Role of Circular RNA DLEU2 in Human Acute Myeloid Leukemia

Dong-Mei Wu,^{a,b} Xin Wen,^{a,b} Xin-Rui Han,^{a,b} Shan Wang,^{a,b} Yong-Jian Wang,^{a,b} Min Shen,^{a,b} Shao-Hua Fan,^{a,b} Zi-Feng Zhang,^{a,b} Qun Shan,^{a,b} Meng-Qiu Li,^{a,b} Bin Hu,^{a,b} Gui-Quan Chen,^c Jun Lu,^{a,b} Yuan-Lin Zheng^{a,b}

^aKey Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Jiangsu Normal University, Xuzhou, People's Republic of China

^bCollege of Health Sciences, Jiangsu Normal University, Xuzhou, Jiangsu Province, People's Republic of China

^cState Key Laboratory of Pharmaceutical Biotechnology, MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, Nanjing, People's Republic of China

ABSTRACT In the current study, we were interested in exploring the molecular mechanism of circular RNA DLEU2 (circRNA-DLEU2) (hsa_circ_0000488) and microRNA 496 (miR-496), as well as PRKACB, in human acute myeloid leukemia (AML) cell activities. The RNA expression levels of circRNA-DLEU2, hsa-miR-496, and PRKACB were assessed by quantitative real-time PCR (qRT-PCR). The proliferation and apoptosis abilities of the cells were determined by CCK8 assay and flow cytometry analysis. Target relationships between circRNA-DLEU2 and miR-496, as well as PRKACB, were analyzed by luciferase reporter assay and probe assay. Immunoblotting assays were used to detect the protein expression level of PRKACB. We also did *in vivo* experiments to observe tumor formation after overexpression of circRNA-DLEU2. Our data showed that circRNA-DLEU2 was upregulated in AML tissues and cells, which promoted AML cell proliferation and inhibited cell apoptosis. circRNA-DLEU2 promoted AML tumor formation *in vivo*. miR-496 was inhibited by circRNA-DLEU2 and was downregulated in AML tissues. circRNA-DLEU2 inhibited miR-496 expression and promoted PRKACB expression. miR-496 antagonized the effects of PRKACB on MOLM-13 cell proliferation and apoptosis. Collectively, circRNA-DLEU2 accelerated human AML by suppressing miR-496 and promoting PRKACB expression.

KEYWORDS AML, PRKACB, circRNA-DLEU2, hsa-miR-496

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and shows significantly multifaceted biological and clinical heterogeneity within different patients (1). AML is a heterogeneous hematological malignancy with poor prognosis, due in part to rapid disease progression and frequent emergence of drug resistance (2). The current therapeutic approach to leukemia is generally chemotherapy to achieve complete remission. However, severe side effects and complications, such as serious infection and bleeding owing to anticancer drugs, are major problems (3). Several AML prognostic biomarkers have been identified as molecular genetic advances; among them, molecular abnormalities were considered the most crucial factors (4–6). However, more prognostic or predictive biomarkers are required to contribute to AML therapy.

Circular RNAs (circRNAs), a class of endogenous noncoding RNAs (ncRNAs), have a closed-loop structure because of a covalent junction between their 3' and 5' ends (7). With the development of high-throughput sequencing technology, a large number of circRNAs have been reported to play indispensable roles in human fetal development, myocardial infarction, and cancer, including hematopoietic malignancies (7–9). Additionally, circRNAs can sponge microRNA (miRNA) to regulate gene expression (10). For instance, hsa_circ_0018289 is upregulated in cervical cancer, which promotes cell

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Address correspondence to Jun Lu,
lu-jun75@163.com, or Yuan-Lin Zheng,
ylzheng@jnu.edu.cn.

proliferation and metastasis by targeting miRNA 497 (miR-497) (11). Memczak et al. demonstrated that circRNA UBAP2 was notably increased in osteosarcoma tissues, which enhanced cell growth and impeded apoptosis both *in vitro* and *in vivo* by inhibiting miR-143 expression (12). Recently, evidence regarding the function of circRNAs in AML patients has emerged. Hirsch et al. delineated the expression pattern of nucleophosmin 1 (NPM1) circular RNA in different AML subgroups (13), L'Abbate et al. observed significant overexpression of circPVT1 in amplicons involving chromosome band 8q24 in 23 cases of AML (AML-amp) cases (14), and Li et al. reported that hsa_circ_0004277 can be a novel biomarker for AML treatment (4). To further investigate the roles of circRNAs in the development of AML, we first performed *in silico* analysis of the AML-specific circRNA microarray and ascertained the differentially expressed circRNA-DLEU2 in cytogenetically normal AML (CN-AML) patients compared with healthy controls. Our study also investigated the effects of circRNA-DLEU2 on the proliferation and reproduction of AML-related cells and its functions in regard to miR-496 and PRKACB.

miRNAs are a subset of small noncoding RNAs that control gene expression in a site-specific manner (15). Accumulating evidence has identified the distinct expression patterns and biological functions of miRNAs in biological processes, including cell differentiation, apoptosis, and metabolism (16). Overexpression of miR-217 inhibited cellular proliferation and enhanced cell chemosensitivity to doxorubicin by the cell apoptosis pathway in AML cells (17). Some studies have clarified the potential roles of miRNAs as diagnostic and prognostic biomarkers in leukemia (18). For example, Li et al. showed that increased expression of miR-181a or miR-181b was significantly associated with longer overall survival (OS) in AML patients (19). Wang et al. found that miR-216b was upregulated in AML and independently conferred a poor prognosis (20). Based on *in silico* analysis, we selected miR-496 to conduct our study. A previous study verified that miR-496 was downregulated in prostate cancer (21). However, the function of miR-496 in leukemia remains largely unknown.

The PRKACB gene encodes cAMP-dependent protein kinase A (PKA) catalytic subunit β and is located on chromosome 1p31.1 (22). The PRKACB protein belongs to the Ser/Thr protein kinase family and is a pivotal modulator of the cAMP/PKA pathway, which is intimately correlated with a host of cellular processes, including cell reproduction, gene transcription, and metabolism (23). Some studies have reported the interaction of PRKACB and miRNAs in human cancers. For instance, PRKACB could be directly regulated by miR-200c in breast cancer cells (24). Wang et al. demonstrated that inhibition of miR-372 led to reduced translational repression of PRKACB in liver cancer (25). However, few studies have reported the effects of PRKACB on AML development. We explored the relationship of PRKACB and miR-496 in AML.

The current study investigated the molecular mechanism of circRNA-DLEU2 in AML cell activities. We also confirmed that circRNA-DLEU2 acts as an miR-496 sponge, which is negatively correlated with the expression of PRKACB. The present findings have therapeutic implications and may provide novel therapeutic targets for AML.

RESULTS

circRNA-DLEU2 is upregulated in AML. Based on the circRNA microarray [GSE94591](#), we screened for 30 remarkably differentially expressed circRNAs by fold change value and *P* value between AML patients and a healthy control via bioinformatics analyses. The data were visualized in a clustered heat map with 10 circRNAs upregulated in the healthy control, 15 in better-risk patients, and 15 in poor-risk patients, among which circRNA-DLEU2 (hsa_circ_0000488; chromosome 13, 50680743 to 50681192) was highly expressed in AML samples compared with healthy samples, showing no significant difference between better- and poor-risk AML patients (Fig. 1A). During the validation of our bioinformatics analyses, we observed that circRNA-DLEU2 levels in AML bone marrow (BM) were significantly higher than those in healthy controls (Fig. 1B) ($P < 0.001$). Additionally, circRNA-DLEU2 in AML cell lines (MOLM-13, HL-60, and MV-4-11) was remarkably upregulated compared with normal cell lines (Fig. 1C) ($P < 0.001$). Therefore, we

FIG 1 circRNA-DLEU2 is upregulated in AML. (A) Heat map showing the differential expression of circRNAs in a healthy control group, a better-risk group, and a poor-risk group. (B) circRNA-DLEU2 expression in AML tissues was higher than that in healthy tissues as detected by qRT-PCR. ***, $P < 0.001$ compared with the healthy control group. (C) Expression levels of circRNA-DLEU2 in AML cell lines (MOLM-13, HL-60, and MV-4-11) were higher than that in healthy human cells (BMSC). **, $P < 0.01$; ***, $P < 0.001$ compared with BMSC. The data are shown as means and SD.

decided to focus on the function of circRNA-DLEU2 and selected MOLM-13 and MV-4-11 cell lines to conduct the subsequent experiments.

circRNA-DLEU2 promoted the proliferation of AML cells and inhibited cell apoptosis. In order to determine whether circRNA-DLEU2 has a function in AML, we first transfected MOLM-13 and MV-4-11 cells with a circRNA-DLEU2 vector and a si-circRNA-DLEU2 vector (Fig. 2A and B). According to the quantitative real-time (qRT)-PCR results, circRNA-DLEU2 and si-circRNA-DLEU2 recombinant plasmids were successfully expressed in MOLM-13 and MV-4-11 cells (Fig. 2C and D) ($P < 0.001$). CCK8 assay (Biotechwell, Shanghai, China) results showed that overexpressed circ-DLEU2 promoted cell proliferation, which was impeded by silencing circRNA-DLEU2 using si-circRNA-DLEU2 (Fig. 2E and F) ($P < 0.01$). Flow cytometry analysis revealed that the overexpression of circ-DLEU2 impaired the apoptosis rate of AML cell lines, which was completely reversed by the knockdown of circ-DLEU2 (Fig. 2G to H) ($P < 0.001$). All these results showed that the altered circRNA-DLEU2 influences the proliferation and apoptosis rates of MOLM-13 and MV-4-11 cells.

circRNA-DLEU2 promoted AML tumor formation *in vivo*. In order to further investigate the role of circRNA-DLEU2 in AML-related tumors, we designed and conducted *in vivo* experiments. Two weeks after injection, we found the tumor volume in mice injected with circRNA-DLEU2-transfected MOLM-13 and MV-4-11 cells was larger than that in mice injected with circ-vector (control [ctrl])-transfected cells (Fig. 3A and B) ($P < 0.01$). At the same time, we confirmed that the circRNA-DLEU2 expression of mice injected with circRNA-DLEU2-transfected cells was higher than that of mice transfected with circ-vector-transfected cells (Fig. 3C and D) ($P < 0.01$). Taken together,

FIG 2 circRNA-DLEU2 promotes the proliferation of AML cells and inhibits cell apoptosis. (A and B) Construction of circRNA-DLEU2, si-circRNA-DLEU2, and si-ctrl vectors. (C and D) Results of qRT-PCR indicating that cells were successfully transfected with circRNA-DLEU or si-circRNA-DLEU. **, $P < 0.001$; ***, $P < 0.001$ compared with a control group. (E and F) Proliferation rates of MOLM-13 cells and MV-4-11 cells in the circ-DLEU2-OE group were higher than in the circ-vector group, and the proliferation rate in the si-DLEU2 group was lower than in the si-ctrl group. *, $P < 0.05$ compared with the circ-vector group; **, $P < 0.01$ compared with the si-ctrl group. (G and H) The apoptosis ratio (annexin V-positive cell numbers/total cell numbers) of circ-DLEU2-OE-transfected MOLM-13 cells and MV-4-11 cells was lower than that of the circ-vector-transfected group, and the apoptosis ratio in the si-DLEU2 group was higher than that in si-ctrl group. ***, $P < 0.001$ compared with the circ-vector group; **, $P < 0.01$ compared with si-ctrl group. The data are shown as means and SD.

FIG 3 circRNA-DLEU2 promotes AML tumor formation *in vivo*. (A and B) Tumor volumes of mice injected with MOLM-13 (A) and MV-4-11 (B) cells transfected with circ-DLEU2. *, $P < 0.05$; **, $P < 0.01$ compared with the circ-vector group. (C and D) circRNA-DLEU2 expression in mice injected with MOLM-13 (C) and MV-4-11 (D) cells transfected with circ-DLEU2. **, $P < 0.01$ compared with the circ-vector group. Five mice were used in each group. The data are shown as means and SD.

these data demonstrated that circRNA-DLEU2 accelerates AML-related tumor formation *in vivo*.

miR-496 is inhibited by circRNA-DLEU2 and is downregulated in AML tissues.

Considering our results, circRNA-DLEU2 is very likely to be a functional circRNA in AML. As previously reported, one of the common regulatory functions of circRNA is to act as an miRNA sponge and to competitively inhibit its activity. For this purpose, we assumed that circRNA-DLEU2 could serve as an miRNA sponge and might regulate a specific AML-related circRNA-miRNA-mRNA network. To explore the potential miRNA candidates of circRNA-DLEU2, we used the Circular RNA Interactome website (<https://circinteractome.nia.nih.gov/bin/mirnasearch>) to predict the possible miRNA candidates that can bind circRNA-DLEU2. Four miRNAs (hsa-miR-496, hsa-miR-892a, hsa-miR-487a, and hsa-miR-485-3p) were screened for the screening criteria were as follows: context+ score percentile, >95 , and context+ score, less than -0.2 and are listed in Table 1. Then, we used the OncomiR database (<http://www.oncomir.org/oncomir/index.html>) to analyze the expression levels of the 4 miRNAs in cancers (Table 2). The result showed that all four miRNAs were downregulated, and miR-496 was expressed at a low level in at least 6 types of carcinomas. Thus, we chose miR-496 as our target to investigate its relationship with circRNA-DLEU2. In MOLM-13 and MV-4-11 cells, miR-496 expression was notably suppressed by the ectopic expression of circRNA-DLEU2 and significantly enhanced by the silencing of circRNA-DLEU2 with si-DLEU2 ($P < 0.001$), while the expression of miR-892a and miR-487a showed no significant difference after the cells were transfected with either circRNA-DLEU2 or si-DLEU2 (Fig. 4A and B). Meanwhile, miR-485 expression was upregulated after knocking down circRNA-DLEU2 (Fig. 4B) ($P < 0.05$). Subsequently, we used qRT-PCR to preliminarily detect the expression of miR-496 and miR-485-3p in BM samples from five AML patients who belonged to the same

TABLE 1 miRNA prediction^a

CircRNA/Mirbase ID ^b	CircRNA (5'→3')/miRNA (3'→5') pairing ^c	Site type	CircRNA position		Context + score	Context + percentile
			Start	End		
hsa_circ_0000488/ hsa-miR-496	AAAUAGUUUGAAAUUACUCACUCUAACCGGUACAUUAGAGU	8mer-1a	110	117	-0.346	99
hsa_circ_0000488/ hsa-miR-579	AAGAUAGCAGUUCCCA--CAAUAGAA/UUAAGCGCCAAUAUUGUUUACUU	8mer-1a	146	153	-0.187	99
hsa_circ_0000488/ hsa-miR-892a	UUCUUUGCAGCCUA-ACACAGUUU/GAUGCGUUCUUCUGUCAC	7mer-m8	438	444	-0.271	98
hsa_circ_0000488/ hsa-miR-487a	GUUUUUCAUAUAUAGUAUGAUU/UUGACCUACAGGGA CAUACUA A	7mer-m8	359	365	-0.246	98
hsa_circ_0000488/ hsa-miR-570	UAAUCUUAACCUAGUUUGUUUUCU/CGUUUCCAUUAACG CAAAAGC	7mer-m8	419	425	-0.139	98
hsa_circ_0000488/ hsa-miR-495	CAGUAGUUAGGUCUGUUUGUUUUU/UUCUUCACGUGGUACAA CAAA	7mer-m8	174	180	-0.087	97
hsa_circ_0000488/ hsa-miR-485-3p	UGUUAUUUCAUAUAUAGUAUGAUU/UCUCUCUCCUGCGC ACAUACUG	7mer-m8	358	364	-0.217	96
hsa_circ_0000488/ hsa-miR-628-3p	GGUUUUCUUAUCCAUUACUAGU/AGCUGACGGUGAGAAU GAUCU	7mer-m8	30	36	-0.195	96
hsa_circ_0000488/ hsa-miR-494	CAGUUCUAGGUUUGAUUUUUCU/CUCCAAAGGGCACAU ACAAAGU	7mer-m8	327	333	-0.088	95
hsa_circ_0000488/ hsa-miR-548g	CUUUGACGCCUAACACAGUUUUU/CAUGUUUUUAUUAU UGUCAA A	7mer-m8	440	446	-0.116	94
hsa_circ_0000488/ hsa-miR-607	UAUUUAUAAUGAUGUUUGAAAA/CAUAUUCUAGACCU AAACUUG	7mer-1a	103	109	-0.024	94
hsa_circ_0000488/ hsa-miR-1258	UGUUUUUUUUGACAGCCUUAACAC/AAGGUGCUGGAUU AGGAUUGA	7mer-1a	434	440	-0.164	92
hsa_circ_0000488/ hsa-miR-1305	AUUUAUUAAUGAUGUUUGAAAA/AGAGAGGGUAUCUC ACCUUUU	7mer-1a	104	110	-0.02	92
hsa_circ_0000488/ hsa-miR-1264	UCAAAUUCACACUCAGACUUUAU/UUGUCCACGAGUUU UUCUGAAC	7mer-1a	52	58	-0.105	91
hsa_circ_0000488/ hsa-miR-1257	UCCAUUACUAGUCAAAUUCACAA/CCAGUCUUGGGU AGUAGUGA	7mer-1a	41	47	-0.095	90
hsa_circ_0000488/ hsa-miR-183	CAAAACAGGAAGUAGCCAUU/UACAUAAAGUAGU ACGGUUAU	7mer-1a	282	288	-0.163	89
hsa_circ_0000488/ hsa-miR-1304	AUAAGUUUUUAUAU CCUCAA C/GUGUAGAGUACAUC CGAGUUU	7mer-1a	204	210	-0.128	88
hsa_circ_0000488/ hsa-miR-571	UACUAGUCAAUUCAACU CACUC AG/GAGUGAGU CUACCGUUAGU	7mer-1a	46	52	-0.137	87
hsa_circ_0000488/ hsa-miR-520f	UUCAGUUUUUCAUAGAAG CACUG /UUGGAGAUUUCC UUCGUGAA	7mer-m8	239	245	-0.128	85

^aTable generated using CircInteractome (<https://circinteractome.nia.nih.gov/bin/mirnaearch>).
^bmiRNAs in boldface were filtered for a context+ score percentile of >95 and a context+ score of less than -0.2, which meant a higher possibility for RNA binding.
^cBinding sites are in boldface.

TABLE 2 Expression of miRNAs in cancers

miRNA name	Cancer abbreviation ^a	Log rank		Z-score	Source of upregulated miRNA	Mean expression (log ₂)		t test	
		P value	FDR ^b			Deceased	Living	P value	FDR
hsa-miR-496	BLCA	1.95E-02	4.96E-01	2.508	Deceased	2.27	1.12	2.10E-01	6.46E-01
hsa-miR-496	HNSC	3.65E-02	1.51E-01	2.126	Deceased	1.42	1.2	7.08E-02	3.72E-01
hsa-miR-496	KICH	1.94E-02	3.17E-01	2.715	Deceased	1.86	0.1	3.60E-01	5.73E-01
hsa-miR-496	KIRC	8.20E-03	4.14E-01	3.097	Deceased	0.47	0.14	1.27E-01	3.29E-01
hsa-miR-496	KIRP	1.25E-02	1.99E-01	3.15	Deceased	0.14	0.03	8.77E-02	4.58E-01
hsa-miR-496	PAAD	2.07E-02	6.89E-01	2.182	Living	1.89	3.12	5.31E-02	3.09E-01
hsa-miR-496	PRAD	1.02E-02	2.56E-01	0.2	Living	0	0.18	1.15E-17	4.07E-15
hsa-miR-496	THCA	4.46E-02	1.47E-01	2.585	Deceased	0.44	0.14	5.20E-02	2.64E-01
hsa-miR-892a	TGCT	4.04E-02	9.36E-01	0.173	Living	0	1.61	3.01E-04	5.25E-03
hsa-miR-487a-3p	ESCA	7.45E-03	5.10E-01	1.806	Living	0.06	0.47	3.56E-02	6.09E-01
hsa-miR-487a-3p	KICH	4.07E-03	3.08E-01	0	Deceased	0.96	0	3.47E-01	5.73E-01
hsa-miR-487a-3p	KIRC	2.90E-02	8.44E-01	2.798	Deceased	0.21	0.01	1.35E-01	3.37E-01
hsa-miR-487a-3p	LGG	3.44E-02	9.79E-02	2.205	Deceased	1.36	1.19	3.38E-01	5.15E-01
hsa-miR-487a-3p	PAAD	1.20E-03	5.28E-01	2.441	Living	0.23	0.78	7.12E-02	3.36E-01
hsa-miR-485-3p	HNSC	4.69E-03	2.34E-01	2.876	Deceased	2.38	2	3.92E-02	2.92E-01
hsa-miR-485-3p	KICH	7.25E-03	3.30E-01	1.982	Deceased	3.37	0.12	3.51E-01	5.73E-01
hsa-miR-485-3p	KIRC	1.78E-04	4.82E-02	4.37	Deceased	2.01	0.53	5.88E-02	2.30E-01
hsa-miR-485-3p	KIRP	1.79E-04	3.49E-02	4.369	Deceased	0.71	0.25	1.20E-02	1.43E-01
hsa-miR-485-3p	UCEC	4.76E-02	9.51E-01	2.117	Deceased	3.07	1.91	3.36E-01	6.40E-01

^aBLCA, bladder urothelial carcinoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; THCA, thyroid carcinoma; TGCT, testicular germ cell tumors; ESCA, esophageal carcinoma; LGG, brain lower grade glioma; UCEC, uterine corpus endometrial carcinoma.

^bFDR, false-discovery rate.

cohort as our 20 AML patients. The results demonstrated that miR-496 expression of AML BM samples was much lower than that of healthy controls (Fig. 4C) ($P = 0.0361$), while no apparent difference in miR-485-3p expression was observed between the AML samples and those from healthy controls (Fig. 4D) ($P = 0.1450$). Therefore, we examined the expression of miR-496 in all 20 AML BM samples and found that miR-496 expression was decreased in the AML samples compared with healthy controls (Fig. 4D) ($P = 0.0008$). Next, we decided to explore the downstream target of miRNA-496. The possible downstream target mRNAs of miR-496 predicted by the miRDB database and microRNA.org are listed in Table 3 and Table 4, respectively. The results were displayed in Venn diagrams through the intersection of two subsets of mRNAs predicted by the miRDB database and microRNA.org. Nine intersected predicted target mRNA candidates (CPEB3, MAN1A2, MCTP1, GRAMD3, PLSCR1, PRKACB, ASXL2, EDIL3, and CLUL1) are shown in Fig. 4F. Meanwhile, we up- or downregulated miR-496 to investigate the expression of circ-DLEU2, and the results showed that the regulation of circ-DLEU2 by miR-496 had no statistical significance (Fig. 4G and H). Next, we used the STRING database (<https://string-db.org>) to predict the protein-protein interaction network of our selected candidates, and because the PRKACB gene is the hub gene in this network, we picked PRKACB for the molecular mechanism study (Fig. 5).

circRNA-DLEU2 inhibited miR-496 expression and promoted PRKACB expression. Our results indicated that the overexpression of circRNA-DLEU2 promoted the expression of PRKACB, while si-DLEU2 inhibited the expression of PRKACB in MOLM-13 and MV-4-11 cells (Fig. 6A and B) ($P < 0.001$). In accordance with the *in vitro* results, PRKACB was found to be upregulated in AML samples compared with healthy controls (Fig. 6C) ($P = 0.0002$). Next, we transfected miR-496 mimics and miR-496 inhibitors into MOLM-13 and MV-4-11 cells before investigating the effects of miR-496 on PRKACB expression, and the expression of the mimics and inhibitors was confirmed by qRT-PCR (Fig. 6D) ($P < 0.001$). We found that miR-496 mimics inhibited the expression of PRKACB ($P < 0.001$), which was drastically reversed by the knockdown of miR-496 by its inhibitors in the MOLM-13 and MV-4-11 cell lines, respectively ($P < 0.01$) (Fig. 6E and F). Altogether, we verified that PRKACB is one of the targets of miR-496 in AML cell lines.

We were eager to find the exact sites of interaction between circRNA-DLEU2, miR-496, and PRKACB. We applied TargetScan (http://www.targetscan.org/vert_50/) to

FIG 4 miR-496 was inhibited by circRNA-DLEU2 and downregulated in AML tissues. (A and B) hsa-miR-496 expression in the circRNA-DLEU2 group was significantly decreased compared to the circ-vector group. ***, $P < 0.001$; ns, not significant. hsa-miR-496 expression in the si-DLEU2 group was significantly increased compared to the si-ctrl group. *, $P < 0.05$; ***, $P < 0.001$. (C and D) Expression of hsa-miR-496 and miR-485-3p in AML and healthy tissues; $n = 5$. (E) hsa-miR-496 expression in AML tissues was lower than in healthy tissues; $n = 20$. (F) Venn diagram showing the 9 common target mRNAs of miR-496 predicted by the miRDB database and microRNA.org. (G and H) circRNA-DLEU2 expression in MOLM-13 and MV-4-11 cells. The data are shown as means and SD.

TABLE 3 High possible targets for hsa-miR-496 in miRDB^a

Target rank	Target score	miRNA name	Gene symbol	Gene product description
1	100	hsa-miR-496	CPEB3	Cytoplasmic polyadenylation element binding protein 3
2	98	hsa-miR-496	FLRT2	Fibronectin leucine-rich transmembrane protein 2
3	97	hsa-miR-496	MAN1A2	Mannosidase, alpha, class 1A, member 2
4	94	hsa-miR-496	MCTP1	Multiple C2 domains, transmembrane 1
5	94	hsa-miR-496	KCMF1	Potassium channel modulatory factor 1
6	92	hsa-miR-496	STRN	Striatin, calmodulin binding protein
7	92	hsa-miR-496	TMEM255A	Transmembrane protein 255A
8	92	hsa-miR-496	MGAT2	Mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase
9	91	hsa-miR-496	CCNT2	Cyclin T2
10	91	hsa-miR-496	GRAMD3	GRAM domain containing 3
11	90	hsa-miR-496	PPP6C	Protein phosphatase 6, catalytic subunit
12	90	hsa-miR-496	ARHGAP32	Rho GTPase-activating protein 32
13	89	hsa-miR-496	NR5A2	Nuclear receptor subfamily 5, group A, member 2
14	88	hsa-miR-496	PLSCR1	Phospholipid scramblase 1
15	88	hsa-miR-496	ELMO2	Engulfment and cell motility 2
16	87	hsa-miR-496	DACT2	Disheveled-binding antagonist of beta-catenin 2
17	87	hsa-miR-496	PRKACB	Protein kinase, cAMP dependent, catalytic, beta
18	86	hsa-miR-496	SUV39H2	Suppressor of variegation 3-9 homolog 2 (<i>Drosophila</i>)
19	86	hsa-miR-496	PTBP3	Polypyrimidine tract binding protein 3
20	86	hsa-miR-496	ASXL2	Additional sex combs like 2 (<i>Drosophila</i>)
21	86	hsa-miR-496	ANKRD12	Ankyrin repeat domain 12
22	84	hsa-miR-496	EDIL3	EGF-like repeats and discoidin I-like domains 3
23	83	hsa-miR-496	RANBP9	RAN binding protein 9
24	83	hsa-miR-496	KIAA1033	KIAA1033
25	82	hsa-miR-496	TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2
26	81	hsa-miR-496	CLUL1	Clusterin-like 1 (retinal)

^aThere are 152 predicted targets for hsa-miR-496 in miRDB (<http://www.mirdb.org/>); targets with a target score of >80 are listed. EGF, epidermal growth factor.

predict binding sites between miR-496 and circRNA-DLEU2 or miR-496 and PRKACB. The results showed that circRNA-DLEU2 had a potential binding site of miR-496, and PRKACB had two possible target sites (around 1,843 bp and 2,911 bp from its 3' untranslated region [UTR]) of miR-496; thus, we constructed a set of mutated or wild-type circRNA-DLEU2 and PRKACB luciferase reporters (Fig. 7A). The sequence information for circRNA-DLEU2 and PRKACB is presented in Table 5 and Table 6. A luciferase reporter assay verified that circRNA-DLEU2 directly targeted miR-496 and that miR-496 could target PRKACB (Fig. 7B and C) ($P < 0.01$). We also performed RNA precipitation assays to verify the interaction between circRNA-DLEU2 and miR-496, as well as PRKACB. First, we confirmed the expression of probe-PRKACB and probe-circ-DLEU2 in MOL-13 cells, and the results are shown in Fig. 7D and 2C, respectively. The results indicated that the enrichment of miR-496 in the overexpressed circRNA-DLEU2 group was lower than that in the circ-vector group, while miR-496 enrichment in the PRKACB overexpression group was higher than that in the vector group (Fig. 7E and F) ($P < 0.05$). Hence, it can be concluded that circRNA-DLEU2 can inhibit miR-496 expression and promote PRKACB expression and that miR-496 can inhibit PRKACB expression.

miR-496 antagonized the effects of PRKACB on MOLM-13 cell proliferation and apoptosis. We further investigated the molecular mechanisms of miR-496 and PRKACB in AML cells. Western blotting revealed that miR-496 impeded PRKACB protein expression while circRNA-DLEU2 increased the expression of PRKACB (Fig. 8A and B) ($P < 0.001$). AML cell proliferation was enhanced by PRKACB ($P < 0.05$), while PRKACB-mediated AML cell proliferation was reversed by miR-496. Nevertheless, the inhibition of cell proliferation induced by suppressed PRKACB was reversed by overexpressed circRNA-DLEU2 ($P < 0.01$) (Fig. 8C). Analogously, PRKACB significantly inhibited AML cell apoptosis ($P < 0.001$), which was neutralized by cotransfection with miR-496. Cotransfection of circRNA-DLEU2 and si-PRKACB showed results similar to those in the control group ($P < 0.01$) (Fig. 8D). Altogether, these results verified that miR-496 antagonized the effects of PRKACB on MOLM-13 cell proliferation and apoptosis.

TABLE 4 mRNAs targeted by hsa-miR-496 in microRNA.org^a

mRNA	mirSVR score
MCTP1	−3.12
KLHL5	−2.94
HIVEP2	−2.7
CPEB3	−2.47
STARD3NL	−2.47
LRRIQ3	−2.37
ASXL2	−2.34
ZNF41	−2.26
LOC728323	−2.22
PLSCR1	−2.15
DMXL1	−2.13
GRAMD3	−2.11
GTDC1	−2.1
ATP5F1	−2.06
SDHC	−2.01
TGFBR2	−2
LOC642502	−1.98
LLGL1	−1.87
PRSS35	−1.83
C5orf25	−1.83
EDIL3	−1.76
CEPT1	−1.71
PRKACB	−1.59
RPAP2	−1.69
C21orf34	−1.63
FAT4	−1.69
ANKRA2	−1.65
RNF138	−1.61
UGT2A3	−1.53
IKZF5	−1.6
GARNL3	−1.59
MTOR	−1.57
MAN1A2	−1.56
GALNT1	−1.52
NAB1	−1.51
TCERG1	−1.5
CCDC129	−1.49
ATP5G3	−1.47
CLUL1	−1.47
PHF12	−1.47
PRTFDC1	−1.46
TYW1B	−1.45
NCOA1	−1.45
MAPRE1	−1.44
ZNF280D	−1.44
TMEM47	−1.44
RGS18	−1.42
EML4	−1.42
RER1	−1.41
TOM1L1	−1.41

^aThere are 4,672 predicted targets for hsa-miR-496 on the microRNA.org website (<http://34.236.212.39/microna/home.do>) Targets and Expression (August 2010 release); the top 50 targets according to the miRNA support vector regression (mirSVR) score are listed.

DISCUSSION

It has been reported that circRNAs play an important role in regulating various biological processes, including leukemogenesis (4), and distinct molecular mechanisms, including the circRNA-miRNA-mRNA axis. In our present study, circRNA-DLEU2 was found to be overexpressed in both AML patients and cell lines; it promoted AML cell proliferation and inhibited apoptosis *in vitro* and promoted tumor formation *in vivo*. circRNA-DLEU2 targeted has-miR-496, and has-miR-496 targeted PRKACB. miR-496 antagonized the effects of PRKACB on MOLM-13 cell proliferation and apoptosis.

Of note, circRNAs are widely expressed in human cells, and their expression can be

FIG 5 STRING database (<https://string-db.org>) network diagram illustrating the relationship between the 9 target mRNAs.

higher by 10-fold or more than that of their linear counterparts (26). Compared with other noncoding RNAs, the unique structure of circRNAs has been characterized as the ideal prognostic biomarker and potential therapy target due to its high stability and specific expression patterns. Some studies have reported the correlation of circRNA and AML. For example, Jeck et al. revealed that has_circ_0004277 served as a new biomarker for AML (27). In our study, circRNA-DLEU2 was found to be upregulated in AML cells and promoted cell proliferation and inhibited cell apoptosis. The roles of DLEU2 in leukemia were reported in some studies. For example, Morenos et al. demonstrated that the DLEU2 locus and embedded miRNA cluster miR-15a/16-1 were commonly deleted in adult cancers and were shown to induce leukemogenesis (28). Kasar et al. revealed that the DLEU2 promoter may augment chronic lymphocytic leukemia therapy by decreasing miR-15a/16-1 expression (29). These studies investigated the role of DLEU2 lncRNA or the DLEU2 protein as tumor suppressors, and our study first explored the molecular mechanism of circRNA-DLEU2 in AML.

Certain circRNAs can bind and negatively influence miRNAs, which are substantially involved in the competing endogenous RNA (ceRNA) network, thereby regulating linear-RNA transcription and protein production. Hansen et al. reported that circRNA ciRS-7 could strongly suppress miR-7 activity, resulting in increased levels of miR-7 (30). circRNA_100290 plays a role in oral cancer by functioning as a sponge for the miR-29 family (31). The circular RNA MYLK, as a competing endogenous RNA, promotes bladder cancer progression by modulating the vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling pathway (32). In our study, circRNA-DLEU2 inhibited the expression level of miR-496 by targeting miR-496, which was downregulated in AML.

To further investigate the molecular network of circRNA-DLEU2 and miR-496, we

FIG 6 PRKACB is upregulated by circRNA-DLEU2 and downregulated by miR-496. (A and B) PRKACB mRNA expression in MOLM-13 and MV-4-11 cells after transfection with circ-DLEU2-OE or si-DLEU2. ***, $P < 0.001$ compared with circ-vector; **, $P < 0.01$ compared with si-ctrl. (C) Expression of PRKACB in AML is higher than that in a healthy control group. (D) Transfection efficiencies of miR-496 mimics and inhibitors verified by qPCR. ***, $P < 0.001$ compared with a mimic control group. **, $P < 0.01$; ***, $P < 0.001$ compared with an inhibitor control group. (E and F) PRKACB mRNA expression in MOLM-13 and MV-4-11 cells. ***, $P < 0.001$ compared with a mimic control group; **, $P < 0.01$ compared with an inhibitor control group. The data are shown as means and SD.

found a downstream target of miR-496: PRKACB. The experiments we performed indicated that PRKACB could be downregulated by miR-496, while circRNA-DLEU2 promoted PRKACB expression in AML cells. Some publications have reported a role of PRKACB in cancer development. For instance, PRKACB was downregulated in non-small-cell lung cancer, and exogenous PRKACB inhibited proliferation and invasion of LTP-A2 cells (22). In contrast, PRKACB induced the cell migration of breast cancer (24). Wang et al. demonstrated that inhibition of miR-372 led to reduced translational repression of PRKACB in liver cancer (25).

To investigate the roles of circRNA-DLEU2, miR-496, and PRKACB, our next study will

FIG 7 Target relationships between circ-DLEU2/miR-496 and PRKACB/miR-496. (A to C) TargetScan was used to predict the binding sites of circ-DLEU2/miR-496 and PRKACB/miR-496. Blue, circ-DLEU2 binding sequence; red, miR-496 binding sequence; purple, PRKACB binding sequence. **, $P < 0.01$ compared with a mimic control group. (D) PRKACB mRNA expression in the PRKACB-OE group was higher than that in the vector group. ***, $P < 0.001$ compared with the vector group. (E and F) Enrichment of circ-DLEU2 and miR-496 in MOLM-13 cells after transfection with circ-DLEU2 probe and PRKACB probe. *, $P < 0.05$ compared with the circ-vector group. The data are shown as means and SD.

focus on the effects of these three molecules' cotransfection on AML cell activities, such as proliferation, metastasis, and the cell cycle. The pathway of circRNA-DLEU2 in AML is also worthy of study.

In summary, our study verified that circRNA-DLEU2 was upregulated in AML and that it promoted the proliferation of AML cells and inhibited cell apoptosis. Additionally, the target relationships of circRNA-DLEU2/miR-496 and miR-496/PRKACB were clarified by a series of experiments. Although we do not know what the *in vivo* role of circ-DLEU2

TABLE 5 Hsa_circ_0000488 (circRNA-DLEU2) sequence^a information

Parameter	Value
circRNA ID	hsa_circ_0000488
Position (genome browser link)	Chromosome 13, 50680743–50681192
Genomic length	449
Spliced length	449
Gene symbol	DLEU2

^aSequence, ATGGAGTTGGCTTGGTTTCTTATTCCATTACTAGTCAAATTCACAACTCAGACTTATAGCTCCTTACTTGT TTAGGGGCTACCTATTATTAATGATGTTTGAAAACTCAACTTTTACAACAAAGATAGCAGTTCACAAATGAAA ACAGCAGTAAGTTAGGTCTGTTTCTTCTTGGATAAGTTGTATAATCCTCAAAGTACTCAACTATTCAAGTTTTCAT AGAAGCACTGCATGGTTGCACTTCTGAAATCAAAAACAGGAAGTATGCCATATAATGTCAGTAAACTAAACTCAGT TCTATGGTTTGATGTTTCTCTCTGCTGATTTTCATATTATGTATGATTTTATGTGACTTAGAATGACTGCGTAAAGGC AAACACTAATCTTACTGATTGTTTCTTTGACAGCCTAACACAGTTTAG.

in the pathophysiological genesis of human AML might be, this could be an interesting question to answer in future research. Our study suggested that circRNA-DLEU2 may serve as a novel biomarker and therapeutic target of human AML by targeting the miR-496/PRKACB channel.

TABLE 6 PRKACB sequence^a information

Parameter	Value
NCBI gene ID	5567
GenBank accession no.	NM_182948
Gene symbol	PRKACB
3' UTR length (bp)	3,209
Gene product description	Protein kinase, cAMP dependent, catalytic, beta

^a3' UTR sequence, AGAGGAACAA GATGACATCT GAGCTCACAC TCAGTGTGTTG CACTCTGTG AGAGATAAGG₆₁ TAGA GCTGAG ACCGTCCTTG TTGAAGCAGT TACCTAGTTC CTTCATTCCA ACGACTGAGT₁₂₁ GAGGTCTTTA TTGCCATCAT CCCGTGTGCG CACTCTGCAT CCACCTATGT AACAGGCGAC₁₈₁ CGTAAGCAA GCATTGTCTG TGCCATAACA CAGTAC TAGA CCACTTTCTT ACTTCTCTT₂₄₁ GGGTTGTCTT TCTCTCTCC TATATCCATT TCTCCTTTT CCAATTTTCAT TGGTTTT CTC₃₀₁ TAAACAGTGC TCCATTTTAT TTTGTTGGTG TTTCAGATGG GCAGTGTAT GGCTACGTGA₃₆₁ TATTTGAAGG GAAGGATAAG TGTTGCTTTC AGTAGTTATT GCCAATATTG TTGTTGTCA₄₂₁ ATGGCTTGAA GATAAACTTT CTAATA ATTA TTATTTCTTT GAGTAGCTCA GACTTGGTT₄₈₁ TGCCAAAAC CTGGTAATT TTTGAAGATA GACTGTCTTA TCAC CAAGGA AATTATACAA₅₄₁ AATTAAGACT AACCTTCTTG GAATTCACCTA TTCTGGCAAT AAATTTTGGT AGACTAATAC₆₀₁ AGTACAGCTA GACCCAGAAA TTGGAAGGC TGATAGTACAG AGGTTCTAGT TCCCTTCCC₆₆₁ TCCTTTTATA TCCTCC TCTC CTGAGTAAT GAAGTGACCA GCCTGTGTAG TGTGACAAAC₇₂₁ GTGTCTCATT CAGCAGGAAA AACTAATGAT ATGGATCATC ACCCAGATTC TCTCACTTGG₇₈₁ TACCAGCATT TCTGTAGGTA TTAGAGAAGA GTTCTAAGTT TTCTAA ACCT TAACGTCTC₈₄₁ TTAAGGAATT TAGCCAGTAT TTTAATAGAA CATGATTAAT GAAAGTGACA AATTTTAAAT₉₀₁ TTTCTCTAAT AGTCTCTATC ATAACTTTT TAAAGGAAAA TAAGCAAAC AAAAAGAACA₉₆₁ TTGGTTTGA TAAATA CTTA TACTTTGCAA AGTCAAAAAT GGCCTGATT TTGGAAACA₁₀₂₁ TATAGAGGTA TTCATATTTA AATGAGGGTT TACATTTGTT TTGTTTTGTA ACCGTTAAAA₁₀₈₁ AGAAGTTGTT TCCAGCTAAT TATTGTGGTG TACTATATTT GTGAGC CTAG GGTAGGGGCA₁₁₄₁ CTGCTGCAAC TTCTGCTTTC ATCCCATGCC TCATCAATGA GGAAAGGGAA CAAAGT GTAT₁₂₀₁ AAAACTGCCA CAATTGTATT TTAATTTTGA GGTATGATAT TTTCAGATAT TTCATAATTT₁₂₆₁ CTAACCTCTG TTCTCTCAGT AACACGAATG TCTGATGCAT CATGCAGATA CAATGTTGGT₁₃₂₁ ATTTGAGAGG TTAGTTTTTT TCCTAC ACTT TTTTTTGCCA ACTGACTTAA CAACATTGCT₁₃₈₁ GTCAGGTGGA AATTTCAAGC ACTTTTGAC ATTTAGTTCA GTG TTGTTTG AGAATCCATG₁₄₄₁ GCTTAACCCA CTGTTTTGC TATTTTTTC TTGCTTTTA ATTTTCCCA TCTGATTTA₁₅₀₁ TCTCTGCGTT TCAGTGACCT ACCTTAAAC AACACACGAG AAGAGTTAAA CTGGGTTTCA₁₅₆₁ TTTAATGATC AATTTA CCTG CATATAAAAT TTATTTTAA TCAAGCTGAT CTTAATGTAT₁₆₂₁ ATAATCATT TATTTGCTTT ATTATCGGTG CAGG TAGGTC ATTAACACCA CTCTTTTCA₁₆₈₁ TCTGTACCAC ACCCTGGTGA AACCTTGAA GACATAAAAA AAACCTGTCT GAGATGTTCT₁₇₄₁ TTCTACCAAT CTATATGCTT TTCGGTTATC AAGTGTCTT GCATGGTAAT GTCATGTAAA₁₈₀₁ TGCTG ATATT GATTTCAGT GTCCATCTAT ATTTAAAACG TGCAAGAAAA AATAAAATA₁₈₆₁ **CTCTGCTCTA** GCAAGTTTTG TGTAACAAAG GCATATCGTC ATGTTAATAA ATTTAAAACA₁₉₂₁ TCATTCGTAT AAAATATTTT AATTTCTTG TATTTT CATT AGACCCAAGA ACATGCTGAC₁₉₈₁ CAATGTGTTT TATATGTAAA CTACAAATTC TATGGTAGCT TTGTTGTATA TTATTGTAA₂₀₄₁ ATTATTTTAA TAAGTCATGG GGATGACAAT TTGATTATTA CAATTAGTT TTCAAGTAATC₂₁₀₁ AAAAA GATTT CTATGAATTC TAAAAAATAT TTTTCTCTAT GAAATTAATA GTGCCAGCT₂₁₆₁ GTAGAATCTA CCTTAGGTAG ATGATCCCTA GACATACGTT GGTTTGAGG GCTATTCAGC₂₂₂₁ CATTCATTT TACTCTCTAT TTAAGGCCG TGAGCA AGCT TGTGATGAGC AAATATGTCA₂₂₈₁ AGGGAGTCAA TTCTTGACCA ATCAAGTACA CTAAATTAGA ATATTTTAA AGTATGTAAC₂₃₄₁ ATTTCCAGTT TCAGCCACAA TTAGCCAAG AATAAGATAA AAATTTGAAT AAGAAGTAAG₂₄₀₁ TAG CATAAAT CAGTATTTAA CCTAAAATAT TTTTCTCTAT GAAATTAATA GTGCCAGCT₂₄₆₁ CTAGTAAAT AATTAAGAGA TGGCATTGTG TAAGAAGGAG CCCTAGACTG AAAGTCAAGA₂₅₂₁ CATCTGAATT TCAGGCTGGA AAATATCAG TATGA TCTCA GCCTCAGTTC TCTGTCTGT₂₅₈₁ AAAATGGAAG AACTGGATTA GGCAGTTTGT AAGATTCTCT CTAACTTTCA CAGTCGATGA₂₆₄₁ CAAGATGTC TTTTATCTG ATATTTTGAA GGGTATATTG CTTTGAAGTA AGTCTCAATA₂₇₀₁ AGGC AATATA TTTTAGGGA TCTTCTCT TATCTCTGAC AGTGTCTTA AAATATTGT₂₇₆₁ AATATCATA GAGCCTTGGT GTCTGCTCTA ATTCCTTCT CACTCACCGA TGCTGAATAC₂₈₂₁ CCAGTTGAAT CAACTGTCA ACCTACCAAA AACGAT ATTG TGCTTATGG GTATTGCTGT₂₈₈₁ CTCAATCTTG GTATATTCT GTGTAACTG CCCATTGGCC TGAA**AATACT CAT** TGTAAGC₂₉₄₁ CTGAAAAAA AAATCTTCC CACTGTTTT TCTGCTTGT GTAAGAATCA AATGAAATAA₃₀₀₁ TGTATG TGAA AGCACCTGT AAACCTGAAC CTATCAATGT AAAATGTAA GGTGTGTTG₃₀₆₁ TATTTCAATTA ATTACTTCT TGTT TAGAAT GGAATTTCT ATGCACACT GTAGCTAGGA₃₁₂₁ AATGCTGAAA ACAACTGTGT TTTTAAATTA ATCAATAACT GCAAAATTA AGTACCTCA₃₁₈₁ ATGGATAAGA CAAAAAAA AAAAAAAA. The targets of has-miR-496 are in boldface.

FIG 8 miR-496 antagonizes the effects of PRKACB on MOLM-13 cell proliferation and apoptosis. (A and B) miR-496 mimics inhibit PRKACB protein expression. **, $P < 0.01$ compared with the NC group, compared with the PRKACB group, and compared with the si-PRKACB group. (C) The proliferation rate in the miR-496 mimic group was lower than that in the NC group, and the proliferation rate in the PRKACB group was higher than that in the NC group. **, $P < 0.01$ compared with the NC group. (D) miR-496 mimics improve the apoptosis ratio. **, $P < 0.01$ compared with the NC group; **, $P < 0.01$ compared with the PRKACB group; *, $P < 0.05$, and **, $P < 0.01$ compared with the si-PRKACB group. The data are shown as means and SD.

MATERIALS AND METHODS

Tissue samples. BM samples were collected from a cohort of 20 CN-AML patients and 20 healthy controls between 2012 and 2016 at Gulou Hospital Affiliated to Medical College of Nanjing University without prior chemotherapy or radiation treatment. Informed consent was obtained from all the patients and healthy controls before surgery. This study was approved by the Ethical Committee of Gulou Hospital

TABLE 7 Clinical information for acute myeloid leukemia patients

Characteristic	No. of patients
Age (yr)	
<15	2
>15	18
Gender (male/female)	12/8
Myeloid progenitor cell	
<60%	6
>60%	14
Fusion gene anomaly	11
Chromosome abnormality	12

TABLE 8 Characteristics of AML patients

Sample no.	Gender ^a	Age (yr)	Diagnosis of FAB ^b subtype	FLT-ITD3 ^c	NPM1	Karyotype	Risk status
1	F	44	M4	+	—	Normal	Poor
2	M	56	M5	+	+	Normal	Poor
3	M	51	M5	+	—	Normal	Poor
4	F	47	M5	+	—	Normal	Poor
5	M	49	M5	+	—	Normal	Poor
6	M	34	M5	+	—	Normal	Poor
7	F	28	M5	+	+	Normal	Poor
8	F	41	M4	+	—	Normal	Poor
9	F	54	M4	+	—	Normal	Poor
10	M	39	M5	+	—	Normal	Poor
11	F	43	M5	—	+	Normal	Better
12	F	37	M5	—	+	Normal	Better
13	M	27	M4	—	+	Normal	Better
14	M	55	M5	—	—	Normal	Better
15	M	40	M5	—	+	Normal	Better
16	F	59	M4	—	+	Normal	Better
17	M	46	M5	—	+	Normal	Better
18	F	29	M5	—	+	Normal	Better
19	F	35	M5	—	+	Normal	Better
20	M	36	M5	—	+	Normal	Better
21	M	29	Healthy control	NA ^d	NA	NA	NA
22	M	36	Healthy control	NA	NA	NA	NA
23	F	55	Healthy control	NA	NA	NA	NA
24	M	31	Healthy control	NA	NA	NA	NA
25	F	51	Healthy control	NA	NA	NA	NA
26	F	47	Healthy control	NA	NA	NA	NA
27	M	49	Healthy control	NA	NA	NA	NA
28	F	38	Healthy control	NA	NA	NA	NA
29	M	45	Healthy control	NA	NA	NA	NA
30	F	36	Healthy control	NA	NA	NA	NA
31	M	26	Healthy control	NA	NA	NA	NA
32	M	34	Healthy control	NA	NA	NA	NA
33	F	40	Healthy control	NA	NA	NA	NA
34	M	52	Healthy control	NA	NA	NA	NA
35	F	39	Healthy control	NA	NA	NA	NA
36	F	41	Healthy control	NA	NA	NA	NA
37	M	43	Healthy control	NA	NA	NA	NA
38	F	54	Healthy control	NA	NA	NA	NA
39	F	58	Healthy control	NA	NA	NA	NA
40	M	46	Healthy control	NA	NA	NA	NA

^aM, male; F, female.^bFAB, French-American-British classification.^cFLT-ITD3, FMS-like tyrosine kinase 3.^dNA, not applicable.

Affiliated to Medical College of Nanjing University. Clinical information for the AML patients is shown in Table 7 and Table 8. All BM samples were independently reviewed by three clinicians.

Cell culture. Three human AML cell lines (MOLM-13, MV-4-11, and HL-60), normal cells (bone marrow stem cells [BMSC]), and HEK293 cells were purchased from the BeNa Culture Collection (Beijing, China). AML and BMSC were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and lipopolysaccharide from *Escherichia coli* (10 μg/ml). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (vol/vol) FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). All the cells were maintained in an incubator with a 5% CO₂ atmosphere at 37°C.

Microarray analysis. Affymetrix microarray platform [GPL19978](#) and microarray data set [GSE94591](#), used for bioinformatics analyses, were obtained from the NCBI Gene Expression Omnibus (GEO) database. The [GSE94591](#) data set contained 10 samples: 4 healthy controls and 6 CN-AML patients' samples (3 poor-risk AML samples and 3 better-risk AML samples). A log₂-fold change of >1.5 or less than -1.5 and a *P* value of <0.05 were set as inclusive criteria to search for differentially expressed circRNAs.

Transfection. circ-DLEU2 and PRKACB were cloned into pLCDH (Genesee Biotech, Shanghai, China) and pcDNA3.1 (BioVector NTCC Inc., Beijing, China), respectively. circ-DLEU2 small interfering RNA (siRNA), hsa-miR-496 mimics, hsa-miR-496 inhibitors, si-ctrl, and circ-vector were purchased from Gene-Pharma Co., Ltd. (Shanghai, China). The sequences of all the recombinant plasmids were confirmed by DNA sequencing. circRNAs were confirmed by both convergent and divergent primers and Northern

TABLE 9 Primer sequences

Primer	Orientation	Sequence
hsa_circ_0000488	Forward	5'-TGACTGCGTAAAGGCAAACAC-3'
	Reverse	5'-AGGGTAGCCCTAAAACAAGT-3'
hsa-miR-496	Forward	5'-TGAGTATTACATGGCCAATC-3'
	Reverse	5'-GAATACCTCGGACCCTGC-3'
PRKACB	Forward	5'-TGGCAGCTTATAGAGAACCACC-3'
	Reverse	5'-ACTCTTTCATTGATCTGTCCCA-3'
GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'-GGATCTCGCTCCTGGAAGATG-3'
U6	Forward	5'-CTCGCTTCGGCAGCACATA-5'
	Reverse	5'-AACGATTCACGAATTTGCGT-3'
hsa-miR-496 mimics		5'-UGAGUAAUACAUGGCCAAUCUC-3'
hsa-miR-496 inhibitor		5'-ACUCAUAAUGUACCGGUUAGAG-3'

blotting. Cells were seeded onto petri dishes or 6-well plates for 24 h, and when the cells reached 80% confluence, transfections were carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Then, the transfected cells were cultured for 24 to 72 h with 5% CO₂ at 37°C before being used in other experiments.

qRT-PCR. Total RNA from 20 samples was isolated using TRIzol reagent (Invitrogen). For extracting circRNA, the extracted total RNA was treated with RNase R (Epicenter Inc.) and digested at 37°C for 20 min. Then, the RNA was quantitated using a NanoDrop 2000 (Thermo Fisher Scientific Inc.) and reverse transcribed into cDNA using a cDNA synthesis kit (Thermo Fisher) with a total mass of 200 ng total RNA. Quantitative real-time PCR was conducted using Thunderbird SYBR qPCR mix (Toyobo, Japan). The PCR conditions were as follows: denaturation at 94°C for 2 min, followed by a further 30 cycles of denaturation at 94°C for 30 s and finally annealing at 56°C for 30 s. All reactions were performed in four independent trials. The primer sequences are listed in Table 9. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for mRNA and circRNA qRT-PCR, while the miRNA expression was normalized to that of U6 small nuclear RNA (U6). The relative circRNA, mRNA, and miRNA expression fold changes were calculated using the $2^{-\Delta\Delta CT}$ method.

CCK8 assay. In the wells of a 96-well plate, 8×10^3 MOLM-13 or MV-4-11 cells were seeded and incubated overnight. At 0, 24, 48, and 72 h after transfection, cell growth and viability rates were measured using a CCK8 kit (Biotechwell, Shanghai, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm in a SpectraMax M5 multimode plate reader (Molecular Devices). All experiments were conducted with six replicates and repeated in triplicate.

Apoptosis analysis. MOLM-13 and MV-4-11 cells were treated with a phycoerythrin (PE)-annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit (BD) for 48 h after transfection. The cells were analyzed with a FACSCalibur (BD), and the data were processed using FACS Diva (BD). All experiments were conducted with six replicates and repeated in triplicate.

Luciferase reporter assay. The luciferase vector psiCHECK (Hanbio, Shanghai, China) was used to construct recombinant plasmids, including wild-type and mutant circRNA-DLEU2, wild-type PRKACB, and mutant PRKACB 3' UTR. Different recombinant plasmids were cotransfected into HEK293 cells with either miR-496 mimics or mimic control. The luciferase activity of each transfection group was detected using a luciferase activity detecting kit (Luc-100; Biothrive, Shanghai, China) 48 h after transfection with a SpectraMax M5 multimode plate reader (Molecular Devices).

RNA precipitation. Biotin-labeled circRNA-DLEU2 probe (5'-TGACAGCCTAACACAGTTTATAGTGGAGTTTGGCTTGGTTTCTT-3'-biotin) and biotin-labeled PRKACB probe (5'-AGAAGTTGTTCCAGCTAATTATTGTTGGTACTATATTTGTGAGCCTAG-3'-biotin) were synthesized by Sangon Biotech. circRNA-DLEU2-overexpressing (OE) and PRKACB-overexpressing MOLM-13 cells were fixed with 1% formaldehyde for 10 min, lysed, and sonicated. After centrifugation, 50 μ l of the supernatant was retained as input, and the remaining part was incubated with circRNA-DLEU2- and PRKACB-specific probes attached to streptavidin-coupled Dynabeads (M-280; Invitrogen). The mixture was incubated overnight at 30°C. The next day, the M-280 Dynabead-probe-RNA mixture was washed and incubated with 200 μ l lysis buffer and proteinase K to reverse the formaldehyde cross-linking. Finally, TRIzol was added to the mixture for RNA extraction and detection.

Western blotting. Transfected cells were washed with phosphate-buffered saline (PBS) before total protein was extracted with radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), separated on SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Following overnight hybridization with monoclonal anti-PRKACB and anti-GAPDH antibodies (Abcam, Cambridge, MA) at 4°C, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Abcam) at room temperature for 1 h. Finally, enhanced chemiluminescence (ECL Plus; Life Technology, Shanghai, China) was used for protein signal detection, and Lab Works 4.5 was applied for image capture and ImageJ

(<https://imagej.nih.gov/ij/>) for relative quantification to calculate the mean gray value of immunoreactive bands against GAPDH bands (as an internal control).

Subcutaneous xenografts. Male C57BL/6 mice (7 weeks old; $n = 5$) were obtained from the Experimental Animal Center of Zhejiang University (Zhejiang, China). For the subcutaneous xenograft model, 3 groups of 5 mice each were injected subcutaneously at the same site with 10^7 prepared cells. Tumor volume was calculated using the following formula: $V = (L \times W^2) \times 0.5$, where V refers to volume, W refers to the short axial length, and L refers to the long axial length. Five weeks after injection, the mice were sacrificed and examined.

Research involving human subjects or animal experimentation were in line with the Requirements and Regulations for Human Subjects or Animal Experiments of the National Health Ministry (article no. 55, 2001, or no. 17, 1999) and the principles of the Ethics Committee of the Affiliated Municipal Hospital of Xuzhou. All efforts were made to minimize the pain of the animals.

Statistical analysis. All data are represented as means and standard deviations (SD). A P value of <0.05 was considered statistically significant. Unpaired Student t tests were used to compare the differences between two groups. One-way analysis of variance (ANOVA) with the Bonferroni *post hoc* test was used to compare the differences in multiple groups. Each experiment was independently repeated at least three times.

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We declare that we have no conflict of interest.

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