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Review Article

Methylation-independent *CRIP1* expression is a potential biomarker affecting prognosis in cytogenetically normal acute myeloid leukemia

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Abstract: Abnormal expression of *CRIP1* has been identified in numerous solid tumors. However, *CRIP1* expression and its regulation are little known in acute myeloid leukemia (AML). The purpose of this study was to evaluate the expression and regulation of *CRIP1* and the clinical implications of *CRIP1* aberration in AML. Real-time quantitative PCR was carried out to detect the level of *CRIP1* expression in 138 AML patients and 38 controls. *CRIP1* methylation was detected by methylation-specific PCR and bisulfite sequencing PCR. Five public available AML datasets from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) were further analyzed. The level of *CRIP1* expression was up-regulated in AML patients compared with controls ($P = 0.045$). *CRIP1*^{high} patients had a significantly lower complete remission (CR) rate than *CRIP1*^{low} patients ($P = 0.020$). *CRIP1*^{high} group had a shorter overall survival (OS) and leukemia-free survival (LFS) than *CRIP1*^{low} group in cytogenetically normal AML (CN-AML) patients ($P = 0.007$ and 0.012, respectively). Multivariate analysis further confirmed that high *CRIP1* expression was an independent risk factor for LFS in CN-AML patients ($P = 0.005$). However, we found that *CRIP1* expression was not associated with the status of its promoter, which was nearly fully unmethylated both in controls and AML patients. Furthermore, our results were validated using the published GEO datasets and TCGA datasets. Our findings suggest that high *CRIP1* expression is independently related with unfavorable prognosis in CN-AML.

Keywords: AML, *CRIP1*, biomarker, prognosis, methylation

Introduction

Acute myeloid leukemia (AML) is a cytogenetically, molecularly, and clinically heterogeneous malignant clonal disease of hematopoietic stem cells. It is characterized by clonal leukemia cells accumulated in bone marrow and other hematopoietic tissues, which is caused by uncontrolled proliferation, differentiation, and blocked apoptosis [1]. This biological heterogeneity makes it difficult for risk stratification and targeted therapy of the disease. At present, long-term survivors among young people and the elderly account for 40% and 10% respectively [2]. Acquired chromosomal abnormalities are the most important independent predictors of complete remission (CR), disease-free survival, and overall survival (OS) for patients with AML [3-5]. However, cytogenetically normal AML (CN-AML) accounts for approximately 45%, and it is difficult to define risk in patients with CN-AML. It has become apparent that the identification of biological markers is important for diagnosis, classification, prognostic evaluation, and guiding individual treatment in AML, especially in CN-AML.

Cysteine-rich intestinal protein 1 (*CRIP1*) belongs to the LIM/double zinc finger protein family. *CRIP1* is abnormally expressed in several types of tumors, including osteosarcoma, breast cancer, cervical cancer, prostate cancer,

Table 1. The sequences of primers used in *CRIP1* expression, MSP and BSP

	Forward (5'→3')	Reverse (5'→3')	Product (bp)
CRIP1 expression	GCTGAGCACGAAGGCAAACC	AAGGGAGCCCTGGGCATCT	196
CRIP1 MSP	CGGAATTGGATTCGGGAGATATTATAG	TCGCGTTTCGTTTTTAGTTAGGTT	128
CRIP1 BSP	TAGTGATGTTTGGTTTAGTTTTGG	GTGTTTTAAGTGTAATAAGGAGGTG	511

pancreatic cancer and colorectal cancer [6-12]. In osteosarcoma, negative *CRIP1* expression was associated with metastases and inferior survival [6]. The lack of *CRIP1* expression is significantly associated with a worse prognosis in breast cancer and down-regulation of *CRIP1* expression increases cell proliferation and cell invasion in breast cancer cell lines [7]. Moreover, *CRIP1* has been identified as a promising biomarker for the staging of breast cancer [7]. However, high *CRIP1* expression was identified as a novel and independent adverse prognostic factor in gastric cancer [13].

Epigenetic modification, especially DNA methylation, can control gene expression by causing changes in chromatin structure, DNA conformation, DNA stability, and the way DNA interacts with proteins.

DNA hypomethylation is often associated with activation of affected genes, while DNA hypermethylation is closely related to gene silencing and loss of tumor suppressor function in cancer [14, 15]. *CRIP1* is regulated at the transcriptional level in prostate cancer by promoter hypomethylation [10]. The methylation frequency of *CRIP1* increases with tumor stage in breast cancer, and its methylation status is related to poor clinical prognosis [16].

However, *CRIP1* expression and methylation patterns as well as its role in AML were rarely investigated. Therefore, we focused on *CRIP1* expression and methylation in AML and further determined the significance of *CRIP1* in predicting prognosis.

Materials and methods

Patients and treatment

A total of 38 healthy donors and 138 de novo AML patients were included in the study, which was approved by Institutional Review Board and the Ethics Committee of the Affiliated People's Hospital of Jiangsu University. Bone marrow (BM) specimens were collected, after the informed consents were signed, from healthy donors and all de novo AML patients as well as 9 patients who achieved CR after induction therapy. Treatment protocols for AML patients have been described previously [17].

Gene mutation detection, RNA isolation and reverse transcription

Gene mutation detection, RNA isolation and reverse transcription were conducted as reported previously [18-26].

Real-time quantitative PCR

The primers for *CRIP1* expression were shown in **Table 1**. *CRIP1* expression was examined by real-time quantitative PCR (RQ-PCR) in 7500 Thermo Cycler (Applied Biosystems, Foster, CA, USA) using TB GreenPremix Ex TaqII (Takara). RQ-PCR program was carried out at 95°C for 5 minutes, followed by 40 cycles at 95°C for 5 seconds, 62°C for 30 seconds, 72°C for 32 seconds and 85°C for 32 seconds to collect fluorescence, finally followed by 95°C for 15 seconds, 60°C for 60 seconds, 95°C for 15 seconds and 60°C for 15 seconds. Relative *CRIP1* expression levels were calculated by $2^{-\Delta\Delta CT}$ method.

DNA isolation, bisulphite modification and real-time quantitative methylation-specific PCR

Genomic DNA from AML patients and healthy donors were isolated using a genomic DNA purification kit (Gentra) and then modified using the CpGenome DNA modification kit (Chemicon). Methylation-specific PCR (MSP) was used to detect *CRIP1* methylation status by the methylation primers (**Table 1**) with TB Green Premix Ex Taq II (Takara). The reaction conditions were 95°C for 30 seconds, 40 cycles for 5 seconds at 95°C, 30 seconds at 59°C, 30 seconds at 72°C, and 75°C for 32 seconds. The quantification of *CRIP1* methylation was calculated as *CRIP1* expression using the reference gene ALU [17].

Bisulfite sequencing PCR

A 511 bp fragment was amplified from the *CRIP1* promoter region, using specific primers

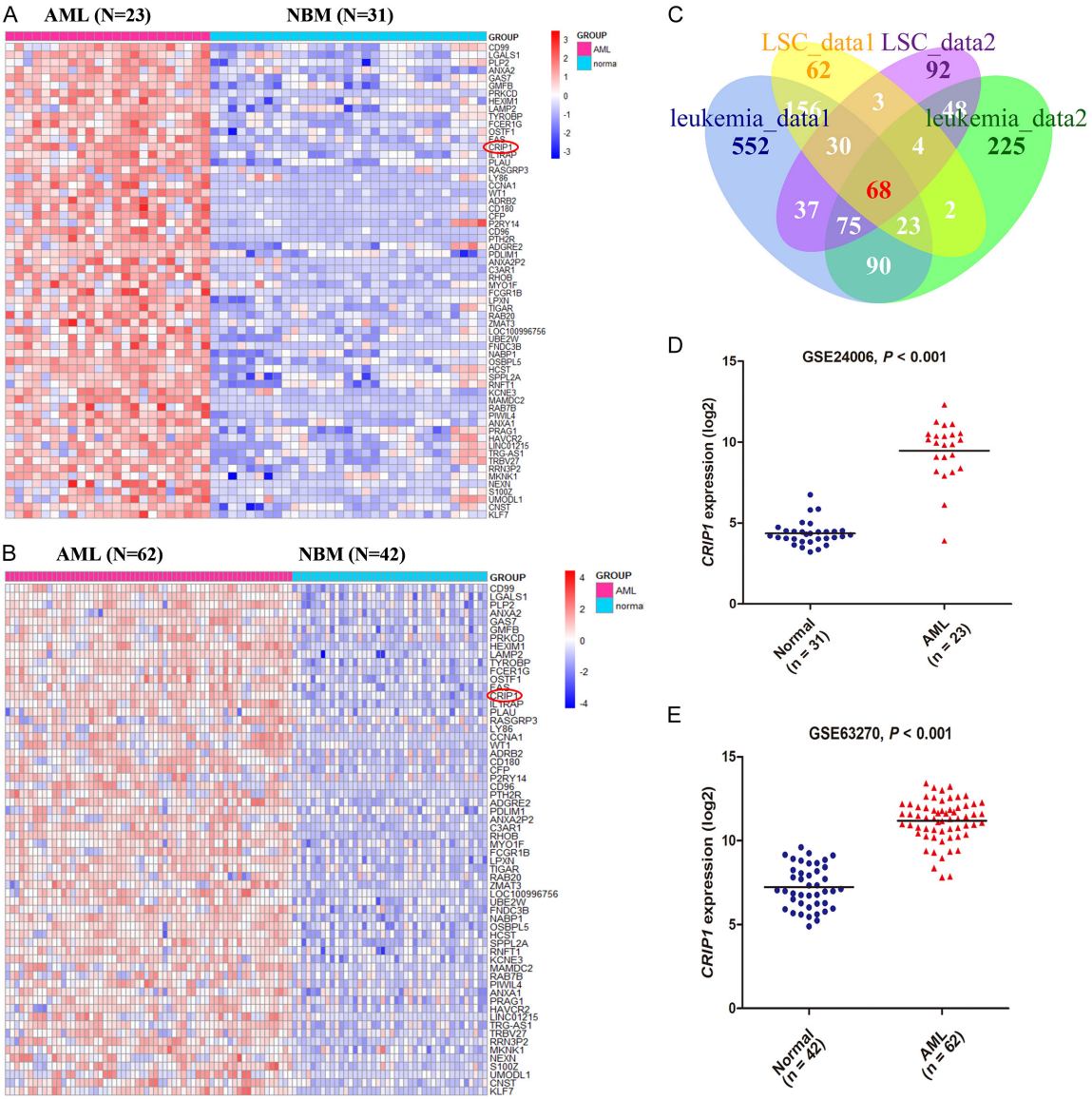

Figure 1. Identification of potential oncogenes in AML. A, B: Heatmaps showing 68 up-regulated genes in controls versus AMLs, from GSE24006 and GSE63270, respectively. Log2 fold changes of gene expression (log2 FC expression) are displayed as bar graphs on the right. C: Venn diagram showing the overlap of four gene sets including: 1031 up-regulated genes (FDR < 0.05, log2 FC > 2) in controls versus all AML patients and 348 up-regulated genes in LSCs versus HSCs in GSE24006 datasets (data 1), 535 up-regulated genes in controls versus all AML patients and 357 up-regulated genes in LSCs versus HSCs in GSE63270 datasets (data 2). The overlapping region (68) represents the finally screened oncogene. D, E: *CRIP1* expression levels in the GSE24006 and GSE63270 databases, respectively.

for bisulfite sequencing PCR (BSP) (**Table 1**). The reaction conditions were 98°C for 10 seconds, 40 cycles for 10 seconds at 98°C, 30 seconds at 58°C, 30 seconds at 72°C, and followed by 7 minutes extension at 72°C. AxyPrep DNA gel extraction kit (AxyGen) was used to purify BSP products, ligated into pMD®19-T Vector (Takara), and then transfected into DH5α competent cells (Vazyme) for cloning. Finally, six independent clones of each sample were sequenced timely (BGI Tech Solutions Co., Shanghai, China).

Public datasets

Five available public AML datasets from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) were used in this study. Two of them consisted of expression data for bulk primary AML samples (GSE12417

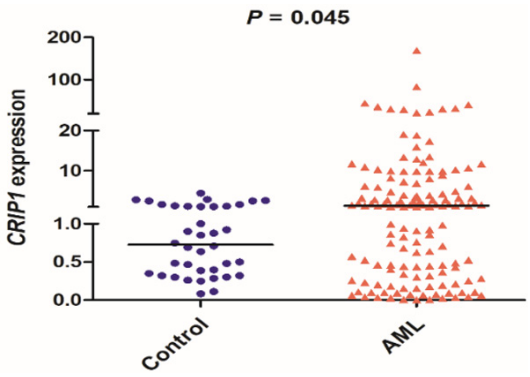

Figure 2. *CRIP1* expression was up-regulated in AML patients compared with controls ($P = 0.045$).

and the TCGA datasets) and three datasets contained both healthy and AML BM samples sorted by fluorescence-activated cell sorting (GSE24006, GSE63270 and GSE63409).

Statistical analysis

The median level was applied to distinguish the expression level of *CRIP1*. SPSS software version 20.0 was used to carry out the statistical analysis. For example, Mann-Whitney's U test was performed to compare the differences of continuous variables. Pearson's chi-squared analysis was conducted to detect the difference of categorical variables. Kaplan-Meier method and Cox regression (univariate and multivariate) analysis were used to analyze the effect of *CRIP1* on prognosis. $P < 0.05$ was defined as statistically significant and all tests were two sided.

Results

Identification of candidate oncogene in AML

To identify candidate oncogene, the dataset GSE24006 (n = 54, data 1) and GSE63270 (n = 104, data 2) were utilized. In the two groups of GEO databases, we performed differential expression analysis in controls versus AML patients and LSCs versus HSCs, respectively, and screened for significantly up-regulated genes (FDR < 0.05, log2 FC > 2, Supplementary File 1). Sixty-eight up-regulated genes, including *CRIP1*, were obtained by the intersection of four sets of data (**Figure 1A-C**). Increased expression of *CRIP1* was shown in the two GEO datasets ($P < 0.001$, **Figure 1D**, **1E**).

Upregulation of CRIP1 in AML

The expression level of *CRIP1* in controls ranged from 0.084 to 4.384 (median 0.728). *CRIP1* transcript level in AML patients ranged from 0.004 to 169.190 (median 1.330). *CRIP1* was significantly up-regulated in AML patients ($P = 0.045$, **Figure 2**).

Clinical and laboratory characteristics of AML patients

The whole cohort of AML patients were divided into two groups according to the median value of 1.330 (**Table 2**). No significant differences were observed in sex, age, platelets, BM blasts and karyotype finding between two groups ($P > 0.05$). There were no correlations between *CRIP1* expression and the common gene mutations ($P > 0.05$). However, patients in *CRIP1*^{high} group showed higher white blood cells (WBCs) than patients in *CRIP1*^{low} group ($P = 0.002$).

Effect of CRIP1 expression on chemotherapy response in AML

Compared with *CRIP1*^{low} group, patients in *CRIP1*^{high} group had a lower CR ($P = 0.020$, **Table 2**). Clinical characteristics of patients with CR and non-CR were further compared. Significant differences were found in *CRIP1* expression, age, WBCs, BM blast, risk group and karyotype ($P < 0.05$, **Table 3**). *CRIP1* expression was further found to be obviously decreased in AML patients achieved CR (n = 54) compared with patients relapsed (n = 21) (**Figure 3**). Moreover, *CRIP1* expression was analyzed in 9 patients with serial samples, which also confirmed that *CRIP1* expression significantly decreased after CR ($P = 0.005$, **Figure 4**).

The relationship between CRIP1 expression and prognosis in AML patients

130 AML patients with available follow-up data were analyzed to explore the impact of *CRIP1* expression on clinical prognosis. Kaplan-Meier analysis indicated that *CRIP1*^{high} group had a shorter OS time than those *CRIP1*^{low} group both in the whole-cohort AML and CN-AML patients ($P < 0.05$, **Figure 5A**, **5E**). There was a trend that patients with *CRIP1*^{high} group had a shorter OS than those in *CRIP1*^{low} group in no-M3 AML ($P = 0.057$, **Figure 5C**). We also found that leukemia-free survival (LFS) time of the *CRIP1*^{high}

Table 2. Comparison of clinical manifestations and laboratory features between AML patients with high and low *CRIP1* expression

Patient's parameters	*CRIP1*^{high} (n = 65)	*CRIP1*^{low} (n = 65)	*P* value
Sex, male/female	37/28	39/26	0.859
Median age, years (range)	61 (18-88)	54 (18-85)	0.097
MedianWBC, $\times10^9$/L (range)	34.5 (0.3-186.9)	7.0 (0.8-528)	0.002
Median hemoglobin, g/L (range)	74 (34-141)	83 (27-144)	0.046
Median platelets, $\times10^9$/L (range)	42 (3-382)	32 (3-415)	0.096
BM blasts, % (range)	42.5 (1-99)	49.5 (3-97.5)	0.962
CR (+/-)	20/45	34/31	0.020
Karyotype classification			0.494
Favorable	10 (15.4%)	17 (26.2%)	
Intermediate	38 (58.5%)	35 (53.8%)	
Poor	8 (12.3%)	6 (9.2%)	
No data	9 (13.8%)	7 (10.8%)	
Karyotype			0.152
normal	24 (37.0%)	30 (46.3%)	
t (8;21)	4 (6.2%)	3 (4.6%)	
t (16;16)	0 (0%)	1 (1.5%)	
t (15;17)	6 (9.2%)	14 (21.5%)	
11q23	3 (4.6%)	1 (1.5%)	
+8	2 (3.1%)	0 (0%)	
-5/5q-	1 (1.5%)	0 (0%)	
-7/7q-	2 (3.1%)	0 (0%)	
t (9;22)	1 (1.5%)	0 (0%)	
others	3 (4.6%)	0 (0%)	
complex	6 (9.2%)	5 (7.7%)	
No data	13 (20.0%)	11 (16.9%)	
Gene mutation			
CEBPA (+/-)	4/38	8/45	0.540
NPM1 (+/-)	8/34	6/47	0.385
FLT3-ITD (+/-)	5/37	8/45	0.768
c-KIT (+/-)	0/42	2/51	0.501
NRAS or KRAS (+/-)	4/38	3/50	0.696
IDH1/2 (+/-)	1/41	0/53	0.442
DNMT3A (+/-)	6/36	3/50	0.177
U2AF1 (+/-)	2/40	0/53	0.193
SRSF2 (+/-)	0/42	1/52	1.000

group was shorter than that of the *CRIP1*^{low} group in CN-AML patients (P = 0.012, **Figure 5F**). These results were further demonstrated in TCGA dataset and GSE12417 dataset (P < 0.05, **Figures 6** and **7**). Cox regression analysis was performed to determine the prognostic value of *CRIP1* expression in AML patients. Multivariate analysis included variables in univariate analysis with P < 0.200 [age (≤ 60 vs > 60 years), WBC (≥ 30×10^9/L vs < 30×10^9/L), *CRIP1* expression (high vs low), gene mutations (mutant vs wild type)]. *CRIP1* expression was identified as an independent risk factor in affecting LFS of CN-AML (P = 0.005, **Table 4**).

Correlation between CRIP1 expression and methylation in AML patients

To examine the methylation status of *CRIP1* promoter in AML patients, the MSP and BSP

Table 3. Comparison of clinical manifestations and laboratory features between AML patients with CR and Non-CR

Patient's parameters	CR (n = 54)	Non-CR (n = 76)	*P* value
CRIP1 expression	0.7 (0-32.3)	2.3 (0-169.2)	0.005
Sex, male/female	29/25	47/29	0.372
Median age, years (range)	46.5 (18-73)	61.5 (18-88)	< 0.001
Median WBC, $\times 10^9$/L (range)	6.5 (0.3-528.0)	33.8 (0.9-186.9)	< 0.001
Median hemoglobin, g/L (range)	77.5 (34-144)	81 (27-141)	0.727
Median platelets, $\times 10^9$/L (range)	30 (3-225)	42 (3-415)	0.093
BM blasts, % (range)	38.8 (1.0-97.5)	54.3 (6.5-99.0)	0.010
Karyotype classification			< 0.001
Favorable	20 (37%)	7 (9.2%)	
Intermediate	28 (51.9%)	45 (59.2)	
Poor	4 (7.4%)	10 (13.2%)	
No data	2 (3.7%)	14 (18.4)	
Karyotype			0.003
normal	25 (46.2%)	29 (38.3%)	
t (8;21)	7 (13%)	0 (0%)	
t (16;16)	0 (0%)	1 (1.3%)	
t (15;17)	14 (25.9%)	6 (7.9%)	
11q23	1 (1.9%)	3 (3.9%)	
+8	0 (0%)	2 (2.6%)	
-5/5q-	0 (0%)	1 (1.3%)	
-7/7q-	0 (0%)	2 (2.6%)	
t (9;22)	0 (0%)	1 (1.3%)	
others	0 (0%)	3 (3.9%)	
complex	3 (5.6%)	8 (10.5%)	
No data	4 (7.4%)	20 (26.4%)	
Gene mutation			
CEBPA (+/-)	6/38	6/36	1.000
NPM1 (+/-)	4/40	10/32	0.083
FLT3-ITD (+/-)	5/39	8/34	0.377
c-KIT (+/-)	2/42	0/42	0.494
NRAS or KRAS (+/-)	1/43	6/36	0.055
IDH1/2 (+/-)	0/44	1/41	0.488
DNMT3A (+/-)	3/41	3/39	1.000
U2AF1 (+/-)	0/44	2/40	0.236
SRSF2 (+/-)	0/44	1/41	0.488

primer sets were designed at the CpG islands of the *CRIP1* promoter (**Figure 8A**). Firstly, *CRIP1* methylation status was examined by MSP, and the results showed that no significant differences were observed between controls and AML patients (**Figure 8B**). Then, two controls and two AML patients were selected randomly to verify the MSP results by BSP. The *CRIP1* promoter was almost completely unmethylated not only in healthy donors but also in AML patients (**Figure 8C**). Differential methylation analyses were performed in the dataset GSE63409 (n = 74). Our analyses yielded 1628 significantly down-regulated genes (FDR < 0.05, log2 FC > 2, Supplementary File 2) in controls versus AML patients, however, *CRIP1* was not included. Moreover, there was no correlation between *CRIP1* expression level and *CRIP1* methylation level both in our group and in TCGA datasets (**Figure 9**).

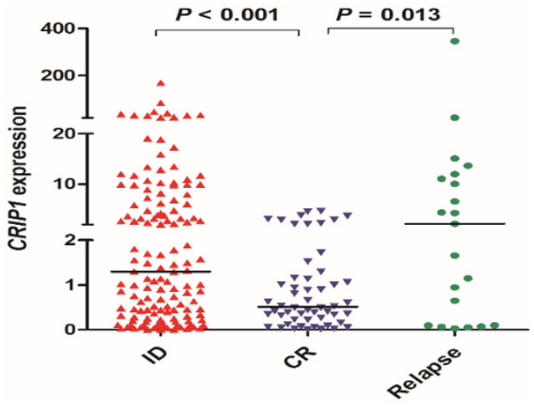

Figure 3. Expression of *CRIP1* in initial diagnosis (ID), complete remission (CR) and relapsed AML patients receiving induction therapy. *CRIP1* expression was increased in AML patients achieved CR than ID patients ($P < 0.001$). *CRIP1* expression was decreased in AML patients achieved CR compared with relapsed AML patients ($P = 0.013$).

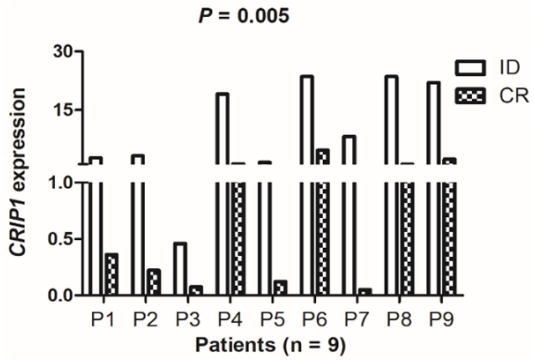

Figure 4. Changes of *CRIP1* expression in follow-up AML patients (n = 9) from the initial diagnosis (ID) to complete remission (CR). *CRIP1* expression significantly decreased after CR ($P = 0.005$).

Discussion

Increasing studies have shown that the abnormal expression of *CRIP1* is connected with the tumorigenesis of various solid tumors. It has been reported that *CRIP1* silencing inhibited cell migration and invasion in colorectal cancer SW620 and HT29 cells [27]. it was reported that the high expression level of *CRIP1* was associated with poor prognosis in endometrial carcinoma [28]. It has been reported that *CRIP1* acted as an oncogene during cell proliferation, migration and invasion in thyroid carcinoma [29]. It has been reported that *CRIP1* had a higher expression in cervical cancer tissues, promotes cell migration and invasion [30]. However, it was also reported that high expression of *CRIP1* in breast cancer suggested a better prognosis, and decreased expression of *CRIP1* can increase cell proliferation and activate cell growth [7]. These results indicate that *CRIP1* plays different roles in different types of tumors.

In the current study, we detected *CRIP1* transcript level in BM samples from AML patients, and observed that higher *CRIP1* expression was associated with lower CR rate. Moreover, *CRIP1* expression was significantly reduced in the patients who achieved CR after induction chemotherapy and significantly increased in the patients who achieved relapsed. These results indicate that *CRIP1* expression could be used to monitoring disease status.

Moreover, we aimed to investigate whether *CRIP1* could act as a potential biomarker for predicting prognosis in AML. It has been reported that *CRIP1* overexpression had a poor prognosis in AML patients, but they only analyzed the effect of *CRIP1* expression on OS, not on LFS [31]. Statistically, they only used univariate Cox proportional hazards regression, without further analyze through multivariate analysis [31]. Our study found that although *CRIP1* expression levels affected OS and LFS in the whole-cohort AML and CN-AML patients in univariate analysis, only LFS was affected in CN-AML patients according to multivariate analysis. In addition, the prognostic value of *CRIP1* expression was also confirmed by online data available in GEO and TCGA datasets. Taken together, above-mentioned data indicate that *CRIP1* expression may be useful as a biomarker to predict a worse chemotherapy response and prognosis in CN-AML patients. Of course, due to small cohort of the patients and different treatment regimen, large and independent cohort of studies and clinical trials are required to validate the prognostic value of *CRIP1* expression before it can be used routinely as a potential biomarker for risk stratification in CN-AML.

In addition to *CRIP1* expression, accumulating studies also showed that aberrant *CRIP1* methylation, regulating *CRIP1* expression, was frequently occurred in diverse human cancers [10, 16, 32]. We used MSP to assess *CRIP1* promoter methylation level in patients with AML. *CRIP1* promoter methylation density was also analyzed by BSP. Unfortunately, aberrant methyla-

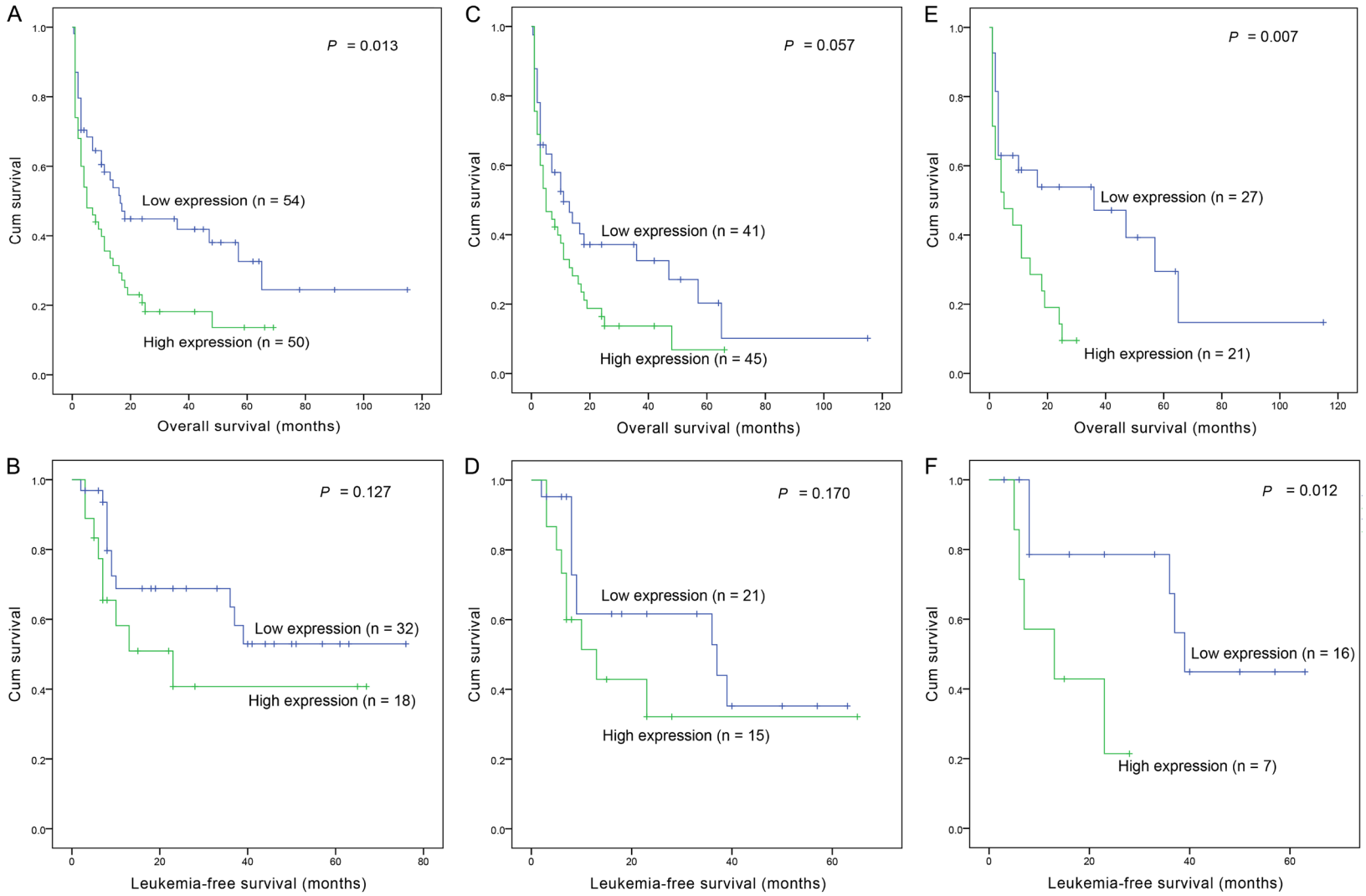

Figure 5. Overall survival (OS) and leukemia-free survival (LFS) between *CRIP1*^{high} and *CRIP1*^{low} groups. A, B: Whole-cohort AML patients; *CRIP1*^{high} group had a shorter OS than those *CRIP1*^{low} group in the whole-cohort AML patients ($P = 0.013$). C, D: Non-M3 AML patients; there was a trend that patients with *CRIP1*^{high} group had a shorter OS than those in *CRIP1*^{low} group in no-M3 AML ($P = 0.057$). E, F: CN-AML patients; *CRIP1*^{high} group had a shorter OS and LFS than those *CRIP1*^{low} group in CN-AML patients ($P < 0.05$).

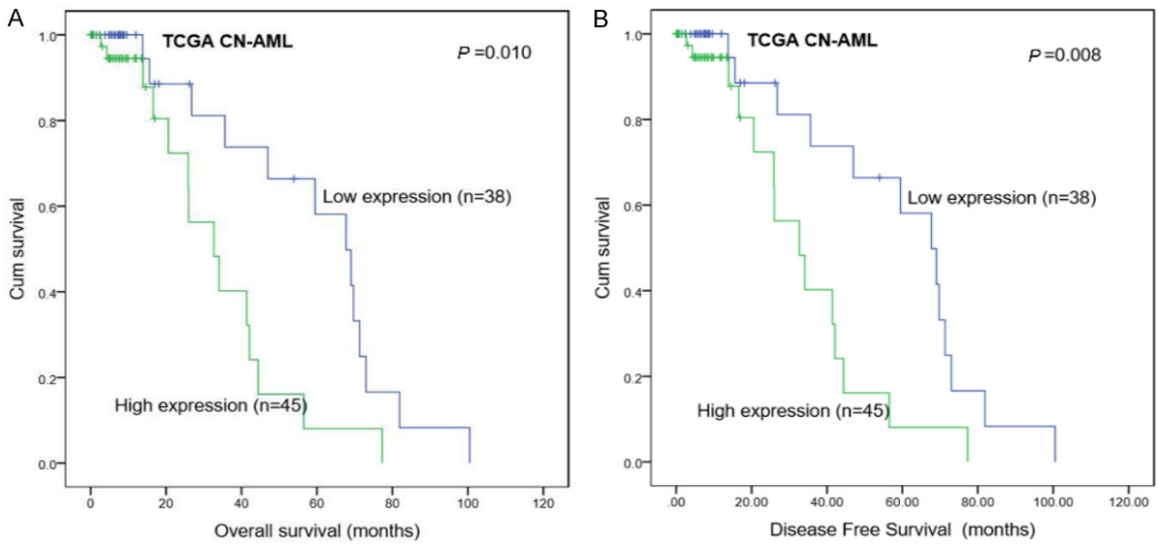

Figure 6. Overall survival and disease-free survival between *CRIP1*^{high} and *CRIP1*^{low} group among CN-AML patients in the TCGA cohort (*P* = 0.010 and 0.008, respectively).

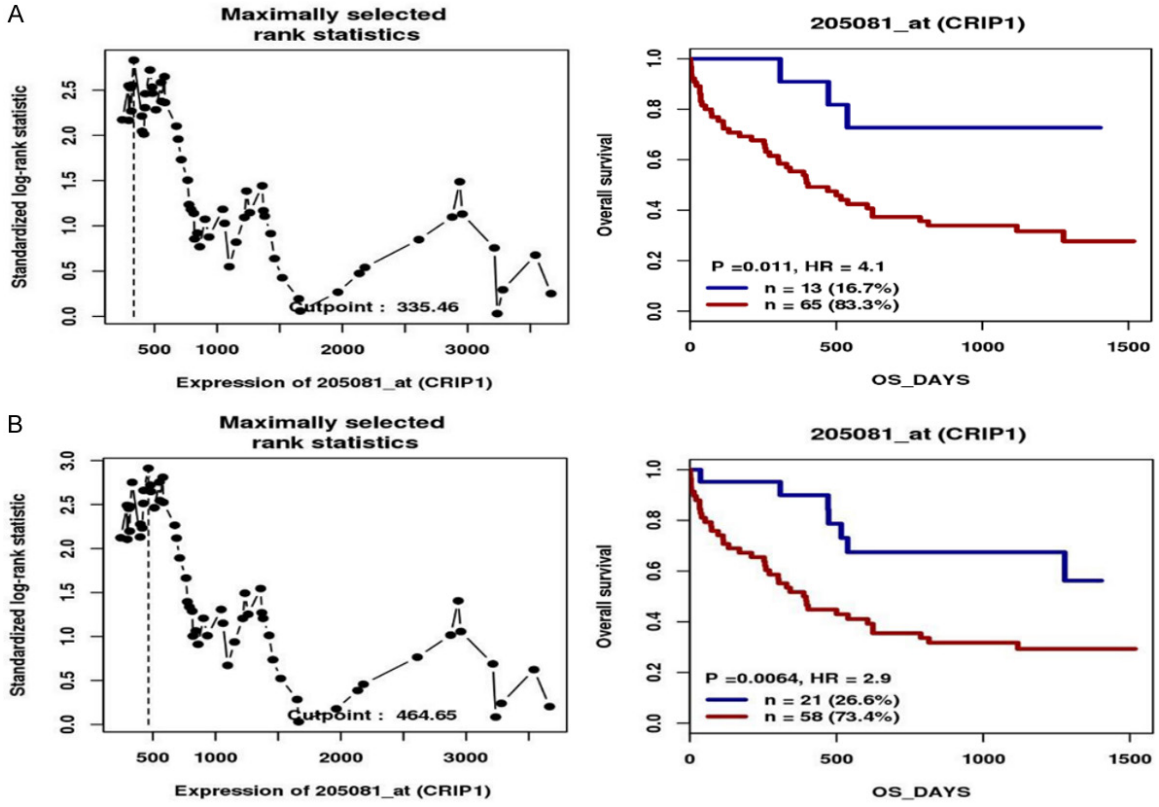

Figure 7. The impact of *CRIP1* expression on overall survival in CN-AML by bioinformatics analysis. An independent cohort of CN-AML patients was obtained from Gene Expression Omnibus data (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE12417). Survival analysis was performed through the online web tool Genomicscape (http://genomicscape.com/microarray/survival.php). A: Affy U133 plus 2; B: Affy U133A.

tion of *CRIP1* promoter was not identified in both AML patients and controls. The result suggested that *CRIP1* expression was not regulated by its promoter methylation in AML. These

Table 4. Univariate and multivariate analyses of prognostic factors for overall survival (OS) and leukemia-free survival (LFS) in CN-AML patients

Variables	OS				LFS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age	2.224 (1.134-4.363)	0.020	1.569 (0.716-3.441)	0.260	3.561 (1.236-10.259)	0.019	2.096 (0.663-6.630)	0.208
WBC	3.205 (1.586-6.479)	0.001	2.510 (1.144-5.508)	0.022	3.475 (1.185-10.192)	0.023	1.640 (0.494-5.443)	0.419
CRIP1 expression	2.526 (1.222-5.223)	0.012	1.720 (0.707-4.180)	0.232	6.986 (1.825-26.741)	0.005	6.986 (1.825-26.741)	0.005
CEBPA mutation	0.982 (0.336-2.869)	0.973	-	-	0.037 (0-36.528)	0.348	-	-
NPM1 mutation	0.776 (0.232-2.595)	0.680	-	-	0.543 (0.069-4.305)	0.563	-	-
FLT3-ITD mutation	0.678 (0.199-2.307)	0.534	-	-	0.716 (0.089-5.736)	0.753	-	-
c-KIT mutation	Undetermined	0.999	-	-	Undetermined	0.999	-	-
N/K-RAS mutation	0.986 (0.283-3.431)	0.982	-	-	0.039 (0-162.382)	0.445	-	-
IDH1/2 mutation	5.531 (0.666-45.960)	0.113	2.468 (0.276-22.059)	0.419	Undetermined	0.999	-	-
DNMT3A mutation	1.164 (0.398-3.402)	0.781	-	-	1.457 (0.307-6.915)	0.636	-	-
U2AF1 mutation	Undetermined	0.999	-	-	Undetermined	0.999	-	-
SRSF2 mutation	2.802 (0.361-21.718)	0.324	-	-	Undetermined	0.999	-	-

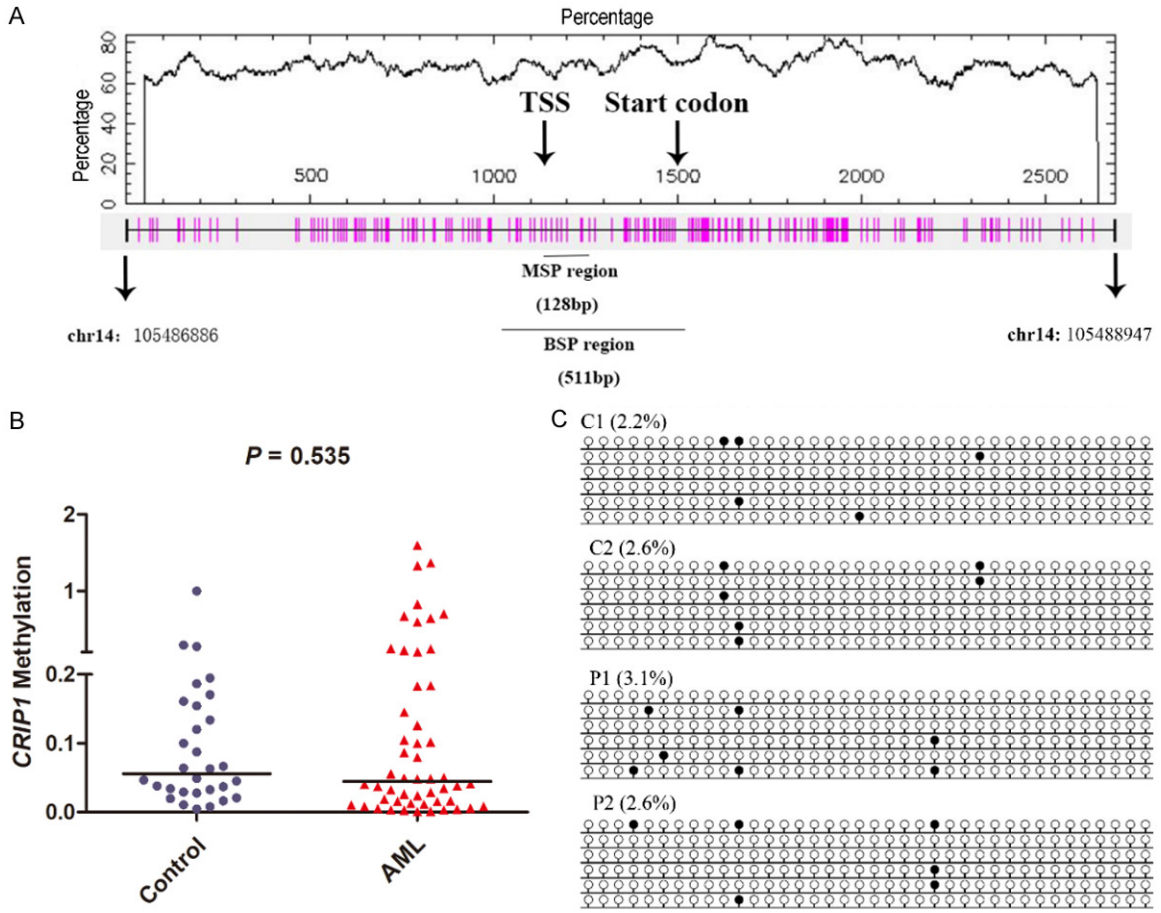

Figure 8. The genomic coordinates (GC) of *CRIP1* promoter region CpG island and primer locations. A. The panel plots the GC content as a percentage of the total. Each vertical bar in the bottom panel represents the presence of a CpG dinucleotide. Black horizontal bars indicate regions amplified by MSP primer pairs and BSP primer pairs. This figure was created using CpGplot (http://emboss.bioinformatics.nl/cgibin/emboss/cpgplot) and Methyl Primer Express v1.0 software. TSS: transcription start site; MSP: methylation-specific PCR; BSP: bisulfite sequencing PCR. B. Relative methylation level of *CRIP1* promoter in AML patients and controls. C. Methylation density of *CRIP1* promoter in AML patients and controls. Methylation density was determined by BSP. White cycle: unmethylated CpG dinucleotide, Black cycle: methylated CpG dinucleotide. C1 and C2: controls; P1 and P2: AML patients.

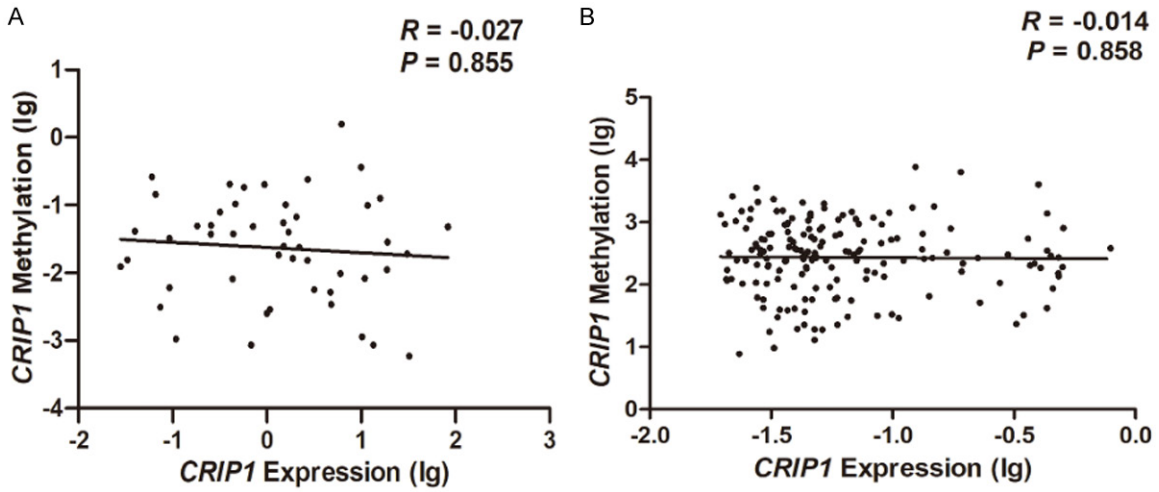

Figure 9. Correlation between *CRIP1* methylation and *CRIP1* expression in our group (A; R = -0.027, P = 0.855) and TCGA datasets (B; R = -0.014, P = 0.858).

results were also confirmed by the analysis of the GEO and TCGA datasets.

In conclusion, methylation-independently high *CRIP1* expression is independently associated with unfavorable prognosis in CN-AML.

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Disclosure of conflict of interest

None.

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