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Original Article

Upregulation of lncRNA CCAT2 predicts poor prognosis in patients with acute myeloid leukemia and is correlated with leukemic cell proliferation

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Abstract: Increasing data have shown that the dysregulation of long non-coding RNAs (lncRNAs) is associated with a variety of human cancers, including acute myeloid leukemia (AML). Colon cancer-associated transcript-2 (CCAT2) gene encodes an lncRNA CCAT2 whose over-activation was observed in many human solid tumors. However the expression and clinical significance of CCAT2 in AML have not been identified. In the study, we found that CCAT2 expression levels in patients with AML were significantly increased compared with healthy individuals. The patients with highly expressed CCAT2 had higher white blood cells than those patients with low CCAT2. The incidence of FLT3/ITD mutation in the patients with high CCAT2 expression was significantly higher than in those patients with low CCAT2 expression. High CCAT2 expression was correlated with more monosomal karyotype and poor risk stratification. Furthermore, patients with high CCAT2 had significantly shorter overall survival times than those patients with low CCAT2. Univariate and multivariate Cox's analyses indicated a poor prognostic value of high CCAT2 in AML patients. Moreover, in vitro assay revealed that overexpression of CCAT2 promoted KG-1 cell proliferation and induced cell cycle arrest at the S phase, whereas CCAT2 knockdown inhibited proliferation by inducing cell-cycle arrest at the G2/M phase. In conclusion, our study demonstrates for the first time that CCAT2 is highly expressed in AML patients, and it associates with poor prognosis and leukemic cell proliferation.

Keywords: AML, lncRNAs, CCAT2, prognosis, KG-1, proliferation

Introduction

Leukemia starts in the blood-forming tissue and produces high numbers of abnormal white blood cells [1], including 4 main types: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML). Among them, AML is the most common malignant myeloid disorder, with an incidence of 3~4 per hundred thousand persons per year [2]. AML is a genetically heterogeneous cancer characterized by somatically acquired genetic changes in hematopoietic progenitor cells altering normal mechanisms of self-renewal, differentiation and growth [3]. Treatment of AML always involves some combination of radiation therapy, chemotherapy, targeted therapy, and bone marrow (BM) transplant [4, 5]. The 5-year survival rate of AML has been greatly improved, but remains unsatisfactory. Therefore, a better understanding of AML will be helpful in identifying new therapeutic approaches for this cancer.

The long non-coding RNAs (lncRNAs) are greater than 200 nucleotides in length and unable to be translated into proteins [6]. Increasing evidence has shown that lncRNAs are aberrantly expressed in human cancer, and function as oncogenes or tumor suppressor genes [7, 8]. For example, Xi, et al [9] showed that lncRNA lncFOXO1 suppresses growth of human breast cancer cells. Chen, et al [10] identified that lncRNA CRNDE promotes hepatic carcinoma cell proliferation, migration, and invasion. Ma, et al [11] found that overexpression of lncRNA AFAP1-AS1 predicts poor prognosis and promotes cells proliferation and invasion in gallbladder cancer. In addition, Chen, et al [12]

demonstrated that upregulation of lncRNA HOTTIP promotes metastasis of esophageal squamous cell carcinoma. However, the clinical significance and biological function of lncRNAs in AML remain largely unexplored.

The colon cancer-associated transcript 2 (CCAT2), a novel lncRNA mapping to human 8q24 genomic region, was first discovered in colon cancer [13]. CCAT2 is highly expressed in colorectal cancer and promotes tumor cell growth and metastasis. In a subsequent study, CCAT2 was found to be upregulated in breast cancer patients and associates with tumor metastasis [14]. Recently, Xu, et al [15] reported that CCAT2 is associated with poor prognosis in hepatocellular carcinoma and promotes tumor metastasis. Wu, et al [16] showed that high expression of CCAT2 indicates poor prognosis of gastric cancer and promotes cell proliferation and invasion. However, to our knowledge, whether CCAT2 expression is abnormal and its clinical significance in AML have not been reported.

Therefore, we performed a study to show the expression profiles of CCAT2 in AML patients, as well as determine the correlation between CCAT2 expression and clinicopathological characteristics. We found that the expression of CCAT2 was upregulated in AML patients, and high CCAT2 expression was associated with higher white blood cells, more FLT3/ITD mutation, more monosomal karyotype, poor risk stratification, and worse overall survival in AML patients. Furthermore, we also observed the effects of CCAT2 on proliferation and cell cycle of KG-1 cells by RNA interference and overexpression. Our results may provide direct evidence for the role of CCAT2 in AML, shedding new light on the treatment for this disease.

Materials and methods

Patients and tissue samples

This study included 46 AML patients with age from 18~81 years and median age at 58.42 ± 9.65 years, including 32 males and 14 females. The patients had a new diagnosis of AML and were treated at Department of Hematology, The First Affiliated Hospital of Bengbu Medical College (Bengbu, China) between January 2010 to March 2014. The diagnosis and classification of AML patients were based on French-America-British (FAB) and World Health Organization (WHO) criteria [17]. Patients were followed up for a median 21 months (range 4~39 months); patients alive at the time of last follow-up were censored on that date. Overall survival (OS) was defined as the time from the diagnosis of AML to any cause of death. A control group of 46 healthy volunteers with no clinical symptoms of cancer or other disease was also taken into account (aged 21~79 years, average aged 60.12 ± 8.47 years). The bone marrow samples were collected from AML patients and healthy individuals who were the hematopoietic stem cell donors. All samples were flash-frozen in liquid nitrogen immediately and stored at -80°C until further use. Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of The First Affiliated Hospital of Bengbu Medical College.

RNA extraction and reverse transcription

Total RNA was extracted using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocols. RNA was reversed transcribed into cDNA using a Primer-Script one step RT-PCR kit (Takara, Japan). The reverse transcription reaction was conducted under the following conditions: 37°C for 30 min, 85°C for 5 s and then hold on 4°C. The cDNA was stored at -80°C until further use.

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR assay was performed on the Roche LightCycler 480 Real-Time PCR System (Roche, USA) by using a SYBR Premix Ex Taq™ kit (Takara, Japan) to determine the expression level of CCAT2. Primers specific for human CCAT2 were 5'-CCCTGGTCAAATTGCTTAAC-3' (forward), and 5'-TTATTCGTCCCTCTGTTTTATGG-3' (reverse). The human GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. The primers for GAPDH were 5'-CCACATCGCTCAGACACC-3' (forward) and 5'-ACCAGGCGCCCAATA-3' (reverse). qPCR conditions consisted of 30 min of DNA polymerase activation at 98°C, followed by 40 cycles of 98°C for 10 sec and 60°C for 30 sec. The results were analyzed using the Applied Biosystems Comparative CT Method [18].

Gene mutation detection

The mutations of IDH1/2, DNMT3A, NRAS or KRAS, NPM1, C-KIT and U2AF1 were detected

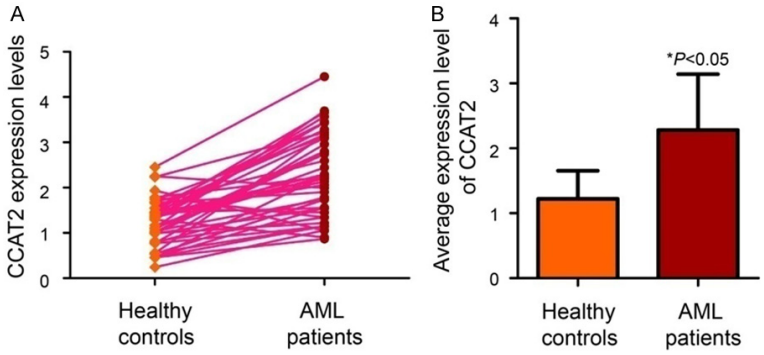

Figure 1. Relative expression levels of lncRNA CCAT2 in AML patients and healthy controls. A. Comparison of CCAT2 expression in bone marrow from healthy volunteers (n = 46) and AML patients (n = 46). CCAT2 expression was detected by qRT-PCR and normalized to the GAPDH. B. The average expression level of CCAT2 in AML patients was 1.87 times higher than that of the healthy individuals. CCAT2: colon cancer-associated transcript-2, qRT-PCR: quantitative Real-time RT-PCR, AML: acute myeloid leukemia. *P < 0.05.

as previously reported [19]. All samples determined positive by high-resolution melting analysis (HRMA) were further confirmed by Sanger DNA sequencing. FLT3/ITD and CCAAT enhancer binding protein alpha (C/EBPA) mutations were directly detected by Sanger DNA sequencing [19].

Cell culture

AML cell line (KG-1) was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). KG-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gaithersburg, MD, USA) and supplemented with 10% fetal bovine serum (FBS, Gaithersburg, MD, USA), 100 U/ml penicillin (Sigma-Aldrich, MO, USA), and 100 mg/ml streptomycin (Sigma-Aldrich, MO, USA) at 37°C and 5% CO_2.

Transfection

The siRNA specifically targeting CCAT2 (siRNA-CCAT2) and siRNA negative control (siRNA-NC) were purchased from RiboBio Co., Ltd (Guangzhou, China). The CCAT2 over-expression plasmid, pcDNA3.1 + CCAT2, was obtained from GenePharma Co., Ltd (Shanghai, China), and pcDNA3.1 + vector was used as an empty control. KG-1 cells (8 × 10^4/well) were plated in 24-well plates overnight. Cells were then transfected with 20 pM siRNA-CCAT2 or siRNA-NC and 2 μg pcDNA3.1 + CCAT2 or pcDNA3.1 + vector for 48 h using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Following the transfections, KG-1 cells were then harvested for qRT-PCR.

Cell proliferation assay

Cell proliferation was quantified daily on days 0~4 with the CCK-8 kit (Dojindo, Japan) according to the manufacturer's protocols. KG-1 cells were plated at a density of 3 × 10^4 cells/well in 96-well plates and cultured in 100 μL of complete medium. The CCK-8 solution (10 μL) was added to each well at the end of the experiment, and the cells were incubated for an additional 4 h. The absorbance in the plates was determined using a microplate reader at 450 nm.

Cell cycle analysis

KG-1 cells were plated at a density of 2 × 10^5 cells/well in 6-well plates and cultured in 2 ml of complete medium overnight. The cells were then transfected with siRNA-CCAT2 or siRNA-NC and pcDNA3.1 + CCAT2 or pcDNA3.1 + vector. At 48 h post-transfection, KG-1 cells were fixed with 70% ice-cold ethanol overnight. The cells were resuspended in PBS containing 10 μg/ml propidium iodide (PI, BD Biosciences, San Jose, CA, USA), 0.1% Triton, and 20 μg/ml RNase A and were incubated for 30 min in the dark. Finally, the samples were detected with BD Accuri C6 Flow Cytometry (BD Biosciences, San Jose, CA, USA) and analyzed with WinMDI version 2.9 software (Scripps Research Institute, La Jolla, CA, USA).

Statistical analyses

Statistical analyses in this study were performed by using SPSS software version 17.0 (SPSS, Chicago, IL, USA). Data are presented as the means ± SD (standard deviation). The significance of differences between groups was estimated by Student's t-test or ANOVA followed by Bonferroni post hoc test, as appropriate. Pearson's chi-square analysis or Fisher's exact

Table 1. Correlation between CCAT2 expression and patient findings

Patient findings	Low CCAT2 (n = 20)	High CCAT2 (n = 26)	*P*
Age, years	57.13 ± 7.42	58.96 ± 8.81	0.67
Sex, male/female	19/8	13/6	0.37
White blood cells counts, × 10^9/L	12.64 ± 3.45	19.13 ± 4.82	0.03
Hemoglobin, g/L	72.56 ± 17.42	75.33 ± 16.15	0.24
Platelet count, × 10^9/L	46.61 ± 9.72	42.07 ± 10.55	0.19
Percentage of blasts, %	52.13 ± 13.66	47.17 ± 11.28	0.15
U2AF1 (+/-) mutation, %	15.38	15.0	0.89
DNMT3A (+/-) mutation, %	19.23	20.0	0.77
IDH1/2 (+/-) mutation, %	7.69	10	0.46
NRAS or KRAS (+/-) mutation, %	26.92	30	0.40
C-KIT (+/-) mutation, %	3.84	5.0	0.63
NPM1 (+/-) mutation, %	23.07	25.0	0.70
FLT3/ITD (+/-) mutation, %	15.38	55.0	0.00
C/EBPA (+/-) mutation, %	7.69	5.0	0.58
FAB classification			
M1~M6	24	17	0.64
M7	2	3	
Risk stratification			
Favorable + intermediate	22	6	0.00
Poor	4	14	
Monosomal karyotype			
Yes	1	9	0.00
No	25	11	

FAB, French-American-British classification; AML, acute myeloid leukemia.

test was used to compare the correlation between CCAT2 level and clinicopathological features. Kaplan-Meier survival curve was used for survival analysis, and the statistical indicators in the survival analysis were included in Cox's proportional hazard regression model analysis. Results were considered significant when *P* was < 0.05.

Results

Upregulation of CCAT2 in bone marrow of AML patients

The CCAT2 expression levels were detected in bone marrow samples from with AML patients and healthy controls by qRT-PCR. As shown in **Figure 1A**, the expression of CCAT2 was markedly upregulated in AML patients relative to healthy controls. The average expression level of CCAT2 relative to GAPDH in AML patients was 1.87 times higher than that of the healthy individuals (**Figure 1B**, $P < 0.05$). This data suggest that CCAT2 upregulation may be associated with the pathogenesis of AML.

Correlation between CCAT2 expression and AML clinical parameters

To evaluate the impact of CCAT2 expression on clinical outcome, the average level of CCAT2 (2.28, 0.87~4.45) in 46 AML patients was used as the cut-off point to define the low CCAT2 expression group (≤ 2.28, n = 20) and high CCAT2 expression group (> 2.28, n = 26). The correlation between CCAT2 expression and AML clinical parameters were analyzed. As shown in **Table 1**, high CCAT2 expression was correlated with white blood cells count, FLT3-ITD mutation, monosomal karyotype and risk stratification. There was no significant association of the CCAT2 expression between the two groups in other clinical features, including sex, age, hemoglobin, platelet count, percentage of blasts in bone marrow (BM), and FAB classification. Our results indicate that that high CCAT2 expression is involved in AML development.

Further analyses found that patients with high CCAT2 expression had a higher white blood cell count than those patients with low CCAT2 expression (**Figure 2A**, $P < 0.05$). Among these mutations, no one except for FLT3/ITD mutation was validated to have a difference between low CCAT2 expression and high CCAT2 expression groups. AML patients with high CCAT2 expression had a significantly higher incidence of FLT3/ITD mutation as compared with those with low CCAT2 expression (**Figure 2B**, $P < 0.05$).

Impact of CCAT2 expression on overall survival of AML patients

To investigate the prognostic impact of CCAT2 expression on AML patients, overall survival

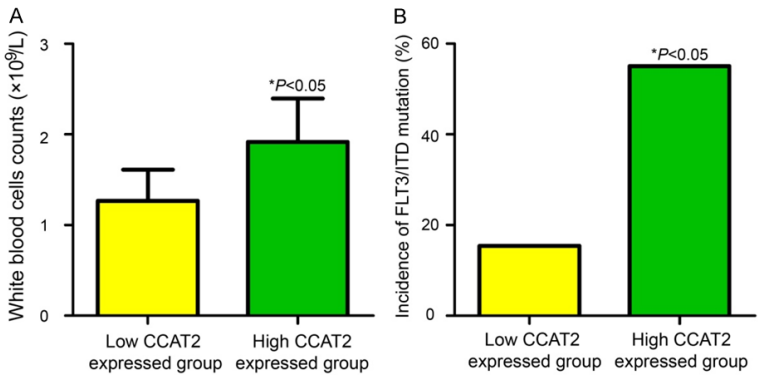

Figure 2. Comparison of the differential counts of white blood cells and differential incidences of FLT3/ITD mutation in high and low CCAT2 groups. A. The patients with highly expressed CCAT2 had higher white blood cell counts than those patients with low CCAT2. The average level of CCAT2 (2.28, 0.87~4.45) in 46 AML patients was used as the cut-off point to define the low CCAT2 group (≤ 2.28, n = 20) and high CCAT2 group (> 2.28, n = 26). B. Statistical analyses showed that AML patients with high CCAT2 expression had a significantly higher incidence of FLT3/ITD mutation compared with those with low CCAT2 expression. *$P < 0.05$.

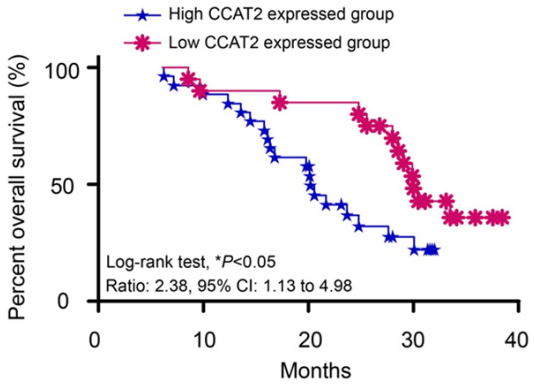

Figure 3. The impact of CCAT2 expression on overall survival (OS) of AML patients. Patients with high CCAT2 had significantly shorter overall survival than those patients with low CCAT2. *$P < 0.05$.

(OS) analysis was performed in 46 cases with follow up data. Kaplan-Meier curve showed that the OS of AML patients with high CCAT2 expression was shorter than those with low CCAT2 expression (**Figure 3**, $P < 0.05$). Furthermore, univariate Cox's proportional hazards regression model analysis was used for the assessment of factors affecting OS, which exhibited that high CCAT2 expression and poor risk stratification were associated with shorter OS in AML patients (**Table 2**, $P < 0.05$). Multivariate Cox's proportional hazards regression model analysis suggested that high CCAT2 expression was an independently predictive factor for worse OS in AML patients (**Table 2**, $P < 0.05$). This finding indicates that CCAT2 can be used as a prognostic biomarker in AML.

Manipulation of CCAT2 expression in AML cells

To investigate the functional effects of CCAT2 in leukemic cells, siRNA-CCAT2 or pcDNA-3.1 + CCAT2 vector were transfected into KG-1 cells to knock down and overexpress CCAT2. qRT-PCR analysis of CCAT2 level was performed 48 h post-transfection. When compared with siRNA-NC treated cells, CCAT2 expression was knocked down by 64.23% in KG-1 cells by siRNA-CCAT2 (**Figure 4A**, $P < 0.05$). The expression level of CCAT2 was up-regulated significantly in KG-1 cells after transfected with pcDNA3.1 + CCAT2 vector, the relative expression of CCAT2 was up-regulated about 8.71-fold over the pcDNA3.1 + vector (**Figure 4B**, $P < 0.05$). Thus, siRNA-CCAT2 and pcDNA3.1 + CCAT2 vector were used in subsequent experiments.

CCAT2 facilitates leukemic cell proliferation and modulates cell cycle

To investigate whether CCAT2 is involved in the development of AML, we evaluated the effect of CCAT2 on cell proliferation. CCK-8 assay revealed that the proliferation ability of KG-1 cells was significantly suppressed at 24 h, 48 h, and 72 h after transfection of siRNA-CCAT2 (**Figure 5A**, $P < 0.05$), whereas overexpression of CCAT2 markedly increased cell proliferation in KG-1 cells (**Figure 5B**, $P < 0.05$). To further evaluate whether the CCAT2-mediated cell proliferation was due to cell cycle change, we then examined the effect of CCAT2 on cell cycle of KG-1 cells. The results showed that knockdown of CCAT2 induced cell cycle arrest at G2/M phase in KG-1 cells (**Figure 6A**, $P < 0.05$). Conversely, overexpression of CCAT2 decreased the percentage of G2/M phase cells and induced cell cycle arrest at S phase (**Figure 6B**, $P < 0.05$).

Table 2. Cox proportional hazards regression model analysis of factors affecting OS

Patients' parameters	Univariate Cox's regression		Multivariate Cox's regression	
	P	HR (95% CI)	*P*	HR (95% CI)
White blood cells counts (> 20/≤ 20 × 10^9/L)	0.28	1.41 (0.63~2.57)	-	-
FLT3/ITD (+/-) mutation	0.67	1.05 (0.50~2.36)	-	-
Risk stratification (Poor/Favorable + intermediate)	0.00	2.17 (1.45~3.98)	0.07	1.94 (1.30~3.66)
Monosomal karyotype (Yes/No)	0.20	1.68 (0.71~3.36)	-	-
CCAT2 expression (High/Low)	0.00	2.38 (1.13~4.98)	0.02	2.26 (0.85~5.33)

OS, overall survival; HR, hazards ratio; 95% CI, 95% confidence interval.

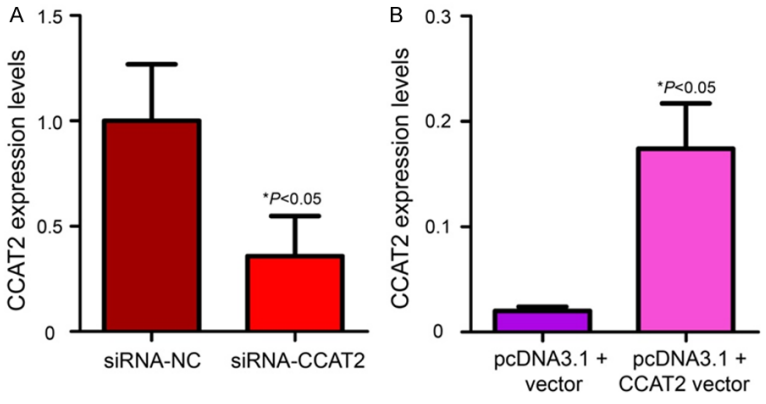

Figure 4. Manipulation of CCAT2 expression in AML cells. A. qRT-PCR analysis of CCAT2 expression after siRNA-CCAT2 or siRNA-NC transfection 48 h in KG-1 cells. B. Comparison of CCAT2 level in KG-1 cells after treated with pcDNA3.1 + CCAT2 vector or pcDNA3.1 + vector. siRNA-CCAT2: siRNA specifically targeting CCAT2, siRNA-NC: siRNA negative control, pcDNA3.1 + CCAT2 vector: CCAT2 over-expression plasmid. *P < 0.05.

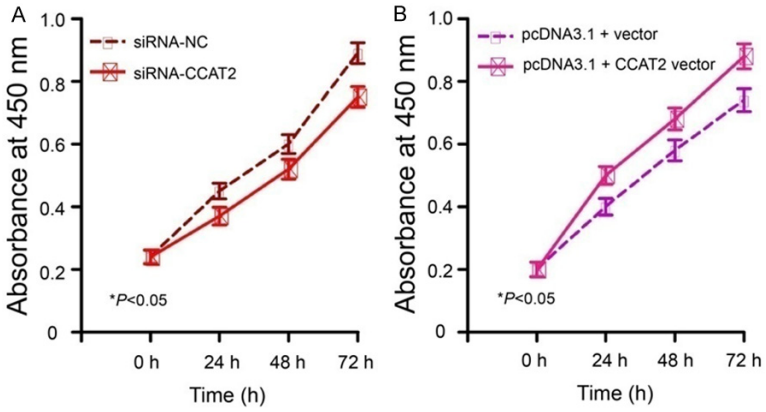

Figure 5. CCAT2 facilitates leukemic cell proliferation in vitro. A. CCK-8 assay showed that proliferation of KG-1 cells was significantly inhibited after transfection of siRNA-CCAT2. B. CCAT2 upregulation promoted the growth of KG-1 cells. *P < 0.05.

Discussion

Extensive studies have established the regulatory roles of protein-coding genes in the initiation, maintenance, and development of AML, which constitutes our main knowledge of the pathogenesis of AML. Since the prognosis of AML is still poor, more efforts are required to develop novel therapies. The latest studies have demonstrated that lncRNAs contribute to many critical signaling pathways in AML development and therapy [20]. For example, lncRNA HOTAIR is upregulated and indicates a poor prognosis in AML patients [21]. Overexpression of lncRNA PVT1 is correlated with leukemic cell proliferation in AML [22]. High expression of lncRNA CRNDE presents as a biomarker for AML and promotes the malignant progression in AML cell line U937 [23]. LncRNA UCA1 promotes cell proliferation, migration and invasion of AML cells [24]. However, the related research of lncRNAs function and clinical significance in AML is far from being fully elucidated.

Recently, lncRNA CCAT2 has been demonstrated to be over-expressed in hepatocellular carcinoma, colorectal cancer, and gastric cancer [25]. Ectopic expression of CCAT2 facilitates tumor cell growth, metastasis, and chromosomal instability [26, 27]. In addition, Wu, et al [14] showed that CCAT2 expression in breast cancer without metastasis is decreased compared with breast cancer metastasis; knockdown of

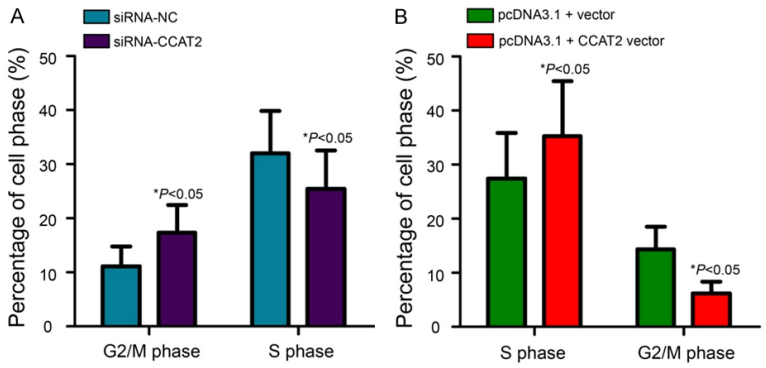

Figure 6. CCAT2 modulates the cell cycle in vitro. A. Analysis of cell cycle transformation was carried out 48 h after siRNA-CCAT2 or siRNA-NC transfection in KG-1 cells. Knockdown of CCAT2 increased the percentages of KG-1 cells in the G2/M phase. B. Analysis of cell cycle transformation in KG-1 cells after treated with pcDNA3.1 + CCAT2 vector or pcDNA3.1 + vector. Elevated expression of CCAT2 increased the percentages of KG-1 cells in the S phase. $*P < 0.05$.

CCAT2 inhibits the proliferation, invasion and migration in breast cancer cells. Although there are a few reports demonstrating that CCAT2 plays an important role in the pathogenesis of several cancers, it is not yet clear whether CCAT2 is involved in the regulation of AML. Thus, the aim of this study was to investigate the correlations of CCAT2 expression with clinicopathologic features and prognosis of AML patients. Our results showed that CCAT2 expression was significantly higher in bone marrow samples of AML patients compared with that in healthy controls. High CCAT2 expression was associated with higher white blood cells, more monosomal karyotype, and poor risk stratification. The presence of increased CCAT2 expression in AML was consistent with results from previous reports [25]. To the best of our knowledge, the study is the first to determine CCAT2 expression in AML. There data suggest upregulation of CCAT2 expression is a common event in AML.

Previous studies have reported that FLT3/ITD mutation, known as a poor prognostic factor in leukemia, was associated with higher peripheral WBC counts and a higher bone marrow blast percentage [19, 28]. Our study showed that patients with high CCAT2 expression had a high incidence of FLT3/ITD mutation. The exact relationship between upregulation of CCAT2 and FLT3/ITD mutation needs further experimental studies. Subsequently, high CCAT2 expression was validated to be associated with shorter OS in AML patients and was proven to be an adverse prognostic factor in AML. We also confirmed in Cox's proportional hazard regression model analysis that high CCAT2 expression could serve as a significant and independent predictor of AML. These results indicate that CCAT2 may be an important modulator involved in AML development, and can be used routinely as a potential biomarker for risk stratification in AML.

Based on the above data, we further elucidated the function of CCAT2 during cell proliferation and the cell cycle. A cell growth experiment in vitro confirmed that overexpression of CCAT2 significantly promoted KG-1 cell proliferation, whereas CCAT2 knockdown markedly suppressed cell proliferation. The analysis of the cell cycle of cells transfected with siRNA-CCAT2 revealed a significantly increased percentage of cells in the G2/M phase and a decreased percentage of cells in the S phase. Transfection of pcDNA3.1 + CCAT2 vector appeared to increase the proliferation of KG-1 cells by inducing cell cycle arrest at the S phase. These results suggest that the CCAT2 is involved in leukemic cell proliferation.

Taken together, our data offer convincing evidence that upregulation of CCAT2 is a common event in AML. High CCAT2 expression associates with advanced disease and poor prognosis in AML patients, and it is correlated with AML cell proliferation. This is, to our knowledge, the first study of a potential role for CCAT2 in AML. Further investigations should focus on how CCAT2 may affect the development of AML at molecular level.

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Disclosure of conflict of interest

None.

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