

## Original Article

# Decreased *SFRP2* expression is associated with intermediate and poor karyotypes in *de novo* acute myeloid leukemia

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**Abstract:** Dysregulation of secreted frizzled-related protein 2 (*SFRP2*) has been found in various cancers. However, it is little known about the pattern of *SFRP2* expression in acute myeloid leukemia (AML). This study was aimed to analyze the expression status of *SFRP2* gene in AML patients and explore its clinical significance using real-time quantitative PCR (RQ-PCR). The level of *SFRP2* expression significantly decreased in AML compared to controls ( $P < 0.001$ ). Receiver operating characteristic curve (ROC) analysis revealed that an area under the ROC curve (AUC) of 0.871 ( $P < 0.001$ ) or 0.902 ( $P < 0.001$ ) in discriminating all patients or cytogenetically normal (CN) patients from controls, respectively. Low level of *SFRP2* expression was found more frequently in cytogenetically intermediate and poor groups (72% and 62%, respectively) than in favorable group (42%) ( $P < 0.05$ ). However, there was no significant difference in the rate of complete remission (CR) and overall survival between the groups with low *SFRP2* and high expression ( $P > 0.05$ ). *SFRP2* expression significantly increased after CR compared to initial diagnosis ( $P < 0.05$ ). These findings suggest that decreased *SFRP2* expression is associated with intermediate/poor karyotypes in AML patients and detection of *SFRP2* expression may be helpful to the diagnosis and disease monitoring in CN-AML.

**Keywords:** *SFRP2*, acute myeloid leukemia, expression

## Introduction

Acute myeloid leukemia (AML), the most common type of leukemia in adults, is characterized by malignant clonal disorders of myeloid progenitor cells [1, 2]. Genetic abnormalities play an important role in the pathogenesis of AML [3]. At present, there are many epigenetic aberrations which contribute to leukemogenesis in AML, for instance, the covalent histone modification pattern, aberrant promoter hypermethylation and miRNA expression [4]. In addition, genetic aberrations, including chromosomal abnormalities (translocation, addition and deletion) and gene alterations (mutation, deletion, amplification and translocation) [5], are associated with leukemia and identified in special types of AML [6, 7]. Collectively, these aberrations are responsible for self-renewal, proliferation, differentiation arrest and impaired apoptosis of leukaemic blasts.

In recent years, a number of researches have indicated that the pathogenesis of AML involves the abnormal activation of Wnt signaling pathway that has crucial roles in extensive cellular processes in differentiation and proliferation as well as hematopoietic cell growth and fate [8-10]. Lots of Wnt signaling aberrations have been detected in solid tumors as well as hematologic malignancies including AML [11, 12]. Wnts regulate multiple signaling pathways through both canonical mechanism ( $\beta$ -catenin dependent) and non-canonical mechanism ( $\beta$ -catenin independent) [13]. Numerous studies have demonstrated that the canonical pathway controls  $\beta$ -catenin mediated transcriptional activation of specific gene expression [13, 14]. The hematopoietic system is constituted from cells with a short-life span renewed by differentiating from a small population of hematopoietic stem cells (HSCs). Many evidences have shown that Wnt signaling is implicated in self-renewal,

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**Table 1.** Clinical characteristics of AML patients divided according to *SFRP2* expression status

Patient's parameters	Status of <i>SFRP2</i> expression			P
	Low (n=60)	High (n=34)	Total (n=94)	
Sex, male/female	34/26	20/14	54/40	1.000
Median age, years (range)	56.5 (15-87)	53.0 (15-76)	56 (15-87)	0.514
Median WBC, ×10 <sup>9</sup> /L (range)	14.6 (0.3-528.0)	9.8 (0.5-136.1)	11.8 (0.3-528.0)	0.902
Median hemoglobin, g/L (range)	76 (34-138)	80.5 (45-131)	77.5 (34-138)	0.192
Median platelets, ×10 <sup>9</sup> /L (range)	39.5 (3-134)	38.5 (10-399)	39.5 (3-399)	0.490
BM blasts, % (range)	49.8 (1.0-97.5)	28.0 (3.0-94.5)	45.5 (1.0-97.5)	0.108
FAB				0.254
M1	4 (7%)	3 (9%)	7 (7%)	
M2	20 (33%)	9 (26%)	29 (31%)	
M3	10 (16%)	13 (38%)	23 (24%)	
M4	18 (30%)	7 (21%)	25 (27%)	
M5	7 (12%)	2 (6%)	9 (10%)	
M6	1 (2%)	0 (0%)	1 (1%)	
WHO				0.192
AML with t(8;21)	1 (2%)	2 (6%)	3 (3%)	
APL with t(15;17)	10 (16%)	13 (38%)	23 (24%)	
AML without maturation	4 (6%)	3 (9%)	7 (7%)	
AML with maturation	19 (32%)	7 (21%)	26 (28%)	
Acute myelomonocytic leukemia	19 (32%)	7 (21%)	26 (28%)	
Acute monoblastic and monocytic leukemia	6 (10%)	2 (6%)	8 (9%)	
Acute erythroid leukemia	1 (2%)	0 (0%)	1 (1%)	
Karyotype classification				0.044
Favorable	11 (18%)	15 (44%)	26 (27%)	
Intermediate	36 (60%)	14 (41%)	50 (53%)	
Poor	8 (13%)	5 (15%)	13 (14%)	
No data	5 (8%)	0 (0%)	6 (6%)	
Karyotype				0.105
normal	30 (50%)	11 (32%)	41 (44%)	
T(8;21)	1 (2%)	2 (6%)	3 (3%)	
T(15;17)	10 (17%)	13 (38%)	23 (24%)	
complex	8 (13%)	3 (9%)	11 (12%)	
others	6 (10%)	5 (15%)	11 (12%)	
No data	5 (8%)	0 (0%)	5 (5%)	
Gene Mutation*				
<i>C/EBPA</i> (+/-)	4/47 (8%)	4/29 (12%)	8/76 (10%)	0.706
<i>NPM1</i> (+/-)	6/45 (12%)	2/31 (6%)	8/76 (10%)	0.320
<i>FLT3-ITD</i> (+/-)	8/43 (16%)	4/29 (12%)	12/72 (14%)	0.757
<i>DNMT3A</i> (+/-)	4/47 (8%)	1/32 (3%)	5/79 (6%)	0.644
<i>IDH1/2</i> (+/-)	3/48 (6%)	3/30 (9%)	6/78 (7%)	0.675
<i>C-KIT</i> (+/-)	1/50 (2%)	1/32 (3%)	2/82 (2%)	1.000
<i>N/K-RAS</i> (+/-)	5/46 (10%)	3/30 (9%)	8/76 (10%)	1.000
CR (+/-)	26/31 (46%)	12/18 (40%)	38/49 (44%)	0.655
<i>SFRP2</i> transcript (‰)	0.00 (0.00-3.67)	19.03 (4.11-856.57)	0.31 (0.00-856.57)	<0.001

WBC, white blood cells; FAB, French-American-British classification; AML, acute myeloid leukaemia; CR, complete remission; \*, percentage was equal to the number of mutated patients divided by total cases in each group.

proliferation and differentiation of normal HSCs in the mediation of  $\beta$ -catenin, while dysregulation of this pathway contributes to the development of leukemia [15-17]. Wnt signaling antagonists, mainly including secreted frizzled-related proteins (SFRPs), Wnt inhibitory factor 1 (WIF1) and Dickkopf proteins (DKKs), have ability to inhibit activity of Wnt proteins, thereby act as modulators of this signaling cascade [18, 19].

SFRPs are the largest family among Wnt antagonists and consist of five members. Four *SFRP* members (*SFRP1/2/4/5*) was identified to contain dense CpG islands around promoter regions. The aberrant methylation of these four *SFRP* genes, which was associated with aberrant Wnt signaling activation [20, 21], was found in AML [22, 23]. Although aberrant methylation of *SFRP2* promoter has been identified as an adverse prognostic factor in core binding factor (CBF) AML [23], the pattern of *SFRP2* expression and its clinical relevance in AML remain unclear so far. Therefore, this study is aimed to detect the status of *SFRP2* expression and to explore the clinical significance of *SFRP2* expression in AML.

## Materials and methods

### Patients' samples and cell lines

The bone marrows derived from 123 samples, including 94 de novo AML diagnosed at the Affiliated People' Hospital of Jiangsu University and 29 normal controls, were obtained after informed consent written. The diagnosis and classification of de novo AML patients were made according to French-America-British (FAB) and World Health Organization (WHO) criteria (blast  $\geq 20\%$ ) [24, 25]. Karyotypes were analyzed by conventional R-banding method. Karyotype risk was classified according to reported previously [26]. The main clinical and laboratory characteristics of the patient cohort were listed in **Table 1**.

Seven human leukemic cell lines (SHI-1, THP-1, U937, HEL, HL60, K562 and NB4) were also studied. All cell lines were cultured in IMDM medium containing 10% fetal calf serum and grown at 37°C in 5% CO<sub>2</sub> humidified atmosphere.

### RNA isolation, reverse transcription and real-time quantitative PCR

The bone marrow mononuclear cells (BMNCs) were separated by Ficoll-Hypaque gradient. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions.

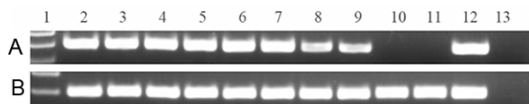
cDNA was transcribed using 2  $\mu$ g of total RNA in a total volume of 40  $\mu$ L including random hexamers 10  $\mu$ M, dNTPs 10 mM each, RNase inhibitor (RNasin) 80 units, and MMLV reverse transcriptase (MBI Fermentas, Hanover, USA) 200 units. The reverse transcription system was incubated for 10 min at 25°C, 60 min at 42°C, and then stored at -20°C.

*SFRP2* was amplified using the primer pair of 5'-TGAGTGCACCGTTTCC C-3' (forward) and 5'-GAGCCACAGCACCGATT-3' (reverse) with expected products of 298 bp. Real-time quantitative PCR (RQ-PCR) was carried out for each sample in a final reaction volume of 20  $\mu$ L, consisting of 0.4  $\mu$ M of primers, 10  $\mu$ L SYBR Premix Ex Taq II, 0.4  $\mu$ L 50 $\times$ ROX (TaKaRa, Japan) and 50 ng of cDNA. RQ-PCR was performed on Step One Plus (Applied Biosystems, CA, USA). Amplification was carried out at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, 62°C for 30 s and 72°C for 30 s, and an fluorescence collection step at 81°C for 30 s, then followed by a melting program at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s. Negative and positive controls were included in all experiments. The specificity of RQ-PCR products was certified by melting curves and DNA sequencing. The housekeeping gene (*ABL*) was used to calculate the abundance of *SFRP2* mRNA. Relative *SFRP2* expression values were obtained according to the following equation:  $N_{SFRP2} = (E_{SFRP2})^{\Delta CT_{SFRP2}(\text{control-sample})} / (E_{ABL})^{\Delta CT_{ABL}(\text{control-sample})} \times 1000\%$ . The parameter efficiency (E) derived from the formula  $E = 10^{(-1/\text{slope})}$  (the slope referred to CT versus cDNA concentration plot).

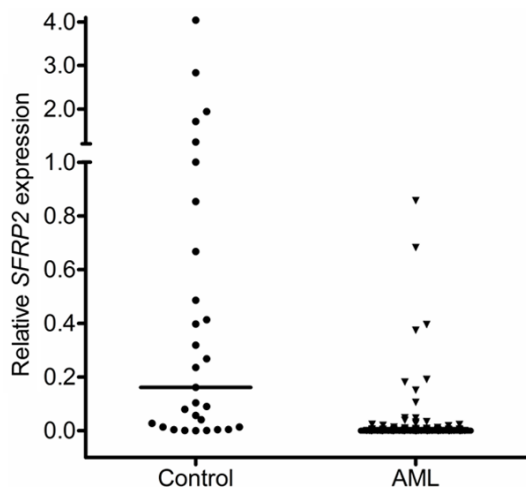
### Gene mutation detection

According to reported previously, *NPM1*, *DNMT3A*, *IDH1*, *IDH2*, *C-KIT* and *N/K-RAS* mutations were detected by high-resolution melting analysis (HRMA) [27-29]. Briefly, genomic DNA samples were amplified using gene-specific primers. Then, mutation scanning was

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**Figure 1.** Electrophoresis results of RQ-PCR products in AML patients. 1: Gene Ruler™ 100 bp DNA ladder; 2-3: normal controls; 4-11: AML samples; 12: *SFRP2* plasmid; 13: negative control. A: *SFRP2*; B: *ABL*.

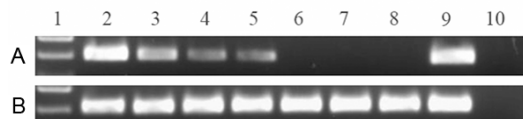


**Figure 2.** Relative expression levels of *SFRP2* in AML and controls.

conducted for PCR products using HRMA with the LightScanner™ platform (Idaho Technology Inc, Salt Lake City, Utah). To confirm the results of HRMA, all positive samples were detected using direct DNA sequencing. *C/EBPA* mutations and *FLT3* internal tandem duplication (ITD) were directly DNA sequenced [30, 31].

### Statistical analysis

All statistics were analyzed with the SPSS 17.0 software package (SPSS, Chicago, IL). Pearson Chi-square analysis or Fisher exact test was carried out to compare the difference of categorical variables between patients groups. At the same time, to compare the difference of continuous variables between patients groups and controls, we used the Kruskal-Wallis test (multiple groups) and Mann-Whitney *U*-test (two groups). The correlation between *SFRP2* expression and the clinical hematologic parameters was analyzed with Spearman's rank correlation. Overall survival (OS) was compared according to the Kaplan-Meier method. Receiver operating characteristic curve (ROC) and area under the ROC curve (AUC) were used to assess the diagnostic value of *SFRP2* expres-



**Figure 3.** Electrophoresis results of RQ-PCR products in leukemic cell lines. 1: Gene Ruler™ 100 bp DNA ladder; 2: SHI-1; 3: THP-1; 4: U937; 5: HEL; 6: HL60; 7: K562; 8: NB4; 9: cloned plasmid; 10: NTC. A: *SFRP2*; B: *ABL*.

sion in discriminating AML patients from normal controls. A *P*-value of less than 0.05 (two-tailed) was determined statistically significant for all analyses.

### Results

#### *SFRP2* expression in de novo AML and leukemic cell lines

We assessed the level of *SFRP2* expression in AML and normal controls. The typical electrophoresis results of RQ-PCR products were shown in **Figure 1**. *SFRP2* level decreased significantly in AML (0-856.57‰, median 0.31‰) compared to controls (0.16‰-4040.54‰, median 161.32‰) ( $P < 0.001$ , **Figure 2**).

Among the tested seven cell lines, SHI-1, THP-1, U937 and HEL cell lines presented variable levels of *SFRP2* transcript (0.28‰-38.36‰, **Figure 3**), the remaining 3 cell lines showed negative *SFRP2* expression.

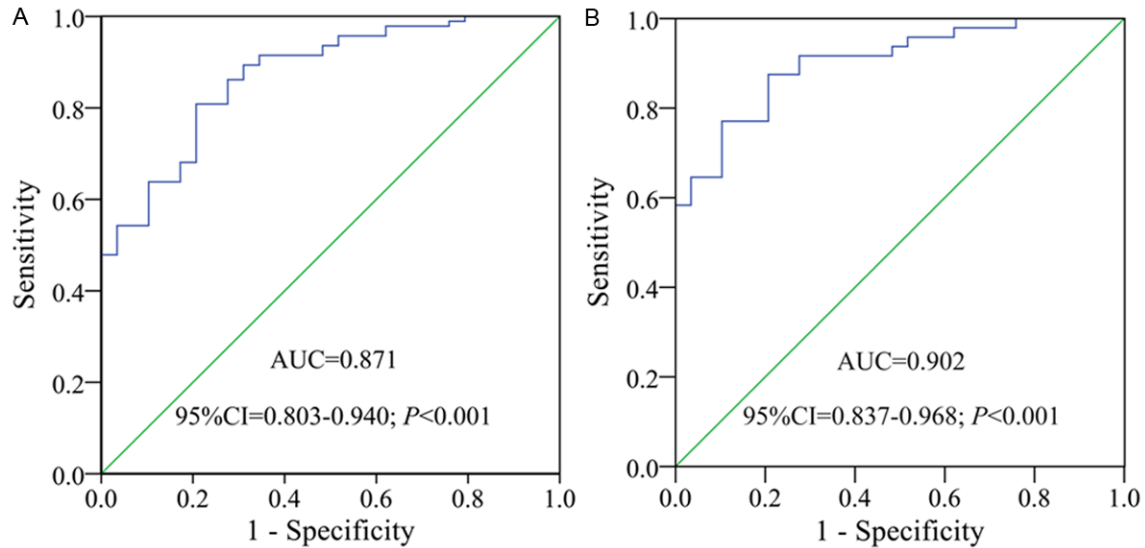
#### Evaluation of *SFRP2* expression as a potential diagnostic marker

The ROC curve was used to evaluate whether *SFRP2* expression can be used as a potential diagnostic marker for de novo AML. It was revealed that the level of *SFRP2* expression could be available for a potential diagnostic biomarker for differentiating AML from controls with an AUC of 0.871 (95%CI: 0.803-0.940;  $P < 0.0001$ ) (**Figure 4A**). At the cut-off value of 3.72‰, the sensitivity and the specificity were 64% and 90%, respectively. Furthermore, ROC curves showed that *SFRP2* level was more powerful to discriminate cytogenetically normal (CN) AML from normal controls (AUC=0.902, 95%CI: 0.837-0.968,  $P < 0.001$ ) (**Figure 4B**).

#### Clinical and laboratory characteristics of AML

According to the set cut-off value of 3.72‰, this cohort of 94 AML patients was divided into

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**Figure 4.** ROC curve analysis using *SFRP2* for discriminating AML patients. A: all patients; B: cytogenetically normal patients.

two groups: low *SFRP2* expression ( $\leq 3.72\%$ ) and high *SFRP2* expression ( $> 3.72\%$ ). There was no significant difference in age, gender, white blood cells, hemoglobin, platelet count, percentage of blasts in bone marrow, WHO or FAB classifications and gene mutations between these two groups (Table 1). However, low *SFRP2* expression was found more frequently in intermediate group (72%) and poor group (62%) than in favorable group (42%) according to karyotype risk ( $P < 0.05$ ).

### Impact of *SFRP2* expression on prognosis

There was no significant difference between low *SFRP2*- and high *SFRP2*-expressing patients in the rates of complete remission (CR) after induction therapy ( $P > 0.05$ ) (Table 1). M3 was excluded from survival analysis due to the different therapy regimen and outcome. Survival data were obtained for 61 non-M3 AML patients, but there was no difference between two groups ( $P > 0.05$ ) (Figure 5). Moreover, there was no significant impact of *SFRP2* expression on overall survival in both patients with intermediate/poor karyotypes and patients with normal karyotypes ( $P > 0.05$ ).

### Significance of *SFRP2* gene expression in the follow-up of AML patients

Five samples of de novo AML were monitored after CR. It was shown that *SFRP2* expression

significantly increased after CR compared to initial diagnosis ( $P < 0.05$ ) (Figure 6).

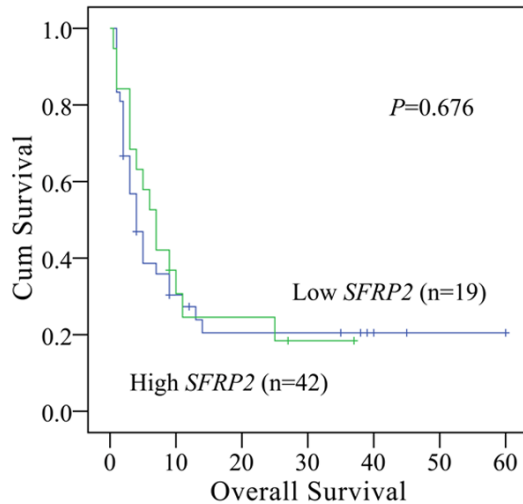
### Discussion

Compared to the extensive studies on hypermethylation of *SFRPs* in various cancers including hematologic malignancies, the pattern of *SFRPs* expression in clinical samples of cancers remains relatively little studied. Zou et al observed the down-regulation of *SFRP2* protein in esophageal adenocarcinoma compared to Barrett's esophagus and normal tissue [32]. Negative/weak *SFRP2* expression was also found in the majority of tumor epithelia of prostate cancer [33]. Furthermore, negative *SFRP2* protein was shown in 60% of Gleason grade 5 carcinomas and was associated with a morphologically diffuse pattern. Moreover, strong reduction of *SFRP2* protein was shown in 74% of primary breast carcinomas and there was a weak association between *SFRP2* loss with unfavorable outcome [34]. The decreased level of *SFRP2* mRNA was also identified in bladder and pancreatic cancer samples compared to their normal counterparts [35, 36]. However, the pattern of *SFRP2* expression has been rarely studied in primary leukemic samples though *SFRP2* hypermethylation has been identified in AML by several studies [21-23, 37, 38].

In this study, we identified the significant reduction of *SFRP2* mRNA in primary AML samples.



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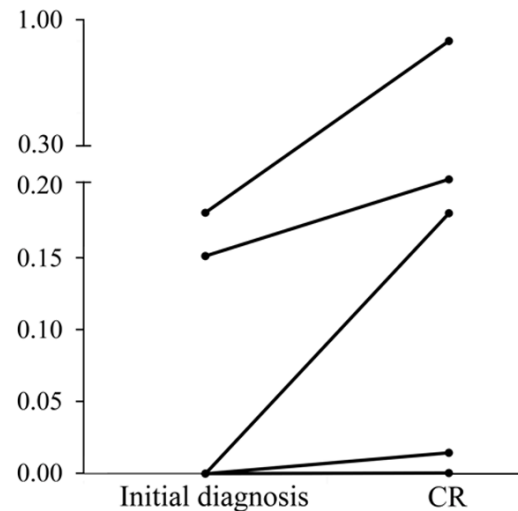


**Figure 5.** Overall survival of non-M3 AML patients.

Furthermore, our results indicated that low *SFRP2* level could serve as a potential biomarker for differentiating CN-AML from controls, while CN-AML often lacks available markers. Moreover, the level of *SFRP2* expression significantly increased in those patients achieved complete remission after chemotherapy, suggesting it can be potentially used as a biomarker for the disease monitoring.

It is worth noting that *SFRP2* down-regulation was associated with intermediate and poor karyotypes. Low *SFRP2* expression occurred more frequently in CN-AML, in accordance with the observation of Cheng et al in which *SFRP2* hypermethylation was predominantly seen in CN-AML [38]. Although Cheng et al found the association between *SFRP2* hypermethylation with *CEBPA* mutations, we did not identify the correlation of *SFRP2* expression with *CEBPA* mutations. Similarly, the association between *SFRP2* expression and *N/K-RAS* mutations was not observed in this study though extensive methylation of *SFRP2* promoter occurred more frequently in *K-RAS* mutated colorectal cancers [39].

The influence of *SFRP2* aberrations on prognosis remains poorly understood. Jost et al identified the adverse effect of aberrant *SFRP2* methylation on overall survival in CBF-AML [23]. Griffiths et al also observed that *SFRP2* methylation was associated with increased risk of relapse and with decreased relapse-free survival in CN-AML [37]. However, Cheng et al found no significant impact of *SFRP2* methyla-



**Figure 6.** Changes of *SFRP2* expression in five AML patients.

tion on survivals in their patient cohort [38]. We did not observe the effect of aberrant *SFRP2* expression on outcome in our AML patients both with normal karyotypes and with intermediate or poor karyotypes. Obviously, more studies should be needed to further determine the clinical significance of abnormal *SFRP2* expression in AML.

Although the fact of aberrant methylation of *SFRP2* promoter is present in various cancers suggests its role of a tumor suppressor, the function of *SFRP2* gene in tumorigenesis remains controversial to date. The studies on gland and renal cancer and angiosarcoma showed that *SFRP2* promotes cell proliferation in vitro and in vivo tumor growth, protects cell from apoptosis and stimulates angiogenesis [40-42]. However, majority of studies indicated that *SFRP2* promotes apoptosis, inhibits proliferation in vitro and tumor growth in vivo, suppresses invasion in colorectal, gastric, cervical, and breast cancers [20, 34, 43, 44]. However, the role of *SFRP2* in leukemogenesis remains unknown. Three aspects indicate the role of *SFRP2* as a tumor suppressor: decreased expression, aberrant promoter methylation and the potential association of *SFRP2* aberration with poor outcome or karyotype risk. It is needed to explore the precise function and related mechanism of *SFRP2* gene in the development of leukemia.

In conclusion, our study shows that the decreased *SFRP2* expression is a common

event and is associated with intermediate and poor karyotypes in AML patients. The detection of *SFRP2* expression may be helpful to the diagnosis and disease monitoring in cytogenetically normal AML.

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### Disclosure of conflict of interest

None.

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