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Clinical Experience

Effect of Qinghuang Powder (青黄散) Combined with Bupi Yishen Decoction (补脾益肾方) in Treating Patients with Refractory Cytopenia with Multilineage Dysplasia through Regulating DNA Methylation*

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ABSTRACT **Objective**: To explore the effect of Qinghuang Powder (QHP, 青黄散) combined with Bupi Yishen Decoction (BPYS, 补脾益肾方) on myelodysplastic syndromes (MDS) patients with refractory cytopenia with multilineage dysplasia (RCMD) and determine the change of DNA methylation in MDS-RCMD patients after the treatment of Chinese medicine formula. **Methods**: All 308 MDS-RCMD patients were treated with QHP combined with BPYS for 2 months at least, absolute neutrophil count (ANC), hemoglobin (Hb), platelets (PLT), primitive bone marrow cells and chromosome karyotype were chosen as the main evaluation indexes to analyze the treatment effect according to criteria from the MDS International Working Group. Then 43 bone marrow samples from 15 MDS-RCMD patients and 28 healthy donors were obtained for the examination of DNA methylation. Gene Ontology (GO) and Pathway analysis were applied to analyze the methylation data. **Results**: The overall MDS response rate to QHP was 61.68% (190/360) including hematologic improvement-neutrophil (HI-N) or hematologic improvement-erythroid (HI-E) or hematologic improvement-platelet (HI-P). Patients with anemia had a better response rate than patients with neutropenia or thrombocypenia (55.88% *vs* 31.54% or 55.88% *vs*. 36.9%). The DNA methylation microarray analysis disclosed that 4,257 hypermethylated genes were demethylated upon the treatment with QHP and BPYS. GO analysis and Pathway analysis showed that these demethylated genes were involved in a lot of tumor-related pathways and functions. **Conclusions**: QHP combined with BPYS could effectively treat MDS-RCMD patients through hematologic improvement (HI-N, HI-P or HI-E) and PLT and RBC transfusion independence due to the demethylation, thereby providing another choice for the treatment of patients with MDS-RCMD.

KEYWORDS Qinghuang Powder, Bupi Yishen Decoction, myelodysplastic syndromes, demethylation, Chinese medicine

Myelodysplastic syndromes (MDS) are a group of bone morrow disorders with symptoms such as peripheral cytopenia, dysplastic marrow cells, and with high risk of developing into leukemia.[1] Patients with MDS have widely variable prognosis and the pathogenesis of this disease is not clearly understood. According to the classification from the World Health Organization (WHO), 30% of the patients with MDS belongs to refractory cytopenias with multilineage dysplasia (RCMD).[2] Although allogeneic hematopoietic stem cell transplantation can cure patients with MDS-RCMD, but the transplantation is limited by its toxicity and donor availability.[3]

Chinese medicine (CM) has an application history of more than 3,000 years and has a special theory for etiology and treatment. CM practitioners think that multiple ingredients lead to the therapeutic effect and the formulae contain several CM herbs in which one affects as the main component, and others represent the adjuvant ones to assist the therapeutic effect.[4,5] Qinghuang Powder (青黄散, QHP, containing arsenic, As_4S_4) combined with Bupi Yishen Decoction (BYD, 补

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脾益肾方), a modern formula according to CM theory, has been applied to treat MDS for more than 30 years. Our previous study indicates that QHP combined with BPYS is effective in the treatment of MDS.[6]

Current studies have demonstrated that the abnormal DNA methylation in MDS patients is crucial to the progression of this disease, and MDS patients have a large number of abnormal hypermethylated genes.[7] Targeted treatment against DNA methylation has gained tremendous attention, and the introduction and approval of 5-azacytidine (azacitidine; AZA) or 5-aza-2'-deoxycytidine (decitabine; DAC) for the treatment of MDS and acute myelocytic leukemia (AML) during the past several years should be the most significant example in this progress.[8] AZA and DAC have revealed better therapeutic efficacy when compared with traditional chemotherapy in the treatment of high risk MDS and some AML patients. However, in clinical trials, it has also been found that a number of patients do not initially respond to AZA or DAC therapy, and that most patients with initial response to AZA or DAC treatment still have eventual relapse.[9,10] Thus, it is urgent to develop novel and effective drugs for DNA methylation-targeted therapy, especially for the treatment of MDS-RCMD. Our previous studies have suggested that As_4S_4-containing CM herbal formula could rescue the dysfunctional methylation status in MDS patients.[11]

Based on the relationship of DNA methylation and MDS-RCMD, the alteration of DNA methylation may be an effective mechanism of QHP coupled with BPYS during the treatment of RCMD according to following experimental protocols. First, we analyzed the data responding to QHP combined with BPYS in 308 MDS-RCMD patients retrospectively. Sequentially, the relationship between the involvement of DNA methylation in MDS-RCMD and the treatment efficacy of QHP combined with BPYS was examined. Finally, we used ChIP-on-chip to check the change of methylation in 15 MDS-RCMD patients and analyzed the corresponding genes through Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

METHODS

Standards for Diagnosis, Inclusion and Exclusion

Patients in this study were diagnosed as MDS-RCMD according to the WHO classification system (2002).[12]

The inclusion criteria in this study included: patients must be more than 14 years old; patients have not been subjected to receive any other therapy for 2 weeks before this study started; patients do not have severe diseases associated with kidney, liver, or heart; and all patients and healthy individuals must provid informed consents.

Patients were excluded if they (1) were pregnant; (2) combined with mental disorders.

Patients

All subjects were MDS-RCMD patients who came from the Outpatient Department of Hematology of Xiyuan Hospital, visited from September 2007 to February 2014, and received and completed the prescribed therapy for at least 2 months. A total of 308 patients were enrolled. They were 156 males and 152 females; 14–81 years old, with illness duration of 3–160 months, and 38±45 months on the average. By International Prognostic Scoring System (IPSS) grading, 232 were in intermediate-1 risk, 76 were in intermediate-2 risk. The karyotype of chromosome was normal in 217 patients and abnormal in 91 patients (37.10%). This study was approved by the Xiyuan Ethical Committees (2012XL032-2).

Treatments

All 308 patients were administrated with QHP capsules combined with BYD orally for 2 months at least. QHP capsule (Xiyuan Hospital, No. 2007001-2014018) was administrated at the dose of 0.4 g/d (containing realgar of 0.16 g, and indigo of 0.24 g), BYD was administrated twice a day, consisting of *Rhizoma diosscoreae* 10 g, *Radix rehmanniae* 15 g, *Fructus corni*, *Poria* 10 g, *Cortex mouta* 10 g, *Rhizoma alismatis* 10 g, *Semen cuscutae* 15 g, *Fructus psoraleae* 15 g, *Radix pseudostellariae* 30 g, *Radix polygoni multiflori* 20 g, *Rhizoma atractylodis macrocephalae* 15 g, *Fructus jujubae* 10 g, *Zingiberis recens rhizoma* 10 g. *Herba ecliptae* 20 g and *Fructus ligustri lucidi* 20 g were provided for patients with yin deficiency; *Radix morindae officinalis* 15 g, *Herba cynomorii* 15 g, *Radix aconiti lateralis preparata* 10 g and *Ramulus cinnamomi* 10 g were provided for patients with yang deficiency. All CM herbs used in this study were provided by Xiyuan Hospital. The response criteria for MDS-RCMD was established by International Working Group which was used to define the

measurements of clinical response.[13,14]

DNA Methylation and Data Analysis

Affymetrix GeneChip Promoter 1.0 Array was used to evaluate the methylation of all 43 samples. Briefly, bone marrow cells were collected from 15 low-risk MDS patients and 28 healthy donors (control group). Methylated DNA Immunoprecipitation Kit (Promega, USA) was used to obtain the methylated DNA fraction. Then, the targeted DNA was amplified through PCR, fragmented, and labeled. After hybridization, washing, and staining, all the methylation chips were scanned according to the manufacturer's instructions. Then, GO and Pathway analysis were applied to analyze the methylation data. At present, the GO project is a major bioinformatics tool used for screening cancer-related abnormal expressed genes and corresponding functions.[15,16] Another important database. KEGG was established to deal with genomes, biological chemicals and enzymatic pathways. KEGG database also can be used to obtain the information of pathways containing signal transduction, membrane transport, cell cycle and so on.[17,18]

Statistical Analysis

Descriptive statistics of demographic data were analyzed for all 308 patients. Binomial 95% exact confidence intervals were provided and response rates were calculated by the modified IWG MDS guideline (2006) with the use of GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA, USA).[13]

GO analysis and Pathway analysis were used to acquire the information associated with genomic function. The difference of methylation levels in different groups and samples were assessed by multiple comparison and *Fisher* test. $P<0.05$ and false discovery rate (FDR) < 0.05 were set as the filtrating cut-point.

RESULTS

Patient Demographics and MDS Disease Characteristics

Table 1 shows the demographics and characteristics of patients with MDS-RCMD. Totally 273 patients (88.6%) belonged to intermediate-1 risk according to the IPSS.[19] Among these 308 patients, normal karyotypes were observed in 224 cases (72.7%), and +8 karyotype was observed in 23 cases (7.5%). The number of patients with bilineage or trilineage cytopenia was 285 (92.5%).

Table 1. Patient Demographics and MDS-RCMD Disease Characteristics

Item	Case (%)
Age	
⩾60	40 (13.0)
<60 years	268 (87.0)
Male	154 (50.6)
IPSS classification	
Low risk	18 (5.9)
Intermediate-1 risk	273 (88.6)
Intermediate-2 risk	17 (5.5)
Cytogenetic findings	
Normal/diploid	224 (72.7)
+8	23 (7.5)
Complex (⩾3 abnormalities)	13 (4.2)
20q-	7 (2.2)
del(7q) or -7	4 (1.3)
Other	37 (12.1)
Cell lineage cytopenia	
Unilineage	23 (7.5)
Bilineage	98 (31.8)
Trilineage	187 (60.7)
Baseline cytopenia(s)	
Anemia	13 (4.2)
Neutropenia	2 (0.6)
Thrombocytopenia	8 (2.6)
Anemia + Neutropenia	41 (13.3)
Neutropenia + Thrombocytopenia	26 (8.4)
Anemia + Thrombocytopenia	27 (8.8)
Anemia + Neutropenia + Thrombocytopenia	191 (62.1)

Administration and Efficacy of QHP Combined with BYD

As shown in Table 2, 152 of 272 patients (55.88%; 95% CI:49.98%-61.78%) were evaluable anemic patients with an hematologic improvement-erythroid (HI-E) response and 79 patients with red blood cell (RBC)-transfusion dependence obtained RBC-transfusion independence. For the patients with neutropenia, an hematologic improvement-neutrophil (HI-N) response was achieved in 81 of 260 and the HI-N rate was 31.53% (95% CI: 25.88%–37.18%). Totally 93 of 252 patients with thrombocytopenia achieved an hematologic improvement-PLT (HI-P) response with an HI-P rate of 36.9% (95% CI: 30.94%-42.86%) in the evaluable group and 19 patients with PLT-transfusion dependence were observed as PLT-

Table 2. Effect of QHP Combined with BYD In the Treatment of Patients with MDS-RCMD

Item	Case (%)	95% CI
HI-E (*n*=272)	152 (55.88)	49.98–61.78
HI-N (*n*=260)	81 (31.53)	25.88–37.18
HI-P (*n*=252)	93 (36.90)	30.94–42.86
Bilineage (HI-N and HI-E, *n*=232)	62 (26.72)	21.03–32.14
Bilineage (HI-P and HI-E, *n*=218)	72 (33.03)	26.79–39.27
Bilineage (HI-N and HI-P, *n*= 217)	41 (18.89)	13.68–24.10
Trilineage (HI-N, HI-E and HI-P, *n*=191)	37 (19.37)	13.77–24.97
HI-E or HI-N or HI-P (*n*=308)	190 (61.68)	56.25–67.11

transfusion independence.

DNA Methylation in MDS-RCMD Patients before and after Treatment

Compared with the control group, the MDS patients had 7,724 abnormal hypermethylated genes and 32 hypomethylated genes before the treatment. However, the number of abnormal hypermethylated genes was decreased to 3,467 from 7,724 and the number of hypomethylated genes reached up to 107 after the treatment with QHP combined with BPYS.

Demethylation Effects of Patients under Going Treatment of QHP Combined with BYD

On the basis of GO and KEGG database, we conducted analysis of the genes, with the ratios of methylation level higher than 1.4 in the MDS-RCMD group before and after treatment.

According to GO analysis of demethylated genes from MDS-RCMD patients, there were 1,895 demethylated genes with the involvement of 640 kinds of biological functions and pathways, including cell proliferation, apoptosis, negative regulation of cell cycle and cell differentiation. The data in Table 3 shows the classification of 640 functions from demethylated genes in 15 patients with MDS-RCMD. As shown in Figure 1A, the top 15 GOs are negative regulation of cell proliferation, positive regulation of MAPK cascade, cell proliferation, apoptotic signaling, Wnt receptor signaling pathway, and so on.

According to the Pathway analysis of the demethylated genes in the MDS -RCMD patients, approximately 861 genes were involved in 125 biological pathways. Table 4 shows some of these biological pathways including tumor pathways, Wnt receptor signaling pathways, and so forth. As listed in Figure 1B,

Table 3. Functions of Demethylated Genes from MDS-RCMD Patients Treated by QHP Combined with BYD

GO_ID	GO_name	diffgene_count	*P* value	FDR
GO:0008285	negative regulation of cell proliferation	22	7.614E-05	0.0120402
GO:0043410	positive regulation of MAPK cascade	6	0.0114884	0.2271009
GO:0002224	toll-like receptor signaling pathway	8	0.0117094	0.2294206
GO:0034162	toll-like receptor 9 signaling pathway	6	0.0206512	0.327854
GO:0097190	apoptotic signaling pathway	7	0.0271119	0.3460925
GO:0030510	regulation of BMP signaling pathway	2	0.0275123	0.3460925
GO:0034138	toll-like receptor 3 signaling pathway	6	0.0322989	0.3886395
GO:0016055	Wnt receptor signaling pathway	9	0.0334706	0.3963893
GO:0007219	Notch signaling pathway	7	0.040663	0.4187346
GO:0008283	cell proliferation	17	0.0079672	0.19332

Notes: this table shows parts of 640 category functions of demethylated genes in 15 MDS patients before and after treatment. go_ id: gene ID number in Gene Ontology database; go_name: category of function of gene; diffgene_count: number of dmethylated genes; *P* value: *P*<0.05 indicating methylation is significant; FDR: false discovery.

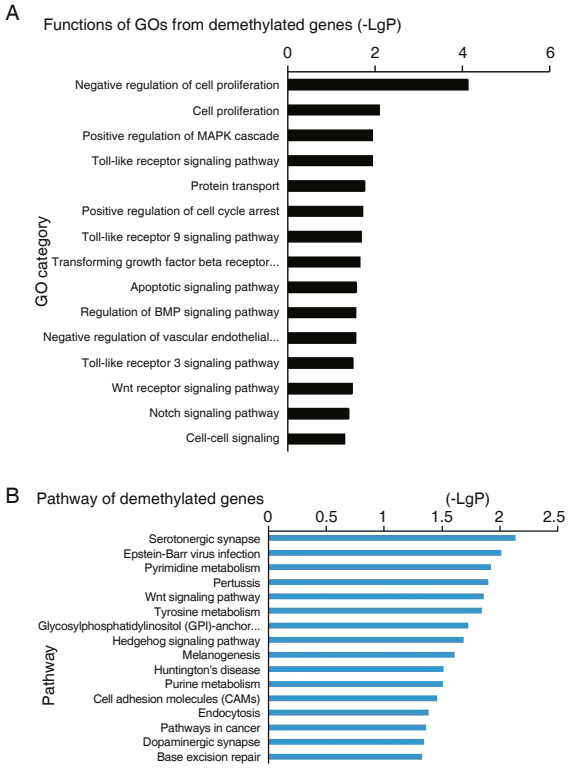

Figure 1. Significantly Changed GOs and Pathways of Demethylated Genes

Notes: A: The functions of GOs from demethylated genes; B: The functions of pathways from demethylated genes. The y axis shows category and the x axis, -LgP. The larger –LgP indicated a smaller *P* value.

Table 4. Pathways of Demethylated Genes from MDS-RCMD Patients Treated by QHP

Path_ID	Path_name	path_diffgene_count	*P* value	FDR
04310	Wnt signaling pathway	12	0.000337	0.0095208
05200	Pathways in cancer	19	0.0005714	0.0117398
05205	Proteoglycans in cancer	12	0.0194746	0.170734
04722	Neurotrophin signaling pathway	8	0.020998	0.170734
04340	Hedgehog signaling pathway	5	0.0210293	0.170734
04151	PI3K-Akt signaling pathway	16	0.0211529	0.170734
00500	Starch and sucrose metabolism	5	0.0312587	0.216995
00051	Fructose and mannose metabolism	4	0.0316851	0.216995
00230	Purine metabolism	14	0.0001333	0.0092841

Notes: This table shows 9 pathways of demethylated genes in 15 MDS-RCMD patients before and after treatment. Path id: gene ID number in KEGG database; Path name: pathway of gene; *P* value: $P< 0.05$ indicating methylation is significant.

the top 15 pathways from demethylated genes included the pathways associated with cancer and Wnt signaling pathway.

DISCUSSION

Our present work has addressed the effects of QHP combined with BPYS during the treatment of MDS-RCMD with a large sample size.

Most patients with MDS-RCMD are died from chronic cytopenias rather than leukemia. Therefore, the management goal for MDS-RCMD patients is to improve the quality of life through treating the complications of cytopenia with supportive care for anemia, neutropenia and thrombocytopenia.[20] In the present study, the overall MDS response rate to QHP was 61.68% which was consistent with our previous study in which complete remission (CR) and partial remission (PR) rates were 23.4% and HI was 49.2% among 61 of 124 patients.[6] In addition, the combinatorial treatment of QHP and BPYS showed an excellent hematopoietic-promoting effect in MDS-RCMD patients and could induce RBC- or PLT- transfusion independence in RCMD patients with RBC- or PLT-transfusion dependence. Bilineage and trilineage responses were also observed. Interestingly, patients with anemia seemed have a better response rate than patients with neutropenia or thrombocytopenia (55.88% *vs*. 31.54% or 55.88% *vs*. 36.9%), implying that this kind of treatment had the highest therapeutic efficacy to erythroid lineage. A randomized and double-blinded clinical trail focusing on clinical responses observed in the current study is highly needed in the future to confirm these results.

Previous studies have shown that abnormal hypermethylated genes had a significant role in MDS.[21] In order to uncover mechanisms of QHP combined with the BYD during the treatment of MDS-RCMD, we compared the methylation level of 15 MDS-RCMD patients with the normal group consisting of 28 healthy individuals. The DNA methylation microarray analysis disclosed the change of 4,257 hypermethylated genes upon QHP treatment combined with the BYD. Meanwhile, through GO and Pathway analysis, these demethylated genes were involved in a lot of tumor-associated pathways and functions and some of which are of particular interest that warrant future futher. We found that the main pathways and functions were negative regulation of cell proliferation, tumor pathways, positive regulation of mitogen-activated protein kinase (MAPK) cascade, cell proliferation, apoptotic signaling, Wnt receptor signaling pathway through the further GO and Pathway analysis. Previous studies have shown that these pathways had significant roles in MDS. For example, abnormal hypermethylation of gene, FZD9, has been demonstrated as an independent adverse prognostic factor of MDS,[22] and FZD9 played an important role in Wnt signaling pathways.[23] MAPK signaling pathway also plays an important role in MDS. Ineffective hematopoiesis of MDS patients at the early stage may be caused by excessive apoptosis of hematopoietic precursor cells, differentiated cells and the abnormal activation of p-38 MAPK signaling pathway (p-38 as a subclass of MAPK) may be correlated with the ineffective hematopoiesis.[24] Taken these observations into consideration, our present data suggests that the effect of demethylation may be the major mechanism for its favorable clinical responses during the treatment of MDS-RCMD.

In summary, QHP combined with BYD can treat MDS-RCMD effectively through hematologic improvement (HI-N, HI-P or HI-E) as well as PLT and RBC transfusion independence due to the demethylation, thereby providing another choice for treatment of MDS-RCMD except azacitidine and decitabine.

Conflict of Interest

The authors report no conflicts of interest in this work.

Author Contributions

Zhou QB, Wang HZ, Wang DX and Xu YG performed the experiments; Zhou QB, Yang XH and MA R designed the experiment. Xu FQ and Hu XM edited and revised manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Tefferi A, Vardiman JW. Myelodysplastic syndromes. N Engl J Med 2009;38:1872-188.
2. Vardiman JW, Harris NL, Brunning RD. WHO classification of tumors of haematopoietic and lymphoid tissues. Blood 2002;100:2292-302.
3. Cutler CS, Lee SJ, Greenberg P, Deeg HJ, Pérez WS, Anasetti C, et al. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. Blood 2004;104:579-585.
4. Anonymous, ed. The Inner Canon of Emperor Huang. Beijing: Chinese Medical Ancient Books Publishing House; 2003.
5. Zhang ZJ, ed. Theory of typhoid miscellaneous disease. Beijing: Chinese Medical Ancient Books Publishing House; 2008.
6. Xu S, Ma R, Hu XM, Xu YG, Yang XH, Wang HZ, et al. Clinical observation of the treatment of myelodysplastic syndrome mainly with qinghuang powder. Chin J Integr Med 2011;17:834-883.
7. Itzykson R, Fenaux P. Epigenetics of myelodysplastic syndromes. Leukemia 2014;28:497-506.
8. Piekarz RL, Bates SE. Epigenetic modifiers: basic understanding and clinical development. Clin Cancer Res 2009;15:3918-3926.
9. Yamazaki J, Issa JP. Epigenetic aspects of MDS and its molecular targeted therapy. Int J Hematol 2013;97:175-182.
10. Issa JP, Kantarjian HM. Targeting DNA methylation. Clin Cancer Res 2009;15:3938-3946.
11. Shuzhen S, Ma R, Hu XM, Yang XH, Xu YG, Hongzhi W, et al. Karyotype and DNA-methylation responses in myelodysplastic syndromes following treatment with traditional Chinese formula containing arsenic. Evid Based Complement Altern Med 2012;5:265-266.
12. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al, The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes, Blood 2009;114:937-951.
13. Tefferi A, Barosi G, Mesa RA, Cervantes F, Deeg HJ, Reilly JT, et al. International Working Group (IWG) consensus criteria for treatment response in myelofibrosis with myeloid metaplasia: On behalf of the IWG for myelo fibrosis research and treatment (IWGMRT). Blood 2006;108:1497-1503.
14. Cheson BD, Bennett JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 2000;96:3671-3674.
15. Berardini TZ. The Gene Ontology in 2010: extensions and refinements. Nucleic Acids Res 2009;38:331-335.
16. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. Nature Genetics 2000;25:25-29.
17. Aoki-Kinoshita KF, Kanehisa M. Gene annotation and pathway mapping in KEGG. Methods Mol Biol 2007;396:71-91.
18. Kanehisa M. The KEGG database. Novartis Found Symp 2002;247:91-103.
19. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079-2088.
20. Cheson BD, Bennett JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 2000;96:3671-3674.
21. Issa JP. The myelodysplastic syndrome as a prototypical epigenetic disease. Blood 2013;121:3811-3817.
22. Jiang Y, Dunbar A, Gondek LP, Mohan S, Rataul M, O'Keefe C, et al. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. Blood 2009;113:1315-1325.
23. Karasawa T, Yokokura H, Kitajewski J, Lombroso PJ. Frizzled-9 is activated by Wnt-2 and functions in Wnt/beta-catenin signaling. J Biol Chem 2002;277:37479-37486.
24. Peng H, Wen J, Zhang L, Li H, Chang CC, Zu Y, et al. A systematic modeling study on the pathogenic role of p38 MAPK activation in myelodysplastic syndromes. Mol Bio Syst 2012;8:1366-1374.

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