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Downregulation of intrinsic apoptosis pathway in erythroblasts contributes to excessive erythrocytosis of chronic mountain sickness

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ABSTRACT

Chronic mountain sickness (CMS) has a higher incidence in the plateau region and is characterized by excessive erythrocytosis and hypoxemia. Bcl-2 family plays an important role in the process of erythropoiesis and the regulation of apoptosis. This study aimed to examine the change in apoptosis of erythroblasts in CMS patients and explore the involvement of Bcl-2 family. Bone marrow mononuclear cells (BMMNCs) were isolated by density gradient centrifugation from 18 CMS patients and 17 control participants. The apoptotic rate, mitochondrial membrane potential (MMP), the protein expression of caspase-3, TNFR, Fas, Bcl-2, Bax and Cyt-C were examined by flow cytometry, and mRNA expression was determined by real-time PCR. The results showed that apoptotic rate of erythroblasts was lower and MMP was higher in CMS group than in control group. The mRNA and protein expression levels of Bcl-2 were higher while Bax level was lower in CMS group than in control group. In CMS group, the apoptosis rate of $CD71^+$ erythroblasts was negatively correlated with the ratio of $CD71^+$ cells in BMMNCs and positively correlated with hemoglobin level. In conclusion, erythroblasts apoptosis is decreased due to the regulation of the expression of Bcl-2 family members in the erythroblasts of CMS patients.

1. Introduction

Chronic mountain sickness (CMS) is a clinical syndrome characterized by excessive erythrocytosis and hypoxemia, and it mainly occurs in people living in high altitude area above 2500 m and maladapted to hypoxia environment [1–3]. Qinghai-Tibet Plateau is the highest, the largest and the most populated plateau in the world, and the incidence of CMS is the highest in the plateau area [4]. With the increase of altitude, the prevalence rate of CMS rises linearly [5].

Previous studies have suggested that the proliferation of erythroid cells in bone marrow plays an important role in the pathogenesis of CMS, which is related with the overexpression of hypoxia inducible factor (HIF) and increased circulating levels of erythropoietin (EPO) [6–8]. Our previous studies indicated the apoptotic index of bone marrow mononuclear cells (BMMNCs) and cultured erythroblasts decreased in CMS patients [9–11]. It was speculated that the downregulation of apoptosis in erythroblasts may be related to the excessive erythrocytosis in CMS. However, the role of the apoptosis of erythroblasts in CMS remains elusive.

There are caspase dependent and independent apoptosis signal pathways, the former includes two main pathways: the extrinsic pathway and the intrinsic pathway [12]. The extrinsic pathway is initiated by the engagement of a transmembrane death receptor. In the intrinsic pathway (also known as the mitochondrial pathway), mitochondrial fragmentation during apoptosis is associated with mitochondrial membrane potential (MMP) collapse, and Bcl-2 family proteins are the most important regulators of outer mitochondrial membrane integrity and function [13]. Bcl-2 family proteins are divided into two classes. Bcl-2 and Bcl-xl inhibit apoptosis, while Bax, Bid, Bad, Bcl-xs, and Bak promote cell apoptosis, and caspase-3 is regarded as the key executor of apoptosis [14,15]. To our knowledge, there is still not report on apoptosis signal pathways involved in CMS. Therefore, this study aimed to determine the effect of erytroblasts apoptosis in bone marrow on the excessive erythrocytosis and to evaluate the changes in the extrinsic and intrinsic apoptosis pathways in the erytroblasts of CMS patients.

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2. Materials and methods

2.1. Subjects

The research protocol was approved by Ethics Committee at the Qinghai University Affiliated Hospital. Informed consent was obtained from each subject. A total of 18 patients with CMS and 17 control subjects participated in this study. All the participants were male Han Chinese (age 31–52 years), and they were born at lowland or moderate altitude and had resided at an altitude of 3000 m–4500 m for 5–20 years in Qinghai province of China. The control subjects were patients without any chronic diseases, who received elective orthopedic surgery to remove remotely placed internal fixation rods. None of the participants had a history of respiratory or cardiovascular diseases, such as chronic obstructive pulmonary disease, pulmonary infection, asthma, shunt valvular disease, congenital heart disease or hypertensive heart disease. The presence and severity of CMS were judged by the "consensus statement on chronic and subacute high altitude diseases" (Qinghai CMS score) [1].

2.2. Blood sampling and assay

2–3 mL blood was drawn from the brachial vein, collected into a tube containing EDTA. The measurement of hematocrit and Hb in venous blood samples were performed in a clinical laboratory using standard procedures (Sysmex XE2100, Japan). Arterial O_2 saturation (SaO_2) was measured by pulse oximetry.

2.3. Bone marrow sampling and assay

Bone marrow specimens were collected according to standard operating procedures. BMMNCs were isolated using ficoll lymphocyte solution. To separate erythroblasts, BMMNCs were incubated with CD71 MicroBeads for 15 min at 4 °C, washed and then placed in the magnetic field of a MACS Separator, and magnetically labeled cells were collected as erythroblasts.

2.4. Flow cytometry analysis of apoptosis

BMMNCs (1×10^5 cells) were incubated with APC-conjugated anti-CD71 (Biolegend) or PE-conjugated anti-CD34 (Ebioscience) for 15 min at room temperature (25 °C) in the dark. $CD34^+$ or $CD71^+$ cells in BMMNCs were analyzed by flow cytometry (BD, USA). For apoptosis detection, cells were washed and resuspended in 1 × binding buffer, then incubated with Annexin V-FITC and propidium iodide (PI) (BD, USA) for 15 min at room temperature in the dark. The apoptotic cells were detected by flow cytometry (BD, USA) within 1 h.

2.5. Flow cytometry analysis of mitochondrial membrane potential (MMP)

$CD71^+$ cells (1×10^6 cells) were incubated with JC-1 Working Solution (BD, USA) for 15 min at 37 °C in a CO_2 incubator. After washing with Assay Buffer, the cells were gently resuspended and MMP was detected by flow cytometry.

2.6. Flow cytometry analysis of apoptosis-related proteins

To detect death receptor pathway related membrane proteins TNFR and Fas, BMMNCs were incubated with 10 μL APC-conjugated anti-CD71, and then incubated with 10 μL PE-conjugated anti-TNFR and FITC-conjugated anti-FAS (BD) for 15 min at room temperature. After washing with cold PBS, the stained cells were analyzed by flow cytometry.

To detect active caspase-3 and mitochondrial pathway related proteins Bcl-2, Bax, and Cyt-C, BMMNCs were first incubated with 10 μL APC-conjugated anti-CD71 for 15 min, washed with cold PBS, and then incubated in Fixation/Permeabilization solution for 20 min at 4 °C, washed twice in 1 × BD Perm/Wash™ buffer, and then incubated with 10 μL PE anti-human caspase-3 (BD), FITC anti-human Bcl-2 (Ebioscience), Alexa Flour 488 anti-human Bax (Biolgend) or FITC anti-human Cyt-C (Biolgend) for 10 min at room temperature in the dark. After washing with Perm/Wash™ buffer, the stained cells were analyzed by flow cytometry.

2.7. Real-time PCR

Total RNA was isolated from $CD71^+$ cells using Trizol Reagent (Life Technologies, Carlsbad, CA, USA). cDNA was generated from 2 μg of total RNA using Quant Reverse Transcriptase (TIANGEN, Beijing, China), and then amplified using SYBR Green PCR Master Mix (Life Technologies) in an Applied Biosystems 7500 Real-Time PCR System as follows: 95 °C for 2 min, 60 °C for 32 s extension, for 40 cycles. The specific primers were purchased QIAGEN: Caspase3 (QT00023947), TNFR (QT00216993), Fas (QT00030618), Bcl-2 (QT00025011), Bax (QT00031192), Cyt-C (QT01021223). 18S rRNA was used as housekeeping gene. Data were analyzed using the $2^{-\Delta\Delta CT}$ method by ABI7500 software v2.0.4.

2.8. Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS, version 19.0) for Windows. Data of the normal distributions were reported as mean ± SD or mean ± SEM and tested using the Student's *t*-test. Correlation analysis was performed by linear correlation analysis. All tests were two-sided and a p value < 0.05 was considered to indicate significance.

3. Results

3.1. General characteristics of the subjects

The general characteristics of the studied participants are shown in Table 1. There were no significant differences in age, height, blood pressure and body-mass index (BMI) between two groups. Compared to control group, Hb ($p < 0.001$), hematocrit ($p < 0.001$), and erythrocyte counts ($p < 0.001$) were significantly higher while SaO_2 was significantly lower ($p < 0.001$) in CMS group. CMS score was 8–17 points in patients with CMS.

3.2. The characteristics of erythroblasts in bone marrow

The bone marrow cells from each group showed normal morphology (Fig. 1A). However, the myeloid: erythroid (M:E) ratio in CMS group (1.39 ± 0.29) was lower than that in control group (2.91 ± 0.54) ($p < 0.001$) (Fig. 1B). The proportions of polychromatic erythroblasts and orthochromatic erythroblasts in bone marrow nucleated cells of CMS patients increased significantly to 15.7 ± 3.7%

Table 1
Clinical characteristics of objects in CMS and control groups.

Characteristics	CMS (n = 18)	Control (n = 17)	*p* value
Age, yr	42.6 ± 5.2	41.5 ± 10.9	0.473
Height, cm	171.9 ± 4.6	170.3 ± 6.0	0.175
BMI, kg/m^2	23.9 ± 2.4	23.1 ± 1.6	0.107
Heart rate, beats/min	80.6 ± 9.3	80.4 ± 10.7	0.749
Systolic blood pressure, mmHg	126.4 ± 15.9	120.5 ± 9.9	0.075
Diastolic blood pressure, mmHg	85.3 ± 8.3	75.4 ± 8.0	0.083
Hemoglobin, g/L	217.1 ± 9.1	142.1 ± 17.9	< 0.001
Hematocrit, %	65.9 ± 4.8	41.8 ± 6.3	< 0.001
Erythrocytes, $\times 10^{12}$/L	7.0 ± 0.7	4.9 ± 0.6	< 0.001
SaO_2, %	85.8 ± 3.3	92.7 ± 2.0	< 0.001
CMS score	11 (8–17)	2 (0–3)	< 0.001

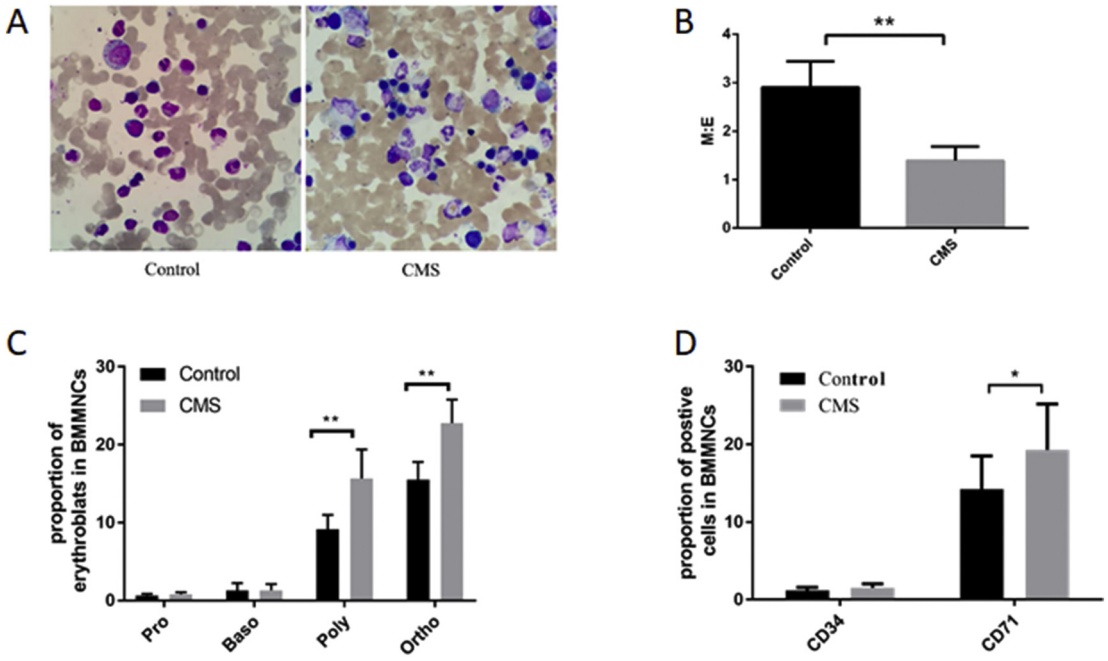

Fig. 1. The characteristics of erythroblasts in BMMNCs. A. Wright's staining of bone marrow cells from CMS and control groups (×1000). B. The myeloid: erythroid (M:E) ratios in bone marrow cells of CMS and control groups. C. The proportions of erythroblasts in different stage in bone marrow of CMS and control groups. Pro, pronormoblasts; Baso, basophilic pronormoblasts; Poly, polychromatophilic normoblasts; Ortho, orthochromatic normoblasts. D. The proportions of $CD34^+$ and $CD71^+$ cells in BMMNCs. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.001$.

and 22.7 ± 3.0%, respectively, in CMS group, compared to 9.2 ± 1.9% and 15.5 ± 2.3% in control group, respectively (Fig. 1C, $p < 0.001$). There were no significant differences in the ratio of pronormoblasts and basophilic pronormoblasts between two groups ($p > 0.05$).

Flow cytometry analysis of $CD34^+$ and $CD71^+$ cells in BMMNCs showed that the proportion of $CD71^+$ cells was significantly higher in CMS group than in control group (20.15 ± 6.77 vs. 15.02 ± 4.21, $t = -2.676$, $p = 0.012$), while the proportion of $CD34^+$ cells was similar in both groups (1.54 ± 0.49 vs. 1.20 ± 0.39, $t = -2.245$, $p = 0.032$, Fig. 1D).

3.3. Apoptotic rate of $CD71^+$ erythroblasts was lower in CMS patients

Based on flow cytometry analysis, the apoptotic rate of erythroblasts in bone marrow was significantly lower in CMS group than in control group (5.54 ± 2.30 vs. 7.32 ± 2.37, $t = 2.257$, $p = 0.031$, Fig. 2A, B). Furthermore, real-time PCR showed that mRNA levels of caspase-3, TNFR, Fas and Cyt-C in erythroblasts had no significant differences between two groups (Fig. 2C), while Bax mRNA level was significantly lower ($t = 2.279$, $p = 0.029$) and Bcl-2 mRNA level was significantly higher ($t = 2.075$, $p = 0.046$) in CMS group than in control group (Fig. 2C). Collectively, these data indicate that apoptotic rate of $CD71^+$ erythroblasts was lower in CMS patients.

3.4. Upregulation of Bcl-2 and downregulation of Bax and caspase-3 in the erythroblasts of CMS patients

To confirm that decreased apoptosis of the erythroblasts is related to the altered expression of Bcl-2 and Bax, we performed flow cytometry analysis of the erythroblasts after staining with respective antibodies. We found that protein levels of TNFR, Fas and Cyt-C in erythroblasts showed no significant differences between two groups (Fig. 3), while Bax and caspase-3 protein levels were significantly lower and Bcl-2 protein level was significantly higher in CMS group than in control group (Fig. 3). The expression levels of the apoptosis related proteins are consistent with their mRNA expression levels and suggest that decreased apoptosis of the erythroblasts is related to the altered expression of factors in intrinsic apoptosis pathway but not in extrinsic apoptosis pathway.

3.5. Higher MMP in the erythroblasts of CMS patients

To confirm that the decreased apoptosis of the erythroblasts is related to intrinsic mitochondrial apoptosis pathway, we detected MMP in $CD71^+$ erythroblasts in bone marrow of CMS and control subjects by flow cytometry with JC-1 fluorescent staining, which detected the loss of MMP (Fig. 4A). We found that JC-1 staining green fluorescence rate of $CD71^+$ erythroblasts in CMS group was 3.26 ± 1.97, significantly lower than that in control group (5.19 ± 3.32) ($t = 2.249$, $p = 0.040$). Thus MMP of $CD71^+$ erythroblasts in CMS group was higher than that in control group. These data indicate that intrinsic mitochondrial apoptosis pathway is inhibited in the erythroblasts of CMS patients.

3.6. Hb concentration was negatively correlated with apoptotic rate of $CD71^+$ erythroblasts of CMS patients

In CMS group, correlation analysis showed that Hb concentration was positively correlated with $CD71^+$ erythroblasts rate in BMMNCs ($r = 0.554$, $p = 0.017$, Fig. 5A) and negatively correlated with apoptotic rate of $CD71^+$ erythroblasts ($r = 0.228$, $p = 0.045$, Fig. 5B).

4. Discussion

Polycythemia is the main characteristic of CMS. Proliferation of erythroid precursor cells in bone marrow is an important factor in excessive accumulation of red blood cells in CMS. In this study, we showed that the myeloid: erythroid (M:E) ratios in CMS group was lower than that in control group, and the proportions of polychromatic erythroblasts and orthochromatic erythroblasts in bone marrow of CMS patients were significantly higher compared to control subjects. We further detected the percentage of $CD34^+$ and $CD71^+$ cells in BMMNCs by flow cytometry. CD34 is a surface marker of immature hematopoietic progenitor cells. CD71 is a transferrin receptor which is mainly

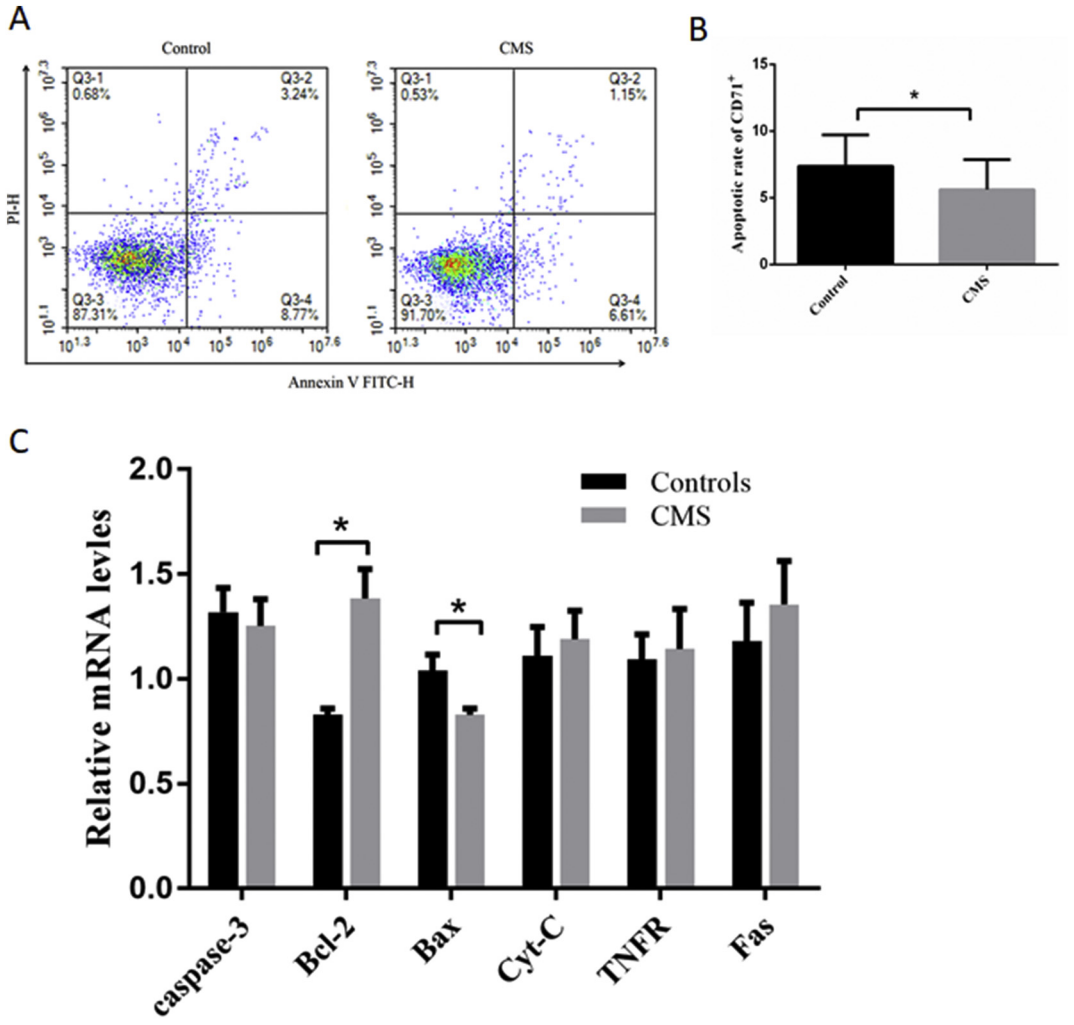

Fig. 2. Analysis of apoptotic rate and gene expression of CD71$^+$ erythroblasts. A. Annexin V and PI staining of CD71$^+$ erythroblasts in CMS patient and control subject. B. The apoptotic rate of CD71$^+$ erythroblasts in CMS group and control group. C. Relative mRNA levels of apoptosis related genes in erythroblasts. Data are presented as mean ± SEM. *$p < 0.05$.

expressed in erythroblasts and their precursors. Our results showed that although there was no significant change in the proportion of CD34$^+$ in BMMNCs between two groups, the ratio of CD71$^+$ erythroblasts in BMMNCs increased in CMS patients and was positively correlated with Hb level in peripheral blood. These results indicated that the proliferation of erythroblasts in CMS was enhanced, and contributed to excessive accumulation of erythrocytes in peripheral blood of CMS, in agreement with previous studies under hypoxia condition [16,17].

The main reason of the proliferation of erythroid cells in CMS bone marrow is increased expression of HIFs, which could induce high expression of many target genes such as EPO and VEGF [18]. These genes promote bone marrow cell proliferation and stimulate microangiogenesis. Meanwhile, HIF-2α and EPO concentrations increased in local microenvironment of bone marrow of CMS, indicating that local autocrine or paracrine mechanism in bone marrow contribute to erythroblast proliferation in CMS [19].

The proliferation of erythroblasts under high altitude environment is considered as a compensatory hematological response to hypoxia. The strengthened proliferation of hematopoietic cells could increase the number of peripheral erythrocytes and Hb level, facilitating the diffusion of oxygen to tissues [20]. However, when the total number of erythrocyte increases to beyond the normal range, it could increase blood viscosity, decrease blood flow velocity, reduce cardiac output, and increase the burden on the heart, even leading to CMS [21]. In physiological condition, there is a dynamic balance in proliferation and apoptosis of erythrocytes. In this study, we found that the apoptosis rate of CD71$^+$ cells in CMS group was lower than in control group, thus there was an unbalance between proliferation and apoptosis in the erythroblasts of CMS. Decreased apoptosis of erythroblasts may contribute to enhanced proliferation of erythroblasts and excessive accumulation of red blood cells in CMS.

Apoptosis is mainly regulated by extrinsic death receptor pathway and intrinsic mitochondrial pathway [12]. In the latter, after the mitochondria receive apoptosis signal, mitochondrial membrane permeability increases, leading to the decrease of MMP and the release of apoptosis-related factors such as Bcl-2 family, Cyt-C to induce apoptosis [13]. In this study we found that MMP was higher in CMS group compared to control group. Furthermore, we found no significant differences in mRNA and protein expression of TNFR and Fas in CD71$^+$ erythroblasts between CMS group and control group. However, anti-apoptotic Bcl-2 expression was significantly higher while pro-apoptotic Bax expression and caspase-3 expression were significantly lower in CMS group than in control group. These results suggest that the inhibition of intrinsic but not extrinsic apoptosis pathway may contribute to decreased apoptosis of erythroblasts in CMS patients.

It was demonstrated that leukemic cells infiltrating bone marrow reside in markedly hypoxic areas compared to cells in healthy bone marrow [22]. The expansion of leukemic cells increased hypoxia condition that promoted their resistance to chemotherapies [23,24]. Interestingly, hypoxia of leukemic cells deregulate apoptotic process and

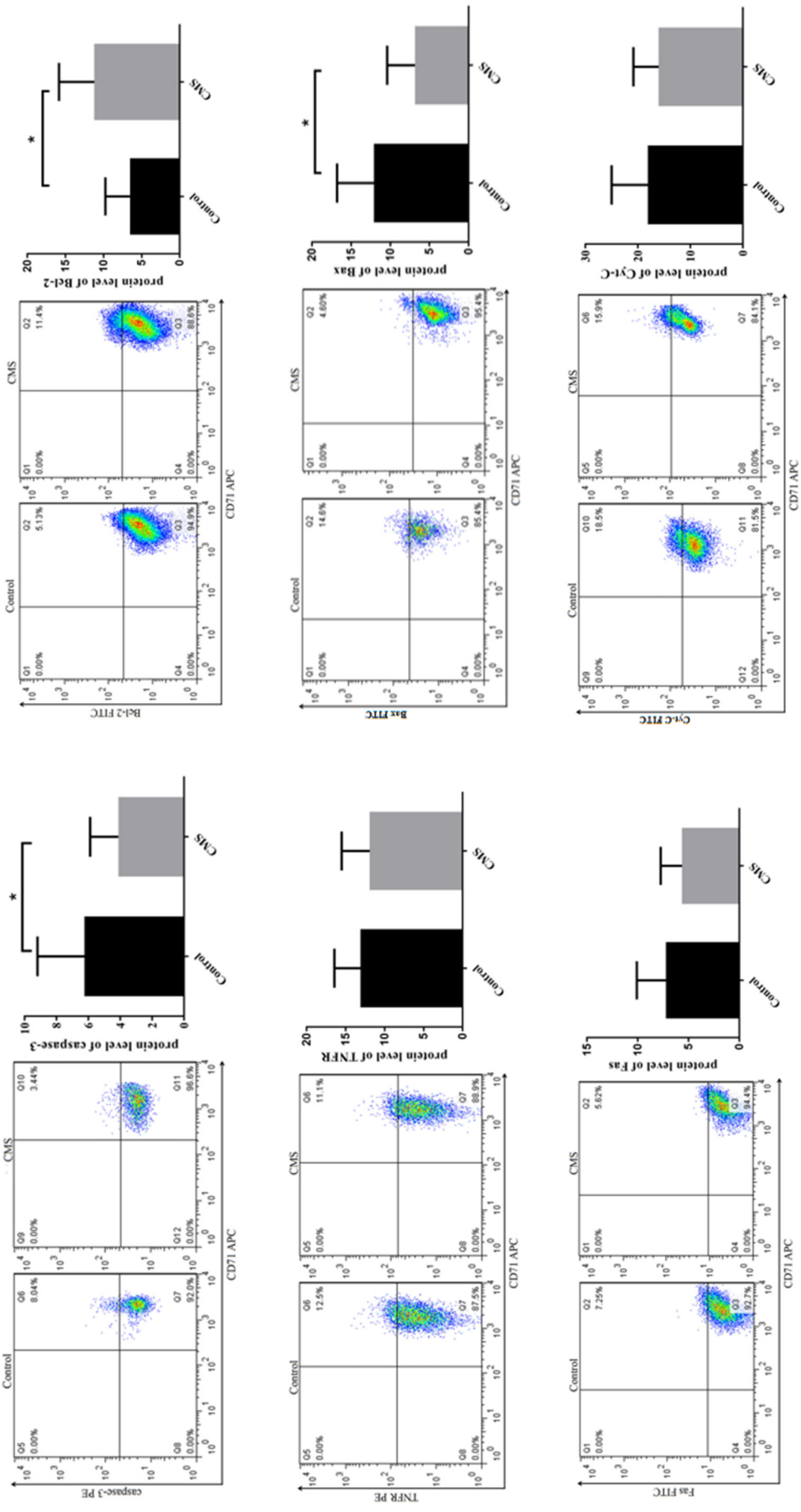

Fig. 3. The expression of apoptosis related proteins in $CD71^+$ erythroblasts. Flow cytometry analysis of protein expressions of caspase-3, TNFR, Fas, Bax, Bcl-2 and Cyt-C in $CD71^+$ erythroblasts of CMS patients and control subjects. Data are presented as mean ± SD. $^*p < 0.05$.

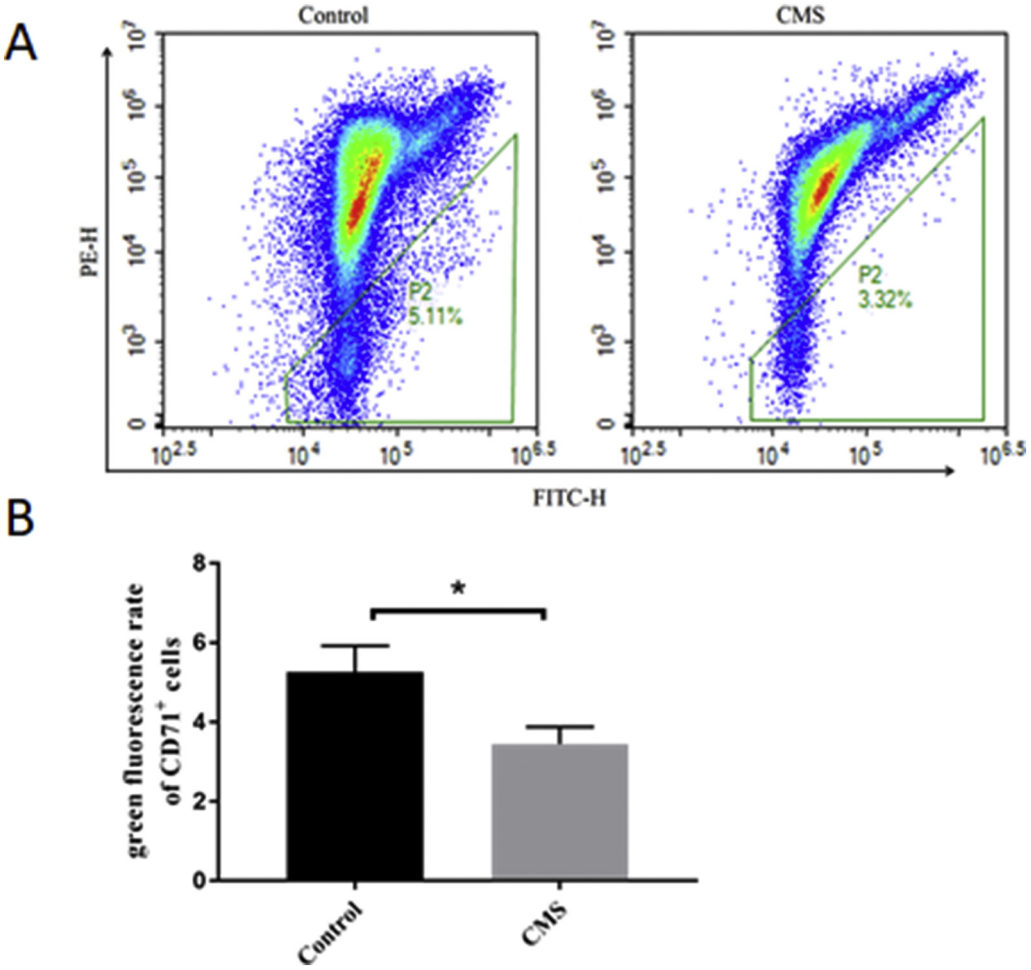

Fig. 4. Analysis of MMP in CD71$^+$ erythroblasts. A. MMP was detected by flow cytometry with JC-1 fluorescent staining in CMS patients and control subjects. B. Quantitative analysis of MMP in two groups. Data are presented as mean ± SEM. $^*p < 0.05$.

has a favorable effect on the expression of anti-apoptotic protein Bcl-2 [25,26]. There is evidence that bone marrow microenvironment contains hypoxic areas that confer survival advantage to hematopoietic cells [24,27].The hypoxic bone marrow microenvironment would be more severe in a highlander. Therefore, changes in Bcl-2 family levels in the bone marrow niche might be involved in the pathogenesis of CMS. In addition, HIF-1α protected cells from apoptosis in hypoxia condition by decreasing Bax expression and increasing Bcl-2 expression [28–31]. It has been reported that EPO has anti-apoptotic effect and upregulates Bcl-2 family expression [32–34]. Our previous study showed that the protein and mRNA expression levels of EPO increased in CMS patients [19]. We speculate that EPO affects the apoptosis of erythroblasts in CMS patients. These studies suggest that hypoxia, HIFs and EPO may play a role in the regulation of apoptosis of the erythroblasts, but

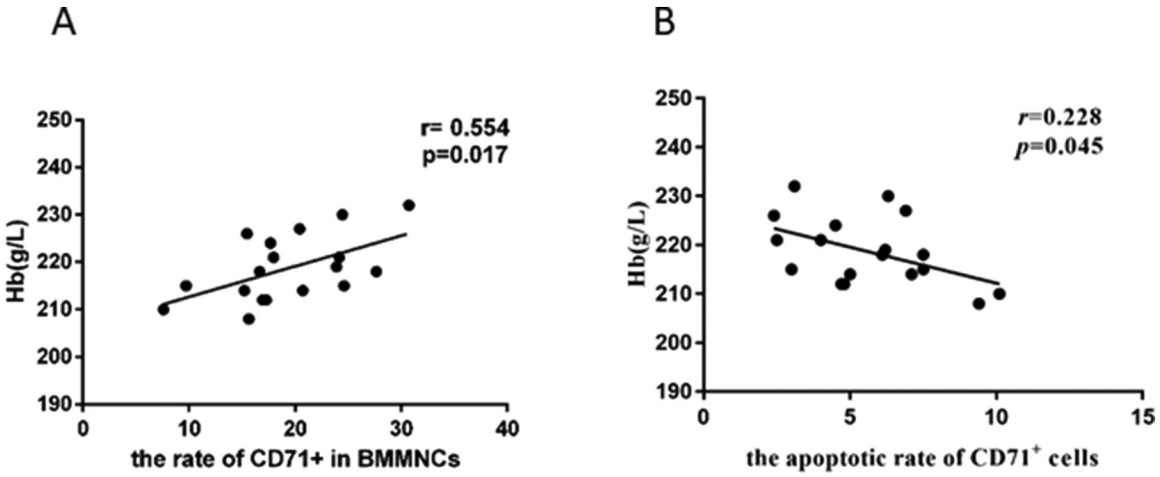

Fig. 5. A. The relationship between Hb level and the rate of CD71$^+$ erythroblasts in CMS patients. B. The relationship between Hb level and the apoptotic rate of CD71$^+$ erythroblasts in CMS patients.

further studies are needed to understand the mechanism by which Bcl-2 expression is upregulated while Bax expression is downregulated in the erythroblasts under the condition of CMS.

In summary, the apoptotic rate of $CD71^+$ erythroblasts was lower in CMS group and was associated to increased Bcl-2 expression and decreased Bax and caspase 3 expression. The apoptotic rate of $CD71^+$ cells was negatively correlated with the ratio of $CD71^+$ cells in BMMNCs, and positively correlated with Hb level. Therefore, hypoxia induced downregulation of intrinsic apoptosis pathway of erythroblasts may contribute to excessive accumulation of red blood cells in CMS.

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Conflict of interest

None.

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