactions that occurred during the experiment, and the experiment was terminated according to the assessment; 3) disease aggravation or other serious diseases that occurred during the experiment; 4) significant deviations in the protocol that led to an inability to evaluate drug efficacy; and 5) active withdrawal from the experiment and loss of follow-up.

During the treatment, the patients followed the diabetes dietary principles: proper intake of total calories, carbohydrates, protein, and fat; appropriate intake of food rich in dietary fiber; regular diet, regular and quantitative meals, and attention to the order of meals.

The above experimental procedures were evaluated and approved by the Medical Ethics Review Committee, Shenzhen Traditional Chinese Medicine Hospital (No. [2016] 6).

2.2 Drugs and reagents

The 10 ingredients, including He-Ye, Huang-Qi, Jue-Ming-Zi, Zhi-He-Shou-Wu, and Shi-Chang-Pu, and their dosages are shown in Table 1. Preparation of the traditional Chinese medicine was authorized to Kangmei Pharma. Co., Ltd.

Table 1: The ingredients in HeQi San.

Chinese name	Botanical name	Herbal name	Dosage
He-Ye	<i>Nelumbo nucifera</i> Gaertn.	Nelumbinis Folium	30 g
Huang-Qi	<i>Astragalus membranaceus</i> (Fisch.) Bge. var. <i>mongholicus</i> (Bge.) Hsiao	Astragali Radix	15 g
Zhi-He-Shou-Wu	<i>Polygonum multiflorum</i> Thunb.	Polygoni Multiflori Radix Praeparata	15 g
Shi-Chang-Pu	<i>Acorus tatarinowii</i> Schott	Acori Tatarinowii Rhizoma	15 g
Cang-Zhu	<i>Atractylodes lancea</i> (Thunb.) DC.	Atractylodis Rhizoma	10 g
Shan-Yao	<i>Dioscorea opposita</i> Thunb.	Dioscoreae Rhizoma	15 g
Dong-Gua-Pi	<i>Benincasa hispida</i> (Thunb.) Cogn.	Benincasae Exocarpium	15 g
Ze-Lan	<i>Lycopus lucidus</i> Turcz. var. <i>hirtus</i> Regel	Lycopi Herba	15 g
Jue-Ming-Zi	<i>Cassia obtusifolia</i> L.	Cassiae Semen	30 g
Gan-Cao	<i>Glycyrrhiza uralensis</i> Fisch.	Glycyrrhizae Radix et Rhizoma	5 g

Fasting plasma glucose (FPG), 2-hour postprandial blood glucose (2hPG), triglyceride (TG), total cholesterol (TC), and low density lipoprotein-cholesterol (LDL-C) determination kits were provided by Roche, Germany; the fasting insulin (FINS) chemiluminescence kit was provided by Beckman Coulter, USA. Reagents used for the determination of HbA1c were U1tra2 original eluent, controls, and consumables provided by Primus, USA.

2.3 Instruments

A low-speed auto balancing centrifuge (LABER, Beijing), high-speed tabletop centrifuge (Beckman, USA), and an ultra-low temperature freezer (THERMO FORMA, USA) were used. Additionally, a Primus Ultra2 affinity chromatography high-performance liquid chromatography (HPLC) detector (Primus, USA), Cobas 8000 automatic biochemical analyzer (Roche, Germany), an ARCHITECT i2000SR automatic immunoassay system (Abbot, USA), and a UniCel DxI800 immunoassay system (Beckman, USA) were used for experiments.

2.4 Therapeutic strategies

Basic therapeutic strategies were applied in the control group, including diabetes health education, diabetic diet, regular exercise, psychological counseling, and individual drug treatment. On the basis of therapeutic strategies applied in the control group, patients in the HQS group were given a total of 180 ml of HQS decoction

per day, of which, 90 ml was orally taken twice per day. The treatment duration was 3 months.

2.5 Determination of blood biochemical indexes

FPG, 2hPG, TC, TG, and LDL-C were determined using the glucose oxidase method. FINS was determined using a chemiluminescent immunoassay, and HbA1c was determined by HPLC.

2.6 Methylation array analysis

After the entire duration of treatment, one female and two male patients were selected from each group, and these patients are labeled in red color in Table S1. For 8 h fasting, elbow venous blood was collected into parallel dry vacuum tubes at 7:00 to 8:00 a.m. the next day, and divided into 300-500 μ L per tube. Blood samples were cold-chain transported to the Medical Laboratory Center, Guangzhou Huayin. Analysis was performed with an Arraystar Human RefSeq Promoter Microarray (4 $\times$ 150 k) to detect apparent methylation and transcription factor binding sites in the RefSeq gene promoter region, covering 23,148 gene promoter regions with approximately 180,000 probes at a 210-bp gap.

Table 2: Blood glucose levels before and after treatment in each group ($\bar{x} \pm s$, $n=30$).

Groups	n	Treatment duration	FPG (mmol·L ⁻¹)	2hPG (mmol·L ⁻¹)	FINS (mU·L ⁻¹)	HbA1c (%)
Control group	30	Pre-treatment	11.80±1.70	16.41±2.09	24.57±5.14	9.51±1.50
		Post-treatment	7.19±1.27 ^{△△}	9.68±1.11 ^{△△}	14.80±5.08 ^{△△}	7.30±0.74 ^{△△}
HQS group	30	Pre-treatment	11.39±1.38	16.38±1.62	24.75±4.81	9.58±1.49
		Post-treatment	6.41±1.04 ^{△△*}	8.47±0.81 ^{△△**}	9.96±4.05 ^{△△**}	5.82±0.49 ^{△△**}

Note: Compared to pre-treatment, ^{△△} $P<0.01$; compared to post-treatment in the control group. * $P<0.05$, ** $P<0.01$.

Table 3: Blood lipid levels before and after treatment in each group ($\bar{x} \pm s$, $n=30$).

Groups	n	Treatment duration	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)
Control group	30	Pre-treatment	9.32±3.99	6.36±2.62	3.37±0.98
		Post-treatment	5.95±2.11 ^{△△}	4.40±1.40 ^{△△}	2.52±0.75 ^{△△}
HQS group	30	Pre-treatment	9.66±3.45	6.68±2.61	3.82±1.09
		Post-treatment	4.79±1.17 ^{△△*}	3.63±1.53 ^{△△*}	2.14±0.51 ^{△△}

Note: ^{△△} $P<0.01$ vs. pre-treatment level; * $P<0.05$, ** $P<0.01$ vs. post-treatment level in the control group.

2.7 Bioinformatics analysis

Molecular functions, biological processes, and cellular components of genes with significant differences in DNA methylation were analyzed in the gene ontology (GO) online database. Signaling pathways were determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and the results were subjected to Fisher's exact test. Differential genes were subjected to enrichment analysis using the DAVID database (<https://da-vid.ncifcrf.gov/home.jsp>).

2.8 Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. The measurement data are expressed as the mean $\pm$ standard deviation ($\bar{x} \pm s$), and the count data are expressed by the composition ratio. The t test was used to compare the inter paired differences. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Decreased blood glucose levels

As shown in Table 2, compared to the control group, the blood glucose indexes including FPG, 2hPG, FINS, and

HbA1c were not significantly different in the HSQ group before treatment. After treatment, blood glucose indexes including FPG, 2hPG, FINS, and HbA1c were significantly reduced in both groups ($P<0.01$). Moreover, compared to the control group, the levels of FPG, 2hPG, FINS, and HbA1cd decreased to 6.41±1.04 mmol/L, 8.47±0.81 mmol/L, 9.96±4.05 mU/L, and 5.82±0.49%, respectively, which were more pronounced in the HSQ group ($P<0.05$, $P<0.01$, $P<0.01$, and $P<0.01$, respectively).

3.2 Decreased blood lipid levels

As shown in Table 3, there were no significant differences in blood TG, TC, and LDL-C between the control and HQS group before treatment ($P>0.05$). After treatment, blood lipid indexes TG, TC, and LDL-C were significantly reduced in both groups ($P<0.01$). Compared to the control group, blood TG (4.79±1.17 mmol/L) and TC (3.63±1.53 mmol/L) decreased more significantly in the HQS group after treatment. No significant difference was observed in LDL-C (2.14±0.51 mmol/L).

3.3 DNA methylation distribution of the CpG island in the gene promoter region

Compared to the control group after treatment, differential DNA methylation distributions in the HQS group are depicted in Table 4. Differential promoter CpG methylation

Table 4: DNA methylation distribution of CpG in the gene promoter.

Chromatin	TCP	HCP	ICP	LCP
chr1	50	30	13	7
chr2	42	28	7	7
chr3	32	20	6	6
chr4	26	21	2	3
chr5	31	25	4	2
chr6	32	21	8	3
chr7	32	19	6	7
chr8	32	19	7	6
chr9	37	31	2	4
chr10	13	7	5	1
chr11	53	26	10	17
chr12	29	21	5	3
chr13	8	6	2	0
chr14	22	13	8	1
chr15	25	16	6	3
chr16	39	23	10	6
chr17	47	31	9	7
chr18	7	7	0	0
chr19	42	22	14	6
chr20	26	15	8	3
chr21	10	2	2	6
chr22	27	17	6	4
X	15	6	7	2
Y	5	0	3	2
Total	682	426	150	106

Note: TCP, total CpG promoters; HCP, high-CpG promoters; ICP, intermediate CpG promoters; LCP, low CpG promoters.

regions were identified in a total of 682 genes, including 426 genes with high-CpG, 150 genes with intermediate CpG, and 106 genes with low CpG.

3.4 GO analysis of genes with differential DNA methylation

Compared to the control group, genes with differential DNA methylation in the HQS group were divided into molecular functions, biological processes, and cellular

components by GO analysis. The ten most significant differential GO items are listed in Figure 1. A total of 725 differential GO items were mainly enriched in regulation of polymer localization, protein degradation, cellular and protein catabolism, cellular distribution, and macromolecular localization.

3.5 KEGG analysis of genes with differential DNA methylation

As illustrated in Figure 2, compared to the control group, genes with differential DNA methylation in the HQS group after treatment were significantly enriched in the Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway, insulin signaling pathway, terpenoid backbone biosynthesis, and renin secretion.

4 Discussion

T2DM is characterized by insulin resistance with a relative or absolute decline of pancreatic islet function. There has been increasing incidence of T2DM worldwide, and the subsequent incidences of diabetic vascular complications are the leading causes of high disability and mortality of T2DM. Hence, it is urgent to develop effective methods for the prevention and treatment of T2DM, as well as alleviation of its complications.

HQS is a TCM that has been verified by strict drug compatibility procedures and long-term clinical observation in the Department of Endocrinology, Shenzhen TCM Hospital. HQS is believed to be an effective medicine that promotes metabolism, accelerates Qi circulation, eliminates phlegm, and removes dampness[6]. These functions are attributed to the main components of HQS, which are He-Ye, Huang-Qi, and Jue-Ming-Zi. He-Ye has the vital functions of removing dampness, dissipating blood stasis, and eliminating phlegm. Modern pharmacological studies found that lotus leaves have multiple physiological effects, such as lipid-lowering, weight-loss, anti-free radical, anti-oxidation, anti-aging, anti-bacterial, and anti-viral effects [13]. Astragalus root is a famous qi and spleen tonic with a diuretic effect. Huang-Qi is also capable of lowering blood glucose levels. Wu et al. [14] pointed out that astragalus stimulates the tyrosine phosphorylation of insulin and its substrate by inhibiting the PTP1B level, thus remarkably decreasing the blood glucose level in diabetic rats. Jue-Ming-Zi excels

Figure 1: GO analysis of genes with differential DNA methylation.

Figure 2: KEGG analysis of genes with differential DNA methylation.

at removing dampness, eliminating phlegm, and exerts a laxative effect. Zheng et al. [15] suggested that cassia seed extracts improve the peroxidation state in the lens of STZ-

induced diabetic mice through scavenging free radicals and inhibiting lipid peroxidation.

T2DM patients with phlegm-blood stasis syndrome are generally obese, accompanied by insulin resistance, hyperglycemia, and hyperlipemia. These patients are at risk for diabetic vascular complications, leading to poor prognosis. HQS is good at ascending the clear and descending the turbid [4]. It burns excess fat, eliminates constipation, and induces diuresis. Hence, syndrome differentiation therapy using HQS is a good choice for T2DM patients with phlegm-blood stasis syndrome. In this study, HQS improved the indexes of both glucose metabolism (FPG, 2hPG, FINS, and HbA1c) and lipid metabolism (TG, TC, and LDL-C) to a greater extent compared with those in control group. Subsequently, we mainly focused on DNA methylation changes between the control group and HQS group.

DNA methylation is one of the important mechanisms underlying epigenetic regulation. S-Adenosylmethionine serves as a donor and is catalyzed by DNA methyltransferase. 5-Methylcytosine occurs at the

same 5' position on the pyrimidine ring where the DNA base thymine's methyl group is located. CpG is mainly located in the promoter and the first exon region. CpG-rich sequences are termed CpG islands, which is the main site of methylation of human genes. In most cases, CpG is in a non-methylated state, whereas free CpG dinucleotides are often in a methylated state. The methylation level of CpG plays a key role in maintaining cell development and tissue stability. Hypermethylation of DNA often leads to gene silencing and maintenance of chromosome stability. Once DNA is hypomethylated, gene activity and expression increase, and chromosomes are unstable. The balance of DNA methylation is of significance in the cell cycle, apoptosis, DNA damage and repair, and other processes [16].

A large number of studies have shown that DNA methylation abnormalities are crucial in the occurrence and progression of many diseases, including malignant tumors, autoimmune diseases, metabolic diseases, schizophrenia, and mental retardation [17, 18]. They are also important in the development of aging. With in-depth studies on epigenomics, abnormal hypermethylation or hypomethylation changes in specific gene promoter regions are considered to be closely related to disease development.

Accumulating evidence indicates that environmental factors and genetic variations can influence the level of DNA methylation, which in turn mediates the activities and expression of related genes [19]. These alterations are capable of inducing abnormal insulin secretion by islet β -cells, changing insulin tolerance, and ultimately leading to the occurrence of T2DM [20]. Therefore, it is of theoretical and clinical significance to uncover the role of DNA methylation in the occurrence and progression of T2DM. It also further provides novel probabilities and directions for developing individualized treatments for T2DM.

In this study, 682 differential methylation regions were identified in the promoter region, including 426 hypermethylation and 106 hypomethylation sites. GO analysis revealed that genes in these differential regions were closely related to molecular and cellular localization, and protein catabolism. Furthermore, KEGG analysis indicated that these genes were mainly involved in the AMPK signaling pathway, insulin signaling pathway, terpenoid backbone biosynthesis, and renin secretion.

AMPK is the key molecule responsible for regulating energy metabolism in the body. The AMPK signaling pathway is well studied in glycometabolism and lipid metabolism, which are necessary for maintaining glucose metabolism in the body. Currently, there is considerable

activity in T2DM research involving the development of drug targets based on the AMPK signaling pathway and its agonists [21, 22]. In addition, the active ingredients of many traditional Chinese medicines targeting the AMPK signaling pathway, such as terpenoids [23, 24], phenols [25], alkaloids [26, 27], and flavonoids [28, 29] have presented promising clinical application in T2DM treatment.

Islet β -cells in the pancreas secrete insulin under the internal or external stimulation of chemical substances. Insulin activates the PI3K/Akt signaling pathway and participates in crucial cellular physiological processes, such as the regulation of glucose homeostasis and lipid metabolism. Insulin is the only hormone that decreases the blood glucose level in the body [30]. Some studies indicate that abnormality in the PI3K/Akt signaling pathway is the most fundamental cause of diabetes, and it has been proposed that development of diabetes drugs should focus on this pathway [31-33]. The monomer composition of many traditional Chinese medicines is able to regulate diabetes metabolism through the PI3K/Akt signaling pathway, including seabuckthorn oil extracts [34], phytol [35], and Chinese catalpa [36].

Terpenoids are derived from mevalonic acid, and these compounds and their derivatives are based on the C5 unit of isoprene as the basic structure. They are widely present in nature, and are the major components of plant flavors, resins, and pigments. The active ingredients of terpenoids have been well studied in China. Stevioside and steviol [37], ginsenoside [38, 39] and tormentic acid [40] are terpenoids that are natural anti-diabetes drugs. Diabetic nephropathy is one of the most common complications of diabetes, and it has become the second leading cause of end-stage renal disease. The renin signaling pathway exerts a key regulatory role in the early stages of diabetic nephropathy [41].

Some limitations should be noteworthy. Firstly, the sample size (only 3 cases out of 30 in each group) in the DNA methylation microarray was relatively small, and the sensitivity was high. Also, we can't Therefore, further experiments should be performed with additional cases. And we have testified the microarray results for all patients. Secondly, the differential sites screened from the microarray and their potential functions in the treatment of T2DM patients with phlegm-blood stasis syndrome using HQS should be explored in the future. Thirdly, a randomized controlled clinical trial with a large sample size is necessary to verify our conclusion.

In conclusion, this study found that HQS remarkably improved both glucose metabolism and lipid metabolism in T2DM patients with phlegm-blood stasis syndrome

through regulating the DNA methylation of genes in the AMPK signaling pathway, insulin signaling pathway and terpenoid backbone biosynthesis. This study provides evidence for the underlying mechanism of DNA methylation in the treatment of T2DM patients with phlegm-blood stasis syndrome using HQS.

Abbreviations

TCM: Traditional Chinese medicine

HQS: He Qi San

T2DM : type 2 diabetes mellitus

FPG : fasting plasma glucose

2hPG : 2-hour postprandial blood glucose

TG : triglyceride

TC : total cholesterol

LDL-C : low density lipoprotein-cholesterol

FINS : fasting insulin

GO : gene ontology

KEGG : Kyoto Encyclopedia of Genes and Genomes

AMPK : Adenosine 5'-monophosphate (AMP)-activated protein kinase

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Competing interests: The authors declare that there is no conflict of interest.

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