Heliyon 9 (2023) e18947

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Upregulation of S100A6 and its relation with CD34$^+$ cells apoptosis in high-risk myelodysplastic syndromes patients

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ARTICLE INFO

Keywords:
S100A6
Myelodysplastic syndromes
CD34$^+$ cells
Apoptosis

ABSTRACT

Objectives: Myelodysplastic syndromes (MDS) are a group of myeloid malignancies characterized by peripheral blood cytopenia and hematopoietic dysplasia that often progress to acute myeloid leukemia (AML). Increased apoptosis of normal hematopoietic cells and decreased apoptosis of malignant clonal hematopoietic cells in patients with MDS is some of the mechanisms leading to ineffective hematopoietic cells in the bone marrow. S100 calcium-binding protein A6 (S100A6) is upregulated in many malignancies. The overexpression of S100A6 in these malignancies has been associated with proliferation, migration, and invasion phenotypes in cancer cells, and we aimed to investigate the expression of S100A6 in CD34$^+$ cells and the relationship between S100A6 expression and apoptosis of CD34$^+$ cells in high-risk patients with MDS.

Methods: We measured S100A6 mRNA expression in bone marrow (BM) CD34$^+$ cells from high-risk patients with MDS using RT-PCR. Next, we examined S100A6 expression in CD34$^+$ cells using flow cytometry. We also analyzed the correlation between CD34$^+$ cell apoptosis and S100A6 expression in high-risk patients with MDS.

Results: Our data showed increased S100A6 mRNA expression in CD34$^+$ cells in patients with MDS (1.05 $\pm$ 0.69 vs. 0.17 $\pm$ 0.12; P < 0.01). The expression of S100A6 in BM CD34$^+$ cells also increased (58.40 $\pm$ 13.18 vs. 45.83 $\pm$ 15.01). The expression of S100A6 in CD34$^+$ cells and apoptosis of CD34$^+$ cells were negatively correlated in patients ($r = -0.75$; $P < 0.01$).

Conclusions: Collectively, S100A6 may be a potential marker of CD34$^+$ cells in high-risk patients with MDS and may participate in the pathological behaviors of CD34$^+$ cells, such as evasion of apoptosis. Thus, S100A6 may be a potential target for eliminating minimal residual disease.

1. Introduction

Myelodysplastic syndrome (MDS) is a group of clonal diseases of hematopoietic stem cells characterized by multilineage dysplasia of immature myeloid cells, ineffective hematopoiesis, peripheral blood cytopenia, and a high risk of transformation to acute myeloid leukemia (AML) [1]. In recent years, azacitidine, decitabine, and hematopoietic stem cell (HSC) transplantation have achieved a certain level of efficacy in the treatment of MDS and have alleviated some patients' symptoms. Despite these advancements, patients with MDS still have a high mortality rate due to the heterogeneity of the disease and lack of specificity in treatment, which seriously affects the prognosis and survival rates of patients. To improve treatment outcomes in patients with MDS, it is vital to conduct an

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https://doi.org/10.1016/j.heliyon.2023.e18947
Received 8 November 2022; Received in revised form 28 July 2023; Accepted 3 August 2023
Available online 5 August 2023

in-depth exploration of the etiology, pathogenesis, and prognosis of MDS. Studies have shown that increased apoptosis of bone marrow cells is one of the primary mechanisms leading to ineffective hematopoietic function in patients with MDS. MDS is a multistep process that originates from repeated genetic or cytogenetic changes leading to clonal expansion of aberrant mutant HSCs [2]. Abnormal HSCs play an essential role in the occurrence and progression of the disease. Owing to their significance in MDS progression, the exploration of these abnormal hematopoietic cells has become the focus of recent clinical and scientific research.

S100A6 is a member of the S100 protein family, which exerts a variety of biological functions through the interaction with calcium ions and target proteins. The protein was found in stem cells and localized mainly in the cytoplasm. As the concentration of Ca^{2+} increases, this protein binds to both the plasma membrane and nuclear membranes [3]. Previous studies have shown that S100A6 regulates various cellular and molecular functions, such as proliferation, apoptosis, cell cycle, differentiation, and participation in cytoskeletal components [4]. A recent study showed that S100A6 is a key regulator of HSCs. In this study, S100A6 was shown to induce resistance to apoptosis in mice [5]. Although the role of S100A6 in many malignancies has been investigated in many malignancies, little research has been conducted on MDS. Furthermore, there are extensive differences in the biological behaviors and clinical prognoses of patients with high- and low-risk MDS. In this study, we detected the expression of S100A6 in bone marrow $CD34^+$ cells derived from high-risk patients, as well as analyzed the relationship between the expression of S100A6 and other clinical indicators. Our objective was to explore whether S100A6 could serve as a potential marker for high-risk MDS. To detect the apoptosis of bone marrow $CD34^+$ cells among patients with MDS, we further analyzed the relationship between the apoptosis of $CD34^+$ cells and the expression of S100A6 to provide a new research direction for the evaluation of MDS pathogenesis and prognosis.

2. Materials and methods

2.1. Patients

Thirty-seven patients diagnosed with high-risk MDS, including 25 men and 12 women with a median age of 68 years (range, 40–84 years), were admitted to the Hematology Department of Tianjin Medical University General Hospital from October 2020 to October 2022. MDS was diagnosed according to the ESMO Clinical Practice guidelines for diagnosis, treatment, and follow-up [6]. Patients assessed as high- or very high-risk using the IPSS-R were included in the high-risk MDS patient group. Patients with leukemia aggravated from MDS were also included in our study. Studies have shown that the S100A6 expression in patients with multiple myeloma is abnormal. Therefore, patients with MDS and multiple myeloma were excluded from our study. The normal controls included 7 men and 25 women with a median age of 51 years (range, 18–80 years). Follow-up data were obtained by consulting cases and telephone follow-up. This study was approved by the ethics committee of the Tianjin Medical University General Hospital, and informed consent was obtained from all patients prior to tissue collection.

2.2. Isolation of $CD34^+$ cells from bone marrow

We collected 10 mL bone marrow samples from the patients and normal controls and diluted them with PBS. BM mononuclear cells were separated using the density gradient method with a lymphocyte separation solution (relative density, 1.077). The volume was fixed to 1 mL and 10 μL was taken for cell counting. According to the counting results, 50 μL of CD34 cells-labeled antibody and 50 μL of FCR blocker (Mitenyi, Germany) were added to each of the 1×10^7 cells, mixed thoroughly, and incubated at 4 °C for 40 min. The total capacity system was adjusted to 500 μL in PBS and then separated by column. The purity of the sorted cells was >95% (Fig. 1).

2.3. Real-time polymerase chain reaction

For $CD34^+$ cells S100A6 mRNA expression analysis, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). $CD34^+$ cells from patients with high-risk MDS and controls were lysed using TRIzol reagent, and the extracted RNA was reverse-transcribed using the complementary (c)DNA Synthesis Kit (Tianshai) according to the manufacturer's protocol. Real-time PCR was

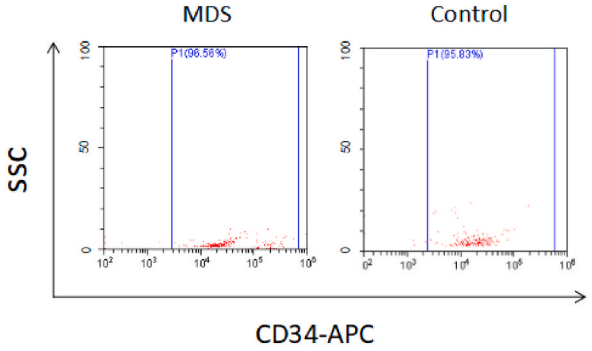

Fig. 1. Detection of $CD34^+$ cells purity by flow cytometry.

performed using the BIORAD iQ5 system (Bio-Rad Laboratories, Inc.). GAPDH was used as the housekeeping gene to standardize targeted mRNA expression. The relative mRNA expression of S100A6 gene in CD34 positive cells was calculated using the $2^{-\triangle\triangle Ct}$ method. The primers used to amplify GAPDH and S100A6 are listed in Table 1.

2.4. Flow cytometry

Anticoagulant tubes containing heparin sodium were used to collect 2 mL of bone marrow blood from the patients with MDS and controls. Bone marrow samples were filtered through 200 mesh filters. Specific CD34-APC (BD Company, USA) was added to the flow tube at 5 μL each, and then 50 μL of the bone marrow samples were added to the corresponding flow tube. Following good mixing, they were incubated at 4 °C in the dark for 15 min. Membrane breaker (BD PharMingen, San Diego, CA, USA) was added after hemolyzed blood was washed. Coralite488-S100A6 antibody (Proteintech) were added to the tubes at 5 μL each. At the same time, 5 μL Coralite488-IgG antibodies were, respectively, added to the control tubes. Samples were put into the machine after washing.

2.5. The apoptosis of $CD34^+$ cells

After collecting and filtering samples as above, specific CD34-APC、FITC-AnnexinV (BD Company, USA) were added to the tubes at 5 μL each. Then, 50 μL of the samples and 100 μL of the buffer were added into each tube, mixed well, and incubated at 4 °C in the dark for 30 min. Add 5 μL PI and mixed well, incubated in the dark for 5 min, and placed into the machine within 1 h.

2.6. Statistical analysis

Data analyses were performed using the GraphPad Prism 8.0 software (GraphPad Software, Inc.). All data passed the normal distribution test, and the difference between the two groups was analyzed using Student's t-test. Spearman's correlation coefficients between the measured data were calculated. Statistical significance was set at $P < 0.05$.

3. Results

3.1. The expression of S100A6 in $CD34^+$ cells with high-risk MDS

Real-time PCR was used to detect the expression of S100A6 in $CD34^+$ cells of high-risk patients with MDS and controls. The $CD34^+$ cells S100A6 mRNA expression of high-risk patients with MDS was significantly higher than that of the control group (1.05 ± 0.69 vs. 0.17 ± 0.12; $P < 0.01$) (Fig. 2B). We then detected the expression of S100A6 in the $CD34^+$ cells of patients with MDS and normal controls by flow cytometry. We use membrane breaker to detect both membrane bound and cytoplasmic S100A6. The expression of S100A6 in $CD34^+$ cells in the MDS group was significantly higher than that of the control group (58.40 ± 13.18 vs. 45.83 ± 15.01; $P < 0.01$, Fig. 2A, C).

3.2. Correlations between S100A6 expression and clinical indicators

Spearman's correlation analysis was performed to investigate the correlation between S100A6 expression and clinical indicators in patients with high-risk MDS and healthy controls. The expression level of S100A6 in $CD34^+$ cells of patients with MDS was negatively associated with WBC count ($r = -0.46$; $P < 0.05$), whereas it was not significantly correlated with hemoglobin (HB), platelet (PLT), or the percentage of bone marrow primitive cells (Fig. 3). Among the high-risk patients with MDS in our study, four patients had complex karyotypes and five patients progressed to leukemia during diagnosis and treatment; however, our analysis showed that there was no significant difference in S100A6 levels between high-risk patients with MDS with and without complex karyotypes, and we found that the expression levels of S100A6 in these patients who progressed to leukemia were not significantly different from those in patients without progression.

3.3. The apoptosis of $CD34^+$ cells in high-risk patients with MDS by flow cytometry

We detected apoptosis of $CD34^+$ cells in high-risk patients with MDS by flow cytometry, and the total apoptosis rate was $9.71 \pm 7.28\%$. Then, the Spearman correlation analysis was performed to investigate the correlation of S100A6 expression with the apoptosis of $CD34^+$ cells in these patients. The total apoptosis rate of $CD34^+$ cells in high-risk patients with MDS was negatively correlated with

Table 1
Primers for real-time quantitative PCR detection.

Primers	Sequence (5′-3′)
S100A6	Forward:GAAGGAGCTCACCATTGGCT
	Reverse:CACCTCCTGGTCCTTGTTCC
GAPDH	Forward:GGAGCGAGATCCCTCCAAAAT
	Reverse:GGCTGTTGTCATACTTCTCAGG

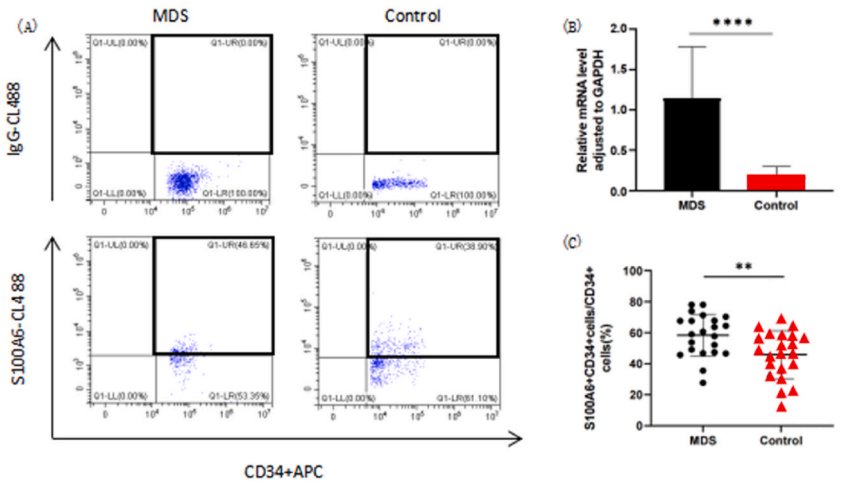

Fig. 2. (A) (C)The expression of S100A6 in CD34$^+$ cells membrane and cytoplasm in MDS patients and normal controls (B) The mRNA levels of S100A6 in CD34$^+$ cells were examined by RT qPCR assay.

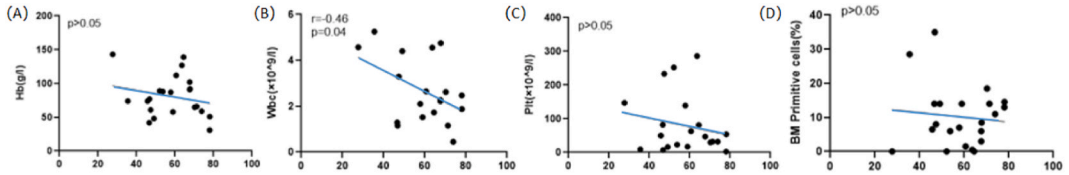

Fig. 3. (A) (B) (C) (D)Correlations analysis of the expression of S100A6 inCD34+ cells membrane and cytoplasm and clinical indicators in high risk MDS patients.

the expression of S100A6 in CD34$^+$ cells ($r = -0.75$; $P < 0.01$, Fig. 4).

4. Discussion

According to previous research exploring the pathogenesis of MDS, it is widely accepted that MDS is a heterogeneous group of clonal myeloid neoplasms where malignant clonal cells originate from HSCs/progenitor cells. Because cancer stem cells are the seeds, we investigated the biology of the MDS HSCs, which drive disease evolution, in an attempt to discover potential therapeutic targets. MDS has two distinct characteristics: excessive apoptosis in early MDS, and excessive resistance to apoptosis in late MDS [7]. This second characteristic, resistance to apoptosis, is the critical factor in the proliferation and progression of malignant tumors in MDS, as well as in leukemia [8].

Studies have shown that the expression of S100A6 is related to the proliferation and invasion of solid tumors. In solid tumors, S100A6 overexpression can promote the proliferation and invasion of non-small cell lung cancer [9], proliferation and migration of cervical cancer cells, and progression of nasopharyngeal carcinoma [10]. S100A6 is also involved in the proliferation of ICC cells and is associated with aggressive ICC behavior [11]. In hematological diseases, S100A6 expression is associated with the occurrence, progression and extramedullary metastasis of multiple myeloma [12]. Despite the prevalence of S100A6 in the occurrence of other similar diseases, it is rarely investigated in MDS. A recent study showed that S100A6 is a critical regulator of HSCs. This study found that

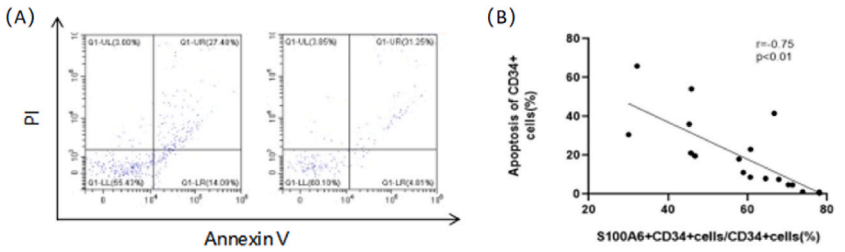

Fig. 4. (A)apoptosis of CD34$^+$ cells membrane and cytoplasm assessed using flow cytometry
(B)Correlations analysis of the expression of S100A6 and apoptosis of CD34$^+$ cells in MDS patients.

S100A6 had antiapoptotic effect of S100A6 in mice HSCs, in which the loss of S100A6 disrupted the HSPC compartment, significantly reducing the number of long-term (LT)-HSC and multipotent progenitor cells, and increasing the number of apoptotic cells in the LT-HSC compartment. It has also been found that S100A6 is upregulated in patients with leukemia with a poor prognosis, and exerts antiapoptotic effects in mixed-lineage leukemia/AF4-positive leukemia cells [5]. To bridge this gap in MDS research, we aimed to explore the occurrence and progression of high-risk MDS and identify potential therapeutic targets.

Throughout our experiments, we detected the expression of S100A6 in the $CD34^+$ cells of both the high-risk MDS patient and control groups using real-time PCR and flow cytometry, respectively, and found that the expression of S100A6 in the MDS group was significantly higher than that in the control group, which was consistent with previous studies on the expression of S100A6 in solid tumors and multiple myeloma [12].

First, our study provides evidence for the upregulation of S100A6 expression in high-risk patients with MDS, suggesting that S100A6 is a potential marker of $CD34^+$ cells in these patients. All the high-risk patients with MDS included in this study have received treatment. The increased expression of S100A6 in $CD34^+$ cells also suggested that S100A6 may be a potential target for eliminating minimal residual disease. Next, we analyzed the correlation between the expression of S100A6 in $CD34^+$ cells and other established clinical indicators of high-risk patients with MDS and found that S100A6 expression was negatively correlated with the absolute WBC count. Furthermore, there was no significant correlation the expression of S100A6 and the absolute counts of HB, PLT, or the percentage of primitive bone marrow cells. In addition, we compared the expression of S100A6 in the $CD34^+$ cells of high-risk patients with MDS with complex karyotypes and the progression from MDS to leukemia in the absence of these factors. Our results showed no significant differences between the groups. Possible explanations for this lack of observed differences may be small sample size, individual heterogeneity, or various treatment recovery times.

A growing body of evidence suggests that programmed cell death (PCD), including apoptosis, necrosis, and pyroptosis, plays important roles in HSC homeostasis [13]. Some scholars have investigated the apoptosis rate (AR) of $CD34^+$ and $CD34^-$ cells in the bone marrow of patients with MDS as compared to a normal control group. The results of this study suggested that the AR of the MDS group was significantly higher than that of the control (11.3 ± 1.5 vs. 6.8 ± 0.7; $P = 0.01$). They also found that the apoptosis/proliferation ratio of $CD34^+$ and $CD34^-$ cells in the low-risk MDS group was significantly higher than that in the high-risk and control groups [14], which may indicate that the malignant clonal cells display resistance to apoptosis, especially in the high-risk MDS group. We detected the apoptosis of $CD34^+$ cells in high-risk patients with MDS via flow cytometry and used these results to analyze the correlation between the expression of S100A6 and the apoptosis of $CD34^+$cells. Previous evidence supports the notion that $CD34^+$ cells undergo progression from excessive apoptosis to insufficient apoptosis in the evolution of low-to high-risk MDS to leukemia, indicating that there is a significant relationship between apoptosis and the evolution of MDS to leukemia. Our experiments further found that the expression of S100A6 in $CD34^+$ cells and apoptosis of $CD34^+$ cells were negatively correlated in high-risk patients with MDS. This suggests that S100A6 expression in high-risk patients has a certain correlated with apoptosis resistance in HSCs.

One key limitation of our study is that our research sample only included high-risk patients with MDS and did not include patients at various stages of MDS. Therefore, more studies will be necessary to explore the role of S100A6 in the pathogenesis, treatment, and prognosis of patients with MDS at various stages of the disease.

The ability of cancers to upregulate the Bcl-2 family of anti-apoptotic proteins to evade apoptosis is a key mechanism underlying treatment resistance and relapse in acute leukemia and MDS. Venetoclax, a targeted Bcl-2 inhibitor, is the primary BH3 mimetic used in contemporary clinical investigations and has been shown to have impressive efficacy in the treatment of several hematologic malignancies. However, the antileukemic activity of BH3 mimetics is limited by drug resistance mechanisms that are gradually being characterized in both *in vitro* and *in vivo* preclinical models [15]. Our results showed that the expression level of S100A6 was negatively correlated with the apoptosis of $CD34^+$ cells in high-risk MDS, which should provide researchers with a new perspective to investigate whether S100A6 is involved in the apoptosis evasion process of $CD34^+$cells in patients with MDS.

In conclusion, our experiments showed that S100A6 expression was upregulated in $CD34^+$ cells of high-risk patients with MDS. We also found that the expression level of S100A6 was negatively correlated with apoptosis of $CD34^+$ cells in high-risk patients with MDS. These results are the first to suggest that S100A6 may serve as a potential marker of $CD34^+$ cells or play a role in the pathological behavior of $CD34^+$ cells, such as apoptosis evasion, in high-risk patients with MDS.

Author contribution statement

Yan Zhai: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. FanQiao Meng; JiaoJiao Li; JunLan Ma; Li Shen: Contributed reagents, materials, analysis tools or data. Wei Zhang: Conceived and designed the experiments.

Funding statement

This study was supported by China Anti Cancer Association.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to thank Wei Zhang, for reviewing the manuscript and for herhelpful suggestions. This study was supported by CHINA ANTI CANCER ASSOCIATION.

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