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Original Article

mRNA expression level and clinical value of hypoxia inducible factor 1 alpha in patients with myelodysplastic syndromes: a fluorescence quantitative real-time RT-PCR study

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Abstract: Objective: The clinical value and prospective function of hypoxia inducible factor 1 alpha (HIF-1α) in myelodysplastic syndromes (MDS) remain poorly understood. Therefore, the objective of the current investigation was to elucidate the expression level and clinical significance of HIF-1α in MDS. Methods: A total of 94 bone marrow tissues were gathered, including 74 from MDS patients and 20 from healthy donors. Morphological examination, fluorescence in situ hybridization (FISH) and karyotype analysis were carried out to categorize MDS into different subgroups. The expression level of HIF-1α mRNA was detected by fluorescence quantitative real-time RT-PCR. Results: HIF-1α mRNA expression in MDS bone marrow samples was clearly upregulated than that in healthy donors (P<0.001). The HIF-1α mRNA levels increased gradually (P=0.040) in different groups based on the IPSS score from low, intermediate-1, intermediate-2 to a high-risk group. HIF-1α expression in the high-risk group, as evaluated by the percentage of BM blasts, was markedly higher than that in the low-risk group (P=0.021). HIF-1α mRNA expression in the FISH-abnormal group was prominently up-regulated compared to the FISH-normal group (P=0.005). HIF-1α expression in the karyotype-abnormal group was apparently increased compared to that in the karyotype-normal group (P=0.026). Furthermore, Spearman analysis disclosed that HIF-1α expression was positively related to patient prognosis, as assessed by karyotype (r=0.330, p=0.022). Conclusion: HIF-1α may participate in the development of MDS and can lead to poor prognosis.

Keywords: Myelodysplastic syndromes (MDS), HIF-1α, mRNA, VEGF, PDGF-BB, prognosis

Introduction

Myelodysplastic syndromes (MDS) consist of a heterogeneous set of myeloid disorders that are specified by peripheral blood cytopenias, dysplasia of one or more lineages, ineffective hematopoiesis, defective bone marrow (BM) hematopoiesis and the incidence of intramedullary apoptosis [1-3]. MDS poses a pivotal hazard for advancement to acute myeloid leukemia. MDS has a complicated pathogenesis, and it is believed that the excessive apoptosis of hematopoietic cells, changes in epigenetics, and abnormal angiogenesis are involved in its development [2, 3]. Great advancements have been made in the diagnosis and therapy of MDS, however, there are no specific drugs for the treatment of MDS due to its complicated pathogenesis. Demethylation therapy with Decitabine has been approved, but it is primarily used to treat high-risk MDS rather than low-risk MDS [4]. The prognosis of MDS patients remains poor, particularly of those affected by high-risk MDS [5]. Hence, there is an urgent need to identify new diagnostic and therapeutic targets for this disease.

Anti-angiogenesis treatment has become an important alternative for patients with low-risk MDS and has a good curative effect [6]. Hypoxia inducible factor 1 (HIF-1) is a critical regulatory factor that controls the expression of more than 100 genes, such as hemopoietin, vascular en-

Table 1. Relationship between HIF-1α expression level and clinicopathological features in MDS

Clinicopathological Features		HIF-1α expression		
		N	Mean ± SD	*P* value
Group	MDS	74	2.556±1.648	<0.001
	Control	20	0.796±0.490	
Gender	Male	48	2.658±1.733	0.471
	Female	26	2.367±1.492	
Age (years)	<51	35	2.694±1.933	0.496
	≥51	39	2.431±1.357	
WHO classification	Early-stage	35	2.126±1.041	0.028
	Advanced-stage	39	2.941±1.981	
IPSS	Intermedia-/low-risk	31	1.945±0.832	0.003
	High-risk	26	3.091±1.628	
BM blasts	Low-risk	36	2.108±1.032	0.021
	High-risk	38	2.980±1.993	
FISH assay[a]	Normal	23	2.078±0.874	0.005
	Abnormal	34	3.210±1.959	
Karyotype[b]	Normal	23	1.903±0.881	0.026
	Abnormal	25	2.663±1.372	
Prognosis based on karyotype[c]	Favorable	25	1.895±0.847	0.003
	Moderate	10	2.103±0.502	
	Poor	13	3.228±1.683	

Notes: [a]Abnormal group: any type of the following conditions: -5/5q-, -7/7q-, +8, 20q- or -Y. [b]Karyotype-abnormal group was defined as any type of chromosomal abnormalities. [c]Favorable group: normal karyotype or karyotype abnormalities with -Y or del(5q) or del(20q); Poor group: complicated (≥3) or chromosomal abnormalities on chromosome 7; Moderate group: other type of karyotype not classified as favorable or poor groups.

dothelial growth factor (VEGF) and glucose transporter (GLUT). HIF-1α, which is modulated by O_2 availability, is a special subunit of HIF-1 that determines the function of HIF-1. HIF-1α, which is widely expressed in human malignancy, can facilitate the proliferation and metastasis of tumor cells and is therefore associated with their growth, invasion and resistance to treatment [7, 8]. In normoxic conditions, the stability of HIF-1α protein is negatively modulated by prolyl hydroxylase domain protein (PHD)-dependent hydroxylation, which causes von Hippel-Lindau protein (VHL)-dependent ubiquitination and proteasome-dependent degradation [9-11]. Under these circumstances, HIF-1α is unstable and cannot be detected. However, under hypoxic environments, the degradation of HIF-1α is blocked, and its concentration increases [12]. Moreover, enhanced expression of HIF-1α generally indicates poor prognosis in some solid tumors [13-15].

The correlation between HIF-1α and malignant hemopathy has been rarely studied. Increased expression of HIF-1α was found to forward the pathogenic processes of acute monocytic leukemia [16-19]. However, only two studies on the correlation between HIF-1α expression and MDS have been performed to date. Qu et al reported that in serum, the HIF-1α protein level was strongly increased in patients with MDS. However, this result might not reflect the endogenous expression level of HIF-1α in BM [20]. Tong et al demonstrated that 49.5% of the examined patients with MDS (40/81) were HIF-1α positive, as determined by immunohistochemistry in BM; however, no normal BM samples were used as a control [21]. To the best of our knowledge, HIF-1α mRNA expression in MDS has not yet been examined. Hence, in the current study, 74 MDS patients and 20 healthy BM donors were enrolled to evaluate the expression level and clinical significance of HIF-1α mRNA by fluorescence quantitative real-time RT-PCR.

Materials and methods

Materials

A total of 94 BM samples, including 74 from MDS patients and 20 from healthy donors, were collected at the First Affiliated Hospital of Guangxi Medical University, People's Republic of China, between October 2013 and July 2016 for use in the current study. The MDS group contained 48 males and 26 females aged between 20 and 86 years old, with a median age of 51 years. All patients were diagnosed based on the expert consensus on the diagnosis and treatment of myelodysplastic syndrome (2014) [5]. The clinical medical records of MDS patients were collected. The control group con-

tained 20 healthy BM donors, including 11 males and nine females. These donors were aged between 18 and 58 years, with a median age of 34 years old. BM cell morphological examination was performed in all MDS patients. Based on the World Health Organization (WHO) classification, patients with MDS were separated into six groups, including refractory anemia (RA, n=3), refractory anemia with ring sideroblast (RARS, n=3), refractory cytopenia with multilineage dysplasia (RCMD, n=28), refractory anemia with excess blast-1 (RAEB 1, n=21), refractory anemia with excess blast-2 (RAEB 2, n=18) and del(5q) syndrome (5q-, n=1). Herein, MDS patients were grouped into: early-stage MDS (RA, RARS, RCMD and 5q-, n=35) and advanced-stage MDS (RAEB-1 and RAEB-2, n=39). In accordance with the International Prognostic Scoring System (IPSS), patients were divided into a low-risk group (IPSS score =0, n=8), intermediate-1 risk group (0.5≤IPSS score≤1, n=23), intermediate-2 risk group (1.5≤IPSS score≤2, n=21) and high-risk group (IPSS score≥2.5, n=5). In the current study, these patients were also grouped into a intermedia-/low-risk group (IPSS score≤1, n=31) and a high-risk group (IPSS score≥1.5, n=26). Furthermore, based on the percentage of BM blasts, patients were grouped into a low-risk group (percentage of BM blasts <5%, n=36) and a high-risk group (percentage of BM blasts >5%, n=38). The morphology of BM cells from MDS patients and healthy donors was examined, and fluorescence in situ hybridization (FISH) test and karyotype analysis were employed. On the basis of the FISH results, patients were separated into FISH-normal (n=23) and abnormal (n=34) groups. Based on the results of karyotype analysis, patients were divided into karyotype-normal (n=23) and abnormal (n=25) groups as well as favorable (n=25), moderate (n=10) or poor (n=13) prognosis groups (**Table 1**).

Separation of BM-derived mononuclear cells (BMDMC)

Two mL of BM was collected via BM puncture from the ilium using a BM puncture needle and was preserved in an Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulative tube. The same amount of Phosphate Buffered Saline (PBS) was added to the EDTA anticoagulative tube, and the resulting mixture was subjected to Ficoll density purification to isolate the BMDMCs using Lymphocyte Separation Medium (Human) (Solarbio, Beijing, China) [22, 23].

Fish

Four mL of BM was collected via BM aspirates from the ilium and preserved in an ethylene diamine tetraacetic acid (EDTA) anticoagulative tube. FISH was performed using a commercial kit from GP Medical Technologies (Beijing, China) in line with the manufacturer's protocol. The results of FISH were assessed using an Olympus fluorescence microscope (Olympus America, Melville, NY, USA) attached to an imaging system (Jena, Germany) with a triple-bandpass filter for DAPI, FITC, and Texas Red fluorescence. Demonstrative cell images were analyzed using a computer-based imaging program, and at least 200 cells were counted in each sample. Interphase cell analysis was conducted in accordance with the manufacturer's instructions.

Karyotype analysis

Four mL of BM was collected via BM aspirates from the ilium and preserved in an EDTA anticoagulative tube. After the nucleated cells were counted, the BM specimens were seeded at a density of 2×10^6 cells/mL in RPMI medium (Dahui, China) supplemented with 10% Fetal Bovine Serum (FBS). Colcemid was added to each flask to a final concentration of 0.05 μg/mL, and the cells were incubated at 4°C overnight (12-14 hrs). Cells were harvested by centrifugation for 10 min at 2000 rpm/min, incubated with 0.075 mol/l KCl at 37°C for 30 min, and fixed with freshly prepared fixative (methanol:glacial acetic acid=3:1, v/v). The G-banding technique (Giemsa dyeing) was used in the karyotyping analysis. At least 20 metaphases were selected to analyze the karyotype of each sample. Chromosomal aberrations were analyzed on the basis of the 2013 International System for Human Cytogenetic Nomenclature (ISCN) guidelines [24]. Abnormal clones were identified by evidence of more than two metaphase spreads with the same numerical or structural abnormality.

Fluorescence quantitative real-time RT-PCR

Total RNA was isolated from all BMDMC samples with Trizol (Invitrogen, USA) based on the manufacturer's protocol. The concentration of total RNA was determined using an ultraviolet spectrophotometer, and the quality of the RNA

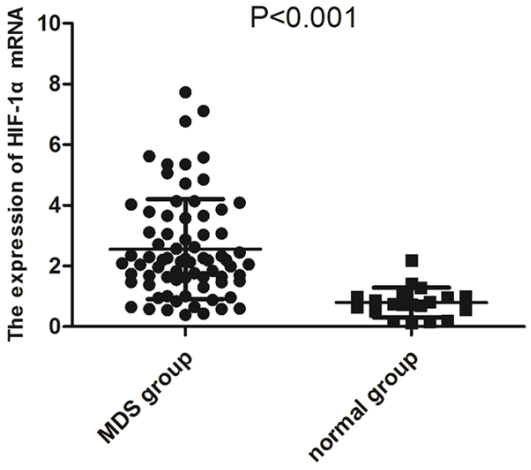

Figure 1. Difference of HIF-1α mRNA level between MDS and normal group.

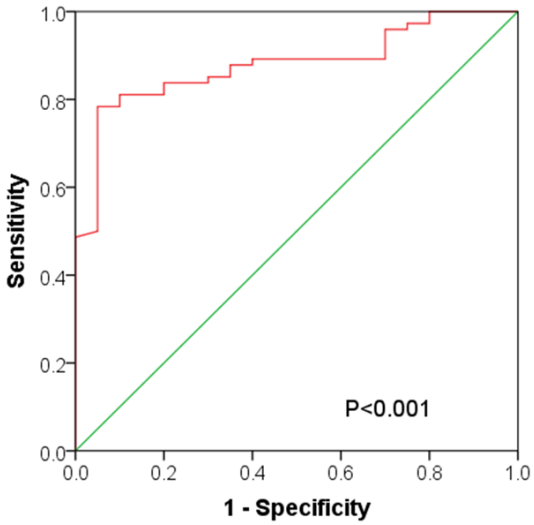

Figure 2. ROC curve of HIF-1α expression in MDS. AUC=0.879.

was assessed according to its OD260/280 ratio. The total RNA was then applied to synthesize cDNA with a PrimeScript™ RT reagent Kit (TaKaRa, Japan). Reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) was taken as an internal reference in this study. Primers were synthesized by TaKaRa as follows: HIF-1α, 5'-GAAGTGTACCCTAACTAGCCGAGGA-3' (forward), 5'-TGAATGTGGCCTGTGCAGTG-3' (reverse); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward), 5'-TGGTGAAGACGCCAGTGGA-3' (reverse). FastStart Universal SYBR Green Master (Roche, German) (12.5 μL), cDNA template (2 μL), forward primer (1 μL), reverse primer (1 μL), and RNase Free dH_2O were added to the reaction system (25 μL total volume). The amplification conditions were as follows: 94°C pre-degeneration for 5 min; 94°C degeneration for 45 s, 60°C annealing for 45 s, and 72°C elongation for 30 s within 40 cycles; and a final 72°C extension for 5 min. All experiments were repeated in triplicate. The relative expression level of HIF-1α was assessed using the $2^{-\Delta\Delta Ct}$ method and normalized based on GAPDH expression.

Statistical analysis

All data were processed using SPSS22.0 statistical software and were presented as the mean ± SD. Student's t-test was employed when comparing the expression levels of HIF-1α mRNA between the MDS and control groups. For experiments with multiple comparisons, one-way ANOVA was performed if the variances were equal; a nonparametric rank sum test was used if the variances were unequal. Correlation between karyotype prognosis and the expression level of HIF-1α mRNA was evaluated by the Spearman rank correlation coefficient if not all variables were normally distributed. A two-tailed test was used during the statistical process and a value of $P<0.05$ was set to be significant.

Results

Expression and diagnostic value of HIF-1α in MDS

All patients were divided into groups in basis of different parameters or criteria (**Table 1**). The level of HIF-1α mRNA in the MDS group (2.556±1.648) was remarkably higher than that in the normal group (0.796±0.490, P<0.001) (**Table 1**; **Figure 1**). Furthermore, we performed ROC curve examination to assess the diagnostic role of HIF-1α in MDS. The area under curve (AUC) of HIF-1α was 0.879 (95% CI: 0.807-0.951, P<0.001, **Figure 2**).

HIF-1α mRNA expression in MDS subgroups

The expression of HIF-1α mRNA in the advanced-stage group (2.941±1.981) was significantly higher than that in the early-stage group (2.126±1.041) (P=0.028) (**Table 1**; **Figure 3**). According to the IPSS, HIF-1α mRNA expression in the high-risk group (3.091±1.628) was markedly higher than that in the intermediate-/low-

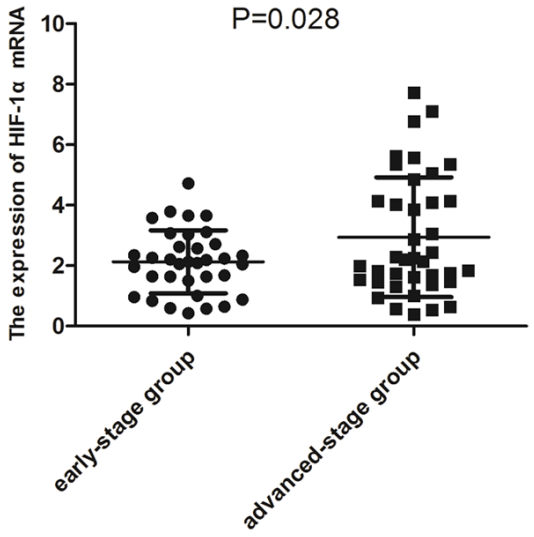

Figure 3. Expression of HIF-1α mRNA in MDS subgroups based on stages.

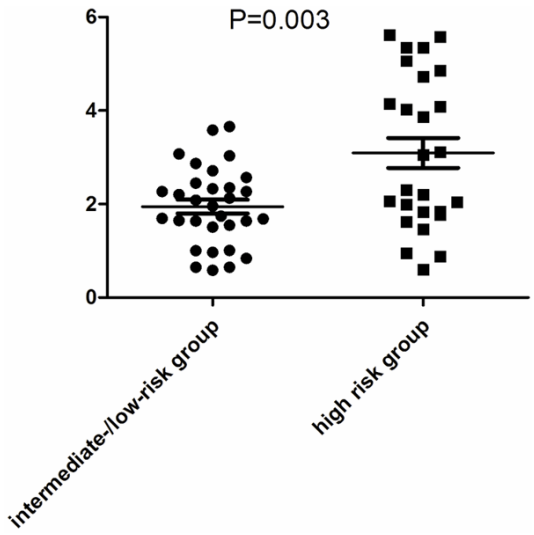

Figure 4. Expression of HIF-1α mRNA in groups based on IPSS.

risk group (1.945±0.832, P=0.003) (**Table 1**; **Figure 4**). On the basis of the percentage of BM blasts, the HIF-1α level in the high-risk group (2.980±1.993) was markedly increased than that in the low-risk group (2.108±1.032, P=0.021) (**Table 1**; **Figure 5**).

FISH assay was performed for all 57 MDS patients. The FISH-abnormal group was characterized by chromosomal abnormalities on chromosome 5, 7, 8, 20 or Y (n=34), whereas the FISH-normal group contained MDS patients

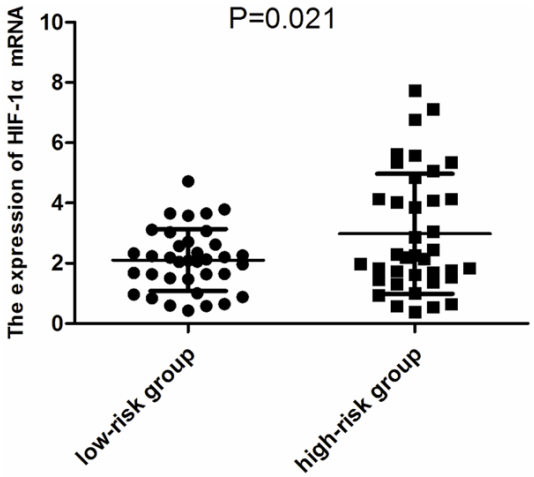

Figure 5. Expression of HIF-1α mRNA in groups categorized by the percentage of BM blasts.

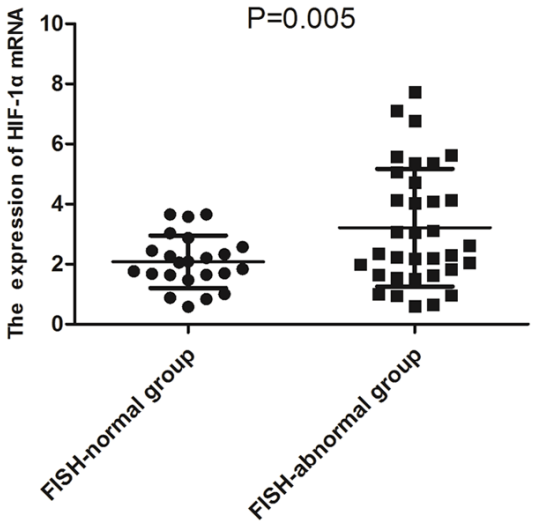

Figure 6. Expression of HIF-1α mRNA in the FISH-normal and abnormal groups.

without chromosomal abnormalities (n=23). HIF-1α expression in the FISH-abnormal group (3.210±1.959) was markedly higher than that in the FISH-normal group (2.078±0.874, P=0.005) (**Table 1**; **Figure 6**).

Karyotype analysis was performed for 48 patients with MDS, which were then subdivided into normal and abnormal groups (**Table 1**). Based on the results of karyotype analysis, the prognosis was categorized into three levels (**Table 1**). The HIF-1α mRNA level in the karyotype-abnormal group (2.663±1.372) was remarkably upregulated than that in the karyo-

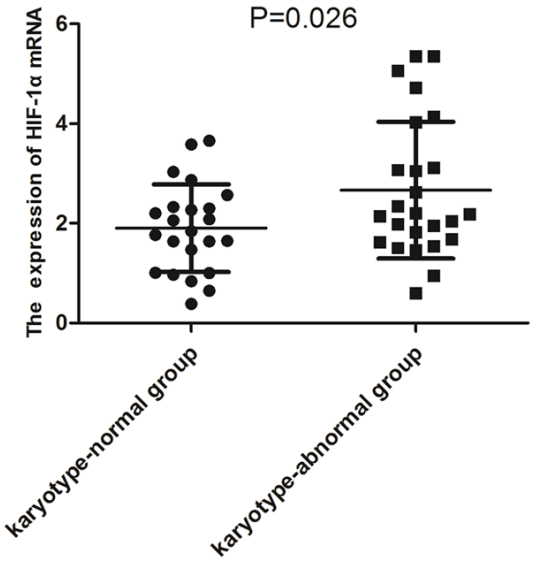

Figure 7. Expression of HIF-1α mRNA in the karyotype-normal and abnormal groups.

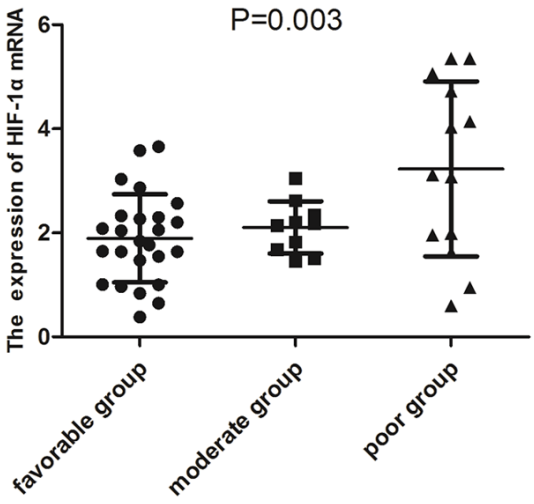

Figure 8. Correlation between the expression of HIF-1α mRNA and karyotype prognosis.

type-normal group (1.903±0.881, P=0.026) (**Table 1**; **Figure 7**).

ANOVA test indicated an apparent distinction of HIF-1α mRNA level among the three groups based on prognosis: favorable group (1.895± 0.847), moderate group (2.103±0.502) and poor prognosis group (3.228±1.683) (P=0.003) (**Table 1**; **Figure 8**). A remarkable correlation was found by the Spearman Correlation test between the HIF-1α mRNA level and karyotype prognosis (r=0.330, P=0.022).

Discussion

In our study, we collected bone marrow tissue samples from 74 MDS patients and 20 healthy donors to examine HIF-1α mRNA expression level via fluorescence quantitative real-time RT-PCR. Together with previous reports, we conclude that HIF-1α may play an essential role in the pathogenesis and progression of MDS.

MDS is a heterogeneous group of myeloid disorders and is now considered one of the most common hematological malignancies, affecting more than 30,000 patients per year in the USA and enhancing the risk of transformation to acute myelogenous leukemia (AML). The drugs that are available for the treatment of MDS, such as azacitidine, decitabine and lenalidomide, are not curative [25]. The cause of MDS is complex and can include multiple genetic alterations and molecular events [26, 27]. Thus, it is imperative to uncover the molecular causes and new therapeutic targets of MDS. HIF-1α is an O_2-dependent transcription factor that is widely expressed in mammals, including humans. HIF-1α protein accumulates in cells due to a block of ubiquitination and the inhibition of degradation under anoxic conditions. HIF-1 is a heterodimer comprising accumulated HIF-1α and HIF-1β subunits that bind to target genes at the core DNA sequence 5'-RCGTG-3' (where R stands for A or G) within the hypoxia response element to enhance the expression of target genes [12]. Abnormal expression of HIF-1α in most normal human tissues has not been found, but increased expression of HIF-1α has been observed in many solid tumors, such as colon cancer, lung cancer and invasive breast cancer, and is associated with the proliferation, apoptosis, invasion, metastasis and resistance of tumor cells [7, 8, 28].

As HIF-1α has become a research hotspot, its association with hematologic malignancy has been explored. It has been reported that abnormal expression of HIF-1α was observed in acute myelocytic leukemia (AML) [18, 29, 30] and B-cell lymphoma [31], as compared to that in no-tumorous controls. In acute promyelocytic leukemia, PML-RARa plays a role in the function of HIF-1α-modulated pro-leukemogenesis, including cellular motility, BM neo-angiogenesis and the self-renewal of acute promyelocytic leukemia (APL) blasts via both *in vitro* and *in*

vivo experiments [18]. Furthermore, after APL cells were treated with all-trans retinoic acid (ATRA), the HIF-1α levels rose obviously. The suppression of HIF-1α in APL mouse models also led to a delay in the development of leukemia. To this end, HIF-1α could function in concert with PML-RARa to facilitate the progression and relapse of acute promyelocytic leukemia [18].

However, only two groups have examined the clinical value of HIF-1α expression in MDS. Qu et al gathered serum samples from 30 MDS patients and 50 healthy individuals to test the expression level of HIF-1α in MDS. The HIF-1α protein level in the serum of MDS patients was markedly higher than that in the normal group, as assessed by ELISA [20]. However, the concentration of HIF-1α in serum might not accurately reflect the endogenous expression of HIF-1α in BM. In addition, the work of Qu et al involved a small sample size (n=30). Tong et al [21] analyzed HIF-1α protein expression by immunohistochemical staining in BM samples obtained from 81 MDS patients and found that only 49.4% of the patients (40/81) showed positive results. Moreover, HIF-1α protein level could be an independent prognostic biomarker for MDS. Furthermore, the expression of HIF-1α protein was notably related to BM blast percentage, hemoglobin count and cytogenetics. However, no healthy controls were enrolled in the study of Tong et al, thus reducing the credibility of the results. Compared to previous reports [20, 21], the current investigation is superior in at least three ways. First, BM samples were employed for the detection of HIF-1α, which can more accurately represent the clinical significance of HIF-1α. Second, 20 healthy donors were enrolled in the study as controls, which makes the reported difference in HIF-1α level more reliable. Third, the HIF-1α mRNA level was assessed, which distinguishes our report from previous reports [20, 21].

Our study proves that the expression of HIF-1α mRNA in the MDS group is markedly higher than that of the normal group, consistent with the findings of Qu et al and Tong et al [20, 21]. IPSS is a crucial index for estimating MDS patient prognosis. A high IPSS score indicates a high likelihood of MDS transforming into leukemia and a poor prognosis. Tong et al found marked differences between the HIF-1α negative and HIF-1α positive groups in IPSS (low risk, intermediate-1, intermediate-2 and high risk group). In addition, they found that MDS patients expressing HIF-1α were more vulnerable to classification in the high-risk group and had a poorer survival time and prognosis than those who did not express HIF-1α [21]. In the present study, our result is largely mirrored by the fact that HIF-1α mRNA expression in the high-risk group is markedly higher than that in the intermediate- and low-risk groups. Thus, HIF-1α may be associated with the prognosis of MDS patients and could become an essential marker of prognosis.

The role of HIF-1α may vary across different MDS groups according to IPSS score. The phenomenon that HIF-1α expression in the high-risk group is pronouncedly higher than that in the low-risk group may be associated with the function of HIF-1α in angiogenesis. HIF-1α can induce the expression of a series of genes, including platelet-derived growth factors-BB (PDGF-BB), basic Fibroblast Growth Factor (bFGF) and epidermal growth factor receptor (EGFR), which are associated with angiogenesis [28]. The marrow microvascular density increases in accord with the elevation of marrow angiogenesis medium.

Up to 40-70% of MDS patients exhibit chromosomal abnormality. The common categories of chromosomal abnormality include -5/5q, -7/7q, +8, 20q and -Y. MDS patients with -7/7q or a complex karyotype are more prone to transform to leukemia. The HIF-1α mRNA level in the FISH-abnormal group was prominently up-regulated compared to that in the FISH-normal group. HIF-1α expression in the karyotype-abnormal group was significantly increased compared to that in the karyotype-normal group. Furthermore, the positive relationship between HIF-1α mRNA level and karyotype prognosis indicates that HIF-1α is strongly linked to the prognosis of MDS.

In summary, HIF-1α plays an indispensable role in the occurrence and development of MDS, which is closely associated with the prognosis of HIF-1α may become an essential marker of prognosis in MDS.

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Disclosure of conflict of interest

None.

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