ORIGINAL ARTICLE

Hepcidin and GDF15 in anemia of multiple myeloma

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Abstract Multiple myeloma (MM) is a malignant disease
of plasma cells and is often accompanied by anemia which
may influence its progression and survival. The mechanism
of anemia of chronic disease (ACD) in which iron
homeostasis is impaired underlies that of MM-related
anemia. In this study, we analyzed the role of hepcidin
which is the main mediator of ACD and ACD-related
cytokines in peripheral blood of MM patients. We showed
that HAMP mRNA and growth differentiation factors 15
(GDF15) mRNA expressions in peripheral blood mono-
nuclear cells (PBMCs) and plasma hepcidin, GDF15,
interleukin-6 and erythropoietin in MM patients all
increased significantly as compared to those in controls. In
MM patients, the expression of HAMP mRNA showed a
positive correlation with serum ferritin level, and a nega-
tive correlation with hemoglobin level. The levels of
plasma hepcidin and GDF15 were significantly decreased
in MM patients who achieved complete remission after six
cycles VD (bortezomib + dexamethasone) regimen che-
motherapy. These data indicated that overexpression of
HAMP mRNA in PBMCs significantly correlated with
increased plasma hepcidin level and may be involved in the
pathogenesis of MM-related anemia. Furthermore, the
levels of plasma hepcidin and GDF15 may be valuable in
assessing the progress of MM.

Introduction

Multiple myeloma (MM) is a malignant disease that char-
acterized by monoclonal proliferation of plasma cells and
overproduction of monoclonal immunoglobulin. Anemia is
a common complication of MM which occurs in approxi-
mately 2/3 patients at diagnosis and could influence the
progression and survival of this disease [1]. Anemia in MM
is usually normocytic/normochromic. The mechanism of
anemia of chronic disease (ACD) in which iron homeostasis
is impaired underlies that of MM-related anemia [2, 3].
ACD is known as inflammatory-related anemia in which
feature is impairment of iron homeostasis [4–6].

The most important mediator of ACD is hepcidin, a
small peptide mainly produced by hepatocytes. Hepcidin is
expressed on many other cells which involved in iron
metabolism (including intestine epithelial cells, monocytic
macrophages, hepatocytes, etc.). Ferroportin is the main
target for hepcidin. Upon binding with hepcidin, the
internalization and degradation of ferroportin could be
induced, thereby preventing iron transport from intestinal
epithelial cells and release from reticuloendothelial cell
systems, finally leading to anemia. Growth differentiation
factors 15 (GDF15) is a member of TGF-beta superfamily.
GDF15 can regulate hepcidin in patients with thalassemia
anemia [7]. In recent, some studies showed that hepcidin
could be produced by monocytic macrophage cells besides
hepatocytes [8–10]. Hepcidin in serum and urine of MM
patients were all elevated [11, 12]. Hence, we suspect
whether the peripheral blood cells in MM, especially
lymphocytes/monocytes, could also express hepcidin or be

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Table 1 MM patients' and disease characteristics

Parameter	Number/level
Patients' number (untreated)	25 (20)
Age median (range)	58 (38–73)
Gender	Male = 16, female = 9
Myeloma type	IgG = 14, IgA = 8, LC = 2, Nsec = 1
Type of prior treatment	MPT = 3, MP = 2
Stage (ISS)	I = 2, II = 7, III = 16
M-component (g/l)	37 (1.97–89.3)
β_2 microglobulin (mg/l)	5.6 (2.3–18.78)
Albumin (g/l)	34 (26–41)
C-reactive protein (mg/dl)	1.53 (1.38–5.68)
HB (g/l)	105 (73–137)
MCV(fl)	91.78 (81.8–104.8)
MCH (pg)	30.37 (25.5–33.2)
MCHC (g/l)	330.44 (310–357)
SF (ng/ml)	581.52 (50.3–1125.51)
SI (μmol/l)	5.9 (4.8–12.9)
TIBC (μmol/l)	50.8 (42.8–72.9)
WBC (10^9/l)	4.2 (3.6–8.9)
Plt (10^9/l)	159 (97–357)

MPT melphalan prednisone thalidomide, *MP* melphalan prednisone, *VD* bortezomib dexamethasone, *ISS* International Scoring System, *M-component* monoclonal component, *HB* hemoglobin, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin, *SF* serum ferritin, *SI* serum iron, *TIBC* total iron binding capacity

involved in the pathogenesis of MM-related anemia. In this study, we detected the expression of HAMP mRNA and GDF15 mRNA in peripheral blood mononuclear cells (PBMCs) of MM patients, and investigated the roles of ACD-related cytokines in MM.

Materials and methods

Patient selection

Twenty-five patients diagnosed with MM (according to the criteria of the International Myeloma Working Group) at the Hematology Department of General Hospital, Tianjin Medical University, Tianjin, China, from 02/15/2013 to 10/30/2013 were included in this study (Table 1). Fifteen healthy blood donors were selected as controls, including 5 males and 10 females (median age 35, age range 26–48).

The patients with MM would be enrolled if they had characteristics as follows: (1) age ≥ 18 years, and (2) untreated or not achieved complete remission (CR) after treatment, and (3) without active infection for at least a week. The patients would be excluded if they had characteristics as follows: renal insufficiency (creatinine > 175 μmol/ml), heart failure (B natriuretic peptide > 100 pg/ml), gastrointestinal hemorrhage, hemolysis, hypersplenism, plasma cell leukemia, bone marrow suppression by chemotherapy (white blood cells < 2 × 10^9/l), iron deficiency (serum ferritin < 14 μg/l), folic acid deficiency (serum folic acid < 3 ng/ml), vitamin B_{12} deficiency (serum vitamin B_{12} < 100 pg/ml).

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

PBMCs were separated from fresh heparinized blood samples (2 ml). Total RNA of PBMCs was extracted using TRIzol reagent (Invitrogen Life Technologies, USA). The reverse transcription reactions to cDNA were performed using the M-MLV reverse transcriptase (Promega, USA). The gene expressions were quantified by Q-PCR (SYBR Green, ABI PRISM-7500 Sequence Detection system, USA). The primer sequences were as follows: β-actin forward 5′-CTC GCT TCG GCA GCA CA-3′, reverse 5′-AAC GCT TCA CGA ATT TGC GT-3′; hepcidin forward 5′-CCA CAA CAG ACG GGA CAA-3′, reverse 5′-GAA TAA ATA AGG AAG GGA GGG-3′; GDF15 forward 5′-GTT AGC CAA AGA CTG CCA CTG-3′, reverse 5′-CCT TGA GCC CAT TCC ACA-3′. The relative quantification (RQ) of gene expression used $2^{-\Delta\Delta C_t}$ method ($\Delta\Delta C_t = (C_{t\ target\ gene} - C_{t\ \beta\text{-actin}})_{patients} - (C_{t\ target\ gene} - C_{t\ \beta\text{-actin}})_{ctrl}$).

Enzyme-linked immunosorbent assay (ELISA)

The levels of plasma hepcidin, GDF15, interleukin-6 (IL-6) and erythropoietin (EPO) were measured by human ELISA kit (R&D Systems, USA).

Statistical analysis

All analyses were performed using SPSS 18.0 software (SPSS Science). Data were presented as mean ± SD. The Student *t* test was used for two independent groups. A paired *t* test was used for two groups of paired data. Pearson correlation test was used for correlation analysis. A *P* value of < 0.05 was considered as statistically significant.

Results

HAMP mRNA and GDF15 mRNA were overexpressed in PBMCs of MM patients

The relative expressions of HAMP mRNA and GDF15 mRNA in MM patients were (4.32 ± 2.45)and (2.41 ± 1.02) folds higher than those in the controls,

Fig. 1 Comparison of expression of HAMP mRNA and GDF15 mRNA in PBMCs among these groups. **a** The expression of HAMP mRNA and GDF15 mRNA in PBMCs of MM patients was significantly higher than those in normal controls (both $P < 0.001$). **b** The expression of HAMP mRNA in PBMCs of ISS-III patients was significantly higher than that in ISS-I/II patients ($P < 0.05$); the expression of GDF15 mRNA had no statistical difference between them ($P > 0.05$). **c** The expression of HAMP and GDF15 mRNA had no statistical difference between IgG and IgA subtype in MM patients (both $P > 0.05$)
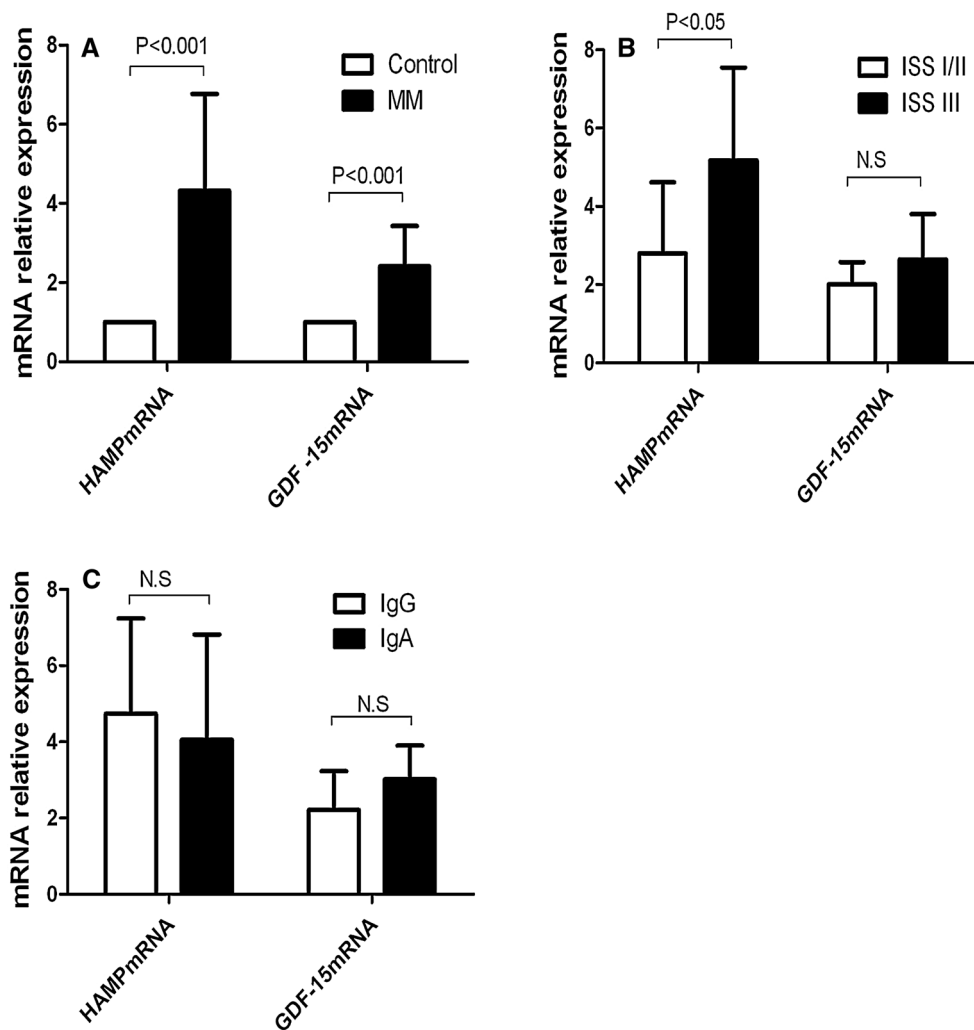
respectively (both $P < 0.001$) (Fig. 1a). The relative expression of HAMP mRNA in ISS-III patients (5.18 ± 2.37) was significantly higher than that in ISS-I/II patients (2.80 ± 1.82, $P < 0.05$), but that of GDF15 mRNA had no significant difference between these two groups (2.64 ± 1.16 vs. 2.01 ± 0.57, $P > 0.05$) (Fig. 1b). The relative expression of HAMP or GDF15 mRNA had no significant difference between IgG and IgA subgroup in MM patients (HAMP mRNA 4.74 ± 2.05 vs. 4.06 ± 2.76, $P > 0.05$; GDF15 mRNA 2.21 ± 1.01 vs. 3.02 ± 0.88, $P > 0.05$) (Fig. 1c).

The plasma hepcidin, GDF15, IL-6 and EPO were increased in MM patients

The MM patients had significantly higher levels of plasma hepcidin (63.73 ± 17.46 ng/ml), GDF15 (1241.65 ± 688.3 pg/ml), IL-6 (11.12 ± 14.46 pg/ml), and EPO (64.44 ± 61.71 μIU/ml) than those in the controls (33.25 ± 16.05 ng/ml, 401.31 ± 92.37 pg/ml, $2.92 \pm$

1.97 pg/ml, 30.70 ± 25.30 μIU/ml, respectively) (Fig. 2a–d). The levels of hepcidin (71.20 ± 16.19 ng/ml) and GDF15 (1452.47 ± 717.87 pg/ml) in ISS-III patients were significantly higher than those in ISS-I/II patients (50.4 ± 10.62 ng/ml, 863.87 ± 460.03 pg/ml, respectively, both $P < 0.01$) (Fig. 2e, f). The levels of both hepcidin and GDF15 had no significant difference between the IgG and IgA subgroups in MM patients (hepcidin 64.17 ± 18.15 vs. 65.64 ± 19.30 ng/ml, GDF15 1087.91 ± 678.82 vs. 1666.39 ± 659.36 pg/ml, both $P > 0.05$) (Fig. 2g, h).

Correlation analysis among the clinic and laboratory parameters of MM patients

In MM patients, the expression of HAMP mRNA had a significantly positive correlation with serum ferritin level ($r = 0.680$, $P < 0.001$, Fig. 3a) and hepcidin level ($r = 0.772$, $P < 0.01$, Fig. 3c), and a negative correlation with hemoglobin (HB) level ($r = 0.596$, $P < 0.01$,

Fig. 2 Comparison of the levels of plasma hepcidin, GDF15, IL-6 and EPO among these groups. **a–d** The levels of plasma hepcidin, GDF15, IL-6 and EPO in MM patients were significantly higher than those in normal controls ($P < 0.001$, $P < 0.001$, $P < 0.05$, $P < 0.05$, respectively). **e–f** The levels of plasma hepcidin and GDF15 of ISS-III patients were statistically higher than those of ISS-I/II patients ($P < 0.01$, $P < 0.05$, respectively). **g, h** The levels of plasma hepcidin and GDF15 had no statistical difference between IgG and IgA subtype in MM patients (both $P > 0.05$)
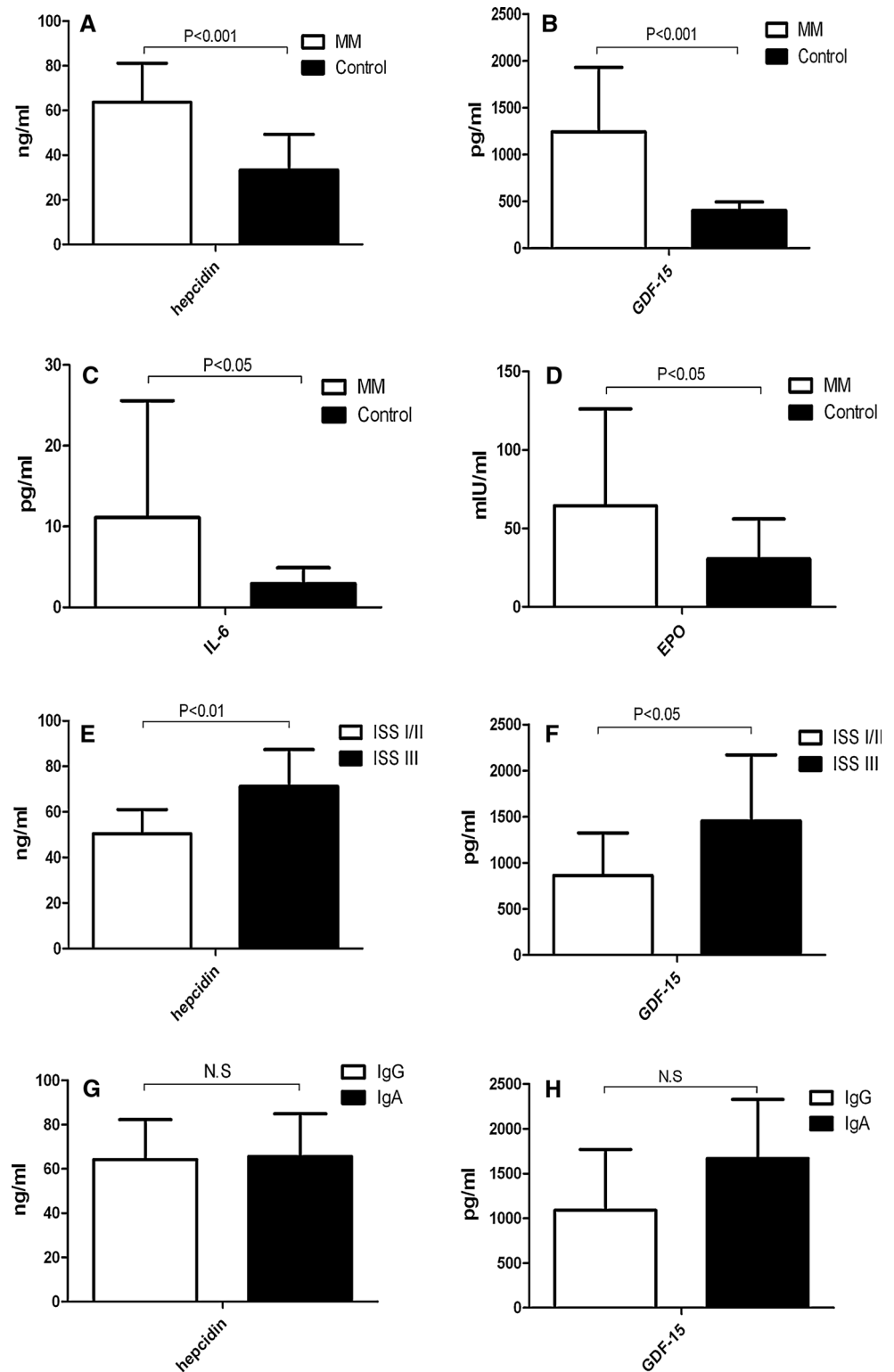

Fig. 3b), but had no correlation with GDF15 mRNA expression (Fig. 3d). The expression of GDF15 mRNA had a positive correlation with plasma GDF15 level ($r = 0.645$, $P < 0.01$, Fig. 3e), and had no correlation with serum ferritin level (Fig. 3f). The EPO level had a negative correlation with HB ($r = 0.439$, $P < 0.05$, Fig. 4a) and no correlation with GDF15 ($r = 0.376$, $P > 0.05$, Fig. 4b). No correlation was found between the plasma IL-6 level and HAMP mRNA expression (Fig. 4c). There was a significantly positive correlation between the plasma IL-6 level

Fig. 3 Correlation between HAMP/GDF15 mRNA and other parameters. **a–c** The expression of HAMP mRNA had a significantly positive correlation with the levels of plasma ferritin ($r = 0.680$, $P < 0.001$) and hepcidin ($r = 0.772$, $P < 0.01$), and had a negative correlation with HB level ($r = -0.596$, $P < 0.01$). **d** There was no significant correlation between the expression of HAMP mRNA and GDF15 mRNA ($r = 0.283$, $P = 0.170$). **e, f** The expression of GDF15 mRNA had a positive correlation with plasma GDF15 level ($r = 0.645$, $P < 0.01$), but was no correlated with plasma ferritin level ($r = 0.333$, $P = 0.104$)
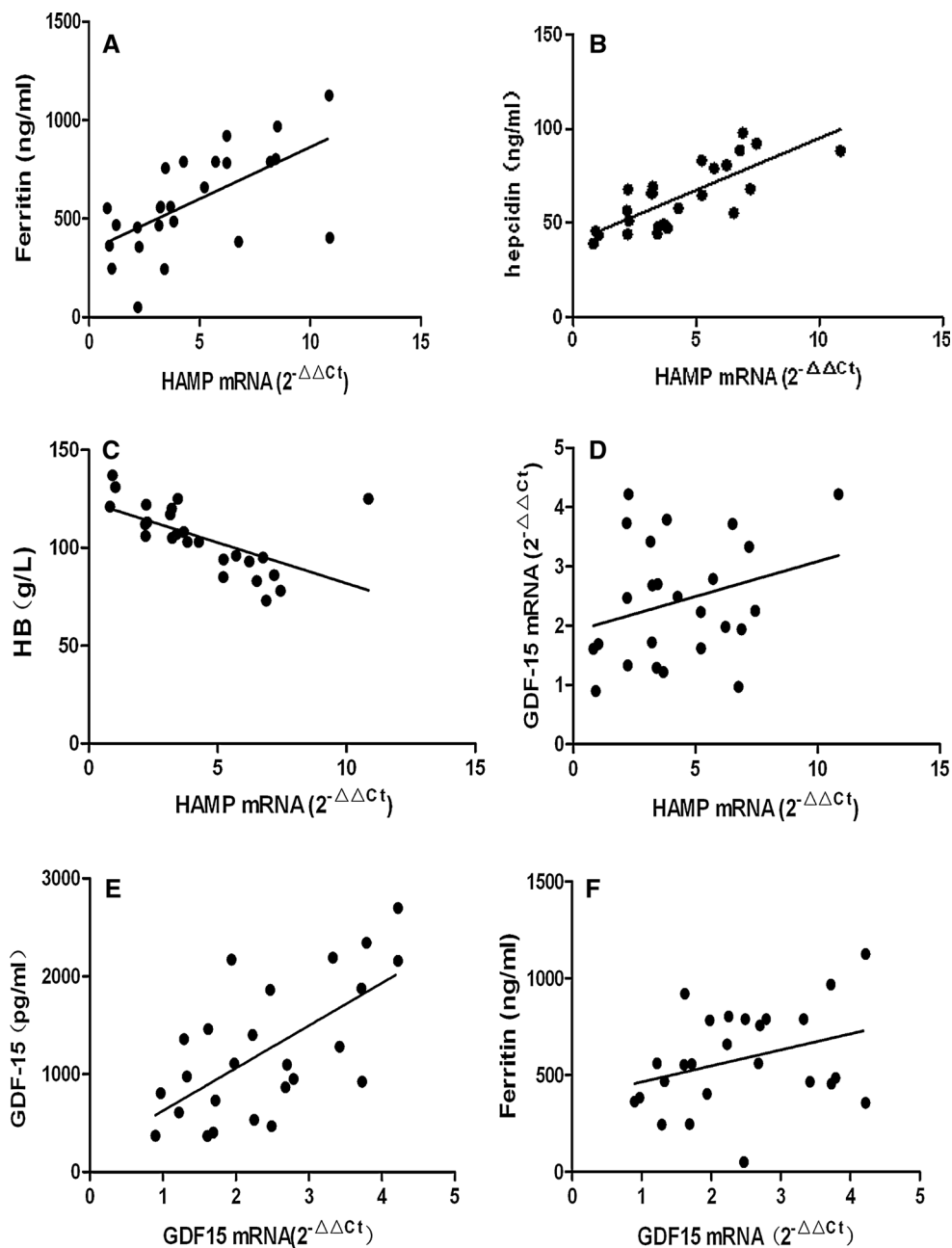
and hepcidin mRNA expression ($r = 0.453$, $P < 0.05$, Fig. 4d).

The changes of plasma hepcidin and GDF15 levels in MM patients who achieved CR after chemotherapy

We detected the levels of plasma hepcidin and GDF15 in 8 MM patients before and after treatment (bortezomib + dexamethasone, VD). After 6 cycles of VD treatment, they all achieved CR both in bone marrow and peripheral blood (with normal level of HB and CRP) (Table 2). The levels of plasma hepcidin ($42.18 \pm$ 12.88 ng/ml) and GDF15 (866.48 ± 407.36 pg/ml) after 6 cycles VD treatment were all significantly decreased compared with those before treatment (66.50 ± 21.14 ng/ml, $P < 0.05$; 1544.83 ± 592.82 pg/ml, $P < 0.01$, respectively) (Fig. 5).

Discussion

Hepcidin is an iron regulatory hormone produced by hepatocytes. Recent researches have proved that increased hepcidin plays an important role in the pathogenesis of

Fig. 4 Correlation between EPO/IL-6 and other parameters. **a**, **b** The plasma EPO level had a negative correlation with HB level ($r = -0.439$, $P < 0.05$), and had no significant correlation with plasma GDF15 level ($r = 0.376$, $P = 0.06$). **c**, **d** The plasma IL-6 level had a positive correlation with plasma hepcidin level ($r = 0.453$, $P < 0.05$), and had no significant correlation with the expression of HAMP mRNA ($r = 0.358$, $P > 0.05$)
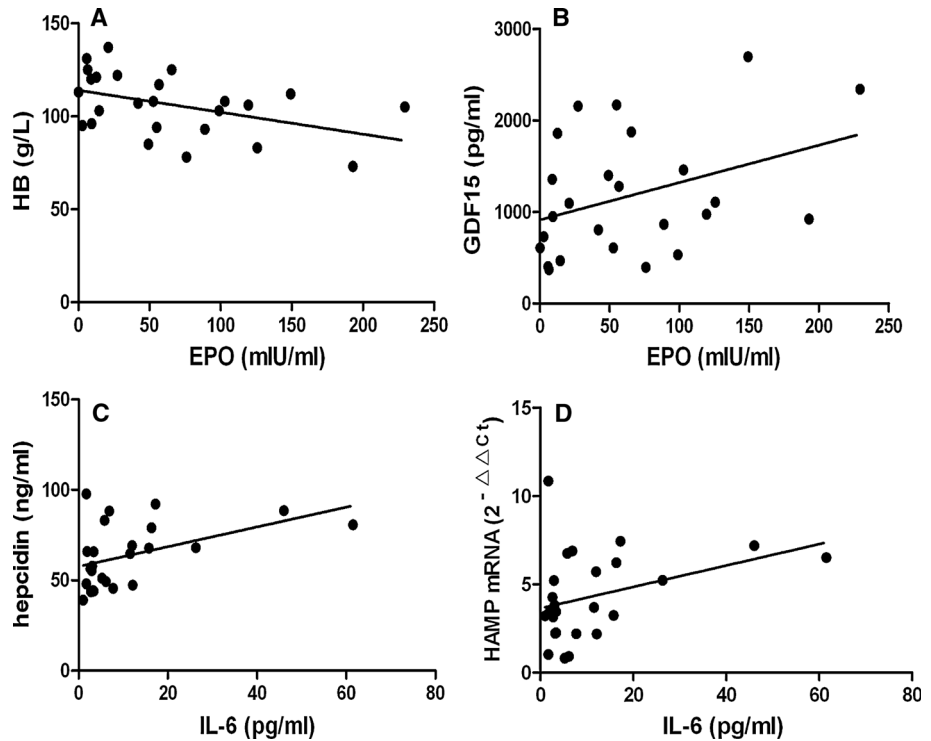
Table 2 Parameters of 8 untreated MM patients before and after VD treatment

Parameters	Before	After
WBC (10^9/l)	5.68 (3.97–8.96)	6.32 (5.36–7.65)
HB (g/l)	83 (76–103)	128 (118–137)
PLT (10^9/l)	235 (189–245)	198 (179–239)
C-reactive protein (mg/dl)	1.56 (1.38–3.39)	0.06 (0.03–0.08)
Albumin (g/l)	34 (28–42)	36 (30–44)

ACD. Studies in humans and animal models suggested that hepcidin overproduction is induced by inflammatory cytokines, such as IL-6, IL-1β, transforming growth factor-β, and bone morphogenic proteins 2, 4, 6 and 9 [13–20].

Some researchers found that hepcidin levels in serum and urine of patients with MM were higher than those in normal controls, which accompanied by higher ferritin levels and lower HB levels [10, 11, 21]. These results indicated that hepcidin might be involved in the mechanisms of MM-related anemia. Our results showed that HAMP mRNA expression of PBMCs in MM patients was significantly higher than those in healthy controls. The plasma hepcidin level significantly increased in MM and had a positive correlation with the expression of HAMP mRNA in the PBMCs. HAMP mRNA expression of PBMCs in MM also had a positive correlation with the serum ferritin level and the severity of anemia. These results indicated that pathologic hepcidin production

Fig. 5 Change of the levels of plasma hepcidin and GDF15 after VD chemotherapy. The levels of plasma hepcidin and GDF15 after 6 cycles of VD chemotherapy were all significantly lower than those before treatment ($P < 0.05$, $P < 0.01$, respectively)
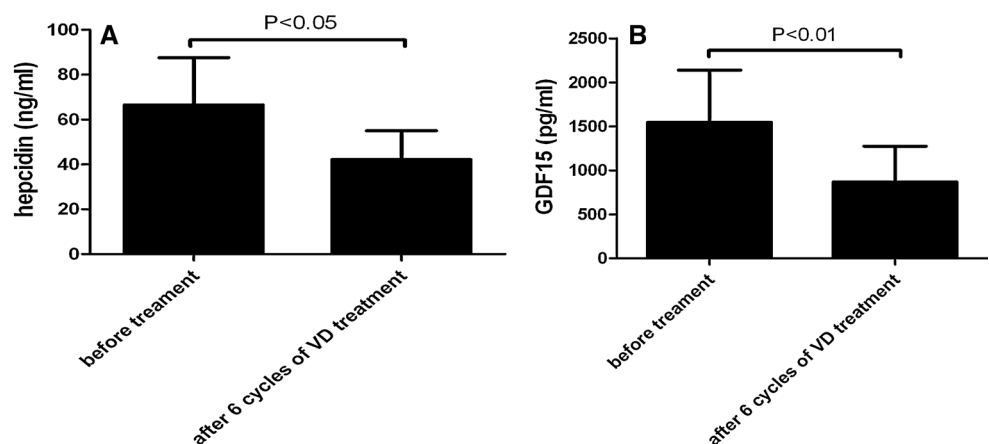
mediated impaired iron homeostasis in MM patients with anemia.

Wrighting and Andrews [22] reported that IL-6 could induce the expression of hepcidin through the JAK-STAT signaling pathway, in the conditions of sepsis, inflammatory bowel disease, malignant tumor, inflammation or chronic infection. Sharma et al. [12] found that hepcidin was upregulated in MM by both IL-6-dependent/independent pathways, and causing the development of anemia. Our study showed that plasma IL-6 levels in MM patients were significantly higher than those in normal controls, and had a positive correlation with the levels of plasma hepcidin. But there was no significant correlation between the IL-6 level and HAMP mRNA expression of PBMCs in MM. We inferred it because hepcidin is mainly produced by hepatocyte, but not PBMCs.

Several studies demonstrated that GDF15 is aberrantly secreted by bone marrow stromal cells and its serum levels are significantly elevated in MM patients, suggesting that GDF15 is a key survival and chemoprotective factor for MM cells [23–25]. Our results showed that GDF15 mRNA expression of PBMCs and the plasma GDF15 level in MM patients were both higher than those in normal controls. The expression of GDF15 mRNA in PBMCs and plasma GDF15 in ISS-III patients was also significantly higher than those in ISS-I/II patients. Katodritou et al. [21] found that serum hepcidin level in anemic MM patients was inversely correlated with HB and PLT counts and positively correlated with the levels of β_2MG, ferritin, TSAT % and ISS. Serum hepcidin levels above the upper normal limit were inversely correlated with duration of response in all studied patients. Tarkun et al. [23] showed that MM patients with high serum GDF15 levels had lower rates of event-free survival and overall survival. Tanno et al. [25] demonstrated that GDF15 could enhance the tumor-initiating and self-renewal potential in MM, which affecting on long-term outcomes. In the current study, we found that the levels of plasma hepcidin and GDF15 in ISS-III patients were all higher than those in ISS-I/II patients. We also observed the changes of plasma hepcidin and GDF15 in 8 patients with MM. And we found that the levels of both plasma hepcidin and GDF15 were significantly decreased after 6 cycles of VD treatment. Our results suggested that the levels of plasma hepcidin and GDF15 might be valuable to assess the progress of MM.

Researches demonstrated that GDF15 was involved in erythroid generation, and EPO could stimulate erythroid precursors to secrete GDF15 [7, 26, 27]. We found that plasma EPO level had a negative correlation with the HB level, and it had a positive correlation with the GDF15 level, though statistical significance was not reached ($P = 0.06$).

Increased expressions of GDF15 were frequently found in ineffective erythropoiesis diseases such as β-thalassemia, RARS, congenital dyserythropoietic anemia type-1 (CDA1), and pyruvate kinase deficiency (PKD) [27] which characterized by erythroid apoptosis. Tanno et al. [7] proved that high GDF15 expression could inhibit the secretion of hepcidin in Mediterranean anemia patients. Tamary et al. [26] studied the role of GDF15 in CDA1, and found that all CDA1 patients with no history of the red blood cells transfusion had higher serum GDF15 levels than those in controls. They suggested that high expression of GDF15 in CDA1 patients contributed to the inappropriate suppression of hepcidin with subsequent secondary hemochromatosis.

Our data, however, showed that there was no correlation between the expression of hepcidin and GDF15 in PBMCs of MM patients. Studies showed that the expression of GDF15 was associated with ages [28], and would be increased in the conditions of stresses, such as hypoxia, inflammation, X-ray exposure, acute injury and cancer [29, 30]. Possibly, only a very high level of GDF15 (> 5000 pg/ml) could suppress the secretion of hepcidin [26], which was very difficult to achieve in normal physiological condition or other diseases except β-thalassemia, CDA1, PKD and RARS.

Recently, Léon et al. [31] reported that erythroid factor erythroferrone plays an important role in iron homeostasis. They found that erythroferrone mRNA expression was greatly increased in bone marrow and spleen after phlebotomy or EPO stimulation which acted on hepatocytes to suppress hepcidin. They also found that erythroferrone mRNA was greatly increased in bone marrow and spleen of β-thalassemia Hbb$^{th3/+}$ mouse model as compared to wild-type controls. Their results suggested that erythroferrone may be an erythroid factor to suppress hepcidin through increasing erythropoietic activity, and may contribute to the pathogenesis of iron-loading anemia. Hence, we inferred that the MM-related anemia might also have other regulatory factors to hepcidin and iron homeostasis besides GDF15.

Since hepcidin is mainly secreted by hepatocytes, the detection of HAMP mRNA expressions in liver through biopsy or operation is ideal, but these procedures are invasive. Our results showed that HAMP mRNA was also expressed by PBMCs, which offered a new way to further studies. However, whether HAMP mRNA expression in PBMCs is consistent with that in hepatocytes is unknown, and which types of cells produce hepcidin and GDF15 in PBMCs are still remain to be determined in our future studies.

In conclusion, our data indicated that overexpression of HAMP mRNA in PBMCs resulting in increased serum hepcidin levels might be involved in the occurrence of the

anemia of MM. The levels of plasma hepcidin and GDF15 might be valuable to assess the progress of MM. As the mechanism of MM-related anemia has become increasingly clear, some new treatment strategies will be possible. The improvement of life quality and prognosis in MM patients is just around the corner.

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