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E-mail: cjim_en@cjim.cn

Original Article

Association of Gene Mutations with Response to Arsenic-Containing Compound Qinghuang Powder (复方青黄散) in Patients with Myelodysplastic Syndromes*

ZHAO Pan[1], LIANG Jun-bin[2], DENG Zhong-yang[1], WANG Ming-jing[1], QIN Jia-yue[2], CHEN Chong-jian[2], and HU Xiao-mei[1]

ABSTRACT **Objective:** To investigate the relationship between gene mutations and response to Compound Qinghuang Powder (复方青黄散, CQHP) in patients with myelodysplastic syndrome (MDS). **Methods:** Forty-three MDS patients after treatment with CQHP for 6 months were genotyped by ultra-deep targeted sequencing and the clinical data of patients were collected and the relationship between them was analyzed. **Results:** Up to 41.86% of patients harbored genet mutations, in most cases with more than one mutation. The most common mutations were in SF3B1, U2AF1, ASXL1, and DNMT3A. After treatment with CQHP, about 88.00% of patients no longer required blood transfusion, or needed half of prior transfusions. **Conlusion:** CQHP is an effective treatment for patients with MDS, especially those with gene mutations in SF3B1, DNMT3A, U2AF1, and/or ASXL1.

KEYWORDS myelodysplastic syndromes, arsenic, next-generation sequencing, realgar

The myelodysplastic syndromes (MDS) are a very heterogeneous group of myeloid disorders characterized by peripheral blood cytopenias and increased risk of transformation to acute myelogenous leukemia (AML).[1] With the development of life sciences, more scholars began to explore the genetic pathogenesis of MDS. It has been found that 70%–80% of MDS patients with at least one gene mutation.[2-4] In recent years, the new recurrent gene mutations have been detected in MDS patients.[5]

Fortunately, next generation sequencing has enabled efficient and cost-effective genotyping, and has become one of the most powerful diagnostic and prognostic tools in the clinic. Consequently, several whole genome or targeted sequencing projects have identified molecular patterns during MDS development.

Arsenicals, including oral arsenicals, have been widely used to treat MDS.[6,7] It was found in our previous studies that Compound Qinghuang Powder (复方青黄散, CQHP), a traditional arsenic-containing formula, has a clonal selection effect on MDS patients.[8-11] We herein report an association of gene mutations with response to compound-QHP in MDS patients. The data highlight the value of CQHP in the treatment of MDS.

METHODS

Inclusion and Exclusion Criteria

MDS was diagnosed based on the Vienna criteria (2007),[12] and classified based on World Health Organization criteria (2008).[13] Patients were included if they met the diagnostic and classification criteria, voluntarily accepted treatment with CQHP, voluntarily participated in the clinical trial, and were between 18 and 80 years old. Patients with serious diseases, including but not limited to disorders of the heart, liver, kidney, and peripheral nerves, were excluded, along with patients who were mentally ill, unable to undergo diagnosis and therapy, or pregnant or lactating. This study was approved by the Clinical Research Ethics Committee of Xiyuan Hospital, China Academy of

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1. Department of Hematology, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing (100091), China; 2. Annoroad Gene Technology Co. Ltd., Beijing (100176), China
Correspondence to: Prof. HU Xiao-mei, Tel: 86-10-62835627, E-mail: huxiaomei_2@163.com; CHEN Chong-jian, E-mail: cchen@annoroad.com

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Chinese Medical Sciences (No. 2014XL-070-2). All patients have signed informed consent.

Karyotyping and Next-Generation Sequencing

Bone marrow cells were aspirated from the posterior superior iliac crest and cultured for a few days, G banded, and karyotyped according to the International System for Human Cytogenetic Nomenclature (2005).[14]

We analyzed 43 hotspot genes, including 19 clinical hotspot genes and 24 research hotspot genes, by ultra-deep sequencing at Annoroad Gene Technology Co. Ltd. NimbelGen SeqCap EZ Choice was used according to the manufacture's protocol with modifications. Multiplexed libraries were sequenced using 75-bp paired-end runs on an Illumina NextSeq 550AR. Reads were aligned using Burrows-Wheeler alignment tool to human genomic reference sequences (HG19, NCBI built 37). To identify single nucleotide polymorphisms and short insertions and deletions, MuTect2 with recommended parameters was performed. All mutations were annotated by ANNOVAR software. A subset of somatic mutations was randomly selected for validation using Sanger sequencing.

Treatment with CQHP

Patients were orally administered with CQHP, prepared by Preparation Laboratory of Xiyuan Hospital, at a daily dose of 2.7 g, including *Realgar* 0.1 g, *Indigo naturalis* 0.2 g, *White peony root* 0.96 g, *Atractylodes macrocephala koidz* 0.48 g, *Pericarpium Citri Reticulatae* 0.48 g, and *Radix Ledebouriellae* 0.48 g.

Of 43 patients, 38 cases were only treated with CQHP, while 5 were treated with a combination of CQHP and stanozolol 6 mg/d (3 cases), danazol 0.4 g/d (1 case), and erythropoietin (1 case) at a *quaque omni die* of 10,000 U, since these patients previously failed to above Western medicines treatment. Three-month was defined as a course of treatment, and all patients were treated for 2 courses.

Blood Arsenic Test

Blood arsenic concentration was measured 10–12 h after treatment with CQHP on days 0, 30, 90, and 180, using CEM microwave digestion (USA) and atomic fluorescence spectrometry (PSA France).

Efficacy Evaluation

Efficacy was evaluated according to international criteria on efficacy of MDS treatments (2006).[15]

Degrees of Side Effects

Side effects were scored based on the Guiding Principles for Clinical Research on New Drugs of Traditional Chinese Medicine (2002)[16] as mild (+), moderate (++), or serious (+++).

Statistical Analysis

Normally distributed data were analyzed by *t* test in SPSS 22.0, while all other data were analyzed by nonparametric tests. *P* value less than 0.05 was considered statistical significant.

RESULTS

Clinical Information

Forty-three MDS patients, consisting of 24 males and 19 females, were enrolled from January 2014 to June 2016. The median age was 49 years (range 18–80 years), and the course of disease lasted 4 to 240 months. Based on MDS classification criteria, 4 patients had refractory anemia (RA), 6 had refractory anemia with ringed sideroblast (RARS), 26 were classified as having refactory cytopenia with multilineage dysplasia (RCMD), 5 had RA with excess blasts (RAEB), and 2 patients were diagnosed with MDS unclassifiable (MDS-U). Further, 25 patients had normal karyotypes, while 18 had abnormal karyotypes, including 8 cases of +8, 2 cases of complex karyotypes, and 1 case each of -Y, t (5; 7; 20), +del (1P), 21P+, 22P+, -X, t (1:9), and +Y. Finally, 7 patients were deemed to be low-risk based on the International Prognostic Scoring System, while 31 were considered intermediate-Ⅰ, and 5 were scored intermediate-Ⅱ (Table 1).

Gene Mutations in MDS

We sequenced 43 hotspot genes associated with MDS and identified 17 mutated genes in the cohort, including SF3B1, U2AF1, PHF6, ASXL1, NRAS, CUX1, DNMT3A, RUNX1, IDH2, EZH1, TET2, JAK2, TP53, SRSF2, ETV6, FAT1 and GNAS. According to mutanted gene classfication, the numbers of patients with mutation were different in the cohort. For instance, SF3B1 mutations were observed in 7 patients, while U2AF1 mutations were found in 5 cases, ASXL1 mutations were present in 4 cases, and NRAS mutations and PHF6 mutations were found in 2 cases, respectively (Figure 1).

Gene Mutations in Various MDS Subtypes

Based on various MDS subtypes, different gene

Table 1. Clinical Characteristics of 43 MDS Patients

Index	Case (%)
Gender	
Male	24 (55.81)
Female	19 (44.19)
Age (Year)	
⩾60	16 (37.21)
40–60	17 (39.53)
< 40	10 (23.26)
WHO classification	
RA	4 (9.30)
RCMD	26 (60.47)
RARS	6 (13.95)
RAEB	5 (11.63)
U	2 (4.65)
IPSS risk	
Low	7 (16.27)
Intermediate-Ⅰ	31 (72.09)
Intermediate-Ⅱ	5 (11.62)
High	0
Karyotype	
Favorable	26 (60.47)
Intermediate	13 (30.23)
Adverse	4 (9.30)

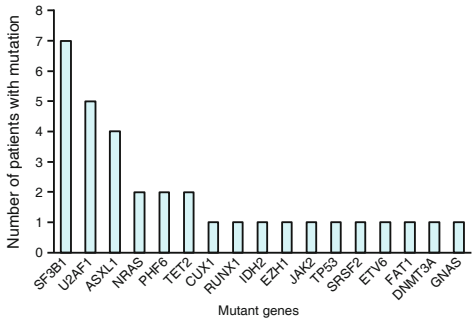

Figure1. Distribution of Mutant Genes in 43 Patients with MDS

Note: Histogram showing the number of patients, according to different gene mutations

mutations were found in the cohort. A U2AF1 mutation was noted in 1 of 4 patients with RA (25%), while gene mutations were found in 10 of 26 patients with RCMD (38.46%). In these 10 patients with RCMD, 8 of 10 patients harbored SF3B1 and CUX1 mutations, SF3B1 and U2AF1 mutations, NRAS and EZH1 mutations, TP53 and FAT1 mutations, a DNMT3A mutation, a U2AF1 mutation, a PHF6 mutation, and a GNAS mutation, respectively. The other 2 patients both carried U2AF1 and ASXL1 mutations. Strikingly, gene mutations were observed in all 6 patients with RARS, of whom one had gene mutations in SF3B1, JAK2, NRAS, SRSF2, TET2 and ETV6. In addition, 3 of 6 patients had SF3B1, CUX1 and RUNX1 mutations, an U2AF1 mutation, and a SF3B1 mutation, respectively. Two patients had mutations both in SF3B1 and ASXL1. Finally, mutations in TET2 and PHF6 were found in 1 of 5 patients with RAEB (Table 2). Collectively, these results indicate a high frequency of mutations among patients with RARS, especially in SF3B1, which was usually found in combination with other gene mutations in the same patient.

Table 2. Mutated Genes in 43 Patients with Various MDS Subtypes

WHO classification (Case)	Mutated gene (Case)	Case (%)
RA (4)	U2AF1 (1)	1 (25.0)
RCMD (26)	U2AF1 (1)	10 (38.5)
	U2AF1 and SF3B1 (1)	
	U2AF1 and ASXL1 (2)	
	GNAS (1)	
	PHF6 (1)	
	DNMT3A (1)	
	NRAS and EZH1 (1)	
	TP53 and FAT1 (1)	
	SF3B1 and CUX1 (1)	
RARS (6)	U2AF1 (1)	6 (100.0)
	SF3B1 (1)	
	SF3B1 and ASXL1 (2)	
	SF3B1, CUX1, and RUNX1 (1)	
	SF3B1, JAK2, NRAS, SRSF2, TET2, and ETV6 (1)	
RAEB (5)	TET2 and PHF6 (1)	1 (20.0)
U (2)	0	0

Gene Mutations and Age

The incidence of MDS was reported to increase with age, although it is unclear whether this is due to the accumulation of mutations with age. Indeed, we found that 23.26% (10/43) of patients in the cohort were under 40 years old, while 76.74% (33/43) patients were over 40 years old (Table 1). All the patients with mutations were in age of over 40 years old, whileas none of the patients under 40 years old harbored gene mutation. Mutations were observed in 9 of 17 (52.94%) patients between 40 and 60 years old, as well as in 9 of 16 (56.25%) patients over 60 years old. There was no significantly difference of the fractions of patients with mutations between the ages of 40 to 60 and over 60 years old (P<0.05).

Gene Mutations and Karyotype

Normal and abnormal karyotypes were observed in 25 and 18 patients, respectively. Among patients with normal karyotypes, 10 had mutations (40.00%), while mutations were observed in 8 patients with abnormal karyotypes (44.44%). Thus, the frequency of mutations was comparable between groups (P>0.05).

Response to CQHP

After 2-course treatment, symptoms improved in 16 patients (37.21%), completely disappeared in 1 case (2.32%), stabilized in 23 case (53.49%), and progressed in 4 case (9.30%). Hence, the overall response rate was 90.70%. Notably, of the 25 patients who required blood transfusions before treatment, 12 (48.00%) did not require transfusions after treatment, and 10 (40.00%) needed only half of prior transfusions. In toatal, the response rate to CQHP in those patients required blood transfusions before treatment, was 88.00%. Moreover, mean hemoglobin concentration in peripheral blood significantly increased from 76.51 ± 22.42 g/L before treatment to 89.49 ± 29.38 g/L after treatment (P<0.05). However, mean hemoglobin concentration was comparable between patients with or without mutations (85.72 ± 17.27 g/L *vs*. 92.20 ± 32.79 g/L, P>0.05).

Arsenic concentrations were detectable in the blood of 39 patients. The average arsenic concentration of patients responded to treatment (37.96 ± 11.30 μg/L) was significantly higher than those unresponded to treatment (2.16 ± 10.04 μg/L, P<0.05).

Gene Mutations and Response to CQHP

Among 25 patients without gene mutations, 11 (44.00%) achieved the hematologic improvement, only 1 (4.00%) had the hematologic progression, and another 13 (52.00%) were stabilized. Among 18 patients with gene mutations, 5 (27.78%) achieved the hematologic improvement, 3 (16.67%) had the hematologic progression, and another 10 (55.56%) were stabilized (Figure 2). In addition, the mean volume of required blood transfusions in patients without mutations decreased 83.33% from 240 mL/month before treatment to 40 mL/month after treatment. In contrast, it declined only 58.82% from 377.78 mL/month before treatment to 155.56 mL/month after treatment in patients with mutations (P<0.05). These data revealed a significantly decreased blood transfusion after treatment with CQHP in patients without mutations (Figure 3).

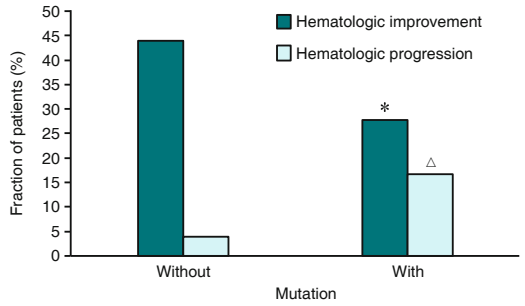

Figure 2. Comparison of Fraction of Patients with and without Gene Mutations in Hematologic Improvement and Hematologic Progression

Note: *P<0.05; △P<0.05 *vs*. without mutations

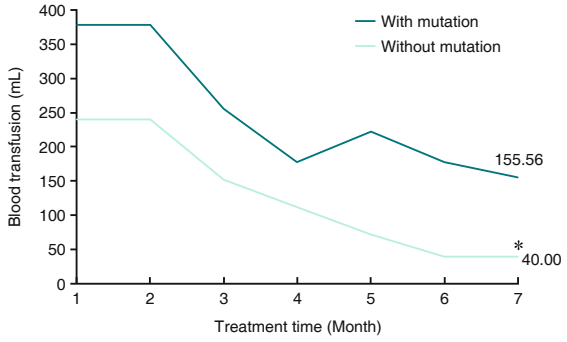

Figure 3. Comparison of Mean Volume of Blood Transfusion during Treatment in Patients with and without Gene Mutations

Note: *P<0.05 *vs*. with mutations

Moreover, we found that all (100%) of 10 patients under 40 years old responded to treatment, while 29 (87.9%) of 33 patients over 40 years old responded to treatment, which was significantly lower than former (P<0.05).

As noted, SF3B1 mutation was the most frequent in the cohort. Strikingly, 6 (85.71%) of 7 patients with SF3B1 mutation responded to CQHP. Furthermore, all 6 patients with U2AF1 mutation, 4 with ASXL1 mutation and 2 with DNMT3A mutation responded to CQHP (Table 3).

Table 3. Gene Mutations and Therapeutic Response

Mutation	Case	Hematologic improvement	Stabilized	Progressed
SF3B1	7	3	3	1
U2AF1	6	1	5	0
ASXL1	4	2	2	0
DNMT3A	2	2	0	0
PHF6	2	–	1	1
NRAS	2	–	1	1

Adverse Reactions to CQHP

Of all patients treated with CQHP, 1 (2.33%)

developed mild diarrhea. Moderate or serious adverse events were not observed. There is no liver or/and kidney dysfunction observed in all of patiennts.

DISCUSSION

Next generation sequencing has been widely used to detect somatic mutations in MDS patients, and data from such surveys have become significantly valuable in diagnosis.[2-4] In this survey, we tested 43 MDS-associated genes, and found frequent mutations in 6 genes, including SF3B1 (16.28%), U2AF1 (13.95%), ASXL1 (9.30%), PHF6 (4.65%), NRAS (4.65%), and TET2 (4.65%). As many as 41.86% of patients harbored gene mutations, in many cases more than one. Remarkably, the frequency of these mutations was much higher in patients over 40 years old and prognosis was much poorer in older patients, likely because of accumulated mutations. Normal and abnormal karyotypes the frequency of mutations was comparable, suggesting that abnormal karyotypes were not associated with gene mutations. Accordingly, we also found a significantly lower hematologic improvement in patients with mutations, specific gene testing may be more informative than karyotyping.

As MDS is heterogeneous, treatments are typically customized.[9] For instance, administration of erythropoietin is suitable for patients with low concentration of blood erythropoietin and who require large blood infusions. What's more, demethylation drugs such as azacitidine and decitabine are conventionally regarded as supplements to compensate for mutations in TET2 and DNMT3A. Immunomodulators such as thalidomide and lenalidomide may also improve hematopoiesis to a certain degree in low-risk or intermediate-risk patients, while chemotherapy is effective for high-risk patients. In any case, transplantation with hematopoietic stem cells is currently the only regimen available to permanently reverse MDS.

The first medicinal use of realgar in China was historically recorded in Shennong's Classic of Materia Medica (*Shen Nong Ben Cao Jing*), the first Chinese Materia Medica before 100 BC. Realgar works as formulae together with other components of medicinal herbs and minerals. Realgar contains > 90% As_2S_2 based on the quality standard in Chinese Pharmacopeia in 2015 edition.

Arsenic-containing formulae QHP, which includes *Realgar* and *Indigo* naturalis, was used as a folk medicine recorded in a famous traditional Chinese medicine book *Effective Prescriptions Handed Down for Generations of Physicians* (Shi Yi De Xiao Fang) published in 1345. QHP used for treating hematologic malignancies has more than 50 years of history in our department.[6,17] Actually, the clinical use of arsenic-containing formulae to treat certain types of leukemia in China did occur as early as As_2O_3.[6] Our clinical investigations showed that CQHP was effective in the treatment of hematologic malignancies with recovery of peripheral blood cells, reducing blood transfusion amount and decreasing blast cells and its therapeutic efficacy could be enhanced by increasing the realgar content in the formula, which could be attributed to As_2S_2.[6] Our clinical observation also revealed that the curative effect of CQHP was associated with the blood concentration of arsenic, the effective blood concentration of arsenic was more than 20 μg/L.[10] Meanwhile, the clinical adverse reactions were obsereved in 15% of MDS patients treated with CQHP characterized by abdominal painful diarrhea, which affected the absorption of arsenic contained in realgar.[6]

For preventing the painful diarrhea caused by CQHP, another Chinese compound formula Tongxieyao Formula (痛泻要方) including *White peony root*, *Atractylodes macrocephala koidz*, *Pericarpium Citri Reticulatae*, and *Radix Ledebouriellae* which was historically used specifically for the treatment of painful diarrhea, was added into CQHP and upgraded to be a new formulae CQHP. Our recent works revealed that CQHP decreased the incidence of painful diarrhea and increased the effective rate in the treatment of MDS, as compared to CQHP.[22,23]

Accordingly, MDS patients in this study were treated with CQHP, and the daily dose of *Realgar* was 0.1 g. The average arsenic concentration of blood was 37.96±11.30 μg/L, which was higher than those treated with CQHP because of preventing the painful diarrhea and then increasing the absorbtion of arsenic, but lower than those reported in other literature.[24,25] Whileas after treatment of CQHP, the overall response rate was 90.70% and the volumes of required blood transfusion were significantly reduced in MDS patients.

Our previous *in vitro* studies indicated that As_2S_2,

the most important component of Realgar, could inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation dose-dependently in MDS-progressed F-36p cells.[26,27] Our previous studies also revealed that As_2S_2 induced cell differentiation negatively regulated by the activation of p38 MAPK in HL-60 cells,[28,29] and MDS (MDS-L) and MDS/AML (F-36P) cell lines were more sensitive to the erythroid differentiation-inducing activity of As_2S_2 than HL-60 cells.[27] These findings provided the precise mechanisms of As_2S_2 action toward its use for clinical application in patients with hematological disorders.

Our previous studies documented that CQHP elicited good outcomes in MDS patients with good oral safety profiles, especially those with normal or +8 karyotype.[11] In the work, we demonstrated that CQHP dramatically increased hemoglobin and reduced the need for transfusion in 88.00% of MDS patients. Such effects could significantly improve patients' quality of life, as well reduce economic and social burdens. Of note, we found that CQHP was more effective in patients without mutations than in patients with gene mutations. Nevertheless, the treatment appears to elicit sufficiently good outcomes in patients with SF3B1, U2AF1, ASXL1 and DNMT3A mutations.

Collectively, the data in the work suggested that CQHP is a safe and effective oral arsenical to treat MDS, especially those with gene mutations in SF3B1, DNMT3A, U2AF1 and ASXL1.

Conflict of Interest

The results of this study were used to prove that the effect of CQHP on MDS patients with different gene mutations were varies, and there was no interest relationship between the results and the enterprise. The authors have no conflict of interest to declare.

Author Contributions

Hu XM and Chen CJ contributed to the study design; Zhao P, Liang JB, Chen CJ and Hu XM wrote the manuscript; Hu XM, Zhao P, Deng ZY and Wang MJ performed the clinical research; Chen CJ, Liang JB and Qin JY performed the next-generation sequencing; Zhao P, Liang JB and Qin JY performed the data processing and statistical analysis. All authors read and agreed on the final version of the manuscript.

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