Relationship of HIF-1α expression with apoptosis and cell cycle in bone marrow mesenchymal stem cells from patients with myelodysplastic syndrome

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Abstract. Myelodysplastic syndrome (MDS) is a group of abnormal clonal disorders with ineffective hematopoiesis, which are incurable with conventional therapy. Of note, MDS features an abnormal bone marrow microenvironment, which is related to its incidence. The hypoxia-inducible factor-1α (HIF-1α) transcriptional signature is generally activated in bone marrow stem/progenitor cells of patients with MDS. To analyze the expression of HIF-1α in bone marrow mesenchymal stem cells (BM-MSCs) and the apoptosis and cell cycle features associated with the disease, BM-MSCs were obtained from 40 patients with a definitive diagnosis of MDS and 20 subjects with hemocytopenia but a negative diagnosis of MDS as a control group. Reverse transcription-quantitative PCR and western blot analyses were used to measure HIF-1α expression in cells from the two groups and apoptosis and cell cycle were also analyzed and compared between the groups using flow cytometry assays. BM-MSCs from both the control group and the MDS group exhibited a fibroblast-like morphology, had similar growth cycles and were difficult to passage stably. It was observed that BM-MSCs from the MDS group had significantly higher HIF-1α expression levels than the control group (P<0.05). Furthermore, the BM-MSCs from the MDS group had a higher proportion of cells in early apoptosis (5.22±1.34 vs. 2.04±0.08%; P<0.0001) and late apoptosis (3.38±0.43 vs. 1.23±0.11%; P<0.01) and exhibited cell cycle arrest. This may be a noteworthy aspect of the pathogenesis of MDS and may be related to high HIF-1α expression under a hypoxic state in the bone marrow microenvironment. Furthermore, the expression of HIF-1α in bone marrow tissue sections from patients with MDS in the International Prognostic Scoring System (IPSS) lower-risk group was higher than that from patients with MDS in the IPSS high-risk group. These results revealed the role of HIF-1α as a central pathobiology mediator of MDS and an effective therapeutic target for a broad spectrum of patients with MDS, particularly for patients in the lower-risk group.

Introduction

Myelodysplastic syndrome (MDS) is a group of abnormal clonal disorders with ineffective hematopoiesis and reduced peripheral blood cells (1). Myeloid cells in the bone marrow of patients with MDS exhibit developmental abnormalities in one or multiple lineages and high-risk patients may progress to acute myeloid leukemia (AML) (2). Bone marrow mesenchymal stem cells (BM-MSCs) are an important constituent of the bone marrow hematopoietic microenvironment. They may support the survival, self-renewal and differentiation of hematopoietic stem cells through direct contact and cytokine secretion (3,4). In MDS, abnormalities in the hematopoietic stem cells and bone marrow hematopoietic microenvironment are present (5,6). There are significant gene expression profile differences between the BM-MSCs from patients with MDS and those of healthy individuals (7,8), which may selectively lead to malignant clonal proliferation of hematopoietic stem cells in MDS. Therefore, changes in BM-MSCs are associated with the pathogenesis of MDS. BM-MSCs from patients with MDS and healthy individuals have a similar cell morphology, proliferation capacity and immune phenotype, and may be induced to transform into osteoblasts and adipocytes *in vitro*. However, the ability of BM-MSCs to support hematopoiesis is decreased in patients with MDS, which is associated with the development and progression of the disease (9).

Oxygen homeostasis is an essential prerequisite for life activities in cells and hypoxia is one of the factors affecting the bone marrow microenvironment. An essential regulatory protein for sensing a hypoxic environment is hypoxia-inducible factor-1 (HIF-1). HIF-1 is composed of the oxygen-sensitive HIF-1α, as well as the constitutively expressed HIF-1β. HIF-1α is an effector to a hypoxic environment that downregulates gene expression in bone marrow cells, which enables the body to adapt to a hypoxic environment (10). HIF-1α has been

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considered to be an oncogenic protein, as hypoxic regions are present in solid tumors and stabilize the HIF-1α protein (11). However, it has been indicated that hypoxia and HIF-1α facilitate the differentiation of AML cells, suggesting that hypoxia has different effects on leukemia (12). MDS comprises heterogeneous hematopoietic disorders, which may be identified based on their genetic, epigenetic, splicing and metabolic aberrations in patients. Mutations in major MDS-associated genes (Dnmt3a, Tet2, Asxl1, Runx1 and Mll1) activate HIF-1α signaling (13). The HIF-1α transcriptional signature was generally activated in bone marrow stem/progenitor cells from patients with MDS (14). *In vitro* experiments indicated that the HIF-1α signature was dysregulated in human patients with MDS; the dysregulation of HIF-1α led to a clinically relevant diversity of MDS phenotypes by functioning as a signaling pathway for MDS-driving mutations. The genetic disruption of HIF-1α resolves MDS phenotypes (15). In addition, specifically inhibiting HIF-1α expression through RNA interference may block hypoxia and HIF-1α-induced cell differentiation (16). BM-MSCs exist under hypoxic conditions and HIF-1α may be identified in the cytoplasm of BM-MSCs, even under normoxic conditions (17). In BM-MSCs in a hypoxic state, IL-1 and TNF-α are activated via the PI3K and MAPK pathways to trigger HIF-1α expression (17,18). In the present study, the difference in the expression of HIF-1α in BM-MSCs between patients with MDS and healthy subjects was examined to evaluate the significance of the difference in HIF-1α expression. Furthermore, the differential expression of HIF-1α was examined in bone marrow biopsy specimens from patients with MDS of different risk groups according to International Prognostic Scoring System (IPSS); those patients may be categorized into different risk groups in order to provide suitable treatment strategies, particularly in terms of HIF-1α targeting therapy in different patients with MDS.

Patients and methods

Patients. The Ethics Committee of Jiading District Central Hospital Affiliated to Shanghai University of Medicine & Health Sciences (Shanghai, China) approved the present study. On recruitment, the subjects were fully informed of the experimental procedures, and the patients signed a consent document for their excess samples to be used for scientific research. The rights and privacy of the subjects were protected to the greatest extent. The present study included 40 patients with MDS (mean age, 62 years; age range, 40-83 years; 28 males and 12 females) and 20 patients with hemocytopenia as the control group. The MDS patients were diagnosed based on the 2008 World Health Organization's criteria. The control group had suspected hematological disease whose diagnosis was ruled out (mean age, 54 years; age range, 35-72 years; 12 males and 8 females). The 40 patients with MDS and 20 patients with hemocytopenia who were recruited at the Jiading District Central Hospital Affiliated to Shanghai University of Medicine and Health Sciences (Shanghai, China) between September 2017 and May 2019, were examined according to the IPSS (19). The patients with MDS were classified into the lower-risk group and higher-risk group (20), as the IPSS score divides patients into a lower-risk subset (low and intermediate-1) and a higher-risk subset (intermediate-2 and high). Among these

MDS cases, 8 cases had MDS with single lineage dysplasia (known as MDS-SLD), 10 cases had MDS with multi-lineage dysplasia (known as MDS-MLD), 4 cases had MDS with ring sideroblasts and MLD (known as MDS-RS-MLD), 8 cases had MDS with excess blasts (EB)-1 (MDS-EB-1) and 10 cases had MDS-EB-2. All MDS cases were treatment-naïve (Table I).

Isolation and culture of BM-MSCs. From each subject, 5 ml of bone marrow was aspirated in a BD vacutainer containing heparin as an anticoagulant (cat. no. 367884; BD Biosciences). After centrifugation, the supernatant was discarded and the cells were resuspended in PBS at 25°C. Ficoll-Paque Plus (cat. no. 17-1440-02; Cytiva) with a density of 1.077 g/ml was used and the cells were centrifuged in a horizontal centrifuge at 400 x g for 30 min at 25°C. Mononuclear cells were collected and washed twice with PBS prior to seeding in the BM-MSC growth culture medium (α-minimum essential medium + 10% FBS + 1% penicillin/streptomycin; all from Gibco; Thermo Fisher Scientific, Inc.) at a density of $1x10^6/cm^2$ in a culture flask (Corning, Inc.). The culture flasks were placed in an incubator a 37°C with 5% CO_2 and saturated humidity for 28 days of culture. The BM-MSCs were adherent to the culture flasks. The BM-MSCs were digested with 1 ml 0.25% trypsin for 3 min. Second- and third-generation cells were used in the experiments.

BM-MSC flow-cytometry assay. BM-MSCs were digested with 0.25% trypsin for 3 min at 25°C and resuspended in PBS at a density of $1x10^6/ml$. The cell suspension was added to test tubes at 500 μl/tube and processed with a test kit, which contained mouse anti-human CD45-PE, CD73-APC, CD90-FITC and CD105-PerCP-Cy5.5 antibodies (cat. no. 562245; BD Biosciences), according to the manufacturer's instructions. Mouse IgG isotype-PE, isotype-APC, isotype-FITC, isotype-PerCP-Cy5.5 (cat. no. 562245; BD Biosciences) were also set up. The tubes were incubated at 4°C for 30 min and washed four times with PBS before they were loaded into the flow cytometer (FACSCalibur; BD Biosciences). Flowjo7.6 software (BD Biosciences) was used for data acquisition and analysis.

HIF-1α expression in BM-MSCs by reverse transcription-quantitative PCR (RT-qPCR). Fluorescence RT-qPCR was used to measure HIF-1α expression in primary BM-MSCs. TRIpure (Aidlab Biotechnologies Co., Ltd, China) was used for RNA extraction. HiScript Reverse Transcriptase (cat. no. R101-01/02; Vazyme Biotech Co., Ltd.) was used for RT of RNA into cDNA according to the manufacturer's instructions. AceQ qPCR SYBR-Green Master Mix (cat. no. Q111-02; Vazyme Biotech Co., Ltd.) was used to analyze target gene expression using the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) The PCR for each gene was conducted as follows: 95°C for 30 sec, and then 95°C for 5 sec and annealing at 60°C for 30 sec for 45 cycles. The primer sequences are provided in Table II. The $2^{-\Delta\Delta Cq}$ method (21) was used to calculate the relative expression level of mRNA.

Western blot analysis of HIF-1α expression in BM-MSCs. Western blot was used to determine the protein expression of HIF-1α in BM-MSCs. Total protein was extracted from

Table I. Clinical manifestation of patients with MDS (n=40).

Item	Value
Sex, female/male	12/28
Age, years	66.9±10.85
IPSS	
Low	1
Intermediate-1	22
Intermediate-2	12
High	5
Diagnosis	
MDS-SLD	8
MDS-MLD	10
MDS-RS	4
MDS-EB-1	8
MDS-EB-2	10

Values are expressed as n or the mean ± standard deviation. IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndromes; MDS-SLD, MDS with single lineage dysplasia; MDS-RS, MDS with ring sideroblasts; MDS-MLD, MDS with multi-lineage dysplasia; MDS-EB-1, MDS with excess blasts-1; MDS-EB-2, MDS with excess blasts-2.

third-generation BM-MSCs with RIPA lysis buffer. The protein from the cell lysate was quantified using the BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 15 μg protein extract was re-suspended in loading buffer prior to resolution by 10% SDS-PAGE in a Tris-glycine buffer, followed by transfer onto a PVDF membrane (EMD Millipore). The PVDF membrane was blocked by 5% skimmed milk (cat. no. 232100; Difco; BD Biosciences) solution at 25˚C for 1h. The membrane was incubated with antibodies to HIF-1α (1:1,000 dilution; cat. no. 79233; Cell Signaling Technology, Inc.) and β-actin (1:1,000 dilution; cat. no. G043; Applied Biological Materials) at 4˚C overnight. Then the membrane was washed thrice with Tris-buffered saline containing Tween-20 (TBST) for 5 min each time. Subsequently, the membrane was incubated with secondary antibody IRDye 800 CW goat anti-mouse (1:10,000 dilution; cat. no. 926-32210; LI-COR Biosciences) or goat anti-rabbit (1:10,000 dilution; cat. no. 926-32211; LI-COR Biosciences) for 1 h at 25˚C, and then washed thrice with TBST. The membrane was then analyzed using a two-color infrared fluorescence imaging system (LI-COR Biosciences).

Cell apoptosis assay. The BM-MCS cells (1x10⁶/ml) from each of the two groups were re-suspended in culture medium and centrifuged at 250 x g for 5 min at 25˚C. PBS (3 ml) was used to re-suspend the cells for 5 min prior to passing them through a 300-mesh nylon sieve once. Alexa Fluor-488 -labeled Annexin V (5 μl; cat. no. 40305ES20; Shanghai Yeasen Biotechnology Co., Ltd.) was added to each tube and cells were incubated in the dark at room temperature for 20 min. Subsequently, 10 μl propidium iodide (PI) and 200 μl working solution were added. The cells were loaded onto the flow cytometer (FACSCalibur; BD Biosciences) within 30 min after they were mixed with the solution and 10,000 cells

were acquired for analysis. According to the manufacturer's instructions, Annexin V⁺ PI⁻ cells are early apoptotic cells and Annexin V⁺ PI⁺ cells are late apoptotic cells. The sum of the proportions of these two types of cells is the apoptosis rate. Annexin V⁻ PI⁺ cells are necrotic cells.

Cell cycle experiment. A 0.25% trypsin solution was used to digest the BM-MSCs at 37˚C for 3 min and the cells were then collected. Subsequently, the cells were centrifuged at 250 x g for 5 min and the culture medium was discarded. PBS was used to wash the cells twice, 1 ml of pre-cooled 70% ethanol was added, and the cells were fixed at 4˚C for 30 min. The samples were then centrifuged at 250 x g for 5 min at 25˚C. Ethanol was aspirated and PBS was added for washing. The tubes were centrifuged at 250 x g for 5 min before the supernatant was discarded. PBS (200 μl) and 2 μl (0.25 mg/ml) RNase A (ST579; Beyotime Institute of Biotechnology) were added and the mixture was incubated at 37˚C for 30 min. PI (5 μl at 50 μg/ml) was added and the tubes were incubated at room temperature in the dark for 30 min. Cells were loaded onto the flow cytometer (FACSCalibur; BD Biosciences) for measurement (FlowJo 7.6; BD Biosciences). 1x10⁶ cells were tested in triplicate and means were calculated.

Immunohistochemistry. Paraffin sections of bone marrow tissues successively underwent three changes of xylenes and two changes of absolute ethanol, 95, 90, 80 and 70% ethanol (5 min each) before immersion in distilled water for 2 min at 25˚C. The thickness of paraffin sections was 4 μm. A 1% trypsin (Gibco; Thermo Fisher Scientific, Inc.) retrieval solution was used for antigen retrieval at 37˚C for 30 min. A 3% hydrogen peroxide solution was added to the tissue sections, followed by incubation at room temperature for 15 min. PBS was used to wash the sections thrice for 3 min each. The glass slides were wiped dry, and the sections were blocked with normal goat serum (1:20 dilution; cat. no. C0265; Beyotime Institute of Biotechnology) at room temperature for 30 min. HIF-1α antibody (1:100 dilution; cat. no. AF1009; Affinity Biosciences) was added, followed by incubation at 4˚C overnight. Absorbent towels were used to dry the slides before HRP-labeled goat anti-rabbit secondary antibody (1:5,000 dilution; cat. no. 074-1506; KPL) was added, after which slides were incubated at room temperature for 20 min. Absorbent towels were used to dry the slices before a freshly prepared DAB color development solution (Shanghai Junrui Biotechnology Co., Ltd.) was added. Color development was stopped by rinsing with distilled water. Harris hematoxylin (cat. no. H9627; MilliporeSigma) was added for 3 min for counterstaining. After washing, 1% of hydrochloric acid-alcohol was used for differentiation before washing with PBS. The slices were put into 75%, 85% and absolute ethanol for 6 min each and then in xylene (Sinopharm Chemical Reagent Co., Ltd) for 5 min for dehydration. The slices were then dried and sealed with neutral gum (Sinopharm Chemical Reagent Co., Ltd). The sections were observed and images were acquired under a microscope imaging system (DS-U3; Nikon Corporation). The immunohistochemistry results were analyzed using Image Pro Plus software 6.0 (Media Cybernetics, Inc.). The mean optical density was obtained by dividing the integrated optical density sum/area sum.

Table II. Primer sequences for reverse transcription-quantitative PCR.

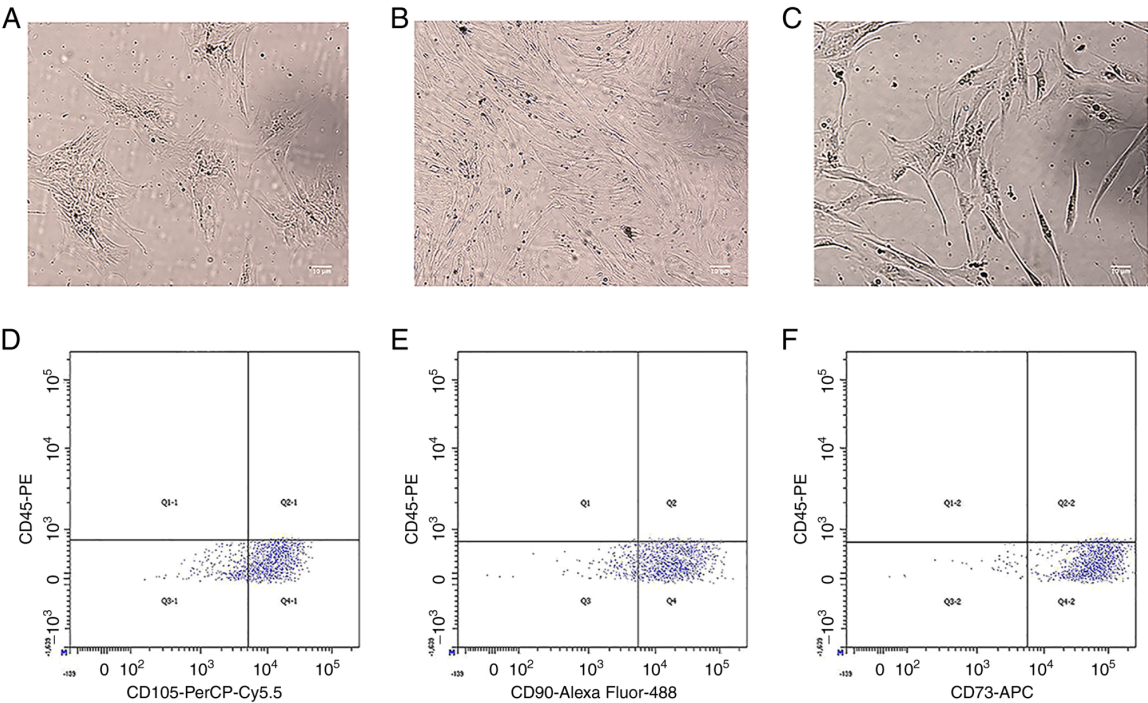| Gene	Primer sequence (5'-3')	
	Forward	Reverse
HIF-1α	TCCAAGAAGCCCTAACGTGT	TGATCGTCTGGCTGCTGTAA
GAPDH	TCAAGAAGGTGGTGAAGCAGG	TCAAAGGTGGAGGAGTGGGT

HIF, hypoxia-inducible factor.

Figure 1. Morphology and marker expression profiles of BM-MSCs from patients with MDS and the control group. Microscopy images of BM-MSCs from (A) patients with MDS at 14 days after inoculation, (B) patients with MDS at 28 days after inoculation and (C) the control group at 28 days after inoculation (original magnification, x100; scale bar, 10 μm). BM-MSCs were positive for (D) CD105 (79.6%), (E) CD90 (83.2%) and (F) CD73 (94.3%). BM-MSCs, bone marrow mesenchymal stem cells; MDS, myelodysplastic syndrome; PE, phycoerythrin; APC, allophycocyanin; PerCP, peridinin-chlorophyll-protein.

Statistical analysis. All of the experiments were repeated three times. Statistical analysis was performed using GraphPad Prism 8 statistical software (GraphPad Software, Inc.). Values are expressed as the mean ± standard deviation and an unpaired t-test was used to compare independent samples between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Morphological characteristics and flow cytometry of BM-MSCs. Morphological examination revealed that the BM-MSCs obtained from 40 patients with MDS and 20 control subjects appeared as clusters of fibroblast-like cells. Fibroblast colony-forming units were observed in 5 out of 40 MDS samples (Fig. 1A). No notable morphological differences were observed between the MDS patient group and control group (Fig. 1B and C). To verify that the obtained cells were BM-MSCs, flow cytometry was used to perform immunophenotypic analysis of primary cells cultured from the bone marrow, and the cells were passaged to the third generation prior to being used in this experiment. The flow cytometry results evaluating the immunophenotype of BM-MSCs isolated from the bone marrow of patients with MDS revealed identical cell immune marker expression to that reported by Flores-Figueroa *et al* (22) on BM-MSCs from normal adult bone marrow. These findings included high expression of CD105, CD90 and CD73 and no expression of CD45. The cells were positive for CD105 (79.6%), CD90 (83.2%) and CD73 (94.3%) (Fig. 1D-F) and negative for CD45.

HIF-1α expression is upregulated in BM-MSCs from the MDS group. To determine the differences in HIF-1α expression between the MDS and control groups, western blot and RT-PCR were used to measure HIF-1α protein and mRNA expression, respectively, in BM-MSCs in the two groups (Fig. 2). The results indicated a statistically significant difference in HIF-1α protein expression between the MDS group and the control group (P<0.05; Fig. 2A and B). BM-MSCs in the MDS group and control group were passaged until the
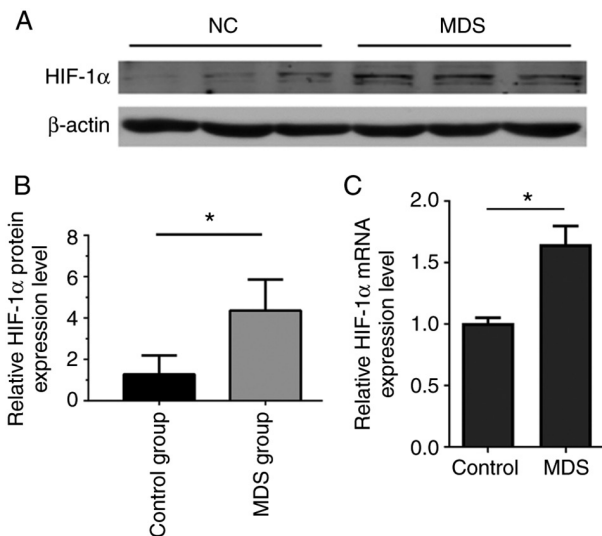
Figure 2. Expression of HIF-1α in BM-MSCs from control and untreated patients with MDS. (A) Representative western blot assessing the expression of HIF-1α protein and (B) semi-quantitative results of HIF-1α protein in BM-MSCs from the MDS and control groups. (C) The mRNA expression in BM-MSCs from the MDS and control groups was determined via reverse transcription-quantitative PCR analysis. Values are expressed as the mean ± standard deviation (n=3). *P<0.05. BM-MSCs, bone marrow mesenchymal stem cells; MDS, myelodysplastic syndrome; HIF, hypoxia-inducible factor.

third generation before RT-qPCR was performed, revealing a significant difference in HIF-1α mRNA expression between the MDS group and the control group (P<0.05; Fig. 2C). These results indicated that HIF-1α expression was altered in MDS BM-MSCs. Changes in the hematopoietic microenvironment may lead to MDS. To determine whether high expression of HIF-1α mRNA in BM-MSCs from patients with MDS (and presumably the corresponding high HIF-1α protein levels) affect apoptosis and the cell cycle, the subsequent experiments were performed.

Apoptotic cell proportion of BM-MSCs. BM-MSCs in the MDS and control groups were passaged until the third generation prior to analysis by flow cytometry with an apoptosis assay kit to measure the proportion of apoptotic cells. Comparison of BM-MSCs from the control and MDS groups indicated that the MDS group had a higher proportion of cells in early apoptosis (2.04±0.08 vs. 5.22±1.34%; P<0.0001) and late apoptosis (1.23±0.11 vs. 3.38±0.43%; P<0.01), but there was no difference in the proportion of necrotic cells (1.99±0.28 vs. 2.38±0.12%; P=0.142; Fig. 3A-C). This demonstrated that MDS BM-MSCs had a higher proportion of apoptosis than normal BM-MSCs.

Cell cycle of BM-MSCs. To further identify changes in the cell cycle in BM-MSCs in MDS, an assay kit and flow cytometry were used to measure the cell cycle distribution of BM-MSCs from the MDS and control groups (Fig. 4A and B). The results suggested that the G_0/G_1 phase populations were not significantly different between the MDS and control groups (Fig. 4C). However, the BM-MSCs of the MDS group had a significantly higher proportion of cells in S phase and G_2/M phase compared with the control group (P<0.05; Fig. 4D and E).

Immunohistochemical analysis. Immunohistochemistry was used to analyze HIF-1α expression in bone marrow tissues from patients with MDS (Fig. 5). According to the prognosis determined by IPSS, the patients with MDS were divided into two groups: A total of 16 patients were assigned to the lower-risk MDS group and 7 to the higher-risk MDS group. Furthermore, 17 hemocytopenia samples were used for analysis. The results indicated that HIF-1α expression in bone marrow tissue sections from the MDS lower-risk group (intermediate-1) (Fig. 5C and D) was higher than that in the MDS higher-risk group (P<0.0001; Fig. 5A and B).

Discussion

MDS is a disease in which abnormalities are present in hematopoietic stem cells and the bone marrow microenvironment (23). BM-MSCs are an essential constituent of the bone marrow microenvironment and possess self-renewal and pluripotent potential (24). BM-MSCs are able to differentiate into osteoblastic, adipogenic and chondroblastic cells (25), promote hematopoiesis and regulate hematopoietic stem cells to ensure they maintain their hematopoietic capabilities throughout their entire lifespan. BM-MSCs also have immuno-regulatory functions, as they maintain the stability of the bone marrow immune microenvironment and have been indicated to reduce the damage caused by stress stimuli on hematopoietic stem cells in *in vitro* experiments (26). BM-MSCs were able to protect intramedullary blast cells from damage caused by NK cells. In animal experiments, gene abnormalities in BM-MSCs have been demonstrated to induce MDS (27). Currently, there is a limited understanding of the genetics and gene expression characteristics of BM-MSCs in MDS. However, the importance of functional senescence in MDS BM-MSCs and its impact on disease progression, prognosis evaluation and treatment effects is widely recognized (28). This may explain why current treatments that only target the multiple mechanisms of clonal hematopoiesis in MDS resulted in poor efficacy in certain patients (29). Therefore, the effect of changes in the bone marrow microenvironment, particularly concerning regulatory mechanisms of BM-MSCs on MDS development, progression and treatment, is worth of further investigation.

Under physiological conditions, there are multiple pathways that regulate biological behavior in numerous stem/progenitor cells to adapt to a hypoxic environment, which has certain effects on gene expression in BM-MSCs (30,31). HIF-1α is an effector in a hypoxic environment. A hypoxic environment may promote endothelial cell proliferation, remodeling and the synthesis of pro-angiogenic factors such as VEGF, bFGF, CXC12 through HIF-1, thereby inducing angiogenesis (32). HIF-1α has also been indicated to participate in enhanced innate immune responses and it regulates the activation of macrophage migration inhibitory factor (MIF) (33). MIF was observed to be highly expressed in AML cells (34) and a number of solid tumor types, including glioblastoma (35-37), neuroblastoma (38) and melanoma (39). However, overexpression of MIF is associated with poor outcome in patients with MDS (40). HIF-1α is associated with AML progression, and if treatment-naïve AML cells contain high levels of HIF-1α, they have an increased tendency toward extramedullary invasion and poor prognosis (41). It may be hypothesized that HIF-1α
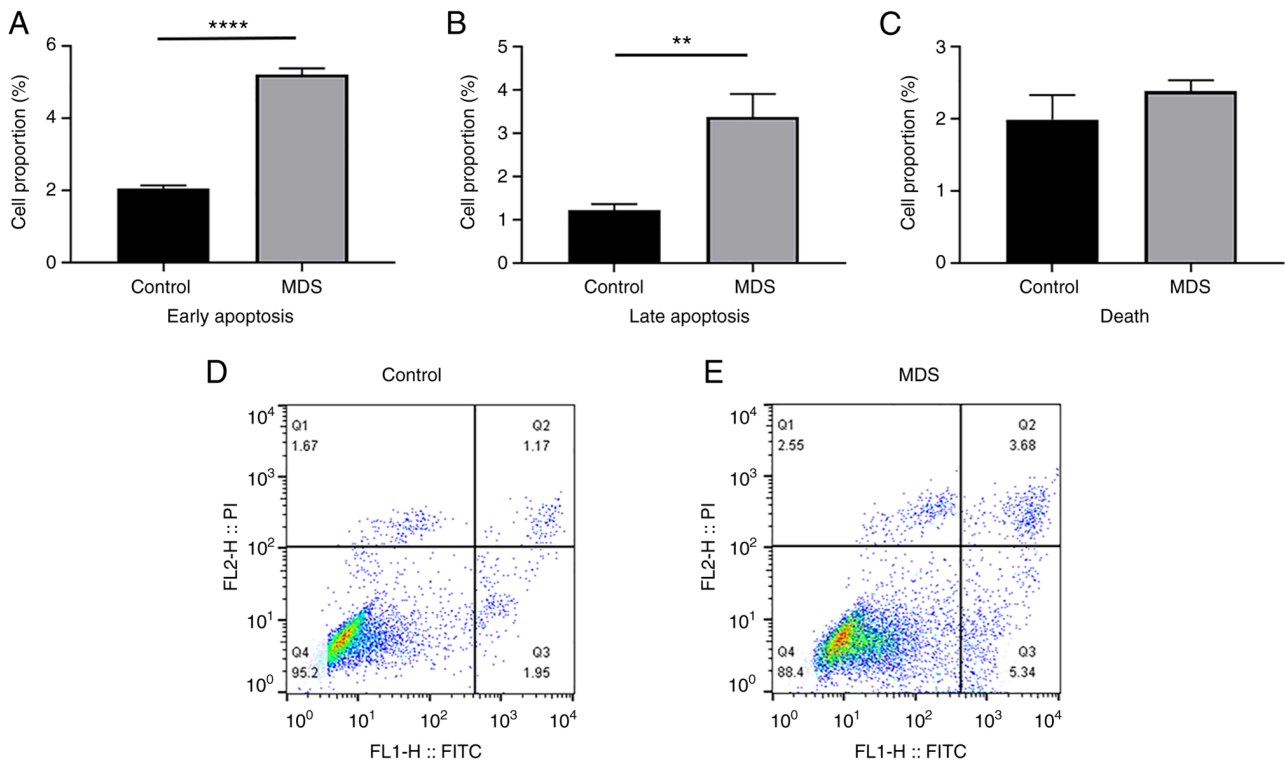
Figure 3. Viability and apoptosis of bone marrow mesenchymal stem cells from the control and MDS patient groups. Proportion of cells in (A) early apoptosis and (B) late apoptosis in the two groups. (C) Dead cells in the MDS and control groups. Flow cytometric dot plots for the (D) and (E) MDS groups with FITC-Annexin V and PI staining. Values are expressed as the mean ± standard deviation (n=3). **P<0.01, ****P<0.0001. MDS, myelodysplastic syndrome; PI, propidium iodide.
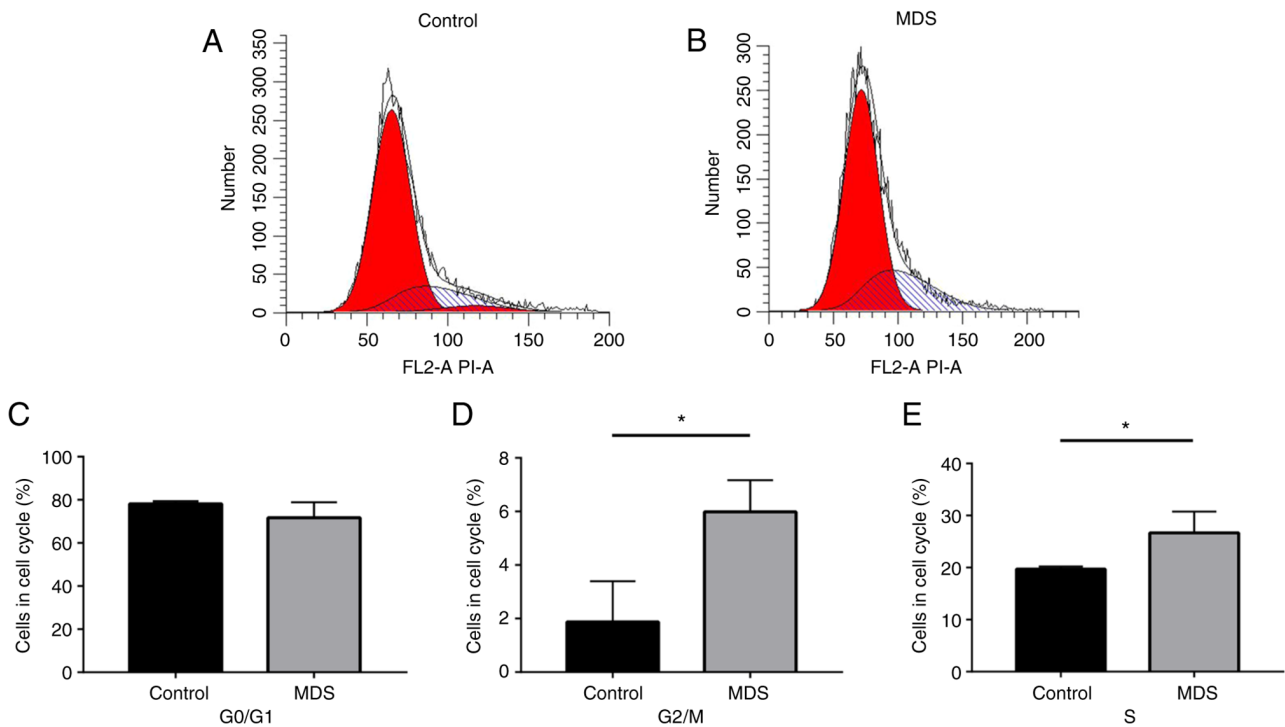

Figure 4. Flow cytometric analysis of the cell cycle. (A and B) Flow cytometry dot plots for the (A) control and (B) MDS patient groups with PI staining. Proportion of cells in (C) G_0/G_1 phase, (D) G_2/M phase and (E) S phase in the control and MDS patient groups. Values are expressed as the mean ± standard deviation (n=3). *P<0.05. MDS, myelodysplastic syndrome; PI, propidium iodide.

participates in the oncogenesis and progression of myeloid leukemia and the specific pathways and effects involved require

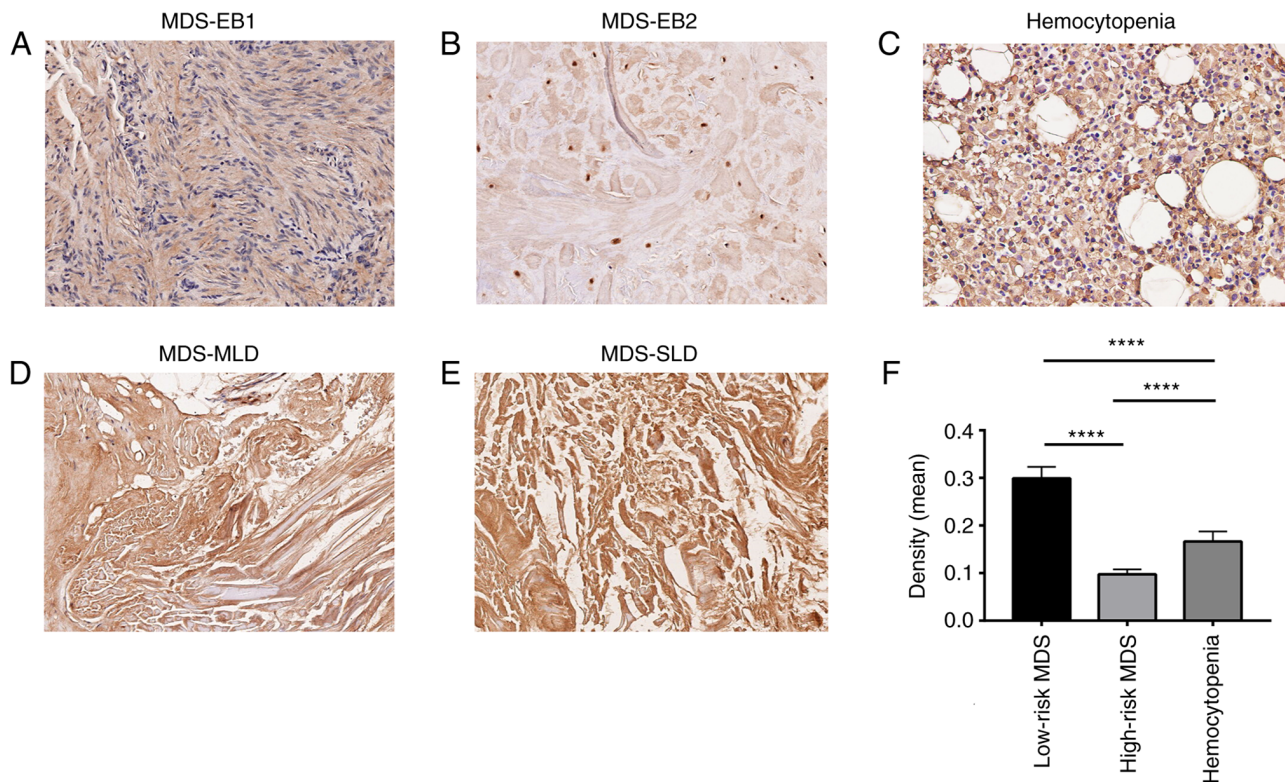further study. Aberrant HIF-1α stabilization and abnormal mitochondrial and autophagic death were observed in the bone

Figure 5. Assessment of bone marrow biopsies using HIF-1α immunohistochemical staining (the orange color indicates the positive staining for HIF-1α). (A) MDS-EB-1 (IPSS intermediate-2 risk), (B) MDS-EB-2 (IPSS high risk), (C) hemocytopenia (D) MDS-MLD (IPSS intermediate-1 risk) and (E) MDS-SLD (IPSS low risk) bone marrow biopsy sample (magnification, x400). (F). Statistical data analysis of the immunohistochemical staining. Values are expressed as the mean ± standard deviation (n=3). ****P<0.0001. IPSS, International Prognostic Scoring System; MDS-SLD, MDS with single lineage dysplasia; MDS-MLD, MDS with multi-lineage dysplasia; MDS-EB-1, MDS with excess blasts-1; MDS-EB-2, MDS with excess blasts-2.

marrow of patients with MDS (41). The expression of HIF-1α was associated with poor overall survival and disease progression of MDS, as well as with the bone marrow blast percentage and cytogenetics (42). Examining and evaluating the effects of hypoxic factors and elucidating their mechanisms of action may provide a potential therapeutic target for the diagnosis and treatment of MDS (43).

In the present study, it was observed that there were no notable morphological differences between BM-MSCs from patients with MDS and those from healthy human bone marrow. BM-MSCs from both groups exhibited long vortex-like fibers and were arranged in a neat and orderly manner, which is similar to what has been reported in the literature. Immuno-phenotype flow cytometry confirmed that the cultured cells were BM-MSCs. Experiments demonstrated that HIF-1α expression was significantly upregulated in BM-MSCs from the MDS group compared with those from the control group (P<0.05). HIF-1α staining of bone marrow biopsy tissues suggested that HIF-1α expression in the low- and intermediate-risk MDS groups was higher than that in the high-risk group (P<0.0001). In addition, a higher proportion of apoptotic BM-MSCs was present in the MDS group compared with that in the control group, and cell cycle distribution differences were simultaneously present. An increased proportion of BM-MSCs from the MDS group in the S-phase and G_2/M phase compared with those from the control group was observed, while the G_0/G_1-phase populations were similar. Although the above results differ from

those reported in the literature (44), the present results suggested that hematopoietic arrest is present in BM-MSCs from patients with MDS. In the present study, the proportion of apoptotic BM-MSCs was higher in the MDS group than in the control group (45). Senescence of BM-MSCs in the MDS bone marrow microenvironment may explain why persistent peripheral blood cytopenia is present in lower patients in the MDS group (45). It is worth noting that there are significant differences in the diagnosis and treatment strategies for low- and intermediate-risk MDS compared with strategies for high-risk MDS. Supportive treatment, immunotherapy and cytokine treatment are the mainstay treatments for the former group, whose main clinical presentation is a reduction in one to three lineages of peripheral blood cells (46). In the present study, HIF-1α expression was measured in the bone marrow biopsy tissues from patients with MDS to reveal that the expression of HIF-1α in the bone marrow biopsy tissues of patients with low- and intermediate-risk MDS was significantly higher than that of patients with high-risk MDS. This suggested that the pathophysiology of low- and intermediate-risk MDS is different from that of high-risk MDS, and accordingly, it was hypothesized that HIF-1α may serve as a potential therapeutic target for MDS. Further research on excessive apoptosis and senescence in BM-MSCs in patients with low- and intermediate-risk MDS is required. In the present study, 40 patients with MDS and 20 patients with hemocytopenia were analyzed, but the number of cases may have been insufficient; thus, the number of subjects may be increased in a future study.

Certain studies observed increased apoptosis in BM-MSCs in the early stage of MDS, resulting in ineffective hematopoiesis and affecting the survival of hematopoietic cells, which in turn resulted in pancytopenia (47). Reduced apoptosis and proliferation of clonal cells occurring in the late stage and persistent clonal proliferation may cause MDS to progress to AML. The pathophysiological mechanisms underlying the early and late stages of this disease have been elucidated. It has been noted that increased apoptosis, which is associated with ineffective progenitors and survival of hematopoietic cells, contributes to cytopenia during the early stages (48). As a consequence of these defective pathways, cytopenia and ineffective hemopoiesis takes place. However, prolonged late-stage MDS may lead to AML, with a decrease in apoptosis, and an increased degree of neoplastic cell survival may be observed (49,50). The mechanisms promoting the conversion of excessive apoptosis in the early stage to reduced apoptosis and accelerated progression in the late stage remain elusive and the effects of the bone marrow microenvironment during progression cannot be ignored. The effects of hypoxia and HIF-1α expression on clonal cells and the bone marrow microenvironment require further investigation.

In the present study, HIF-1α overexpression occurred in primary BM-MSCs from patients with MDS and these BM-MSCs exhibited excessive apoptosis and senescence, which differs from literature reports indicating that HIF-1α overexpression under normoxic conditions may lead to higher survival and inhibit apoptosis in MSCs (51). It was previously reported that increased HIF-1 expression in a hypoxic environment promoted the differentiation of MSCs into endothelial cells as well as bone marrow angiogenesis, leading to increased erythroid proliferation and an increased red blood cell count (51), which differs from the observations of the present study that indicated excessive apoptosis and growth arrest of BM-MSCs from patients with MDS and cannot explain cytopenia in MDS. Senescence of MDS-MSCs may explain cytopenia in low- and moderate-risk patients. The mechanisms underlying the clonal proliferation and the driving factors for transformation in the high-risk group require further investigation to ensure that screening for patients who are more suitable for HIF-1α-targeted therapy will be possible in the future.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BQ participated in writing the manuscript and performed the statistical analysis. XH participated in the experimental design and data acquisition. LZ, FZ and QG performed the experiments. BQ and XH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Jiading District Central Hospital Affiliated to Shanghai University of Medicine & Health Sciences (Shanghai, China) approved the present study and all subjects provided written informed consent for the use of their samples in research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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