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PI3K-Akt Signal Transduction Molecules Maybe Involved in Downregulation of Erythroblasts Apoptosis and Perifosine Increased Its Apoptosis in Chronic Mountain Sickness

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Data Interpretation D
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Background: Chronic mountain sickness (CMS) has a higher incidence in the plateau region. The one of its principal characters is excessive erythrocytosis. The PI3K-Akt pathway plays an important role in the process of erythropoiesis, and could downregulate apoptosis by regulating apoptosis-related molecules. In this paper, we explored the change in apoptosis of erythroblasts and the effect of the PI3K-Akt signal pathway on erythroblasts apoptosis in CMS.

Material/Methods: A total of 22 CMS and 20 non-CMS participants were involved in this study. Bone marrow mononuclear cells were cultured and treated with celecoxib and perifosine *in vitro* for 72 hours. The apoptotic rate, the mRNA expressions of Akt, Bcl-xl, and caspase-9, and the protein expressions of Akt, p-Akt, Bcl-xl, and caspase-9 were determined by flow cytometry, quantitative RT-PCR, and western-blot technique.

Results: The apoptotic rate of cultured erythroblasts was lower in the CMS group than in the non-CMS group. It was increased after perifosine intervention. The mRNA and protein expressions of Akt and Bcl-xl were higher and caspase-9 was lower in the CMS group than the non-CMS group. Perifosine induced decreased Bcl-xl mRNA and proteins and p-Akt proteins, and increased caspase-9 mRNA and proteins *in vitro*. In the CMS group, the hemoglobin concentration was correlated with apoptotic rate negatively and with Bcl-xl mRNA positively in erythroblasts; the erythroblasts apoptotic rate was negatively associated with the Akt mRNA and Bcl-xl mRNA.

Conclusion: The erythroblasts apoptosis was downregulated and the PI3K-Akt signal pathway appeared to be involved in the mechanism of decreased erythroblasts apoptosis in CMS.

MeSH Keywords: Altitude Sickness • Apoptosis • Erythroblasts • Phosphatidylinositol 3-Kinases

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Background

Chronic mountain sickness (CMS) is a clinical syndrome of individuals living in high altitude areas who are maladapted to the hypoxia environment. It is characterized by excessive polycythemia, severe hypoxia, and an array of neurologic symptoms including headache, fatigue, somnolence, and depression. The analysis of bone marrow cells morphology has shown that erythroblasts proliferation is obviously increased in CMS patients [1]. The expressions of hematopoietic growth cytokines and signal transduction molecules, such as thrombopoietin (TPO), interleukin-2 (IL-2), signal transducer and activator of transcription (STAT5), mitogen-activated protein kinases (MAPK), GATA-1 and GATA-2 were significantly increased in CMS patients [2]. Meanwhile, growing evidences indicates that the apoptotic mechanism plays a relevant role in the regulation of erythropoiesis under physiologic and pathologic conditions. Under long-term hypoxia, mice were shown to develop polycythemia, and apoptosis of erythroid cells was decreased during erythropoiesis [3]. In CMS patients, it was found in our previous study that the apoptotic rate and the mRNA expression of caspase-3, -8, and -9 of bone marrow mononuclear cells (BMMNCs) were decreased [4]. The apoptotic rate and the mRNA expression of Bax and Bid were decreased, while the mRNA expression of Bcl-2 and Bcl-xl were increased in erythroblasts cultured *in vitro* [5]. Therefore, the downregulated apoptosis in hematopoietic cells might be involved in the mechanism of CMS.

EPO/EpoR signaling is a critical regulator of multiple aspects of mammalian primitive erythropoiesis. EPO/EpoR provides anti-apoptotic signals in definitive erythroid progenitors and promotes primitive erythroblasts survival during the terminal stages of erythroblast maturation by regulating the expression of pro- and anti-apoptotic genes [6]. The binding of EPO to EPOR on the cells surface bridged and activated dimeric EPOR/JAK2 complexes, which phosphorylates and activates phosphatidylinositol 3-kinase (PI3-kinase). The pleckstrin homology (PH) domain of Akt/PKB shares a similarity to those found in other signaling molecules that bind 3-phosphoinositides [7,8]. The PH domain interacts with membrane lipid products such as phosphatidylinositol (3,4,5) trisphosphate (PIP3) produced by PI3-kinase. In the case of Akt/PKB, the PH domain is required for its recruitment to the plasma membrane through high-affinity binding to PIP3; and PIP3 recruits Akt/PKB to the plasma membrane to alter its conformation and allow subsequent phosphorylation by the phosphoinositide-dependent kinase-1 (PDK1). BAD is a member of the Bcl-2 family of proteins that binds to Bcl-2 or Bcl-xl and inhibits their anti-apoptotic potential. But once BAD is phosphorylated on Ser136 by Akt/PKB, it is released from a complex with Bcl-2/Bcl-xl that is localized on the mitochondrial membrane, and forms a complex with 14-3-3 proteins in the cytosol, thus inactivates its pro-apoptotic function. Caspase-9 acts as an initiator and an effecter of apoptosis [9]. Human caspase-9 has been reported to be phosphorylated on Ser196 by Akt/PKB, resulting in attenuation of its activity [10]. Akt can augment HIF-1α expression by increasing its translation [11]. The HIF-1α expression, through activating PI3K/Akt pathway under both normoxia and hypoxia, has protective effects for cells against hypoxia-induced apoptosis [12]. Therefore, PI3K/Akt signaling pathway plays a center role to anti-apoptosis in physiological and pathological conditions. For breast cancer and gliomas, Akt inhibitors such as celecoxib and perifosine increases its cells apoptosis by inhibited PI3K/Akt signaling pathway.

However, the effect of the PI3K-Akt signal transduction pathway on hematopoietic cells apoptosis and excessive erythrocytosis in CMS patients is rarely considered. To address this issue, the PI3K-Akt signal transduction pathway change and its effect on hematopoietic cells apoptosis in CMS patients were studied.

Material and Methods

Patients

The research protocol was approved by the Human Subject Protection Committee at the Affiliated Hospital of Qinghai University. Informed consent was obtained from each participant. Twenty-two patients with CMS (men; Han Chinese; mean age 50.14±10.45 years) and twenty control participants (men; Han Chinese; mean age 47.36±15.80 years) were included in this study. These participants came from villages located at altitude of 3,400–4,300 m in Qinghai province of China. They were born at lowland or moderate altitude and were residing at high altitude for 13.2±7.4 years. The control participants (non-CMS) were patients without any chronic diseases, who were undergoing elective orthopedic surgery to remove remotely placed internal fixation rods. None of the participants had a history of respiratory or cardiovascular disease, such as chronic obstructive pulmonary disease, asthma, infectious diseases, congenital heart disease, shunt, valvular disease, or hypertensive heart disease.

A CMS self-report questionnaire and a complete clinical examination were performed for each participant. The evaluation of the presence and severity of CMS was made by the "consensus statement on chronic and subacute high altitude diseases" (Qinghai CMS score) [13,14], established during the VI World Congress of Mountain Medicine and High-Altitude Physiology in 2004, which is based on the symptoms and hemoglobin levels. The symptoms included headache, dizziness, breathlessness, palpitations, sleep disturbance, cyanosis, tinnitus, paresthesia, and veins dilatation. Each criteria was graded on a scale of 0 to 3 (0 was no symptoms; 1 was mild

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symptom; 2 was moderate symptoms; and 3 was severe symptoms). Hemoglobin concentration was dichotomized as either 0 or 3 points with a cutoff of 210 g/L for males and 190 g/L for females. The participants were considered to have CMS if hemoglobin levels were ≥210 g/L and the CMS score was >5.

Blood and bone marrow samples and assay

Blood samples and bone marrow were collected from every participant. The measurement of hematocrit and hemoglobin (Sysmex XE2100, Japan) and blood gas analysis (Nova, USA) were performed in a clinical laboratory using standard procedures in venous and arterial blood samples, respectively. The bone marrow was drawn from the posterior superior iliac spine of all participants, and 15 mL of the bone marrow fluid was collected into one serum separator tube and two tubes containing heparin. All the bone marrow samples were transported on ice to Xining within eight hours of collection, and then analyzed within one hour after arrival. The BMMNCs were isolated using Ficoll and cultured in erythroid progenitor cells culture system. The apoptotic rate of cultured erythroblasts was determined by flow cytometry analysis. The mRNA expressions of Akt, Bcl-xl, caspase-9 in cultured erythroblasts was determined with RT-PCR technique. And the protein expressions of Akt, p-Akt, Bcl-xl, and caspase-9 were detected with western blot technique. The bone marrow supernatant was harvested for measuring the EPO levels.

The culture and drug intervention of erythroblasts *in vitro*

BMMNCs were isolated and the viable cell rate was counted with trypan blue staining under the light microscope. BMMNCs were cultured in 5 mL of IMDM erythroid progenitor culture system [15] (30% FCS, 2-ME 55 umol/L, 1% penicillin and streptomycin, IL-3 20 ng/mL, EPO 3 U/mL) with 2×10^5 cells/mL, and divided into four groups according to different intervention. The cells of these four groups were pretreated with dimethyl sulfoxide (DMSO), Akt inhibitor celecoxib (Pfizer Pharmaceuticals, USA) (100 μmol/L, 125 μmol/L) and perifosine (Selleck, USA) (45 μmol/L) [16], respectively. And the cells were collected after incubation for 72 hours at 37°C with 5% CO_2 in an incubator (Thermbo Scientific, USA) (Figure 1).

The apoptotic rate was determined with flow cytometry analysis

The cultured cells were collected, washed twice with cold PBS and resuspended with 1x binding buffer at a concentration of 1×10^6 cells/mL. Then 100 μL of the solution (1×10^5 cells) was transferred to a 5 mL culture tube and 5 μl of FITC Annexin V (BD, USA) and 5 μL PI (BD, USA) were added into the tube. The cells were gently vortexed and incubated for 15 minutes at room temperature (25°C) in the dark. Then 400 μL of 1× binding buffer was added into each tube and analysis of apoptotic rate was performed by flow cytometry (Beckman, USA) within one hour.

The mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured erythroblasts by TRIzol reagent (Life Technologies) followed by removal of contaminating DNA (DNase treatment); first strand complementary DNA was synthesized from 1 μg total RNA (quantified with a NanoDrop ND-1000 spectrophotometer) using RETROscript (Ambion). The mRNA expressions of Akt, caspase-9, and Bcl-xl were determined using RT-PCR technique (ABI7500, USA). Reactions were initiated at 95°C for 15 minutes, followed by 40 cycles consisting of 15 seconds at 94°C, 15 seconds at 58°C, and 15 seconds at 72°C, with the final cycle of 5 minutes at 72°C to extend the amplified products. Data were analyzed using the $2^{-\Delta\Delta CT}$ method by ABI7500 software v2.0.4. The Akt primer sequence: forward primer 5'ATGAGCGACGTGGCTATTGT3', reverse primer 5'TGAAGGTGCCATCATTCTTG3' (106 bp); caspase-9 primer sequence: forward primer 5'AGGTTCTCAGACCGGAAACA3', reverse primer 5'CTGCATTTCCCCTCAAACTC3' (93 bp); Bcl-xl primer sequence: forward primer 5'CAGGCCTCCTGTGGGAC3', reverse primer 5'GGTAGGAGCTGTGGCGACT3' (105 bp); GAPDH primer sequence: forward primer 5'AAGGTGAAGGTCGGAGTCAA3', reverse primer 5'AATGAAGGGGTCATTGATGG3' (108 bp).

Protein extraction and western blot analysis

For protein isolation, the cultured erythroblasts were collected, washed twice with cold PBS, and lysed with the RIPA Lysis Buffer (Beyotime, China) [0.2–0.4 mL; 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS], 100x protease inhibitor cocktail (Beyotime, China) (2–4 μL; 200 mM AEBSF, 30 μM aprotinin, 13 mM bestatin, 1.4 mM E64 and 1 mM leupeptin in DMSO) and 50× phosphatase inhibitor cocktail A (Beyotime, China) (2–4 uL; 250 mM sodium fluoride, 50 mM sodium pyrophosphate, 50 mM β-glycerophosphate, and 50 mM sodium orthovanadate in H2O) in ice for 30 minutes. The mixture was centrifuged at 12,000 g and the supernatants were retained, which were boiled for five minutes. Protein concentrations were determined using a BCA protein assay kit (Beyotime, China).

For western blotting, equal amounts of total protein were run in each lane of an SDS-PAGE gel (12% acrylamide). Each protein sample was mixed with an equal volume of 4× Laemmli buffer and boiled for five minutes before loading onto the gel. After completion of gel electrophoresis, protein was transferred to a Hybond nitrocellulose membrane (Beyotime, China) over two hours using a blotting apparatus. Membrane were block with 3% bovine serum albumin (BSA) for one hour at room

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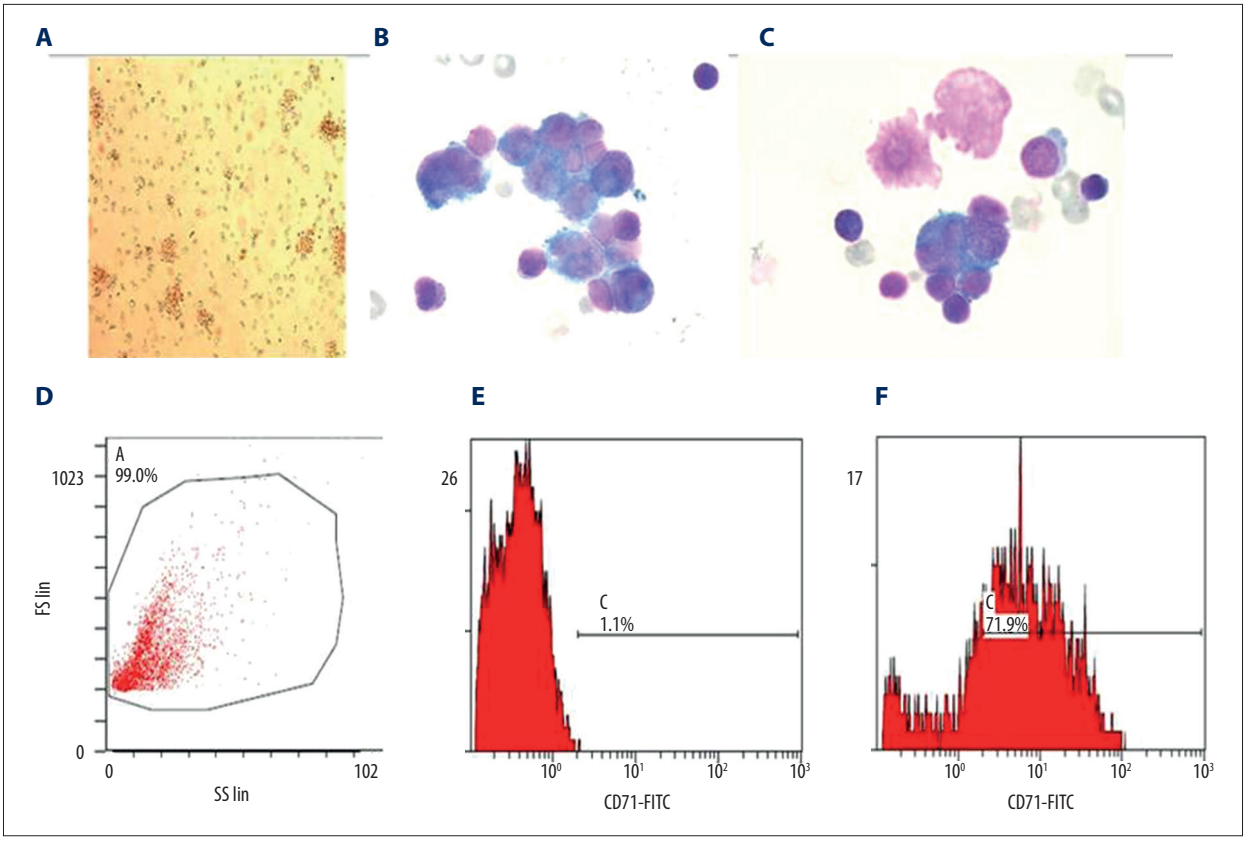

Figure 1. Morphology of cultured erythroblasts in microscope and the rate of CD71 positive cells (FCM). (**A**) Morphology of erythroblasts cultured for 72 hours (1×40). (**B, C**) Morphology of erythroblasts cultured for 72 hours by Wright's staining (1×400). The rate of erythroblasts was determined by FCM with CD71 staining (**D–F**). Erythroblasts can be grow in suspended cell medium including EPO and IL-3, and morphology of cultured erythrocytoblasts were round and bright, the cells grew well. The nuclei can be seen clearly by Wright's staining. The rate of erythrocytoblast was 70.5±2.67%). FCM, flow cytometry.

Table 1. Clinical characteristics of CMS and Non-CMS ($\bar{\chi}$±s).

	CMS (n=22)	Non-CMS (n=20)	*p* Value
Age, yr	50.1±10.5	47.4±15.8	0.533
Heigh, cm	172.5±4.7	170.3±5.6	0.176
BMI, kg/m^2	25.3±1.9	23.2±2.1	0.092
Systolic blood pressure, mmHg	126.5±16.3	119.5±8.9	0.076
Diastolic blood pressure, mmHg	84.3±9.4	74.5±7.9	0.083
Hemoglobin, g/L	223.9±17.2	146.1±18.6	<0.001
Hematocrit, %	66.9±5.8	43.3±5.5	<0.001
Erythrocyte, ×10^{12}/L	6.9±0.8	4.8±0.8	<0.001
SaO2, %	86.5±2.3	93.7±2.4	<0.001
CMS-score	11 (9–17)	2 (0–3)	<0.001

Values are means ±SD unless otherwise specified; *N* – number of subjects; CMS – chronic mountain sickness; SaO2 – arterial O2 saturation.

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temperature, and incubated with antibodies against caspase-9, Bcl-xl, total Akt, and phospho-AKT (antibodies from Abcom, USA) overnight at 4°C. After the membranes were washed three times with 1× TBST, the secondary antibody of goat anti-rabbit monoclonal antibody (Abcom, USA) were incubated for one hour at room temperature and a chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, USA) were add to membranes. The images were obtained using the LAS4000 system (Alpha Innotech).

The level of EPO was detected by sandwich enzyme immunoassay kits

The bone marrow supernatant was isolated from bone marrow sample. Specific sandwich enzyme immunoassay kits were used for determining of bone marrow supernatant EPO (BD, USA). The standard sample storage and analysis procedure described by the manufacturer was followed for each kit running each sample in duplicate.

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS, version 17.0) for Windows. Normality test was performed with Kolmogorov-Smirnov. The data of normal distribution were reported as mean ±SD in four different group, Two-sided student's unpaired *t*-test and ANOVA were used for statistical analyses. Correlation analysis was performed by linear correlation analysis. The data of non-normal distribution were reported as median (quartile spacing) [M (QR)]. The Mann-Whitney U test was performed in the two groups and the Kruskai Wallis test in four different groups. The correlation analysis was performed using Spearman Rank correlation analysis. A *p* value less than 0.05 was considered to indicate statistical significance.

Results

General characteristics

The general characteristics of the study participants are shown in Table 1. There was no difference in age (p=0.533), height (p=0.176), blood pressure (p=0.076, 0.083) or body-mass index (p=0.092) between the two groups. But hemoglobin (p<0.001), hematocrit (p<0.001), and erythrocyte counts (p<0.001) were significantly higher, while SaO_2 was lower in the CMS group than those in the non-CMS group (p<0.001), respectively. The CMS-score was 9–17 points in patients with CMS based on Qinghai CMS scoring guidelines.

The cultured erythroblasts and the rate of cells with CD71 positive

The cells grew well after cultured for 72 hours, the morphology of cultured erythrocytoblasts was round and bright, and the nuclei was seen clearly by Wright's staining. The rate of erythrocytoblasts was 70.5±2.67% by flow cytometry with CD71 staining (Figure 1).

The apoptotic rate of cultured erythroblasts was lower in CMS patients than in non-CMS participants, which was increased by perifosine pretreatment *in vitro*

The apoptotic rate of cultured erythroblasts was detected in 21 patients with CMS and 18 patients with non-CMS. The apoptotic rate of cultured erythroblasts underwent pretreatment with placebo DMSO in the CMS group was lower than that in the non-CMS group (t=2.726, p=0.011, Table 2, Figure 2), and it was increased after treatment with perifosine in these two groups. However, celecoxib treatment induced a significant increase in the non-CMS group by higher concentration of celecoxib 125 μmol/L. And there was no significant different between CMS group and non-CMS group in apoptotic rate of cultured erythroblasts after treatment with celecoxib or perifosine (Table 2, Figure 2).

The expressions of Akt and Bcl-xl mRNA were higher and caspase-9 mRNA was lower in the CMS group than in non-CMS group, and perifosine pretreatment decreased Akt and Bcl-xl mRNA and increased caspase-9 mRNA in erythroblasts *in vitro*

The expressions of Akt, caspase-9, and Bcl-xl mRNA in cultured erythroblasts were determined in 21 patients with CMS and 18 non-CMS patients. The expressions of Akt and Bcl-xl mRNA were higher and caspase-9 mRNA was lower in the CMS group than the non-CMS group. The Bcl-xl mRNA expression was decreased and Akt and caspase-9 mRNA were increased in the two groups after perifosine intervention, and Bcl-xl mRNA expression was decreased in the two groups after the celecoxib 125 umol/L pretreatment, but there was no significant change in the expression of Bcl-xl, Akt, and caspase-9 mRNA in the two groups after celecoxib 100 umol/L pretreatment (Figure 3).

The Bcl-xl protein was higher and caspase-9 protein was lower in the CMS group than the non-CMS group, and perifosine inhibited Bcl-xl expression and induced caspase-9 expression of erythroblasts *in vitro*

The protein expressions of Akt, p-Akt, Bcl-xl, and caspase-9 in cultured erythroblasts were determined in eight patients in the CMS and non-CMS group, respectively. The Bcl-xl protein was higher and caspase-9 protein was lower in the CMS

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Table 2. The apoptotic rate of cultured erythroblasts after drug intervention *in vitro* ($\bar{\chi}$±s).

Group	CMS (n=21)	Non-CMS (n=18)
DMSO	7.94±3.01*	11.79±4.94
Celecoxib 100 μmol/l	10.48±4.91	14.78±3.49
Celecoxib 125 μmol/l	12.12±5.49	17.55±3.30#
Perifosine	19.28±11.39#	23.26±6.59#
F	8.822	14.30
p	<0.001	<0.001

Values are expressed as mean ±SD. * $p<0.05$ (Compared with the DSMO in Non-CMS group) and # $p<0.01$ (Compared with the DMSO in same group).

group than the non-CMS group. There was no significant difference in Akt and p-Akt protein expression between these two groups. After perifosine intervention, the caspase-9 protein was increased and the p-Akt and Bcl-xl proteins were decreased. However, the celecoxib treatment did not induce any significant change in the protein expression of Bcl-xl, Akt, p-Ak, and caspase-9 in the two groups (Figures 4, 5).

The erythropoietin (EPO) levels in the bone marrow supernatant were similar between two groups

The levels of EPO were determined in 21 patients with CMS and 20 patients with non-CMS. The EPO level in the bone marrow supernatant was 23.40 (16.99–33.51) (IU/L) in the CMS group and was 28.40 (16.51–66.92) (IU/L) in the non-CMS group, which was not significant different between the two groups (Z=–0.680, $p=0.496$). The EPO level was not correlated with the hemoglobin concentration, the apoptotic rate of erythroblasts, and the mRNA expressions of Akt, Bcl-xl, and caspase-9.

The hemoglobin concentration was correlated with apoptotic rate negatively and with Bcl-xl mRNA positively in erythroblasts of CMS

In CMS group, correlation analysis showed that the hemoglobin was inverse correlated with the apoptotic rate of erythroblasts ($r=–0.5748$, $p=0.0064$, Figure 6A) and positively correlated with the Bcl-xl mRNA ($r=0.6942$, $p=0.0018$, Figure 6B). In all participants of the study, hemoglobin was also negatively correlated with the apoptotic rate ($r=–0.3817$, $p=0.0311$, Figure 6C) and caspase-9 mRNA ($r=–0.5465$, $p=0.0007$, Figure 5E) of erythroblasts, while was positively correlated with the Bcl-xl mRNA ($r=0.4071$, $p=0.0152$, Figure 6D). But there was no significant correlation between hemoglobin and Akt mRNA ($r=–0.2701$, $p=0.2364$), caspase-9 mRNA ($r=0.1549$, $p=0.5024$) in

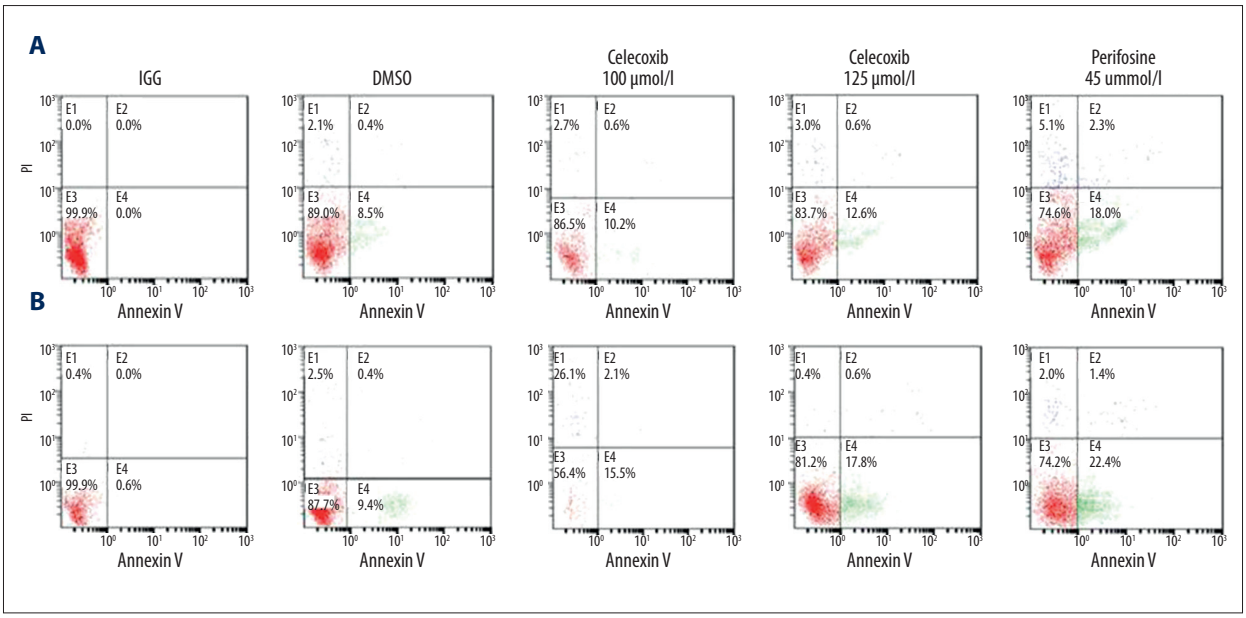

Figure 2. FCM analysis of the apoptotic rate with Annexin V and PI staining of erythroblasts in patients with CMS and non-CMS patients (**A**) CMS, (**B**) Non-CMS. The apoptosis rate of cultured erythroblasts in the CMS group was lower than that in the non-CMS group, and it was increased after treatment with perifosine (perifosine 45 μmol/L) in CMS patients and non-CMS patients. In non-CMS patients, it was increased after treatment with celecoxib 125 μmol/L, but was not increase in CMS patients. Meanwhile, it was not increased after treatment with celecoxib 100 μmol/L in two group. CMS, chronic mountain sickness; FCM, flow cytometry.

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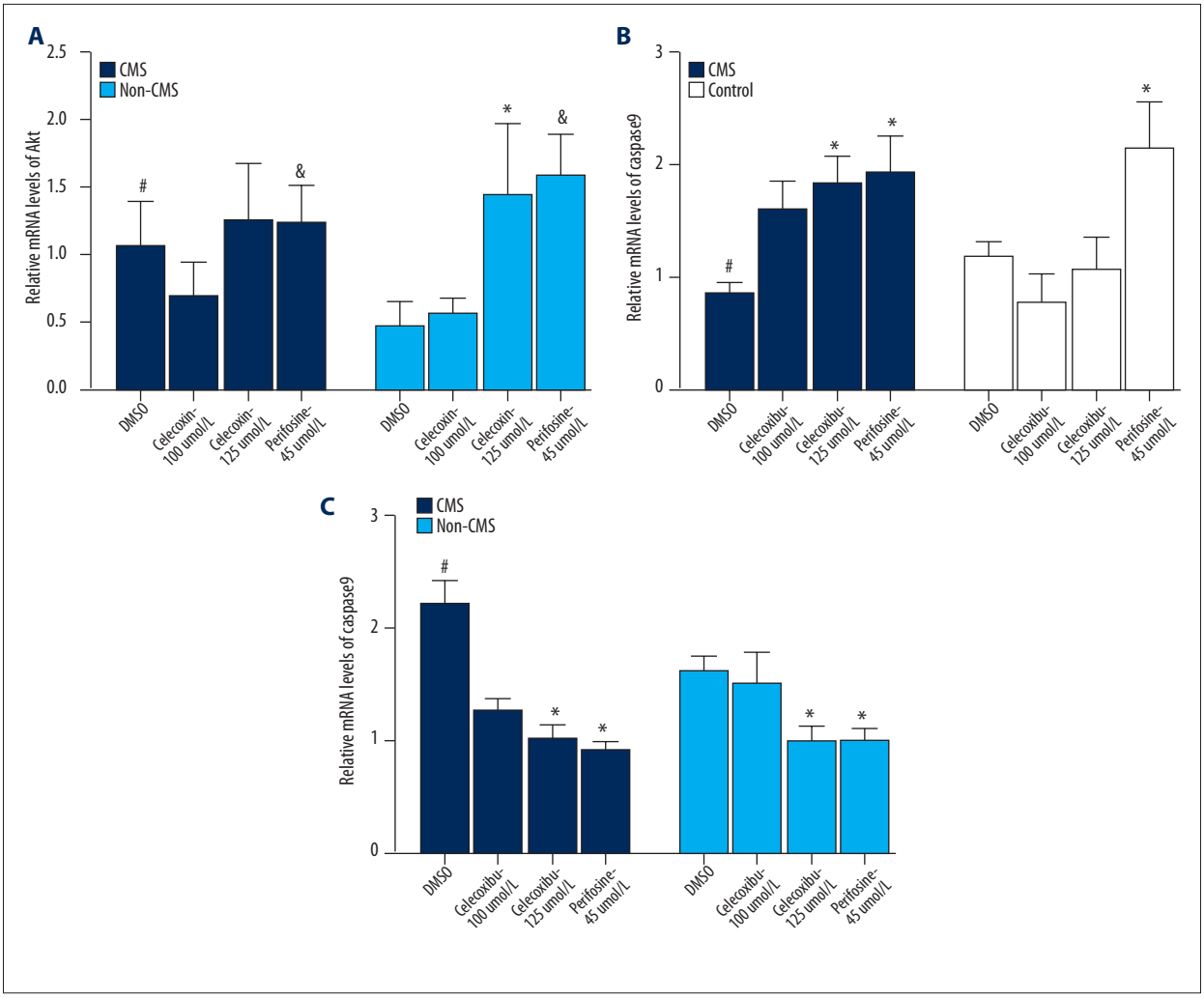

Figure 3. The mRNA expressions of Akt (**A**), caspase-9 (**B**) and Bcl-xl (**C**) in cultured erythroblasts after drug intervention *in vitro*. Values are expressed as mean ±SEM. # $p<0.05$ (compared with the DMSO in non-CMS group); * $p<0.05$ (compared with the DMSO in same group) and & $p<0.01$ (compared with the DMSO in same group). CMS, chronic mountain sickness.

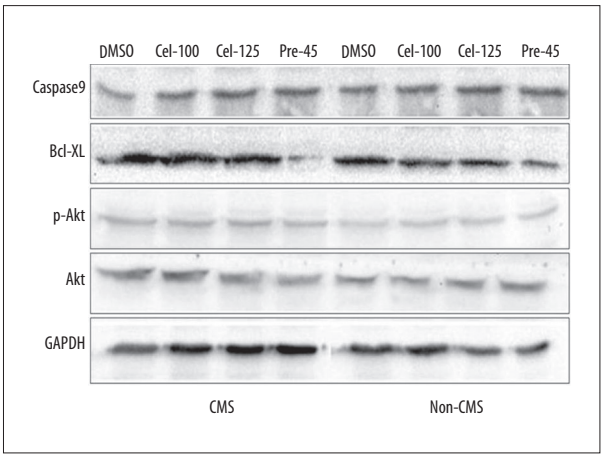

Figure 4. The protein expressions of Akt, p-Akt, caspase-9, and Bcl-xl in cultured erythroblasts after drug intervention *in vitro*. The protein expressions of Akt and p-Akt were not different between CMS patients and non-CMS patients. The protein expression of Bcl-xl was higher and caspase-9 protein was lower in CMS patients than in non-CMS patients. The protein expression of caspase-9 was increased and the p-Akt and Bcl-xl proteins were decreased after perifosine intervention, but there was no significant difference in Bcl-xl protein, Akt protein, and caspase-9 protein between the two groups after the celecoxib treatment. CMS, chronic mountain sickness.

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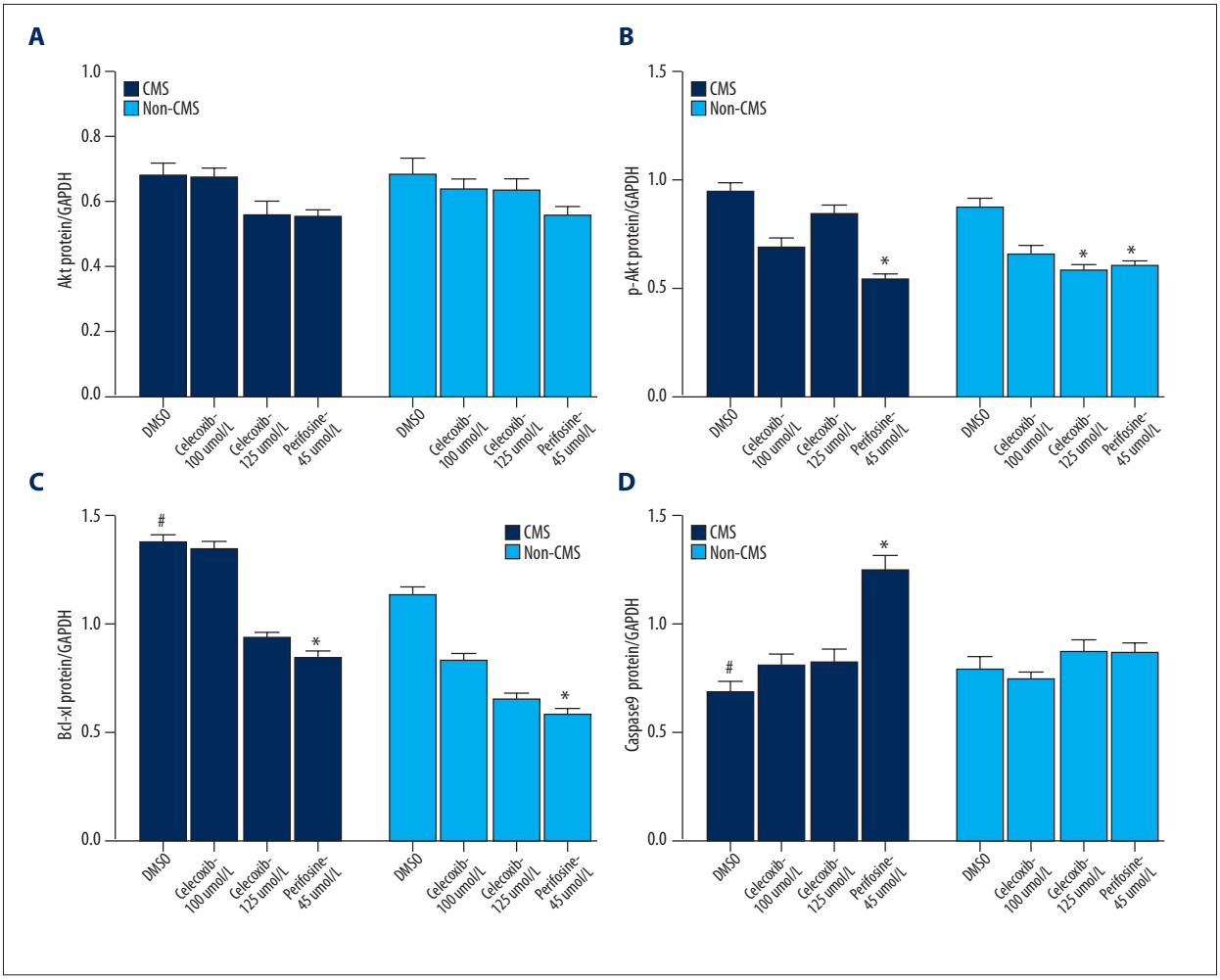

Figure 5. The level of protein expression relative to GAPDH after perifosine and celecoxib in the two groups. The protein expression of Akt and p-Akt was not different between the two group (**A, B**), but p-Akt protein was decreased after perifosine intervention in the two groups and after celecoxib 125 μmol/L intervention in the non-CMS group (**B**). The protein expression of Bcl-xl was higher and caspase-9 was lower in CMS group than non-CMS group (**C, D**), and Bcl-xl was decreased and caspase-9 was increased after perifosine intervention in the two group (**C, D**). Data are presented as mean ±SED. The statistical differences were calculated using the ANOVA and *t*-test. # $p<0.05$ (compared with the DMSO of control group), * $p<0.05$ (compared with the DMSO in same group). CMS, chronic mountain sickness.

the CMS group, and Akt mRNA (r=0.3058, p=0.0835) in all participants of the study.

The increased expressions of Akt mRNA and Bcl-xl mRNA were involved in the downregulation of apoptotic rate of erythroblasts in CMS

In CMS patients, the apoptotic rate of erythroblasts was negatively associated with the Akt mRNA (r=–0.5888, p=0.0050, Figure 7A) and Bcl-xl mRNA(r=–0.6942, p=0.0005, Figure 7B), and Bcl-xl mRNA was positively related with Akt mRNA (r=0.5262, p=0.0143, Figure 7C). But there was no significant correlation between the apoptotic rate of erythroblasts and caspase-9 mRNA (r=4714, p=0.0889).

Discussion

The present study showed that apoptosis of erythroblasts was downregulated in CMS patients. Polycythemia is the main characteristic of CMS. Previous studies of bone marrow cells morphology showed that the hematopoietic cells, especially proliferation of erythroid cells, were obviously enhanced in CMS [1]. And the expressions of hematopoietic growth cytokines, such as EPO, TPO, interleukin-2 (IL-2), and signal transduction factors (STAT5, MAPK, etc.) were significantly increased in CMS patients [2]. Meanwhile, the studies of physiologic erythropoiesis have clearly indicated that immature erythroid precursors are sensitive to apoptotic triggering mediated by activation of the intrinsic and extrinsic apoptotic pathway [6]. Mice under

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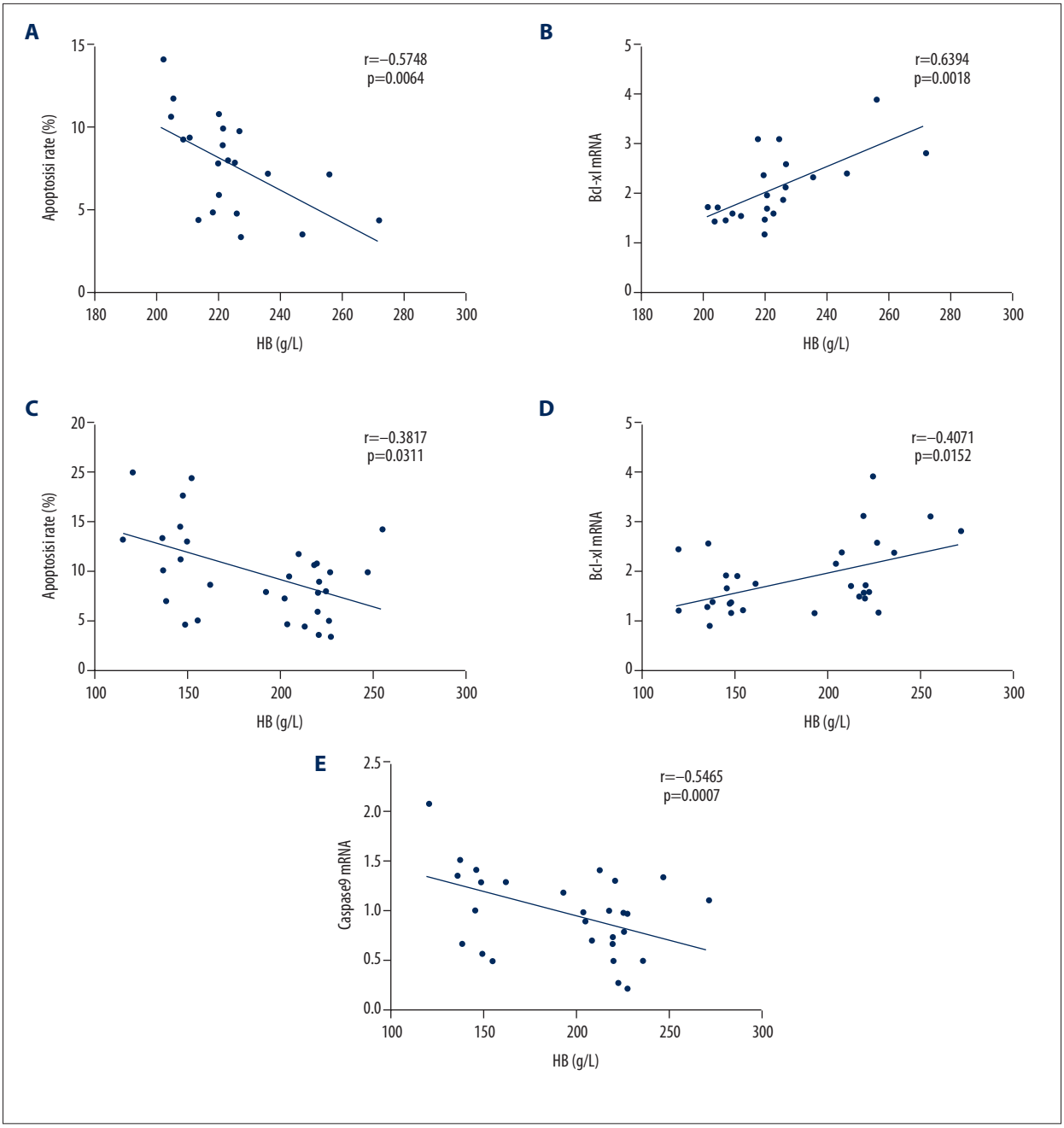

Figure 6. The relationship between the concentration of hemoglobin and apoptotic rate of erythroblasts in CMS patients (**A**) and in all participants (**C**), and the mRNA expressions of Bcl-xl in CMS patients (**B**) and in all participants (**D**), and the mRNA expression of caspase-9 in all participants (**E**). CMS, Chronic mountain sickness.

long-term hypoxia (three months) showed polycythemia and the apoptosis of erythroid cells was decreased [3]. In this study, the results showed that the apoptotic rate of cultured erythroblasts was lower in the CMS group than that in the non-CMS group, and the hemoglobin concentration was inversely correlated with the apoptotic rate of erythroblasts in CMS patients. This was consistent with the result of our previous study about apoptotic rates of BMMNCs and erythrocytoblasts in CMS patients [4,5]. Therefore, the downregulated apoptosis of erythroblasts contributed to the excessive accumulation of red blood cells in CMS.

The lower expression of caspase-9 might be related with downregulation of apoptosis of erythroblasts in CMS. In mammalian cells, the apoptotic response is mediated through either the intrinsic (mitochondrial) pathway or extrinsic (death receptor)

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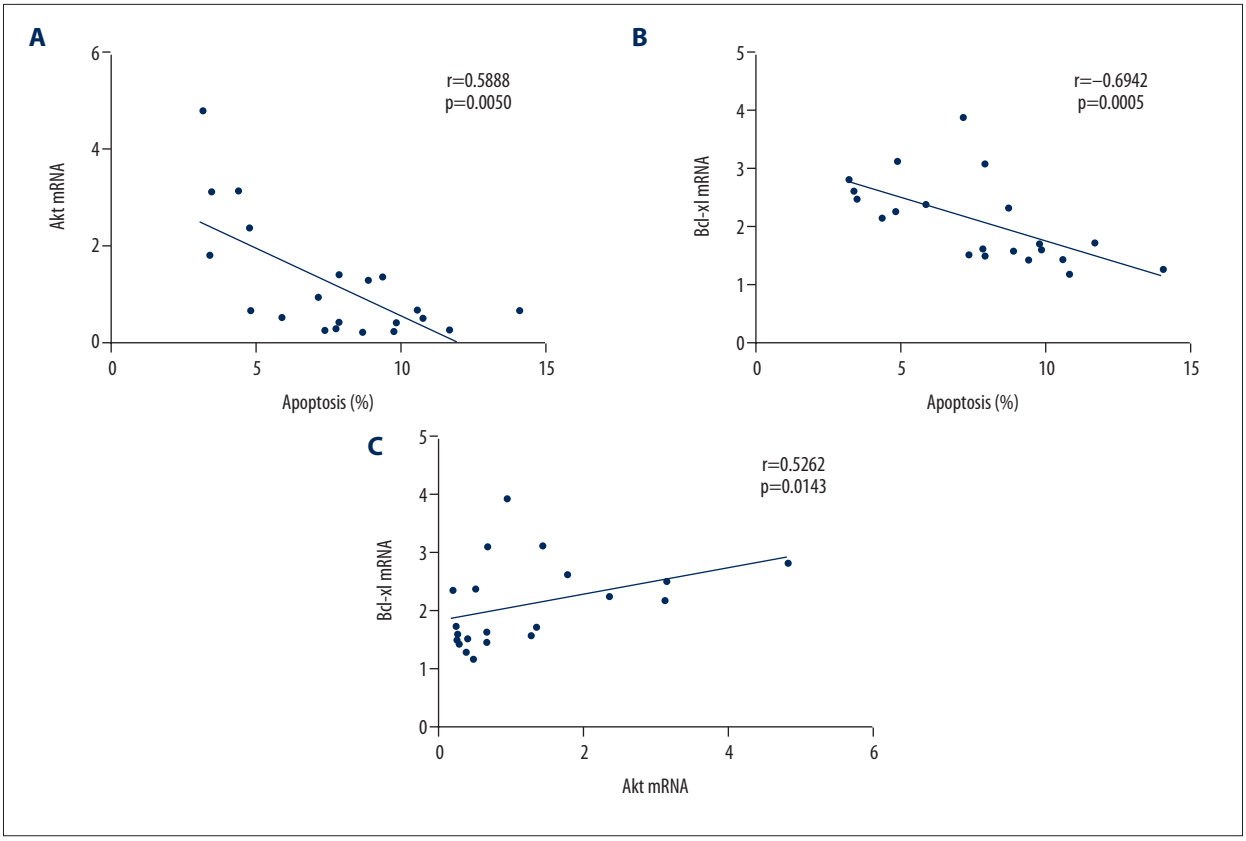

Figure 7. The relationship between the apoptotic rate of erythroblasts and the mRNA expressions of Akt (**A**) and Bcl-xl (**B**) in CMS patients, and between the mRNA expressions of Akt and Bcl-xl (**C**) in CMS patients. CMS, Chronic mountain sickness.

pathway, depending on the origin death stimuli [17] and these are caspase-dependent. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. This proteolytic cascade amplifies the apoptotic signaling pathway and thus leads to rapid cell death. To date, 12 major caspases have been identified and broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5,-11,-12) [18,19]. Caspase-8 and caspase-9 are the most important initiators of the extrinsic and intrinsic pathway of apoptosis, respectively. Our previous study showed that the mRNA expression of caspase-3,-8, and -9 were decreased in BMMNCs of CMS patients [4]. And the present study showed further that the expression of caspase-9 mRNA and protein were all decreased in erythroblasts of CMS patients and hemoglobin was negatively correlated with the apoptotic rate of erythroblasts and caspase-9 mRNA in the entire population. This indicated that decreased apoptosis might be related with decreased expression of caspase-9 in CMS patients.

The increased expression of Bcl-xl may contribute to the downregulation of erythroblasts in CMS patients. Bcl-2 family proteins are associated with the intrinsic apoptosis pathway. The intrinsic pathway is activated by the formation of a cytosolic apoptosome composed of apoptotic protease activating factor-1, pro-caspase-9, and cytochrome C, which are released from the mitochondria [20]. Mitochondrial fragmentation during apoptosis is associated with mitochondrial membrane potential collapse, which is a point of no return in the cell death cascade [20]. The Bcl-2 family proteins regulate outer mitochondrial membrane integrity and function [21]. Therefore, downregulation of Bcl-2 and Bcl-xl decreased the mitochondrial membrane potential and resulted in the formation of the cytosolic apoptosome. In our previous study, we found that the expressions of Bcl-2 and Bcl-xl mRNA increased and the expressions of Bax and Bid mRNA decreased in BMMNCs of CMS patients [5]. And we further found in the present study that the expressions of Bcl-2 mRNA and protein also increased in erythroblasts of CMS patients and the Bcl-xl mRNA was negatively association with the apoptotic rate of erythroblasts and positively association with the hemoglobin in CMS patients. These results indicated that higher expression of anti-apoptosis factors and lower expression of pro-apoptosis factors of Bcl-2 families contributed to the mechanism of downregulation of apoptosis of hematopoietic cells in CMS.

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As for the reason of increased Bcl-xl expression, research has reported that the mechanisms of increased expression of Bcl-2 and Bcl-xl and its protection of hematopoietic cells seems to be related to an EPO- and VEGF-mediated upregulation of Bcl-2 and Bcl-xl expression, associated with a consequent inhibition of caspase activation [22,23]. CMS is a disease related with hypoxia. Due to insufficient oxygen supplied in the hypobaric hypoxic environments at high altitudes, tissue hypoxia induces increased expression of HIFs, stimulating an increase secretion of hormone EPO and VEGF [24–26], which play important roles in hypoxia-induced polycythemia. In spite of the level of EPO found in our study that was not different between the CMS and the non-CMS group (which is consistent with many investigations), the EPO receptor (EPOR) signaling pathway is thought to play an important role in erythropoiesis and anti-apoptosis of erythroblasts. Our previous study showed that EPO concentration in bone marrow supernatant was significantly higher in CMS patients than in controls [27]. The animal model with HAPC has shown that mRNA and protein expression of EpoR on the myeloid $CD71^+$ cell were significantly increased [28] and pre-incubation of neurons with NO results in hypoxia upregulating the expression of EpoR [29]. In the meantime, hypersensitivity to EPO play a central role in human erythropoietic disorders [30,31] and erythroblasts exhibited EPO hypersensitivity and enhanced cellular proliferation in polycythemia vera [32]. EPO/EPOR binding upregulates Bcl-xl expression via inhibition of caspase activities, thus resulting in the protection of erythroid cells from apoptosis [33,34]. A rat study showed that EPO stimulation resulted in reduced apoptotic cells death of late-stage erythroblast accompanied by decreased caspase-3 and caspase-9 activities and upregulated the Bcl-xl mRNA, resulting in decreases in the mRNA ratios of Bak, Bax, Bad, to Bcl-xl, which is indicative of the induction of apoptosis through mitochondrial pathway in rat late-stage cultured erythroblasts derived from bone marrow [35] and Bcl-xl and Mcl expression increase and Bid, Bax, and Bim decrease in bone marrow-derived definitive erythroblasts [36,37].

PI3K/Akt signaling pathway were activated and involved in the protection of apoptosis in erythroblasts of CMS. In this study, we used a liquid culture system to investigate the expression of Akt mRNA and Akt protein in CMS erythroblasts. Our results showed that the mRNA expression of Akt increased in the CMS group, and the apoptotic rate of erythroblasts was negatively associated with the Akt mRNA expression. After treatment with perifosine, the apoptotic rate of erythroblasts was increased and the protein expression of p-Akt was decreased in CMS patients. These results indicated that there was abnormal expression of PI3K/Akt signaling factors and which was related to the decreased apoptosis of erythroblasts in CMS. Hypoxic preconditioning has been reported to activate the PI3K/Akt pathway and promote survival of BMSCs [38,39] and the PI3K/Akt signaling pathway plays a central role in during erythropoiesis, mega-karyocytopoiesis, and granulocytopoiesis/monocytopoiesis [40]. EPO binds to EPOR on the cells surface, bridges and activates dimeric EPOR/JAK2 complexes, which phosphorylates and activates PI3K, and exerts its role in the development of erythroid progenitors. In the PI3K/Akt signaling pathway, the PH domain of Akt/PKB shares similarities to those found in other signaling molecules that bind 3-phosphoinositides [7,8]. The PH domain interacts with membrane lipid products such as phosphatidylinositol (3,4,5) trisphosphate (PIP3) produced by PI3K. In the case of Akt/PKB, the PH domain is required for its recruitment to the plasma membrane through high-affinity binding to PIP3 [41]. PIP3 recruits Akt/PKB to the plasma membrane and alters its conformation to allow subsequent phosphorylation by the phosphoinositide-dependent kinase-1 (PDK1). AKT transduces a differentiation-specific signal downstream of EpoR in erythroid cells, and downregulation of expression of AKT kinase inhibits significantly fetal liver-derived erythroid-cell colony formation and gene expression in wild-type and JAK2-deficient fetal liver cells [42]. And the p-Akt was involved in myeloproliferative neoplasia (MPNs) megakaryocytes and thrombocythemia patients [43]. So AKT play an important role in myeloproliferative. Bad and caspase-9 are pro-apoptotic proteins. Bad is a member of the Bcl-2 family, which accelerates apoptosis via the formation of heterodimers with pro-survival factors, Bcl-2 and Bcl-xl. Phosphorylation of Bad at Ser112 and Ser136 by PI3K/AKT signaling pathways blocks its binding with Bcl-2 or Bcl-xl and promoting cell growth [44,45]. Caspase-9 induces cell death via mitochondria-mediated initiation of caspases [46]. It has been reported that AKT is involved in the inactivation of caspase-9 by phosphorylating caspase-9 at Ser196 [10]. Thus, caspase-9 is a target for AKT to prevent cells from apoptosis. In EC109 human esophageal cancer cells, vitamin E succinate reduced the levels of active AKT, and promoted the activation of Bad and caspase-9 to mediate cell apoptosis [47]. Hepatocyte growth factor also prevented cell injury and death by increasing the expression of the anti-apoptotic Bcl-xl protein by Akt-dependent pathways in endothelial cells [48]. A dual PI3K/mTOR inhibitor (BEZ235) inhibited the proliferation and induced cell cycle arrest and apoptosis by reduced Bcl-xl expression levels of mouse and human JAK2V617F mutated cell lines in MPN [49,50]. Meanwhile, Akt can augment HIF-1α expression by increasing its translation [11] and HIF-1α expression through activating PI3K/Akt pathway under both normoxia and hypoxia, and had protective effects against hypoxia-induced apoptosis in L02 cells [12]. And Akt inhibitor such as celecoxib and perifosine were increased in breast cancer and lung cancer cell apoptosis by inhibited PI3K/Akt signaling pathway [51,52]. In this paper, we found the apoptotic rate of erythroblasts was negatively associated with the mRNA expression of Bcl-xl, there was positive association between the mRNA of Bcl-xl and Akt in CMS patients. And the mRNA and protein expressions were decreased

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in Bcl-xl and were increased in caspase-9 with the p-Akt decreased after perifosine intervention. These findings indicating the anti-apoptosis effect of PI3K/Akt may be completed by regulating apoptosis-related molecules, such as caspase-9 and Bcl-xl of erythroblast in CMS.

Conclusions

In summary, in this paper we investigated the expression of PI3K-Akt signal transduction factors and apoptosis-related molecules, and the effect of Akt inhibitor on the apoptotic rate and apoptosis molecules in erythroblasts of CMS patients. The results showed that the apoptotic rate of cultured erythroblasts was lower in the CMS group than that in the non-CMS group, and that Akt and Bcl-xl increased in the CMS group. However, the p-Akt and the caspase-9 decreased in the CMS group. The apoptotic rate of erythroblasts was negatively associated with the Akt and Bcl-xl mRNA expression. After perifosine intervention, the apoptotic rate of erythroblasts and caspase-9 increased, meanwhile Bcl-xl increased in CMS patients, indicating Akt inhibitor increased the apoptosis of cultured erythroblasts. Therefore, downregulation of apoptosis of erythroblasts induced by hypoxia contributed to the excessive accumulation of red blood cells in CMS, and the activation of PI3K-Akt signal transduction pathway maybe involved in this mechanism.

The limitation of this paper

The precise mechanism of PI3K-Akt regulates apoptosis of hematopoietic cells in CMS is very complex. We detected fewer molecules in PI3K-Akt signal transduction, and this was the only *in vitro* intervention study of erythroblasts from CMS patients. Next, we need to study the effect of Akt inhibitor to hematopoietic cells apoptosis in CMS animal models.

Conflict of interest

None.

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