

Over-expression of RPL23 in myelodysplastic syndromes is associated with apoptosis resistance of CD34⁺ cells and predicts poor prognosis and distinct response to CHG chemotherapy or decitabine

Lingyun Wu · Xiao Li · Feng Xu · Chunkang Chang · Qi He · Zheng Zhang · Yan Zhang

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Abstract Ribosomal protein (RP) L23 has been suggested to be a negative regulator of cell apoptosis. In the present study, we analyzed RPL23 expression in 169 patients with myelodysplastic syndrome (MDS) by using real-time PCR. The apoptosis of CD34⁺ marrow cells was examined by flow cytometry, and the correlation between RPL23 expression levels and apoptosis in CD34⁺ cells was assessed. We then analyzed the clinical significance of RPL23 expression for predicting disease progression and patient survival as well as therapeutic response in patients administered with a cytarabine, homoharringtonine, and G-CSF (CHG) regimen or decitabine therapy. Increased RPL23 expression was found in patients with higher-risk MDS than in patients with lower-risk disease ($p=0.004$). RPL23 expression levels were found being inversely correlated with decreased apoptotic ratio of CD34⁺ cells in higher-risk patients ($r=-0.672$, $p<0.001$). Compared to patients with normal RPL23 expression levels, those with increased RPL23 expression presented higher rates of transformation to acute myeloid leukemia ($p=0.005$) and reduced 2-year survival rates ($p=0.012$). Multivariate regression analysis showed that RPL23 expression level was an independent predictor of prognosis, regardless of patient age, IPSS score, or hemoglobin level. Moreover, patients with RPL23 over-expression appeared to have lower response rates to CHG chemotherapy ($p=0.027$) but similar response rates to decitabine treatment. In conclusion, the over-expression of RPL23 might confer apoptosis resistance in CD34⁺ cells, which may lead to disease progression and adverse prognosis

in MDS. Increased RPL23 expression was an inverse indicator for CHG regimen, but not for decitabine treatment.

Keywords Myelodysplastic syndrome · Ribosomal protein L23 · Apoptosis · Chemotherapy · Decitabine

Introduction

Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal hematopoietic stem cell disorders that are characterized by peripheral cytopenias, hypercellular bone marrow (BM), and increased mortality due to the evolution of acute myeloid leukemia (AML) [1]. In lower-risk MDS patients, the significantly increased rate of apoptosis among hematopoietic cells contributes to BM hypercellularity and peripheral cytopenia. By contrast, BM hematopoietic cells in patients with higher-risk MDS demonstrate resistance to apoptosis, and some of these patients subsequently develop AML [2, 3]. However, this apoptotic resistance observed in higher-risk MDS patients is poorly understood.

Ribosomal proteins (RPs) are ubiquitous RNA-binding proteins that are believed to perform multiple auxiliary extra-ribosomal functions and have been suggested to play a role in the occurrence of cancer [4]. RPL23 is a protein component of the 60 S large ribosomal subunit that interacts functionally with murine double minute 2 protein (MDM2 or HDM2 in humans) [5]. Upon binding to MDM2, RPL23 inhibits MDM2-mediated p53 degradation, thus activating p53 and inducing cellular apoptosis [5, 6]. Intriguingly, RPL23 has also been suggested to be a negative regulator of apoptosis by suppressing the Miz1-induced transcription of the cell cycle inhibitors p15^{INK4B} and p21^{CIP1} [7]. A microarray

L. Wu · X. Li (✉) · F. Xu · C. Chang · Q. He · Z. Zhang · Y. Zhang
Hematology Division,
Sixth Hospital Affiliated to Shanghai Jiaotong University,
Shanghai 200233, China
e-mail: lixiao3326@yahoo.com.cn

analysis conducted by Sridhar et al. [8] supported the anti-apoptotic characterization of RPL23 and demonstrated that a substantial number of RP genes, including RPL23, are over-expressed in the CD34⁺ marrow cells of higher-risk MDS patients (particularly those with transformed MDS). However, the two opposing roles of RPL23 in cellular apoptosis may be the result of differences in individual cells and disease stages.

In this retrospective study, we measured the mRNA expression levels of RPL23 in a large cohort of MDS patients with different disease stages and subtypes. Additionally, this was the first study to analyze the association between RPL23 expression and the rate of apoptosis among CD34⁺ marrow cells. The results indicated that RPL23 expression was predictive of disease progression and survival. Moreover, considering the significant role of RPL23 in apoptosis, we assessed whether the expression of RPL23 could predict the efficacy of MDS treatments, including a low-dose cytarabine and homoharringtonine priming with granulocyte colony-stimulating factor (G-CSF) (CHG) chemotherapy regimen, which exerts cancer-killing and proapoptotic effects) and decitabine administration (which acts mainly as a hypomethylating agent at low doses).

Patients and methods

Patients

All patients enrolled in this study had been diagnosed with MDS at our department according to the FAB/WHO classification criteria [9, 10] and the minimal diagnostic standards for MDS [11]. The BM samples were obtained at diagnosis of MDS before initiation of any treatment between 2006 and 2011 following approval by the local ethics committee and according to institutional guidelines. Each of the 169 MDS patients and 25 normal donors provided written informed consent. The characteristics of the MDS patients studied are listed in Table 1. As of December 2011, the median follow-up period for these subjects was 24 months (range of 3–52 months).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from 2 ml of bone marrow mononuclear cells (BMNCs) using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. cDNA was synthesized by random priming from 10 µL of total RNA using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions. The PCR was performed in a final volume of 10 µL, including 1 µL of cDNA, 0.5 µM of each primer, 4 mM MgCl₂, and 2 µL of the supplied enzyme mix containing the reaction buffer, FastStart Taq DNA polymerase, and DNA double-strand specific SYBR Green I dye for PCR product detection. The PCR was performed in a fluorescent quantitation PCR cycler

Table 1 Characteristics of the 169 MDS patients

Characteristics	Category
Median age (range)	56 (17–86)
Sex, <i>n</i> (%)	
Male	103 (61)
Female	66 (39)
WHO/FAB classification, <i>n</i> (%)	
RA	8 (5)
RAS	6 (4)
RCMD	102 (60)
RCMD-RS	6 (4)
RAEB-1	21 (12)
RAEB-2	13 (8)
RAEB-t	6 (4)
5q- syndrome	3 (1)
CMML	4 (2)
International prognostic scoring system, <i>n</i> (%)	
Low	14 (8)
Int-1	117 (69)
Int-2	22 (13)
High	16 (10)

(LightCycler, Roche, Switzerland). The primer sequences for RPL23 were as follows: 5'-TGGTGATGGCCACAGTCAA G-3' (sense) and 5'-GCTACTGGTCCTGTAATGGCAGA-3' (antisense). The primer sequences for GAPDH were as follows: 5'-GCACCGTCAAGGCTGAGAAC-3' (sense) and 5'-GTGGTGAAGACGCCAGTGGGA-3' (antisense). The PCR conditions were as follows: a 30-s preincubation at 95°C and then 40 cycles of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C. The threshold cycle (Ct) value was subsequently determined, and the relative quantification of mRNA expression was calculated using the comparative Ct method. The relative quantification value of the target, which was normalized to that of an endogenous control (GAPDH gene) and relative to that of a calibrator (the mean expression level of normal controls), was expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where $\Delta Ct = Ct$ of the target gene - Ct of the endogenous control gene (GAPDH), and $\Delta\Delta Ct = \Delta Ct$ of the samples for the target gene - ΔCt of the calibrator for the target gene.

Flow cytometry analysis of apoptosis in CD34⁺ marrow cells in MDS patients

For the flow cytometry analysis, we used the following fluorescent-labeled monoclonal antibodies CD45-PerCP, CD34-APC, and Annexin V-FITC (BD Biosciences, USA), in addition to the corresponding isotype controls. All samples were subjected to flow cytometry analysis within 12 h of collection. A flow cytometer (FACS Calibur, Becton Dickinson, USA) equipped with CellQuest software was used for

logarithmic (Log) sampling, and at least 10^5 cells were acquired and analyzed for each sample. The BMNCs were stained with anti-CD34 conjugated to APC and anti-CD45 conjugated to PerCP at room temperature for 20 min and then stained with 5 μ L of Annexin V conjugated to FITC in combination with 2 μ L of PI for 15 min. The cells were then washed once and analyzed within 2 h of staining.

CHG regimen or decitabine treatment

Twenty-nine patients with higher-risk MDS accepted treatment with a priming CHG regimen. The CHG chemotherapy regimen consisted of an intravenous continuous infusion of low-dose cytarabine (25 mg/d) (Pharmacia, Italy) and homoharringtonine (1 mg/d) (Minsheng Pharma, Hangzhou, China) in combination with G-CSF (300 μ g/d) (Kirinkunpeng, Japan), which was administered by subcutaneous injection from day 0 until peripheral neutrophil counts had recovered to 2.0×10^9 /L. G-CSF was administered intermittently when the peripheral white blood cell count increased beyond 20×10^9 /L. Thirty-seven patients with higher-risk MDS were treated with decitabine (Johnson & Johnson Inc, USA), which was administered at a dose of 20 mg/m² by continuous intravenous infusion for 1 h and repeated daily for 5 days. The cycle was repeated every 4 weeks, depending on each patient's recovery from myelosuppression. BM aspirates were performed 4 weeks after the CHG regimen or decitabine treatment was completed. The treatment response was assessed after two cycles of the CHG regimen or decitabine therapy and was determined according to the standardized

response criteria for MDS [12]. Detailed information regarding these treated patients was shown in Table 2.

Statistical methods

Statistical analyses were performed using the Statistical Package for Social Sciences 10.0 (SPSS Inc., Chicago, IL, USA). Two independent sample populations were compared using Student's *t* test, and multiple sample populations were compared using one-way ANOVAs. The Pearson χ^2 or Fisher's exact test was applied to compare the enumeration data between groups. The Pearson correlation analysis was used to examine the correlations between two parameters, and the Kaplan–Meier method was used to estimate the median overall survival. The log-rank test was used to compare Kaplan–Meier survival estimates between groups, and the Cox regression analysis was used for the multivariable analysis. *p* values <0.05 were considered statistically significant.

Results

Patient characteristics

A total of 169 MDS patients, consisting of 103 males and 66 females, were involved in this study. Their median age was 56 years (range 17–86 years). These patients were classified as having MDS with isolated del(5q) (*n*=3), refractory anemia (RA) (*n*=8), RA with ringed sideroblasts (RAS) (*n*=6), refractory cytopenia with multilineage dysplasia (RCMD) (*n*=102), RCMD with ringed sideroblasts (RCMD-RS) (*n*=6),

Table 2 Baseline characteristics of patients treated with the CHG regimen or decitabine

Characteristics	CHG regimen (<i>n</i> =29)	Decitabine (<i>n</i> =37)	<i>p</i> value
Median age (range)	60 (21–81)	57 (20–81)	0.815
Sex ratio (male/female)	17:12	22:15	0.945
FAB/WHO classification, <i>n</i> (%)			
RAEB-1	12 (41)	18 (48)	0.894
RAEB-2	13 (45)	14 (38)	
RAEB-t	3 (11)	3 (8)	
CMML	1 (3)	2 (6)	
Karyotype risk group			
Good	14 (48)	18 (49)	0.942
Intermediate	7 (24)	10 (27)	
Poor	8 (28)	9 (24)	
IPSS score			
Int-1	11 (38)	22 (59)	0.221
Int-2	11 (38)	9 (24)	
High	7 (24)	6 (17)	
RPL23 expression, <i>n</i> (%)			
Over-expression	14 (48)	17 (46)	0.851
Normal expression	15 (52)	20 (54)	

refractory anemia with excess blasts-1 (RAEB-1) ($n=21$), RAEB-2 ($n=13$), RAEB in transformation (RAEB-t) ($n=6$), chronic myelomonocytic leukemia (CMML)-1 ($n=3$), and CMML-2 ($n=1$). One hundred thirty-one patients with international prognostic scoring system (IPSS) [13] scores ≤ 1.0 were included in the lower-risk MDS group (including 14 low-risk and 117 int-1 patients), whereas 38 MDS patients with IPSS scores >1.0 were included in the higher-risk MDS group (22 int-2 and 16 high-risk patients). Detailed information regarding these MDS patients is shown in Table 1. Twenty-nine patients received CHG chemotherapy regimens, and 37 patients received decitabine treatment (Table 2).

RPL23 expression levels were significantly elevated in higher-risk MDS patients

RPL23 mRNA expression levels, as defined by the $2^{-\Delta\Delta Ct}$ values (mean \pm SE), were evaluated for 169 MDS samples. The expression level of RPL23 ($2^{-\Delta Ct}$) in normal controls was 0.48 ± 0.08 . The RPL23 expression levels for patients with various clinical forms of MDS were as follows (in increasing order): CMML patients, 0.25 ± 0.06 ; 5q-/RA/RAS patients, 0.43 ± 0.08 ; RCMD/RCMD-RS patients, 0.65 ± 0.07 ; RAEB-1 patients, 1.21 ± 0.22 ; RAEB-2 patients, 1.47 ± 0.38 ; and RAEB-t patients, 2.57 ± 0.77 ($F=10.716$, $p<0.001$) (Fig. 1). Patients with higher-risk MDS (IPSS scores >1.0) demonstrated increased RPL23 expression levels compared to patients with lower-risk MDS (IPSS scores ≤ 1.0) (1.49 ± 0.27 vs. 0.83 ± 0.09 , $t=2.929$, $p=0.004$) (Fig. 2).

Over-expression of RPL23 was more common in higher-risk MDS patients

We used a cutoff value of 1.0, with samples having a $2^{-\Delta\Delta Ct}$ values greater than 1.0 considered positive for over-expression.

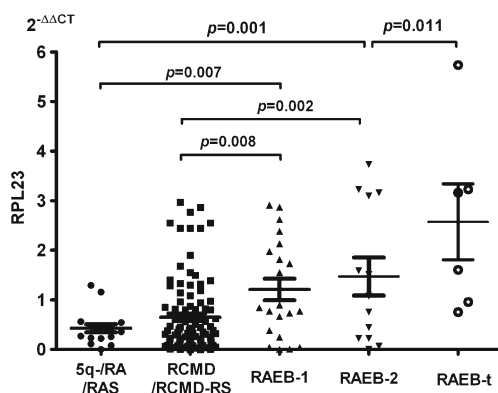


Fig. 1 RPL23 mRNA expression level in patients with MDS and MDS-AML. The RPL23 mRNA expression levels gradually increased from 0.43 ± 0.08 and 0.65 ± 0.07 in patients with 5q-/RA/RAS and RCMD/RCMD-RS, respectively, to 1.21 ± 0.22 , 1.47 ± 0.38 , and 2.57 ± 0.77 in patients with RAEB-1, RAEB-2, and RAEB-t, respectively ($F=10.716$, $p<0.001$)

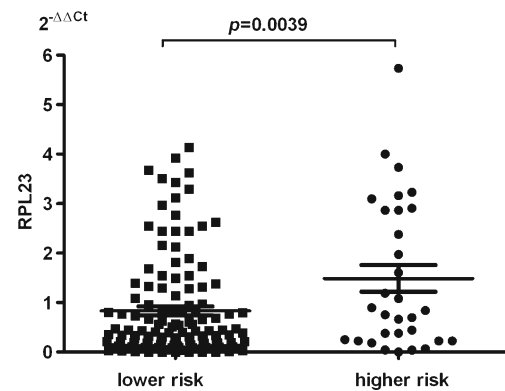


Fig. 2 RPL23 expression in patients with lower-risk and higher-risk MDS. Patients with higher-risk disease (IPSS scores >1.0) demonstrated higher RPL23 expression levels when compared to those with lower-risk MDS (IPSS scores ≤ 1.0) (1.49 ± 0.27 vs. 0.83 ± 0.09 , $t=2.929$, $p=0.004$)

This number for cutoff is in accordance to the Relative Quantification Software (Roche Molecular Diagnostics) Recommendations for the LightCycler. RPL23 over-expression was observed in 51 of the 169 patients (30.2 %). The percentage of RAEB-1/RAEB-2/RAEB-t patients with RPL23 over-expression (20/40, 50.0 %) was significantly higher than that of patients with 5q-/RA/RAS (2/17, 11.8 %) or RCMD/RCMD-RS (29/108, 26.9 %) ($p=0.007$ and 0.008 , respectively). Notably, none of the patients with CMML showed elevated RPL23 expression. Also, 47.4 % (18/38) of patients with higher-risk MDS demonstrated RPL23 over-expression, which was significantly greater than the frequency of over-expression among lower-risk patients (25.2 %, 33 of 131, $\chi^2=6.876$, $p=0.009$).

RPL23 expression levels were inversely correlated with the percentage of apoptotic CD34⁺ cells

Apoptotic CD34⁺ marrow cells were detected in 33 cases (Fig. 3a–e), and the percentage of apoptotic CD34⁺ cells was significantly greater among lower-risk MDS patients than higher-risk MDS patients (34.3 ± 3.1 vs. 11.8 ± 2.4 %, $p<0.001$) (Fig. 3f–h). Moreover, in MDS patients, RPL23 expression was significantly inversely correlated with the percentage of apoptotic CD34⁺ marrow cells ($r=-0.672$, $p<0.001$) (Fig. 4).

Over-expression of RPL23 predicted disease progression and poor survival

During a median follow-up period of 24 months (range 3–52 months), 37.1 % (13/35) of patients with RPL23 over-expression progressed to AML, compared to 14.7 % (14/95) of patients with normal RPL23 expression levels ($\chi^2=7.803$, $p=0.005$). Moreover, there was a significant difference in the 2-year survival rates between patients with RPL23 over-expression and patients with normal RPL23 expression levels (49.3 vs. 71.6 %, $p=0.012$) (Fig. 5). For

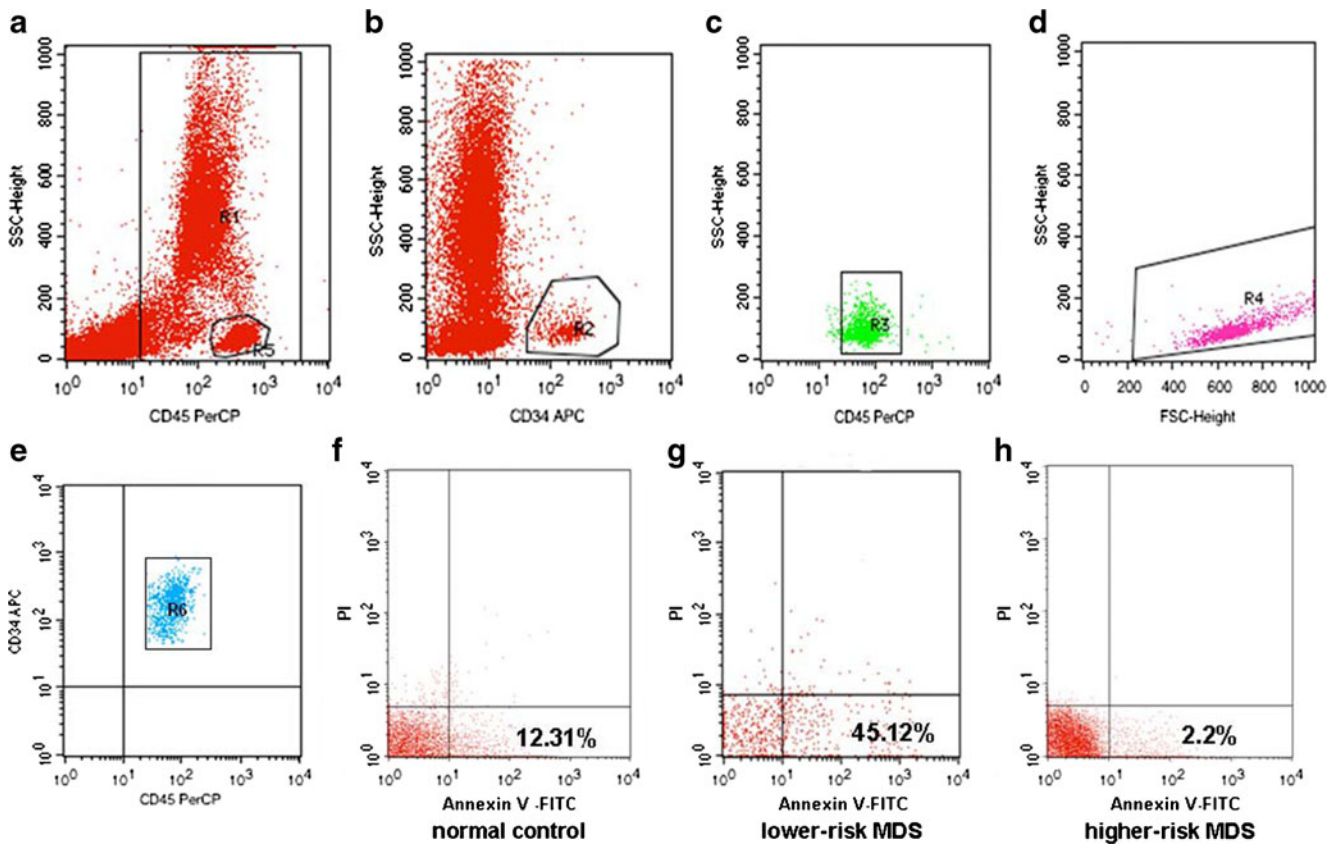


Fig. 3 Flow cytometric analysis of bone marrow CD34⁺ cells and apoptosis ratio in CD34⁺ cells for MDS patients and normal control. (a) On a CD45 vs. side-scatter plot, CD45- positive events (R1) were selected. (b) On a CD34 vs. side-scatter, region R2 represents CD34⁺ cells (gated by R1). (c) CD45 vs. side-scatter analysis of CD34⁺ cells gated by R1 and R2. (d) Region R4 represents a blast region (cells gated by R1, R2 and R3). (e) CD45 vs. CD34 analysis of CD34⁺ cells

(R6) gated by R1, R2, R3 and R4. (f–h) Apoptosis ratio in CD34⁺ cells (gated by R6) was determined on annexin V-FITC versus PI plots. The lower right quadrants in panel f–h represent the apoptotic cells, FITC-Annexin V positive and PI negative. Compared to the apoptotic ratio in CD34⁺ cells of normal controls (f), the percentage of apoptotic CD34⁺ cells increased in lower-risk MDS patients (g) and decreased in patients with higher-risk MDS (h)

patients with lower-risk MDS, the 2-year survival rate of patients with RPL23 over-expression was strikingly lower than that of patients with normal RPL23 expression levels (59.9 vs. 82.1 %, $p=0.005$) (Fig. 6a). Among higher-risk

patients, the median survival time of patients with RPL23 over-expression was also significantly shorter than that of patients with normal RPL23 expression levels (11 vs. 22 months, $p=0.018$) (Fig. 6b). Using multivariate regression

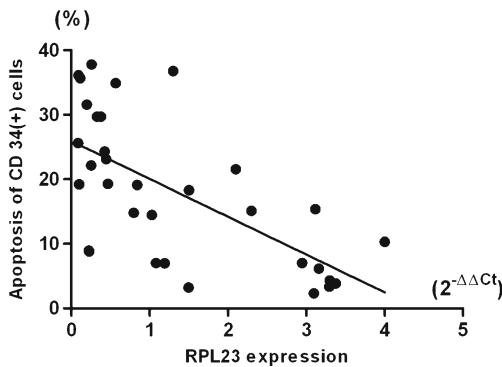


Fig. 4 Correlation between RPL23 expression and apoptosis of CD34⁺ cells in MDS patients. In MDS patients, RPL23 expression was significantly inversely correlated with the percentage of apoptotic CD34⁺ marrow cells ($r=-0.672$, $p<0.001$)

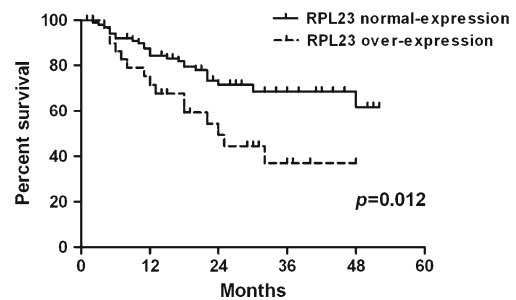


Fig. 5 Overall survival of MDS patients, grouped according to RPL23 expression level. Over a median follow-up period of 24 months (range 3–52 months), the 2-year survival rates of patients with RPL23 over-expression were significantly lower than those of patients with normal RPL23 expression levels (49.3 vs. 71.6 %, $p=0.012$)

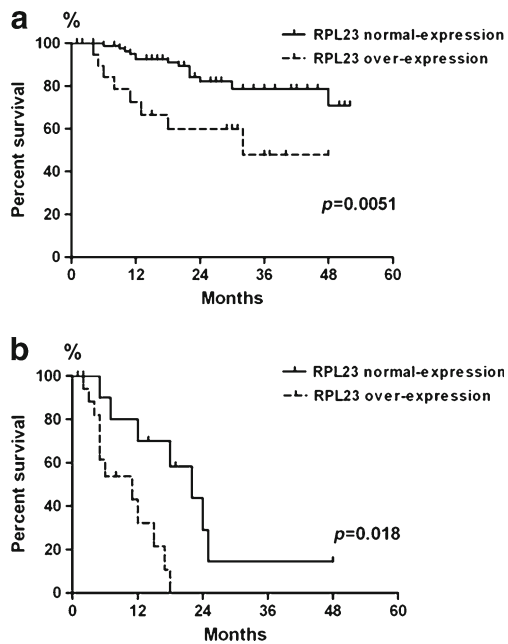


Fig. 6 Overall survival of lower-risk (a) and higher-risk MDS (b) patients, grouped according to RPL23 expression level. Among patients with lower-risk MDS, the 2-year survival rates of those with RPL23 over-expression were significantly lower than those of patients expressing normal levels of RPL23 (59.9 vs. 82.1 %, $p=0.005$) (a). Among higher-risk patients, the median survival time of those with RPL23 over-expression was significantly shorter than that of patients expressing normal levels of RPL23 (11 vs. 22 months, $p=0.018$) (b)

analysis, we showed that the RPL23 expression level served as an independent prognostic indicator, regardless of patient age, IPSS score, or hemoglobin level (Table 3).

Patients with elevated RPL23 expression levels achieved lower response rates to CHG regimen chemotherapy but not to decitabine treatment

The baseline characteristics (including age, gender, FAB/WHO classification, karyotype, IPSS score) of patients treated with the CHG regimen and decitabine were comparable ($p>0.05$) (see Table 2). Of the 29 higher-risk MDS patients who received the CHG chemotherapy regimen, 2 of

Table 3 Multivariate analysis by Cox Regression for survival

Criterion	β	p value	Exp (β)	95 % CI for β
Age	0.034	0.003	1.034	1.011–1.058
Hemoglobin	−0.023	0.020	0.977	0.958–0.996
IPSS score	0.943	0.000	2.568	1.759–3.750
RPL23 expression	1.340	0.000	3.819	1.834–7.953

Blasts in the BM, the karyotype, neutrophil cell count, platelet count, BM cellularity, and WHO classification were also included in the multivariate analysis and shown to be not independent predictive factors for survival in patients with MDS

14 (14.3 %) patients with RPL23 over-expression experienced complete remission (CR) or marrow CR (CR+mCR) compared to 9 of 15 (60.0 %) patients with normal levels of RPL23 expression ($p=0.027$). By contrast, for the 37 patients who received decitabine treatment (median number of courses was 3), there was no difference in the response rates (CR+mCR) between patients with RPL23 over-expression (6/17, 35.2 %) and those with normal expression (5/20, 25.0 %, $p>0.05$).

Discussion

This study was the first to show that RPL23 is overexpressed at the mRNA level in higher-risk MDS patients and that elevated RPL23 expression is inversely associated with apoptosis in CD34⁺ marrow cells. These results support the findings of a cDNA microarray analysis performed by Sridhar et al. [8], who found RPL23 to be associated with poor outcome and MDS transformation to AML. Our findings are also consistent with those of Wanzel et al. [7], who demonstrated that RPL23 is a negative regulator of apoptosis through its suppression of Miz1-induced transcription of the cell cycle inhibitors p15^{INK4B} and p21^{CIP1}. More importantly, we observed that increased RPL23 expression was associated with disease aggressiveness. Compared to patients with normal expression levels of RPL23, patients with increased RPL23 expression levels experienced much higher rates of transformation to AML ($p=0.005$) and therefore lower 2-year survival rates ($p=0.012$). Furthermore, over-expression of RPL23 correlated with reduced overall survival in both lower-risk and higher-risk MDS patients (as defined by IPSS score). Therefore, these results, in addition to those of the multivariate regression analysis, suggest that RPL23 expression can serve as an independent prognostic indicator, regardless of the age, IPSS score, and hemoglobin level of the patient. The relevance of this protein as an independent negative prognostic factor requires further evaluation and confirmation in a larger cohort of patients and needs to be evaluated prospectively.

In contrast to patients with de novo AML, patients with MDS or MDS-AML typically respond poorly to conventional induction chemotherapy [14, 15], which could be due to the existence of MDS blast cells with primary multidrug resistance (MDR) [16, 17]. It has been reported that RPL23 expression promotes MDR in gastric cancer cells by suppressing drug-induced apoptosis [18], and an enhanced anti-apoptotic phenotype among higher-risk MDS patients has been shown to increase CD34⁺ cell resistance to chemotherapy [19]. Our previous studies showed that the CHG regimen for low-dose chemotherapy was partially effective at treating patients with higher-risk MDS [20, 21], and in the current study, we found that RPL23 over-expression was an inverse indicator of the treatment response to the CHG regimen among higher-risk MDS patients. Patients with

elevated levels of RPL23 expression achieved significantly lower response (CR+mCR) rates when compared to those expressing normal levels of RPL23. As the combination of low-dose homoharringtonine and cytarabine has been shown to synergistically induce the apoptosis of blast cells [22], it is conceivable that elevated RPL23 expression (conferring potential apoptosis resistance, as described above) could predict a poor treatment response to CHG chemotherapy. The hypomethylating agent decitabine has demonstrated clinical efficacy in patients with higher-risk MDS [23]. We found that no differences in decitabine treatment response rates between patients with elevated RPL23 expression levels and those with normal RPL23 expression levels. This similar response rate may have been due to the mechanism of action of low-dose decitabine therapy; this treatment is thought to induce re-expression of tumor-suppressive genes via hypomethylation rather than by inducing apoptosis of blast cells [24]. Thus, it is possible that a hypomethylation agent would be more suitable than proapoptotic therapy for the treatment of MDS patients with increased levels of RPL23 expression.

In conclusion, RPL23 over-expression in patients with MDS is associated with abnormal blast resistance to apoptosis, which likely leads to disease progression and a poor prognosis. It is therefore conceivable that RPL23 over-expression could serve as an inverse indication for certain types of chemotherapy, such as the CHG regimen (but not decitabine therapy), in patients with higher-risk MDS.

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