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Retinoblastoma protein interacting zinc finger 1 gene in hematological malignancies

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Background
Several lines of evidence have shown that inactivation of tumor suppressor genes is closely associated with tumorigenesis in a wide variety of hematological malignancies. The role of downexpression of the RIZ1 gene in hematological malignancy has been reported in many studies, but its levels after receiving induction therapy in these patients have not been evaluated, which was the aim of this study.

Objective
The current study aimed to find the relationship between expression of RIZ1 in cases of hematological malignancies before the start of induction therapy and at the end of induction therapy and its possible role as a risk factor in disease progression.

Patients and methods
The study included two groups of patients: group I included 26 newly diagnosed patients with hematological malignancies before the start of induction therapy (day 0) and group II included 12 patients at the end of induction therapy (day 28). Group III included 10 individuals with another indication for bone marrow (BM) (such as peripheral blood unexplained cytopenia) who were age and sex matched with the patients and were enrolled in the study as a control group. The following investigations were performed for all the participants: complete blood count, BM sample for morphological examination, immunophenotyping, and cytogenetic analysis. The levels of RIZ1 gene expression were detected by real-time PCR (RT-qPCR) at diagnosis (day 0) and after induction therapy (day 28).

Results
The mean levels of RIZ1 gene expression in day 0 patients were significantly lower than those of the control group, and at the end of induction therapy (on day 28), the level was significantly increased to reach close to that in the healthy controls. Furthermore, the levels of the RIZ1 gene were negatively correlated with the blast cell counts in peripheral blood and BM.

Conclusion
We conclude that the RIZ1 gene is downexpressed in leukemic patients and its levels increased after induction therapy, indicating its possible role in disease pathogenesis. The relationship between its level and risk factors suggests its role in disease progression. A large-scale study is recommended for the use of the RIZ1 gene in the therapy of hematologic malignancies.

Keywords:
hematological malignancy, real-time PCR, RIZ1 1 gene

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Introduction

Hematopoietic homeostasis is maintained throughout the lifetime of an individual through self-renewal of hematopoietic stem cells. Defects in self-renewal and differentiation lead to hematopoietic insufficiency and development of malignancies. Leukemic stem cells, which are considered to originate from hematopoietic stem or progenitor cells, not only adopt the regulatory machinery operating in normal haematopoietic stem cell but establish their own mechanisms against apoptosis and senescence. Hematopoietic malignancies are of lymphoid origin such as chronic lymphocytic leukemia and actue lymphocytic leukemia (ALL) and myeloid malignancies such as acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML); in addition, there are disease of the plasma cells such as multiple myelomas [1].

Several lines of evidence have shown that inactivation of tumor suppressor genes is closely associated with tumorigenesis in a wide variety of human tumors [2].

The retinoblastoma protein-interacting zinc finger gene (RIZ) was isolated in a functional screening for Rb-binding proteins. RIZ is also known as a positive regulatory domain methyl transferase (PRDM2) [3].

The RIZ gene maps to the short arm of chromosome 1p36; the gene encodes two products: RIZ1, which contains the PR domain, and RIZ2, which lacks the domain. RIZ1 but not RIZ2 has tumor-suppressive properties. Thus, it is considered a tumor suppressor gene [4].

RIZ1 methylates histone H3 lysine 9, a modification that is important for transcriptional repression, resulting

in the modulation of chromatin structure and inhibition of the transcriptional process [5] as it arrests cell cycle at the G2/M phase and induces apoptosis [6,7].

Deletion or loss of heterozygosity of the RIZ1 gene has been reported in various human cancers, including both hematological and malignant lymphoma, and blastic crisis of chronic myelogenous leukemia and nonhematological malignancies such as breast [7], liver [8], and esophageal carcinoma [5].

A decrease in the expression of RIZ1 has been noted because of inactivation of the RIZ1 gene by methylation of CpG islands in gene promoter regions, which has been reported to be a major molecular mechanism of gene silencing and underlies both cancer development and progression [9].

Silencing or downregulation of a gene is best identified by real-time quantitative PCR (RT-qPCR), which has become a routine and robust approach for measurement of the expression of genes of interest [10].

The role of downexpression of the RIZ1 gene in hematological malignancy has been reported in many studies, but its levels after receiving induction therapy in these patients were not evaluated. Therefore, the present study was designed to determine the relationship between expression of RIZ1 in cases of hematological malignancies before the start of induction therapy and at the end of induction therapy and to establish its possible role as a risk factor in disease progression.

Patients and methods

The current study included 31 patients with diagnosed hematological malignancies who were attending the hematology oncology clinics of Menoufia University Hospital and the National Cancer Institute Cairo University. They were divided into two groups: group I included 26 newly diagnosed cases (15 men and 11 women) of hematological malignancies before the start of induction therapy (day 0) and group II included 12 patients (eight men and four women) at the end of induction therapy (day 28).

Group III included 10 individuals with another indication for bone marrow (BM) (seven men and three women) with normal aspirate morphological findings, and were enrolled in the study as a control group; they were age and sex matched with the patients.

Sampling

Peripheral blood and BM samples were obtained from the patients (group I) at day 0 before the start of induction therapy and from the control group (group III); in addition, peripheral blood samples from patients were taken at day 28 of induction therapy (group II) under complete aseptic conditions.

The study was approved by the Ethical Committee of Menoufia Faculty of Medicine, and a written consent was obtained from the patients included in the study.

Patients and Methods

EDTA samples for a complete blood count by Advia 120 and Leishman-stained peripheral blood and BM smears were examined morphologically to count blast cells and for FAB classification.

EDTA BM samples were used for immunophenotyping by a Becton Dickinson (BD) (Becton, Dickinson and Company1 Becton DriveFranklin Lakes, New Jersey, USA) flowcytometer after staining by fluorescent monoclonal antibodies according to the indicated panels. Cytogenetic analysis and screening for the presence of the most frequent fusion transcripts (BCR-ABL, PML-RARA, and FLT3 mutation) were carried out from BM heparinized samples that were kindly received from their department.

RIZ1 detection by RT-PCR

Fresh BM mononuclear cells were obtained using the Ficoll hypaque separation method and tested for expression of the RIZ1 gene and the GAPDH gene as an internal control gene quantitatively by RT-qPCR.

(1) DNA extraction: Total RNA was extracted using the Gene JET RNA Purification Kit provided by FERMENTSE (Keeptowen, South Africa), which utilizes a silica-based membrane technology in the form of a convenient spin column following the manufacturer's protocol. The purified RNA was used for reverse transcription immediately or stored at -70°C until use.
(2) Reverse transcription was carried out using the RevertAid First Standard cDNA Synthesis Kit (Promega Corporation, Madison, Wisconsin, USA). Briefly, for the preparation of first-strand cDNA, 1 μl of random hexamer primer and 6 μl of nuclease-free water were added to 5 μl of template RNA in a sterile, nuclease-free tube placed on ice, followed by incubation at 65°C for 5 min and addition of 4 μl of reaction buffer, 1 μl of Ribolock RNase inhibitor, 1 μl of dNTP mix, and 1 μl of M-MULV RevertAid (Moloney murine leukemia virus reverse transcriptase). The contents of the tube were mixed gently and centrifuged, and then tubes were placed in a thermal cycler for 5 min at

25°C, followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min.

(3) Quantitative real-time monitoring of PCR product: Master mix was prepared for both the RIZ1 gene (gene of interest) and the GAPDH gene (internal control gene) separately in the following order: 1 μl of forward primer, 1 μl of reverse primer, 0.25 μl of ROX, 5 μl of cDNA, 5 μl of Sybr green (Applied Biosystems Inc., Foster City, California, USA), and 7.75 μl of DNase-free water to reach a total volume of 20 μl. Thermal cycling for 45 cycles was repeated as follows: 50°C for 2 min, 94°C for 12 min, 94°C for 15 s, and 60°C for 1 min.
The sequence of primers for the RIZ1 gene was as follows: forward: 5′-TGGCTGCGATATGTGAATTG-3′ and reverse: 5′-CTTTCCGGCTCTTGGGGG-3′ and primers for the GAPDH gene were forward: 5′-TGCACCACCAACTGCTTAGC-3′ and reverse: 5′-GGCATGGACTGTGGTCATGAG-3′.
The 7300 Real-Time PCR System was used (atitude® Ltd., Sickingenstr. 26 10553 Berlin, Germany).

(4) The calculation of PCR results: Analysis of quantitative real-time reverse transcription PCR (RT-PCR) data was carried out as relative quantization levels using the $2^{-\Delta\Delta CT}$ method. This method relies on setting the crossing threshold (C_T), which is the number of cycles that it takes each reaction to reach an arbitrary amount of fluorescence. It is used to determine RIZ1 and GAPDH genes.

For each sample, the difference in the C_T values of the gene of interest and the endogenous control was calculated (the ΔC_T); then, the following equation was used: $\Delta\Delta C_T$ = ΔC_T of the patient–ΔC_T of the control. The negative value of this subtraction, the $-\Delta\Delta C_T$, was used as the exponent of 2 in the equation and then converted into a percent value to be expressed as a relative quantity [10].

Statistical analysis

Data were analyzed statistically using statistical package for social science program, version 13 (SPSS Inc., Chicago, IL) for windows for all analyses. Qualitative data were expressed as frequency and percent and quantitative data were expressed as mean ± SD. The Student *t*-test was used for comparison of two groups with quantitative normally distributed data and the Mann–Whitney test was used for abnormally distributed data. The one-way analysis of variance test was used for comparison of three or more groups with quantitative normally distributed data. The χ^2-test was used to compare the qualitative variables. The Spearman correlation coefficient was used to determine the correlation between variables. The *P* value was considered statistically significant when it was less than 0.05.

Results

The results of this study are presented in Tables 1–6 and Figs 1–4.

Day 0 before induction therapy

The morphological FAB and immunophenotyping classification was as follows*:* 10 common B ALL patients and 12 AML patients (one case M1, one case M1 with aberrant CD 2, 7, 56, two cases M2, one case M2 with aberrant CD19 expression, two cases M3, one case M3 variant, three cases M4, and one case of M5). One case had Bilineage acute leukemia, two cases had CML in blastic crises and one case had Burkitt lymphoma.

The cytogenetic findings were as follows*:* translocation (9 : 22) was positive in three cases (two cases of CML in blastic crises, one case of common B ALL), negative in nine cases of common B ALL, translocation (15 : 17) was positive in three M3 AML cases, and translocation (8 : 21) was positive in one AML case and negative in four AML cases. The *FLT3* mutation was negative in eight AML cases and no cytogenetic data were available in three cases (data not reported in the table).

The clinical data showed that 11 patients had an enlarged liver, 15 patients had a normal-sized liver, 13 patients had an enlarged spleen, and 13 patients had a normal-sized spleen, 16 patients

Table 1 Comparison between group I (day 0) and controls (group III) of clinical data

The variable	The studied groups [*n* (%)]		Fisher's exacts	*P* value	Significance
	Day 0 cases (*N* = 26)	Controls (*N* = 10)			
Hepatomegaly	11 (42.3)	6 (60.0)	0.91	0.46	NS
Splenomegaly	13 (50.0)	6 (60.0)	0.29	0.72	NS
Fever	16 (61.5)	2 (20.0)	4.98[a]	0.03	S
Lymphadenopathy	9 (34.6)	0 (0.0)	4.61	0.04	S

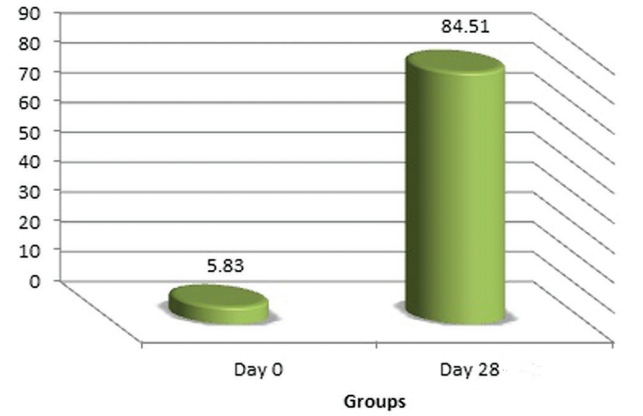
NS, nonsignificant; S, significant; [a]χ^2; Significant difference was considered when $P < 0.05$.

Table 2 Comparison between group I (day 0) and group III (controls) of CBC results

The variable	The studied groups		Mann–Whitney *U*	*P* value	Significance
	Day 0 cases (*N* = 26)	Controls (*N* = 10)			
Hb (g/dl)					
X ± SD	6.95 ± 1.68	10.48 ± 1.66	3.99	<0.001	HS
Median	7.07	10.66			
Range	3–9.8	7.2–13			
Platelets (10^3/μl)					
X ± SD	77.42 ± 58.08	115.30 ± 53.70	1.68	0.09	NS
Median	62.0	112.5			
Range	5–190	20–220			
WBCs (10^3/μl)					
X ± SD	44.77 ± 64.78	7.70 ± 3.46	2.17	0.03	S
Median	14	7.7			
Range	1.78–245	2.7–12.5			

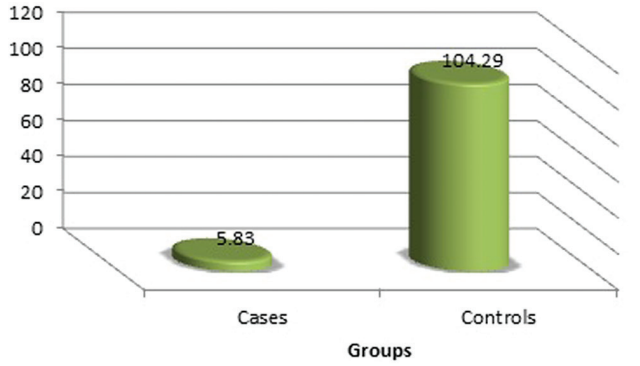
CBC, complete blood count; Hb, hemoglobin; HS, highly significant; NS, nonsignificant; S, significant; WBCs, white blood cells.

Figure 1



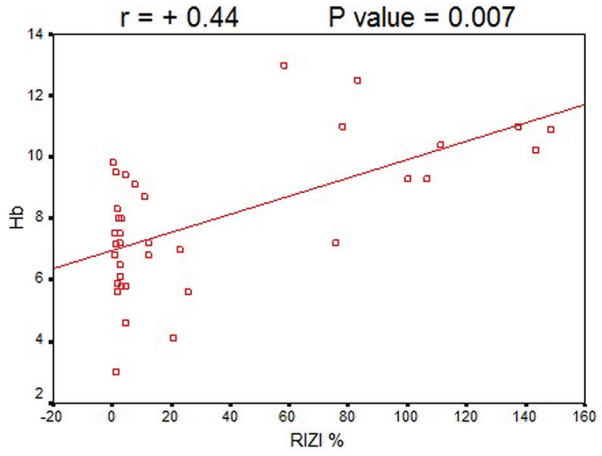
Bar chart representing RIZ levels in group I (day 0) and group III (controls).

Figure 2



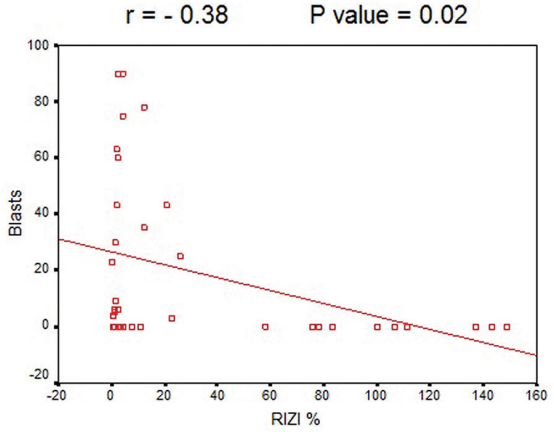
Bar chart representing RIZ levels in group I (day 0) and group II (day 28).

Figure 3



Positive Spearman correlation between RIZI levels in day 0 cases and Hb level. Hb, hemoglobin.

Figure 4



Negative Spearman correlation between RIZI levels in day 0 cases and blast% in peripheral blood smear.

had fever, 10 patients had no fever, nine patients had lymphadenopathy, and 17 patients had no lymphadenopathy (Table 1).

Comparison between group I (day 0) and group III (control) in complete blood count results indicated a significant decrease in the Hb concentration (day 0) compared with the controls. Whereas the white blood

cells (WBCs) count was significantly increased, the platelet count was not significantly different between both the groups (Table 2).

For the RIZ1 levels, a highly significant decrease was detected in the RIZ1 level in patients at day 0 compared with the controls (Table 3).

Day 28 after induction therapy

Treatment response at the end of induction therapy: according to National Comprehensive Cancer Network guidelines.

After induction therapy, seven patients achieved complete morphological remission and two patients showed induction failure, one patient with partial remission and two patients with complete remission incomplete.

After induction therapy (at day 28), a significant decrease was observed in the platelets, WBCs, and peripheral blood and BM blast counts, but the Hb level was increased compared with the patient groups before induction therapy (day 0) (Table 4).

The RIZ1 level was significantly increased in day 28 after induction therapy compared with its level before induction (Table 5).

Spearman correlation between RIZI level, clinical, and laboratory data showed a positive correlation between both the Hb concentration and the RIZ1 level. In contrast, an inverse correlation was found between the RIZI level and each of the blast cell counts in peripheral blood and BM. Meanwhile, no relationship was found between the RIZI level and platelets, WBCs counts, hepatomegaly, splenomegaly, fever, or lymphadenopathy (Table 6).

Table 3 Comparison between group I (day 0) and group III (control) of RIZ1 gene levels

The variable	The studied groups		Mann–Whitney *U*	*P* value	Significance
	Day 0 cases (*N* = 26)	Controls (*N* = 10)			
RIZ1 level					
X ± SD	5.83 ± 7.18	104.29 ± 31.11	4.59	<0.001	HS
Median	2.48	103.51			
Range	0.15–25.65	58.28–148.67			

HS, highly significant.

Discussion

The PRDM family has recently received considerable interest as it has been implicated in fundamental aspects of cellular differentiation and has increasingly been shown to be linked to human diseases. Several PRDM family members are deregulated in human diseases, most prominently in hematological malignancies and solid cancers, where they can act both as tumor suppressors and as drivers of oncogenic processes [11].

The RIZ1 gene is a candidate tumor suppressor gene as it arrests cell cycle at the G2/M phase [6]. Decreased or lost expression of RIZ1 is found in various human solid tumors, including breast cancer, liver cancer, and

Table 4 Comparison between day 0 (group I) and day 28 (group II) of induction therapy of CBC and BM results

The variable	The studied cases		Mann–Whitney *U*	*P* value	Significance
	Day 0 (*N* = 26)	Day 28 (*N* = 12)			
Hb (g/dl)					
X ± SD	6.96 ± 1.68	8.16 ± 3.10	2.03	0.04	S
Median	7.07	8.14			
Range	3–9.8	3–12.2			
Platelets (10^3/μl)					
X ± SD	165.28 ± 80.26	143.29 ± 80.81	2.18	0.03	S
Median	71.5	127			
Range	5–41000	44–323			
WBCs (10^3/μl)					
X ± SD	44.77 ± 64.78	3.62 ± 2.86	3.67	<0.001	HS
Median	14	3.25			
Range	1.78–245	0.46–10.5			
Peripheral blood blasts (%)					
X ± SD	26.46 ± 31.27	0.0 ± 0.0	3.67	<0.001	HS
Median	7.5				
Range	0–90				
BM blasts (%)					
X ± SD	78.90 ± 22.58	8.92 ± 15.88	2.37	0.02	S
Median	90	4			
Range	20–99	0–58			

BM, bone marrow; CBC, complete blood count; Hb, hemoglobin; HS, highly significant; S, significant; WBCs, white blood cells.

Table 5 Comparison between day 0 (group I) and day 28 (group II) of RIZ1 gene levels

The variable	The studied cases		Mann–Whitney *U*	*P* value	Significance
	Day 0 (*N* = 26)	Day 28 (*N* = 12)			
RIZ1 level					
X ± SD	5.83 ± 7.18	84.51 ± 64.60	3.99	<0.001	HS
Median	2.48	80.05			
Range	0.15–25.65	2.73–185.46			

HS, highly significant.

Table 6 Spearman correlation between RIZI levels of group I (day 0 cases) and their clinical and laboratory data (*n* = 26)

The variable	RIZI %		
	r	*P* value	Significance
Hb (g/dl)	0.44	0.007	HS
Platelets (10^3/μl)	0.21	0.23	NS
WBCs (10^3/μl)	−0.29	0.09	NS
Blasts (%)	−0.38	0.02	S
BM blasts (%)	−0.41	0.01	S
Hepatomegaly	−0.13	0.34	NS
Splenomegaly	−0.22	0.20	NS
Fever	−0.25	0.12	NS
Lymphadenopathy	−0.27	0.11	NS

BM, bone marrow; Hb, hemoglobin; HS, highly significant; NS, nonsignificant; S, significant; WBCs, white blood cells.

colon cancer. Similarly, RIZ1 expression is suppressed in some hematological disorders [7].

DNA methylation at CpG sites in promoter regions is a frequent, acquired epigenetic event involved in the pathogenesis of various types of human malignancies. Methylation in the promoter region can induce gene silencing, which may provide an alternative pathway to gene inactivation, in addition to deletions or mutations [12].

RIZ1 is commonly silenced by DNA methylation. The RIZ1 promoter has been shown to have the characteristics of a CpG island, which suggests that RIZ1 is a target of inactivation by an epigenetic mechanism that causes promoter hypermethylation and silencing of RIZ1 gene transcription [4].

The current study aimed to determine the relationship between expression of RIZ1 in cases of hematological malignancies before the start of induction therapy and at the end of induction therapy and to establish its possible role as a risk factor in disease progression.

In this study, the level of RIZ1 genes in day 0 patients was significantly lower than those of the controls ($P < 0.001$), which is not in agreement with the results of Shimura *et al.* [13], who analyzed the expression of the RIZ1 gene in patients with newly diagnosed ALL by RT-PCR, and found that it was decreased compared with normal BM. They also found that forced RIZ1 expression in T-ALL cell lines suppressed cell growth, accompanied by G2/M arrest and apoptosis.

In the same manner, the study by Sasaki *et al.* [14] showed that the expression of RIZ1 was significantly decreased in ALL and AML patients compared with the healthy controls. Moreover, Chen *et al.* [15] found that the expression of RIZ1 mRNA in peripheral blood of AML was significantly decreased compared with the healthy controls; both studies used the RT-qPCR technique.

Shadat *et al.* [16] examined the role of RIZ1 in the cell growth of mouse and human monocytic leukemia cells in case of M5. They found that RIZ1 expression was induced in response to tumor necrosis factor-α. They added that RIZ1 expression induces malignant cell cycle arrest and apoptosis by augmentation of p53 expression.

Phan *et al.* [17] have reported that loss of the 1p36 region that harbors the RIZ1 gene region is commonly found in advanced stages of CML as well as in nonhematological malignancies. Lakshmikuttyamma *et al.* [18] characterized RIZ1 expression in BM biopsies from CML patients in the chronic phase, accelerated phase, and myeloid blast crisis, and found a significant decrease in RIZ1 expression in blast crisis compared with the chronic phase.

In our study, there was a statistically significant increase in the level of the RIZ1 gene at the end of induction therapy compared with the level of the RIZ1 gene before treatment. This finding was not obtained in any recent study, except the nearly similar results in M3 cases reported by Gazzerro *et al.* [19], who concluded that patients with M3 have a low RIZ1 expression both by RT-PCR and by immunocytochemistry, which assess the intracellular distribution of RIZ1 protein in leukemic promyelocyte cell lines; then, RIZ1 gene expression was determined again by both methods after treatment with all-trans-retinoic acid to assess variations in the intracellular distribution of RIZ protein after treatment. They found that all-trans-retinoic acid induced a selective expression of RIZ1 that correlated with the granulocytic differentiation of patients' leukemic promyelocytes. Thus, RIZ1 protein plays a role in granulocyte differentiation and its downregulation is associated with arrest of differentiation.

Altered expression of the RIZ1 gene in both hematological and nonhematological malignancies [4,5,7,8,9,20,21] led to searching for the explanation of its downregulation that is suspected to be a cause outside the gene sequence itself-epigenetic cause. Therefore, methylation-specific PCR was used to investigate the promoter region methylation status and the following was reported.

Chen *et al.* [15] carried out methylation-specific PCR analysis on the peripheral blood of AML, which indicated that 29.7% of cases with reduced RIZ1 expression have RIZ1 promoter methylation and all cases of normal peripheral blood samples are nonmethylated. Another explanation for the decreased RIZ1 expression in CML blast crisis provided by Lakshmikuttyamma *et al.* [18] is that the RIZ1 promoter CpG island is aberrantly hypermethylated; in addition, they used hypomethylating agents as a target therapy, which reversed CpG island hypermethylation and hence reversed downregulation of the RIZ1 gene.

In contrast, Uehara *et al.* [12] reported that RIZ1 promoter CpG islands were not methylated in any of CML patients with blastic crises or in healthy individuals.

Surprisingly, in this study, an inverse correlation was found between each of peripheral blood and BM blast cell counts and the RIZ1 level before therapy. Lakshmikuttyamma *et al.* [18] reported that low RIZ1 expression was correlated with myeloid blast crisis and was not because of low RIZ1 expression in immature hematopoietic cells as they compared RIZ1 expression in immature and mature hematopoietic cells by measuring RIZ1 expression in CD34+ cells, granulocytes, and monocytes. They found that RIZ1 was expressed in mature myeloid and CD34+ cells, indicating that differences in RIZ1 expression in the chronic phase and blast crisis were not a reflection of increased immature cell population in blast crisis, but with the presence of blast cells. Similarly, Xie *et al.* [6] found that the RIZ1 gene mRNA levels decreased obviously in the high-risk group of MDS cases (with a high blast percentage) compared with the low-risk group of myelodysplastic syndrome and healthy controls.

Conclusion

From this study, it can be concluded that RIZ1 gene expression is significantly decreased in leukemic patients on day 0 of treatment and increases significantly on day 28 of treatment. This makes RIZ1 gene expression a valuable tool for the evaluation of development, progression, and treatment response.

For future research, a large-scale study is recommended to determine the possible role of the RIZ1 gene in pathogenesis of hematological malignancies and to study its use as a synergistic target therapy.

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Conflicts of interest

There is no conflicts of interest.

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