Int J Clin Exp Pathol 2016;9(6):6031-6038
www.ijcep.com /ISSN:1936-2625/IJCEP0024948

Original Article

GDF11 is increased in patients with myelodysplastic syndrome

Yu Han, Gaochao Zhang, Huaquan Wang, Rong Fu, Limin Xing, Lijuan Li, Huijuan Jiang, Wei Zhang, Jinglian Tao, Zonghong Shao

Department of Hematology, General Hospital, Tianjin Medical University, Tianjin 300052, China

Received January 26, 2016; Accepted April 23, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Objective: To explore the role of growth differentiation factor 11 (GDF11) in patients with myelodysplastic syndrome (MDS) in erythropoietic functions. Methods: The serum GDF11 in MDS patients was measured using an enzyme-linked immunosorbent assay. The percentage of nucleated erythroid cells (CD235a) in bone marrow was detected via flow cytometry. The correlation between these changes and erythropoietic function were evaluated. GDF11 mRNA expression in bone marrow mononuclear (BMMNC) was detected through real time polymerase chain reaction. Results: The concentration of GDF11 in high-risk MDS patients was significantly higher than that in low-risk MDS patients and higher than that in healthy controls. The concentration of GDF11 in low-risk MDS patients was significantly higher than that in healthy controls. In high-risk MDS patients, the expression of GDF11 was negatively correlated with hemoglobin, red blood cells, and hematocrit and positively correlated with reticulocyte, mean corpuscular volume, CD235a+ cells, and nucleated erythrocytes in bone marrow. In low-risk MDS patients, the expression of GDF11 was positively correlated with CD235a+ cells in bone marrow. The mRNA expression of GDF11 of BMMNC in the MDS group was higher than that in the control group. Conclusion: GDF11 was increased in patients with MDS and negatively correlated with late erythropoiesis.

Keywords: Myelodysplastic syndrome, growth differentiation factor 11, anemia

Introduction

The myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell diseases characterized by cytopenias, dysplasia in one or more of the major myeloid cell lines, ineffective hematopoiesis, and increased risk of development of acute myeloid leukemia (AML). Anemia is the most frequent manifestation in MDS patients [1]. Erythropoiesis is a multistep process by which erythroid precursor cells proliferate and differentiate into mature red blood cells (RBCs). Under the regulation of erythropoietin (EPO), committed progenitor cells divide and differentiate into proerythroblasts. These cells further mature through a series of normoblast stages before undergoing enucleation to form reticulocytes and mature RBCs [2]. MDS are characterized by ineffective erythropoiesis that can lead to overproduction of EPO, erythroid hyperplasia, and bone marrow expansion, as well as dysregulated iron metabolism, including suppressed hepcidin production. The efficiency of recombinant human EPO in MDS patients' anemia is approximately 40%. Furthermore, patients with refractory anemia with ringed sideroblasts or patients who have high levels of EPO in the blood often respond poorly to recombinant human EPO therapy [3].

Growth differentiation factor 11 (GDF11) is a ligand of the transforming growth factor-β superfamily (TGF-β). At present, in mammals, more than 40 members of this superfamily are recognized, and they are divided into several subcategories that include TGF-βs, GDFs, bone morphogenic proteins, Müllerian inhibitory factors, activins, and inhibins. Ligands of TGF-β, and their respective activin receptors, and the intracellular signaling proteins Smad2/3, have been shown to be negative modulators of late-stage erythropoiesis. This mechanism of action is distinct from EPO, which stimulates proliferation of RBC progenitor cells during early stages of erythropoiesis. Increased Smad2 phosphory-

Table 1. The characteristics of MDS patients

Case	Sex/age	Diagnosis	Cytogenetics	IPSS
1	Female/54	5q-	Good	Low
2	Female/68	RAS	Good	Low
3	Female/63	RA	Good	Low
4	Male/32	RAS	Good	Low
5	Male/49	RCMD	Good	Low
6	Male/16	RCMD	int	Int-1
7	Male/50	RCMD	Good	Int-1
8	Female/41	RCMD	Int	Int-1
9	Female/64	RCMD	Good	Low
10	Male/69	RCMD	Int	Int-1
11	Female/61	RCMD	Good	Int-1
12	Female/63	RAEB-1	Good	Int-1
13	Male/42	RCMD	Int	Int-1
14	Male/47	RCMD	Good	Int-1
15	Female/68	RAS	Int	Int-1
16	Male/59	RAEB-1	Good	Int-1
17	Male/27	RAEB-1	Int	Int-1
18	Female/60	RAEB-1	Good	Int-1
19	Male/46	RAEB-2	Poor	high
20	Male/49	RCMD	Poor	Int-2
21	Male/52	RAEB-2	Int	high
22	Male/49	RAEB-2	Good	Int-2
23	Male/78	RAEB-2	Int	high
24	Male/40	RAEB-2	Int	high
25	Female/30	RAEB-2	Poor	high
26	Male/76	RCMD	Poor	Int-2
27	Male/76	RAEB-2	Poor	high
28	Male/81	RAEB-2	Int	high
29	Female/32	RAEB-2	Poor	Int-2
30	Male/37	RAEB-2	Int	Int-2
31	Male/72	RAEB-2	Int	high
32	Male/51	RAEB-2	Poor	high
33	Female/21	RAEB-2	Int	Int-2
34	Female/58	RAEB-2	Good	Int-2
35	Female/63	RAEB-2	Poor	high
36	Male/27	RAEB-2	Int	high
37	Female/21	RAEB-2	Good	Int-2
38	Male/62	RAEB-2	Int	Int-2
39	Male/53	RAEB-2	Good	Int-2
40	Male/66	RAEB-2	Poor	high
41	Male/57	RAEB-2	Poor	high
42	Female/50	RAEB-2	Good	Int-2
43	Male/78	RAEB-2	Good	Int-2
44	Male/50	RCMD	Poor	Int-2

lation and signaling may play a role in conditions of ineffective erythropoiesis, as has been shown in bone marrow biopsies of patients with MDS. GDF11 and GDF8 of the TGF-β superfamily are the major cytokines that regulate late-stage erythropoiesis [4]. In this study, we detected GDF11 expression in the peripheral blood of MDS patients, and investigated the correlation with erythropoiesis.

Materials and methods

Patient selection

Forty-four patients with MDS admitted to the Hematology Department of General Hospital of Tianjin Medical University were enrolled in this study from September 2014 to June 2015. There were 28 males and 16 females, and the median age was 52 (range, 16 to 81) years. According to WHO criteria, these included 1 patient with refractory anemia, 3 with refractory anemia with ringed sideroblasts, 12 with refractory cytopenia with multilineage dysplasia (RCMD), four with refractory anemia with excess blast-1, 23 with refractory anemia with excess blast-2, and 1 with MDS associated with isolated del (5q). Based on the International Prognostic Scoring System for MDS, these samples were divided into groups: low-risk groups (low and intermediate-1 [INT-1]) and high-risk groups (high and intermediate-2 [INT-2]). Among these patients, 6 were at low risk (0), 12 at INT-1 risk (0.5-1.0), 13 at INT-2 risk (1.5-1.0) and 13 at high risk (≥2.5). In general, the low-risk group comprised 18 cases and the high-risk group comprised 26 cases. According to the severity standards of anemia, there were 3 normal, 6 mild (hemoglobin [Hb: 90-110 g/L]), 31 moderate (Hb: 60-90 g/L), and 4 severe (Hb: 30-60 g/L) cases. These patients were separated into two groups: the normal/mild anemia group and the moderate/severe anemia group (**Table 1**). Ten healthy blood donors were selected as controls, including 2 men and 8 women, with a median age of 26 (range, 25-30) years.

Enzyme-linked immunosorbent assay

The levels of plasma GDF11 were measured using a human enzyme-linked immunosorbent assay kit (Cloud-Clone USCN, USA).

Quantity of erythropoiesis in bone marrow

Erythroid cells (CD235a+) were identified using flow cytometry. Herein, 100 μL of whole bone marrow was immunostained using fluorophore-

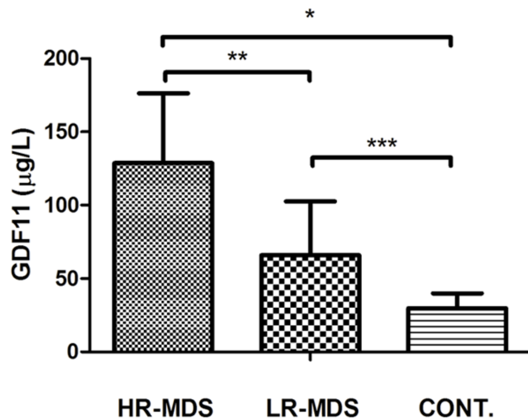

Figure 1. Levels of plasma GDF11 in high risk MDS (n=26), low risk MDS (n=16) and healthy controls (n=10). (*P<0.01, **P<0.05, ***P<0.05). HR-MDS: high risk MDS; LR-MDS: low risk MDS.

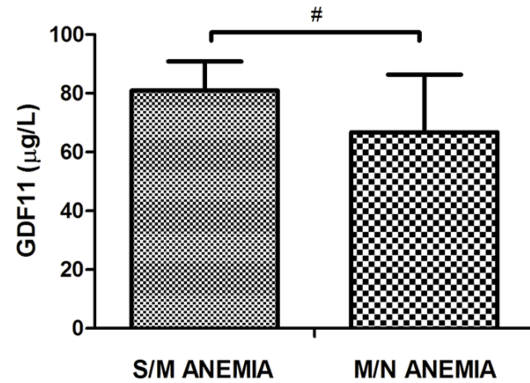

Figure 2. Levels of plasma GDF11 in MDS patients with severe or moderate anemia (n=35) and patients with mild anemia or without anemia (n=9). (#P>0.05). S/M ANEMIA: severe or moderate anemia; M/N ANEMIA: mild or no anemia.

conjugated monoclonal antibodies: CD235a-FITC, and mouse isotype control monoclonal antibody (Becton Dickinson [BD], Franklin Lakes, NJ, USA) in TruCount tubes (BD), followed by red blood cell lysis in 1.0 mL of FACS RBC lysing solution (BD). Samples were acquired on a FACSCalibur (BD) and analyzed using CellQuest version 3.1 (BD).

Real-time quantitative transcriptase-polymerase chain reaction (Q-PCR)

Bone marrow mononuclear cells (BMMNCs) were separated from fresh heparinized bone marrow samples (2 mL). Total RNA of BMMNCs was extracted using TRIzol reagent (Invitrogen Life Technologies, USA). The reverse transcription reactions to cDNA were performed using the QIAGEN OneStep RT-PCR Kit (QIAGEN, Germany). The gene expressions were quantified by Q-PCR (SYBR Green, ABI PRISM-7500 Sequence Detection system, USA). The primer sequences were as follows: GAPDH forward 5'-GCA CCG TCT AGG CTG AGA TG-3', reverse 5'-TGG TGA AGA CGC CAG TGG T-3'; GDF11 forward 5'-TGC GCC TAG AGA GCA TCA AGT-3', reverse 5'-CCC AGT TAG GGG TTT CAG TCG T-3'. The relative quantification (RQ) of gene expression used $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{GAPDH})_{patients} - (Ct_{target\ gene} - Ct_{GAPDH})_{control}$).

Collection of clinical data

The complete blood counts (CBC) of the patients and controls were collected, including RBCs, Hb, reticulocyte, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular-hemoglobin concentration (MCHC). Nucleated erythroblasts in bone marrow were calculated by two hemato-pathologists.

Statistical analysis

All analyses were performed using SPSS Version 21.0 software (SPSS Science). Data were presented as mean ± SD. The Student *t* test was used for two independent groups. The Pearson correlation test was used for correlation analysis. A *P* value of <0.05 was considered statistically significant.

Results

GDF11 was increased in the plasma of MDS patients

The concentration of GDF11 in high-risk MDS patients (128.67±47.62 μg/L) was significantly higher than that in low-risk MDS patients (65.96±36.55 μg/L, P<0.01) and higher than that in normal controls (29.76±10.10 μg/L, P<0.01). The concentration of GDF11 in low-risk MDS patients was significantly higher than that in normal controls (P<0.05) (**Figure 1**).

GDF11 was elevated in the plasma of severe/moderate anemia MDS patients

The concentration of GDF11 in severe/moderate anemia MDS patients (80.97±9.94 μg/L) was higher than that in normal controls/mild anemia MDS patients (66.82±19.52 μg/L).

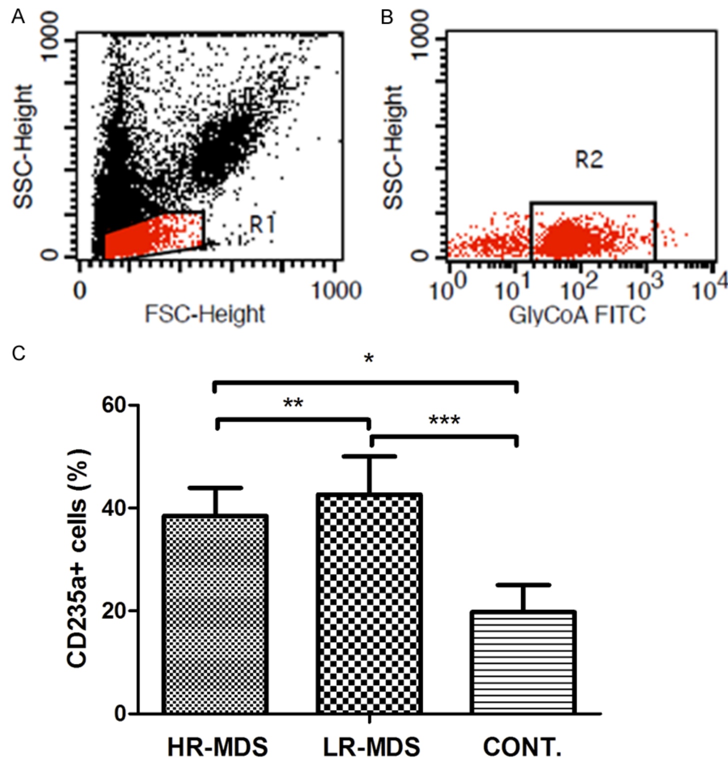

Figure 3. The percentage of CD235a+ cells in bone marrow of high risk MDS (n=26), low risk MDS (n=16) and healthy controls (n=10). A. Flow gates BMMNC with SSC and FSC. B. Flow gates CD235a+ cells with GlyCoA-FITC antibody. C. The percentage of CD235a+ cells in bone marrow of high risk MDS and low risk MDS are higher than that of healthy controls. (*P<0.01, **P>0.05, ***P<0.01). HR-MDS: high risk MDS; LR-MDS: low risk MDS; BMMNC: bone marrow mononuclear cells.

However, there was no statistically significant difference between the groups (P>0.05) (**Figure 2**).

CD235a+ cells increased in the bone marrow of patients with MDS

The percentage of CD235a+ cells was 38.49%±5.42% in the high-risk group and 42.64%±7.36% in the low-risk group, and both were significantly higher than in the controls (19.76±5.27%; both P<0.05). There was no statistically significant difference between the high-risk group and the low-risk group (P>0.05; **Figure 3**).

GDF11 correlated with erythropoiesis in MDS patients

In high-risk MDS patients, the expression of GDF11 was negatively correlated with Hb, RBC, and Hct in peripheral blood (r=-0.437, -0.430, and -0.306, respectively; all P<0.05), and positively correlated with reticulocyte, MCV, CD235a+ cells, and nucleated erythrocytes in bone marrow (r=0.465, 0.392, 0.505, and 0.387, respectively; all P<0.05), but not correlated with MCH (P>0.05) (**Figure 4A**).

In low-risk MDS patients, the expression of GDF11 was positively correlated with CD235a+ cells in bone marrow (r=0.429, p<0.05), and not correlated with Hb, reticulocytes, RBCs, MCHC, MCV, MCH, Hct, or nucleated erythroblasts (all p>0.05) (**Figure 4B**).

GDF11 mRNA was overexpressed in BMMNCs of MDS patients

The relative expressions of GDF-11 mRNA in the MDS group (39.82±14.55) was higher than that in the control group (1.84±0.64, P<0.01) (**Figure 5**).

Discussion

The number of RBCs remains relatively steady through erythropoiesis and destruction. When this equilibrium is disrupted, anemia is the result. The body will activate a series of stress responses to compensate for the drop in Hct and Hb. The proliferation and differentiation of erythroid precursor cells depends on stem cell factor and EPO, but not late-stage erythroid cells. The anemia observed in MDS patients is characterized by ineffective erythropoiesis and excess apoptosis of premature RBCs that can lead to overproduction of EPO. Furthermore, some patients with MDS are not sensitively responsive to EPO [5]. Therefore, it is urgent to explore other mechanisms regulating late-stage erythropoiesis.

The TGF-β superfamily is a potential regulator of EPO and iron metabolism, including GDF11 and GDF8. Suragani *et al.* [4] reported that GDF11-ActRIIB-Smad2/3 signaling plays a key

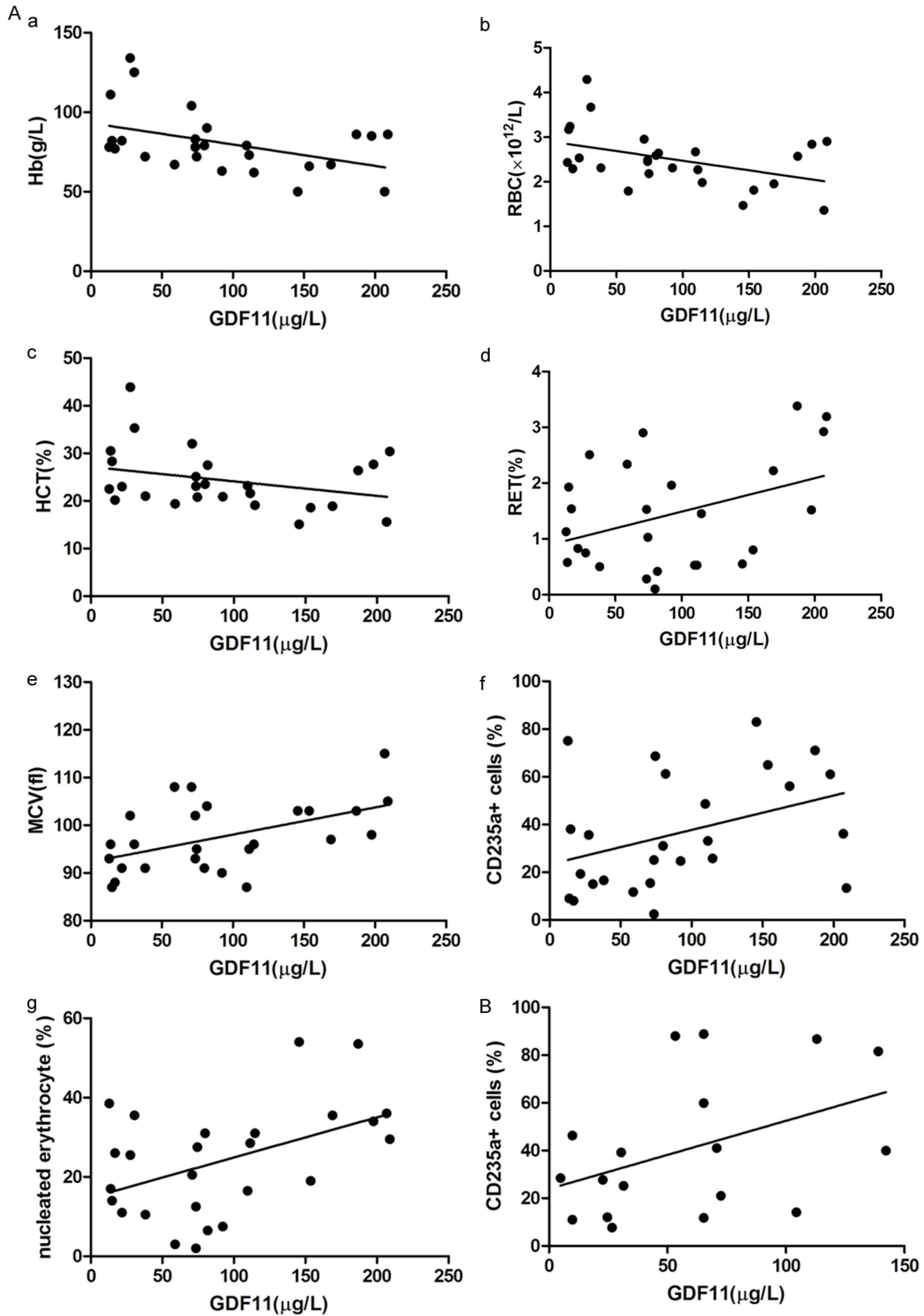

Figure 4. The relationship between plasma GDF11 and erythropoiesis index in MDS patients. A. The relationship between plasma GDF11 and erythropoiesis index in high risk group. a-c: In high-risk MDS patients, the expression of GDF11 was negatively correlated with Hb, RBC and Hct in peripheral blood ((r=-0.437, -0.430, -0.306, all P<0.05).

d-g: The expression of GDF11 was positively correlated with RET%, MCV, CD235a+ cells and nucleated erythrocytes in bone marrow (r=0.465, 0.392, 0.505, 0.387, all P<0.05). B. The relationship between plasma GDF11 and erythropoiesis index in low risk group. In low-risk MDS patients, the expression of GDF11 was positively correlated with CD235a+ cells in bone marrow (r=0.429, *P*<0.05).

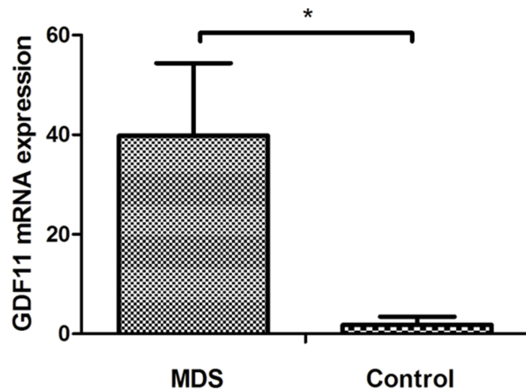

Figure 5. GDF11 mRNA expression of BMMNCs of MDS patients and healthy controls. (*P<0.01).

role in regulating the proliferation and differentiation of late-stage RBCs. Additionally, the levels of plasma GDF11 in MDS patients and NUP98-HOX13 in MDS mice were significantly higher than in normal controls. ACE-536 (human) and RAP-536 (mice) function as ligand traps to block the interaction of GDF11 with activin receptors on developing normoblasts and consequently promote the proliferation and differentiation of late-stage RBCs in normal and anemic patients or mice, thus increasing the levels of RBC and Hb. Dussiot *et al.* [6] studied thalassemia, another disease of ineffective erythropoiesis. Expression of GDF11 was increased in splenic erythroblasts from thalassemic mice and accompanied with the addition of ligands of the TGF-β superfamily, including activin receptor type I (ALK4/5) and type II (ActR IIA and ActR IIB). Activation of GDF11 increased reactive oxygen species and the amount of erythroid precursor cells. Abnormal GDF11 expression blocked terminal erythroid differentiation, which leads to anemia. ACE-011, a recombinant human fusion protein containing the extracellular domain of the human ActR IIA, binds to and inhibits activin and other members of the TGF-β superfamily. GDF11 inactivation decreases oxidative stress, resulting in increased terminal erythroid differentiation. Inactivation of GDF11 also corrects the abnormal ration of immature/mature erythroblasts by inducing apoptosis of immature erythroblasts through the Fas-Fas ligand pathway. Studies have demonstrated that treatment with ACE-011 can increase RBC and MCH levels, and decrease the degree of ineffective erythropoiesis and anemia [7].

Our results indicate the levels of GDF11 in MDS patients are significantly higher than in normal controls. The expression of GDF11 was associated with disease stage and degree of anemia. GDF11 expression is much higher when a patient is in a high-risk state or has severe anemia. The levels of GDF11 were negatively correlated with erythropoiesis, especially in the high-risk group. The expression of GDF11 was negatively correlated with Hb, RBC, and Hct, and positively correlated with reticulocyte, MCV, CD235a+ cells, and nucleated erythrocytes. Therefore, we speculated that the overexpression of GDF11 may be involved in the pathogenesis of anemia in MDS patients. Studies have demonstrated that GDF11 links with activin receptor IIa/IIb on erythroid differentiation, activating and thus bonding with a type I receptor, ALK4 or ALK5. Then, this recombinant protein initiates phosphorylated Smad2/3, which decreases erythropoiesis and differentiation, especially the late-stage erythroid, thus leading to refractory anemia [4]. It may be helpful to treat ineffective erythropoiesis by inhibiting GDF11 and receptors specifically so as to reduce Smad2/3 signaling. An open-label, Phase 2 study of ACE-536 in transfusion-dependent patients with low or intermediate-1 risk MDS confirmed its therapeutic effect. After treatment, 6 of 7 patients with low transfusion burden became transfusion independent. Six of nineteen patients with high transfusion burden saw a reduction in the frequency of transfusion, and 5 of 6 patients became independent [8]. The theory was also confirmed by the study of ACE-011 in patients with low or intermediate-1 risk MDS requiring transfusion. Of the 53 patients evaluable for efficacy, hematological improvement was observed in 21 (40%). Eight of nine patients with low transfusion burden showed red cell count increases. Of these, 6 patients became transfusion independent. Nineteen of forty-four patients with high trans-

fusion burden had responses. Five patients became transfusion independent. Increases in platelet and neutrophil levels were observed in some patients with baseline thrombocytopenia and neutropenia, respectively [9].

GDF11 is not only a regulator of hematopoiesis, but also a rejuvenation factor in other systems. By joining the circulatory system of an old mouse to that of a young mouse, scientists have produced some remarkable results. In the heart, brain, muscles, and almost every other tissue examined, the blood of young mice appears to bring new life to ageing organs [10]. Another study reported that direct infusions of GDF11 alone were sufficient to physically increase the strength and stamina of muscles, as well as to reverse DNA damage inside muscle stem cells [11]. Therefore, GDF11 received wide attention as having a "youth" factor [12-14]. However, when another laboratory attempted to reproduce these experiments they produced entirely different results. They showed that the levels of GDF11 increased during ageing and that it exhibited inhibitory functions on myogenesis and muscle regeneration. Given the data in humans showing an age-related increase, GDF11 could be a target for pharmacologic blockade to treat age-related disorders [15]. However, Poggioli *et al.* [16] disagreed with this result. Their results showed circulating GDF11/8 levels decline with age. GDF11 can increase the ratio of erythroid precursor cells and block the differentiation and maturation of late-stage RBCs in MDS and thalassemia. Thus, we deduced that GDF11 could promote early-stage cell regeneration into other tissues. However, it needs further exploration to determine whether early-stage cells can differentiate and mature into terminal and functional cells. The main reason for these different results was the different methods of detection. When the body was responding to stress, was GDF11 overexpression a protective mechanism? If the overexpression of GDF11 in MDS patients leads to abnormal late-stage erythroid differentiation and maturation, was premature apoptosis a protective mechanism? Can ACE-536 or ACE-011 increase the transformation from MDS to AML? The effective ratio of ACE-536 or ACE-011 in treating anemia was approximately 40% in low transfusion burden patients, while high transfusion burden patients had a lower response ratio. Therefore, we inferred that apart from the abnormal levels of GDF11, there must be other reasons (for example, clonal hematopoietic stem cells inhibiting hematopoiesis) for anemia in MDS.

In conclusion, GDF11 levels are high in MDS patients. The expression of GDF11 is associated with disease stage and degree of anemia. The levels of GDF11 are correlated with erythropoiesis. Treatments targeting GDF11 may help to improve anemia in patients with MDS.

Acknowledgements

This project is partly supported by The National Natural Science Foundation of China (No. 81170472, 81400088, 81570111, 81500101), Application Bases and Advanced Technology Research Program of Tianjin (No. 14JCYBJC27200, 09JCYBJC11200).

Disclosure of conflict of interest

None.

Address correspondence to: Huquan Wang and Zonghong Shao, Department of Hematology, General Hospital, Tianjin Medical University, 154 Anshandao, Heping district, Tianjin 300052, China. Tel: 86-22-60817111; Fax: 86-22-60362085; E-mail: wanghuaquan@tmu.edu.cn (HQW); shaozonghong@sina.com (ZHS)

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