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Research Paper

Epigenetic dysregulation of *NKD2* is a valuable predictor assessing treatment outcome in acute myeloid leukemia

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Abstract

AIM: The present study was aimed to investigate *NKD2* expression as well as promoter methylation and further analyze their clinical significance in patients with acute myeloid leukemia (AML).

METHODS: Real-time quantitative PCR was carried out to detect the pattern of *NKD2* expression in 113 AML patients and 24 controls. Real-time quantitative methylation-specific PCR (RQ-MSP) and bisulfite sequencing PCR (BSP) were carried out to detect *NKD2* promoter methylation in 101 AML patients and 24 controls with available DNA.

RESULTS: The level of *NKD2* transcript in AML patients was significantly down-regulated as compared with controls (P=0.039). *NKD2* methylation level in AML patients was significantly higher than controls (P=0.044). Moreover, *NKD2* methylation negatively correlated with *NKD2* expression in AML patients (R=-0.218, P=0.029). Furthermore, demethylation of *NKD2* could increase *NKD2* expression in the leukemic cell line THP1 (P<0.05). *NKD2* low-expressed and high-expressed patients showed no statistical significance in complete remission (CR) rate among cytogenetically normal AML (CN-AML). However, low *NKD2* expression was associated with shorter overall survival (OS) time and acted as independent risk factor in CN-AML according to Kaplan-Meier (P=0.029) and Cox regression analyses (P=0.022). Furthermore, gene expression (GEP) data also confirmed the prognostic value of *NKD2* expression in CN-AML patients. Moreover, *NKD2* showed significantly increased level in post-CR than initial diagnosis in follow-up AML patients (P=0.024).

CONCLUSION: Decreased *NKD2* expression inactivated by promoter hypermethylation is a common event in AML and is associated with adverse outcome in CN-AML patients.

Key words: *NKD2*, expression, methylation, prognosis, acute myeloid leukemia.

Introduction

Acute myeloid leukemia (AML) is a complex cancer characterized by uncontrolled proliferation and apoptosis of abnormal stem cells in the bone marrow (BM) and inhibition in the growth of normal hematopoietic cells [1,2]. The diagnosis of AML mainly depends on the features of morphology, immunology, cytogenetics, and molecular biology (MICM). Despite the advancements in the treatment of leukemia, clinical outcome of AML remains unsatisfactory [3]. Cytogenetic alterations play crucial roles in the pathogenesis of AML and also act as vital biological markers in predicting prognosis for AML

[4,5]. However, there are approximately 40% *de novo* AML patients do not have clonal cytogenetic abnormalities, which was defined as cytogenetically normal AML (CN-AML) [6,7]. Clinical outcome of CN-AML patients is quite heterogeneous from a few days to complete cure. Recently, several molecular biomarkers including *NPM1*, *CEBPA*, *C-KIT*, and *FLT3* mutations have provided helpful prognostic information for these patients [8]. Therefore, identifying novel genetic and epigenetic alterations which can recognize the patients who are at the risk of poor outcome is warranted to optimize treatment strategies [9,10].

WNT/β-Catenin signaling pathway plays crucial roles in physiological processes including stem cell maintenance, cell differentiation, migration, apoptosis and proliferation during embryonic development [11,12]. At the same time, abnormal regulation of Wnt/β-catenin signaling pathway also contributes to the development of human cancers including leukemia. Increasing studies have indicated that dysregulated Wnt pathway is associated with leukemogenesis and clinical outcome [13-16]. Overexpression of Wnt/β-catenin pathway genes or loss of Wnt antagonists promotes uncontrolled cell growth and survival [17]. Naked family (*NKD1* and *NKD2*) has been reported to function as a negative regulator of Wnt/β-catenin signaling pathway [18]. *NKD* binds and inactivates dishevelled, a scaffold protein that transduces the Wnt signal from the frizzled receptor to the destruction complex [19]. Decreased expression of *NKD2* caused by its promoter hypermethylation has been increasingly demonstrated in several solid tumors [19-22]. However, the pattern of *NKD2* expression and methylation in AML has not been explored yet. Therefore, we focused on the *NKD2* expression as well as methylation and their clinical significance in *de novo* patients with AML in the current study.

Materials and methods

Patients' samples

A total of 113 patients with a diagnosis of *de novo* AML including 42 CN-AML were enrolled into the study approved by the Institutional Review Board of the Affiliated People's Hospital of Jiangsu University. The diagnosis and classification of the patients were based on the revised 2008 World Health Organization (WHO) criteria [23]. Clinical features of the patients were showed in Table 1. Treatment protocol for AML patients was described previously [24]. A total of 9 AML patients achieved complete remission (CR) after induction therapy together with 24 healthy donors were also included. Follow-up data was obtained in 94 patients ranging from 1 to 58 months with a median of 7 months. Bone marrow (BM) were collected form all the patients and participants after written informed consents were obtained.

Table 1. Comparison of clinical manifestations and laboratory features between AML patients with low and high *NKD2* expression.

Patient's parameters	High (n=56)	Low (n=57)	*P* value
Sex, male/female	36/20	31/26	0.340
Median age, years (range)	58 (15-86)	53 (10-93)	0.726
Median WBC, ×10⁹/L (range)	22.7 (1.1-203.6)	6.75 (1.0-197.7)	0.066
Median hemoglobin, g/L (range)	78.5 (40-133)	69.5 (34-142)	0.074
Median platelets, ×10⁹/L (range)	48.5 (3-447)	40 (6-399)	0.916
BM blasts, % (range)	48.5 (5.5-94.5)	38 (3-92)	0.385
CR (-/+)	27/25	28/18	0.419
WHO			0.865
AML with t(8;21)	4 (7%)	4 (7%)	
APL with t(15;17)	9 (16%)	13 (23%)	
AML with inv(16)(p13q22)	0 (0%)	0 (0%)	
AML with less differentiation	0 (0%)	1 (2%)	
AML without maturation	5 (9%)	3 (5%)	
AML with maturation	20 (36%)	17 (30%)	
Acute myelomonocytic leukemia	12 (21%)	11 (19%)	
Acute monoblastic/monocytic leukemia	4 (7%)	7 (12%)	
Acute erythroid leukemia	2 (4%)	1 (2%)	
Karyotype classification			0.726
Favorable	12 (21%)	17 (30%)	
Intermediate	34 (61%)	30 (53%)	
Poor	7 (13%)	8 (14%)	
No data	3 (5%)	2 (3%)	
Karyotype			0.167
normal	31 (56%)	20 (35%)	
t(8;21)	4 (7%)	4 (7%)	
t(15;17)	8 (14%)	12 (21%)	
11q23	1 (2%)	0 (0%)	
complex	6 (11%)	9 (16%)	
others	3 (5%)	10 (18%)	
No data	3 (5%)	2 (3%)	
Gene mutation			
CEBPA (+/-)	10/42	6/44	0.417
NPM1 (+/-)	7/45	6/44	1.000
FLT3-ITD (+/-)	10/42	6/44	0.417
c-KIT (+/-)	2/50	1/49	1.000
NRAS or *KRAS* (+/-)	6/46	2/48	0.148
IDH1 or *IDH2* (+/-)	3/49	4/46	0.713
DNMT3A (+/-)	6/46	3/47	0.264

WBC: white blood cells; AML: acute myeloid leukemia; CR: complete remission.

Cell line, cell culture and 5-aza-dC treatment

Human leukemic cell line (THP1) was cultured in 1640 medium containing 10% fetal calf serum (ExCell Bio, Shanghai, China) and grown at 37°C in 5% CO_2 humidified atmosphere. For epigenetic studies, 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, Steinheim, USA) diluted in dimethyl sulfoxide (DMSO) was added in three flasks of THP1 cells (at a density of 5×10^5 cells/ml in 10 ml) once a day at different final concentrations of 0 μM, 2 μM and 4 μM during 3 days. All cells were cultured until harvested for extraction of RNA and DNA.

RNA isolation, reverse transcription and RQ-PCR

BM mononuclear cells (BMMNCs) were extracted by Lymphocyte Separation Medium (TBD sciences, Tianjin, China). Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was performed as reported previously [25,26].

Real-time quantitative PCR (RQ-PCR) was performed on a 7500 Thermo cycler (Applied Biosystems, CA, USA). The reaction system with 20 μL volume consisted of H_2O 6 μL, 10 μM of AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA), 0.4 μM of ROX Reference Dye 2 (Invitrogen, Carlsbad, CA,USA),and 0.8 μM of primers (forward 5′-ACAGGAGGTTGTCTGCA CACG-3′ and reverse 5′-GACTTGAGGAACTG CTTCTCCG-3′) [20]. The RQ-PCR reaction conditions were 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, 65 °C for 30 s, 72 °C for 32 s, and 86 °C for 32 s to collect fluorescence, finally followed by 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s, and 60 °C for 15 s. Both positive and negative controls were included in each assay. Relative *NKD2* expression levels were calculated using the following equation:

$$N_{NKD2}=(E_{NKD2})^{\Delta CT\ NKD2\ (control\text{-}sample)} \div (E_{ABL})^{\Delta CT\ ABL\ (control\text{-}sample)}.$$

DNA isolation, chemical modification and RQ-MSP

Genomic DNA was isolated using genomic DNA purification kit (Gentra, Minneapolis, MN, USA) and was modified using the CpGenome DNA Modification Kit (Chemicon, Ternecula, Canada) according to the manufacturer's instructions. The primers used for the methylated (M) [(forward) 5′-GATCGTAGGGGATAGTTTCGTGGC-3′; (reverse) 5′-AAAACAACTCTAACACCGCTCCCCG-3′] and unmethylaed (U) [(forward) 5′-TGGATTGTAGG GGATAGTTTTGTGGT-3′; (reverse)5′-CAAAAAC AACTCTAACACCACTCCCCA-3′] [20]. Real-time quantitative methyaltion-specific PCR (RQ-MSP) reaction contained 0.8 μM of primers, 10 μM of AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA), 0.4 μM of ROX Reference Dye 2 (Invitrogen, Carlsbad, CA, USA), and 20 ng of modified DNA was also performed a 7500 Thermo cycler (Applied Biosystems, CA, USA). The reaction condition was 95°C for 5 min, 40 cycles for 10 s at 95°C, 30 s at 62°C (M) or 64°C (U), 72°C for 30 s, and 80°C (M) or 76°C (U) for 30 s, eventually a melting program of one cycle at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s. The normalized ratio ($N_{M\text{-}NKD2}$) was applied to assess the level of *NKD2* promoter methylation in samples. $N_{M\text{-}NKD2}$ was calculated using the following formula:

$$N_{M\text{-}NKD2} = (E_{M\text{-}NKD2})^{\Delta CT\ M\text{-}NKD2\ (control\text{-}sample)} \div (E_{ALU})^{\Delta CT\ ALU\ (control\text{-}sample)}.$$

BSP

Bisulfite sequencing PCR (BSP) reaction system was composed of 1×PCR buffer (KCl 0.25 mM), dNTP Mixture 6.25 μM, primers 0.5 μM, hot start DNA polymerase 0.75 U (Takara, Tokyo, Japan), and modified DNA 20 ng. The primers for bisulfite modified *NKD2* promoter were 5′-TTTTGGAGTTAG TAGTGGGGG-3′ (forward) and 5′-CACCAAAAA CAACTCTAACACC-3′ (reverse). The program was carried out at 98°C for 10 s, 40 cycles for 10 s at 98°C, 30 s at 58°C, 72°C for 30 s, and followed by a final 7 min extension step at 72°C. The PCR products were analyzed on 2% agarose gels. Each purified product was cloned into pMD19-T Vector (Takara, Tokyo, Japan), and was transfected into DH5α competent cells (Vazyme Biotech Co., Piscataway, NJ, USA). Five clones from each sample were sequenced (BGI Tech Solutions Co., Shanghai, China).

HRMA and sequencing

High-resolution melting analysis (HRMA) was performed on the LightScanner platform (Idaho Technology Inc., Salt Lake City, Utah, USA). *NRAS* or *KRAS*, *DNMT3A*, *IDH1/2*, *KIT*, and *NPM1* mutations were detected by HRMA [27-30]. All positive samples were confirmed by DNA direct sequencing. *FLT3-ITD* and *CEBPA* mutations were detected by direct DNA sequencing (BGI Tech Solutions Co., Shanghai, China) [31].

Statistical analyses

Statistical analyses were performed using the SPSS 20.0 software package. Mann-Whitney's *U* test was carried to compare the difference of continuous variables between two groups. Pearson Chi-square analysis or Fisher exact test was employed to compare the difference of categorical variables. Correlation analysis between *NKD2* expression and prognosis was performed by Kaplan–Meier curves and Cox regression analysis. Receiver operating characteristic curve (ROC) and area under the ROC curve (AUC) were used to assess the value of *NKD2* expression in discriminating AML patients from controls. For all analyses, a two-tailed $P<0.05$ was defined as statistically significant.

Results

NKD2 expression in AML patients

NKD2 transcript level in AML patients (range

0.0000-57.9485, median 0.0000) was significantly lower as compared with controls (range 0.0000-3.6715, median 0.0082) which indicated that *NKD2* expression was down-regulated in AML (*P*=0.039, Fig. 1).

Discriminative capacity of NKD2 expression

ROC curve indicated that *NKD2* expression might serve as a biomarker for distinguishing whole-cohort AML patients from normal controls (AUC=0.628, 95% CI: 0.515-0.740, *P*=0.049, Fig. 2).

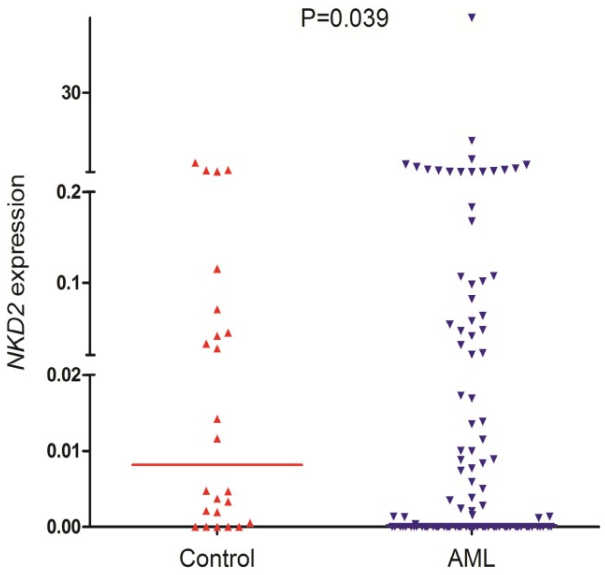

Figure 1. *NKD2* expression in controls and AML patients.

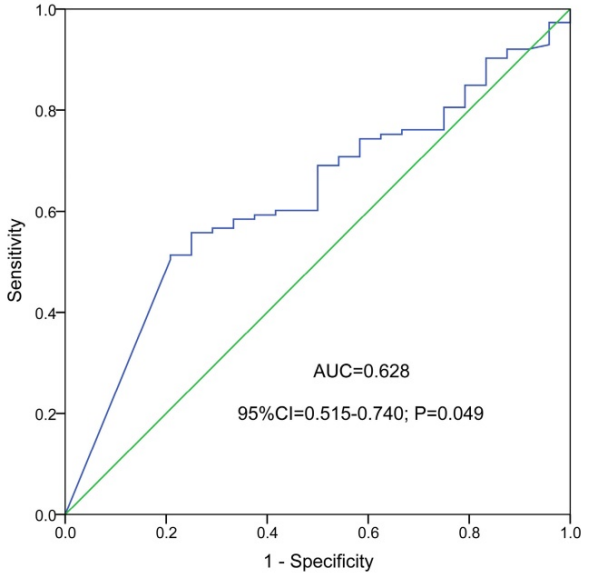

Figure 2. ROC curve analysis using *NKD2* expression for discriminating AML patients from controls.

NKD2 methylation in AML patients

NKD2 methylation was detected in 101 AML patients and 24 controls with available DNA. *NKD2* methylation level in AML patients (median 0.0009, range 0.0000-25.2199) was significantly higher than controls (median 0.0005, range 0.0000-1.0000) (*P*=0.044, Fig. 3) which suggested that *NKD2* was hypermethylated in AML.

To further confirm RQ-MSP result, we selected 6 samples (2 with lowest methylation level, 2 with highest methylation level, and 2 selected randomly) to investigate the *NKD2* methylation density by BSP. Controls presented almost fully unmethylated *NKD2* promoter, while the two methylated AML patients presented higher density of *NKD2* methylation (Fig. 4). Moreover, *NKD2* methylation density in the tested samples was heavily correlated with *NKD2* methylation level (R=0.928, *P*=0.008).

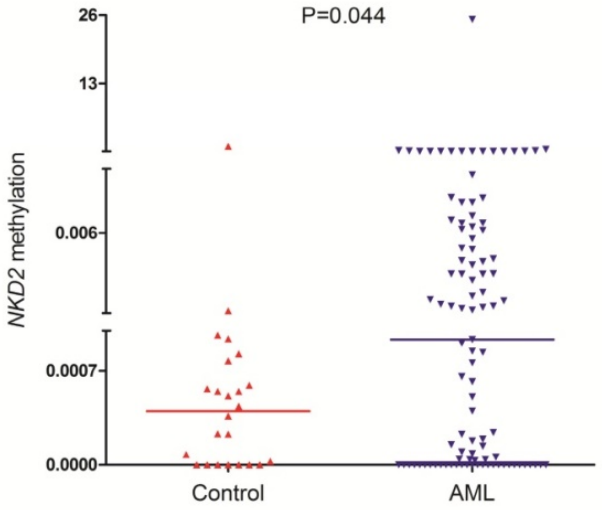

Figure 3. *NKD2* methylation in controls and AML patients.

NKD2 expression was regulated by its promoter methylation

To determine the role of *NKD2* promoter methylation in regulating *NKD2* expression in AML, we analyzed the relationship between *NKD2* methylation and expression in AML patients. Significantly negative correlation was observed between *NKD2* expression and its promoter methylation in AML patients (R=-0.218, *P*=0.029, Fig. 5).

We further selected *NKD2*-methylated THP1 cell line treated by 5-aza-dC to further verify the epigenetic mechanism. Before 5-aza-dC treatment, THP1 showed weakly *NKD2* expression and highly methylated *NKD2* promoter. As expected, *NKD2* promoter methylation level was decreased, meanwhile, *NKD2* expression was significantly up-regulated after 5-aza-dC treatment (Fig. 6).

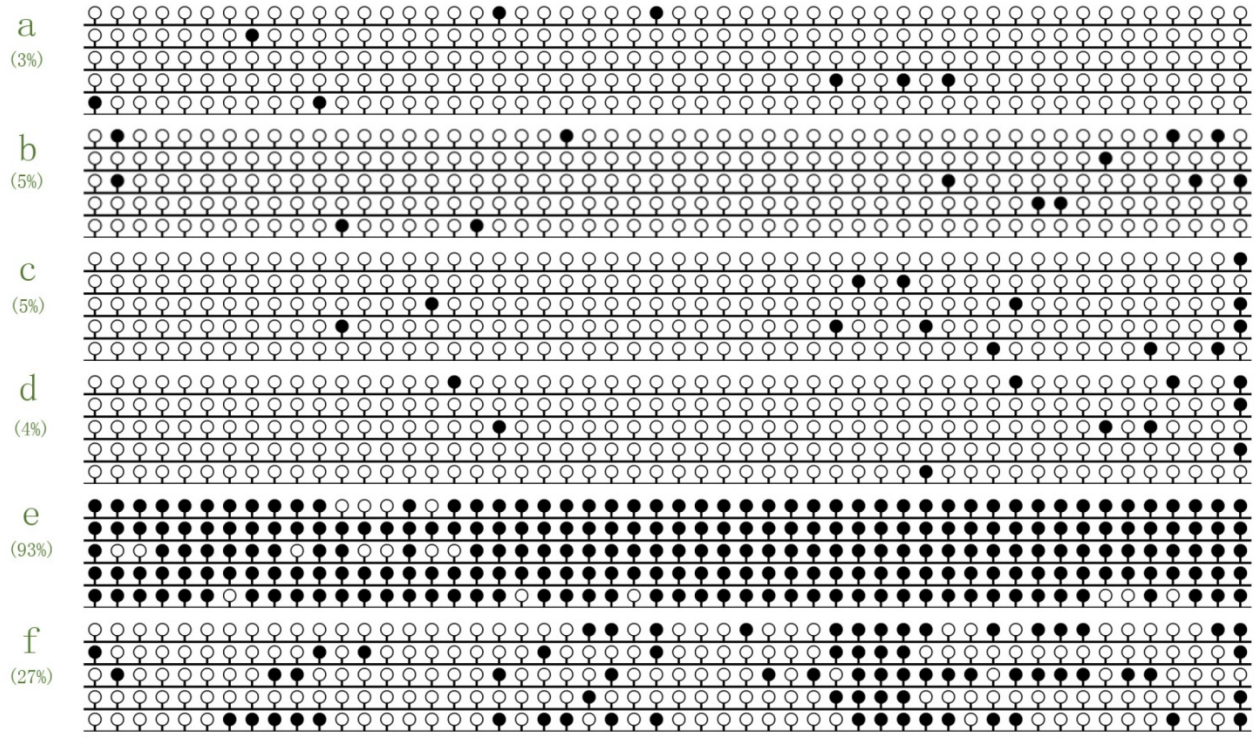

Figure 4. Methylation density of *NKD2* promoter in normal controls and AML patients. White cycle: unmethylated CpG dinucleotide; Black cycle: methylated CpG dinucleotide. a, b: controls; c, d: selected randomly AML patients; e, f: high methylated AML patients.

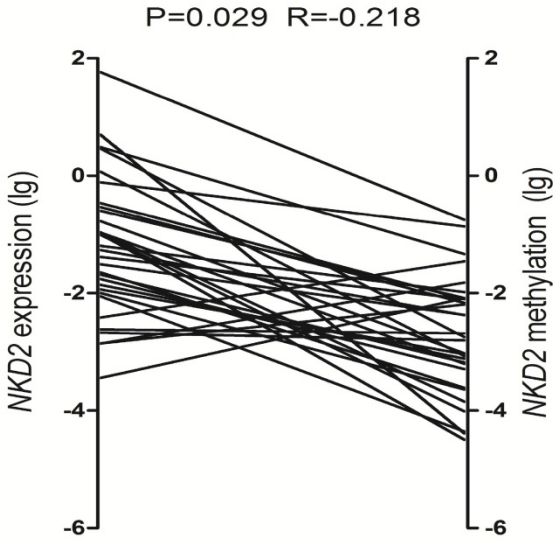

Figure 5. Relationship between *NKD2* expression and its promoter methylation in AML patients.

Clinical and laboratory characteristics of AML patients

The whole cohort of AML patients were divided into two groups at the median level of *NKD2* expression: low *NKD2* expression (*NKD2*^{low}) and high *NKD2* expression (*NKD2*^{high}). The correlation between *NKD2* expression and clinical characteristics was presented in Table 1. There were no significant differences in sex, age, platelets, white blood cells, hemoglobin and BM blasts between the *NKD2*^{high} and *NKD2*^{low} groups (P>0.05). No significant differences were found between the two groups in the distribution of WHO subtypes, and karyotypic classifications. Moreover, no significant correlations were found between *NKD2* expression and nine gene mutations (P>0.05).

Correlation between NKD2 expression and clinical outcome

After induction therapy, there was no significant difference in complete remission (CR) rate between *NKD2*^{low} patients and *NKD2*^{high} patients (P=0.419, Table 1). Moreover, no significant differences were found between the two groups among both non-APL AML [30% (11/26) vs. 37% (16/27), respectively, P=0.636] and cytogenetically normal AML (CN-AML) patients [27% (4/11) vs. 50% (14/14), respectively, P=0.199]. The whole-cohort and non-APL AML showed no significant difference in both the overall survival (OS) (P=0.081 and 0.106) and leukemia-free survival (LFS) (P=0.516 and 0.376). However, among CN-AML patients, cases with low *NKD2* expression had significantly shorter OS (median 3 versus 11 months, respectively, P=0.029, Fig. 7) but not LFS (P=0.178) time than those with high *NKD2* expression. We further performed Cox regression analysis to determine the prognostic value of *NKD2* expression in

CN-AML patients. Multivariate analysis including variables [WBC (≥30×10^9/L vs. <30×10^9/L), age (>60 vs. ≤60 years old), gene mutations (mutant vs. wild-type), and *NKD2* expression (high vs. low)] with P<0.20 in univariate analysis, and disclosed that low *NKD2* expression and WBC were independent risk factor in OS but not LFS analyses among CN-AML patients (Table 2 and 3).

Furthermore, the prognostic impact of *NKD2* expression was also confirmed by gene expression profiling (GEP) data (accession number GSE12417, http://www.ncbi.nlm.nih.gov/geo/) from two independent cohorts of adult CN-AML patients published by Metzeler et al using the online web tool Genomicscape (http://genomicscape.com/microarray/survival.php) [32]. Similarly, patients with low *NKD2* expression showed shorter OS as compared to those with high *NKD2* expression in both two cohorts (162 and 78 patients) (P=0.033 and 0.053, respectively, Fig. 8a and 8b).

Surveillance of NKD2 expression in follow-up AML patients

To further investigated whether levels of *NKD2* factored in patients' response to therapy, we followed *NKD2* expression of 9 patients with AML from the initial diagnosis to CR. Our study revealed that *NKD2* showed significantly increased level in post-CR than initial diagnosis in follow-up AML patients (P=0.024, Fig 9).

Table 2. Univariate and multivariate analyses of prognostic factors for overall survival in CN-AML patients.

prognostic factors	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
age	2.717 (1.504-4.912)	0.001	1.897 (0.823-4.376)	0.133
NKD2 expression	0.444 (0.202-0.973)	0.043	0.328 (0.127-0.849)	0.022
WBC	1.748 (0.991-3.083)	0.054	2.489 (1.057-5.862)	0.037
FLT3-ITD mutation	0.685 (0.272-1.727)	0.423	-	-
NPM1 mutation	0.933 (0.418-2.086)	0.867	-	-
CEBPA mutation	1.346 (0.603-3.004)	0.468	-	-
c-KIT mutation	0.391 (0.054-2.837)	0.353	-	-
NRAS or *KRAS* mutation	1.096 (0.434-2.769)	0.847	-	-
IDH1 or *IDH2* mutation	1.672 (0.809-3.455)	0.165	7.559 (1.897-30.129)	0.004
DNMT3A mutation	0.762 (0.302-1.921)	0.564	-	-

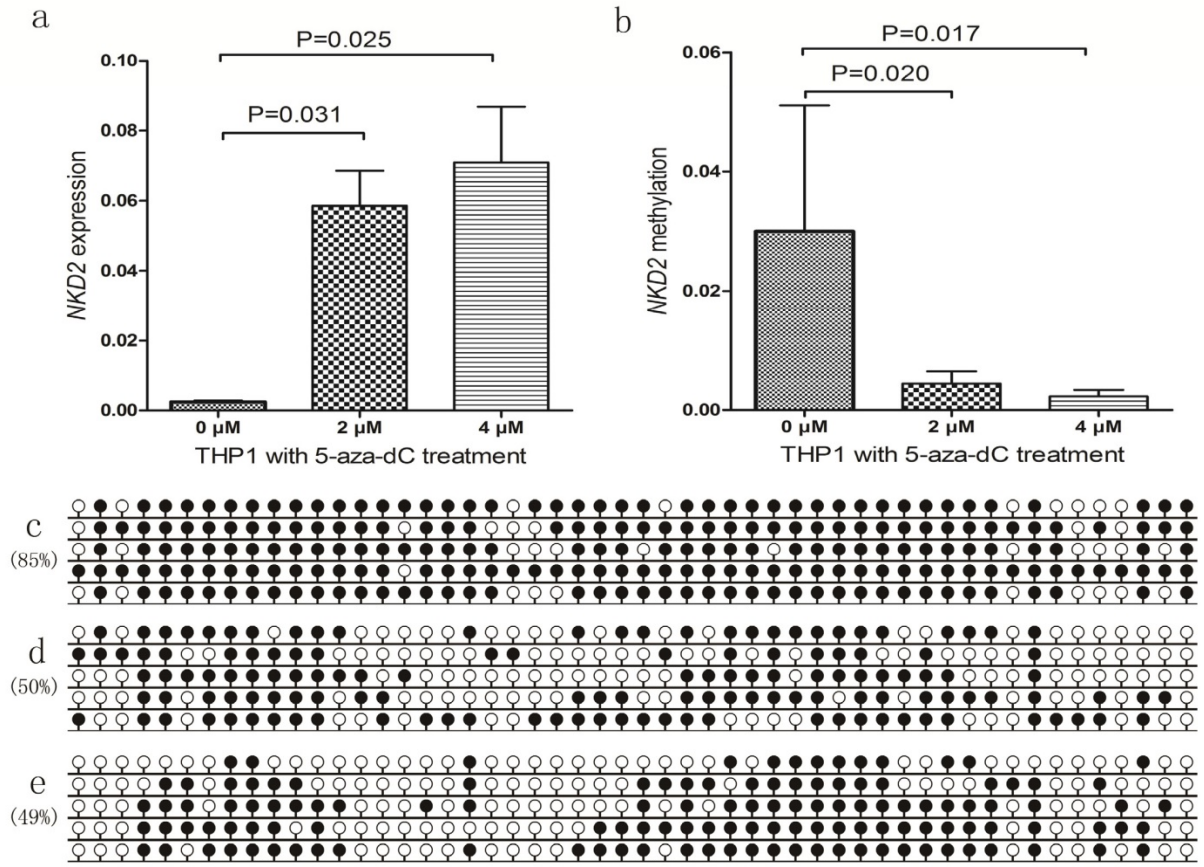

Figure 6. *NKD2* expression and methylation in THP1 cell line before and after 5-aza-dC treatment. a: *NKD2* relative expression levels; b: *NKD2* relative methylation levels; c: *NKD2* methylation density before treatment; d: *NKD2* methylation density after 2 μM treatment; e: *NKD2* methylation density after 4 μM treatment.

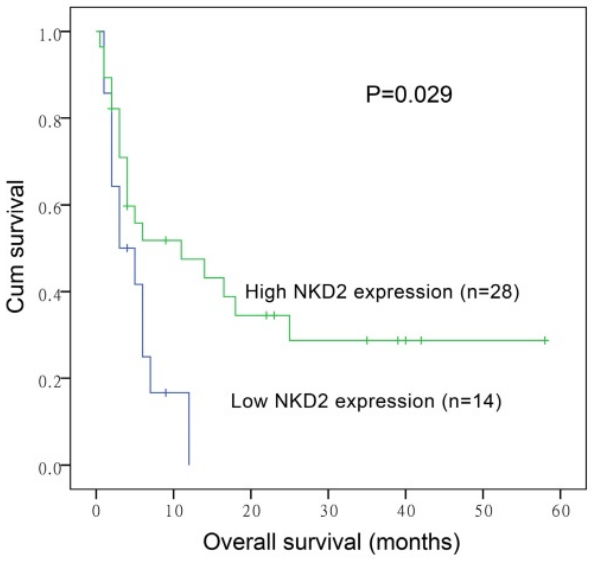

Figure 7. Overall survival of CN-AML patients.

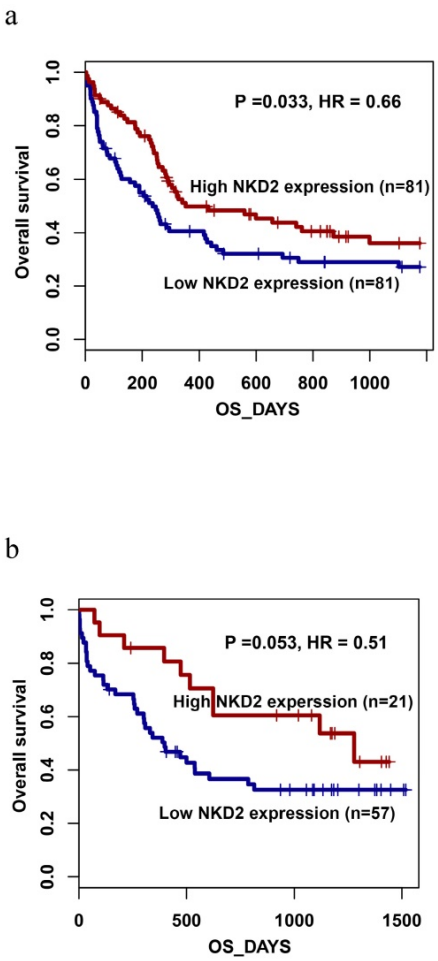

Figure 8. Prognostic value of *NKD2* expression using Gene expression profiling data (accession number GSE12417, http://www.ncbi.nlm.nih.gov/geo/) in CN-AML. a: data with 162 patients; b: data with 78 patients.

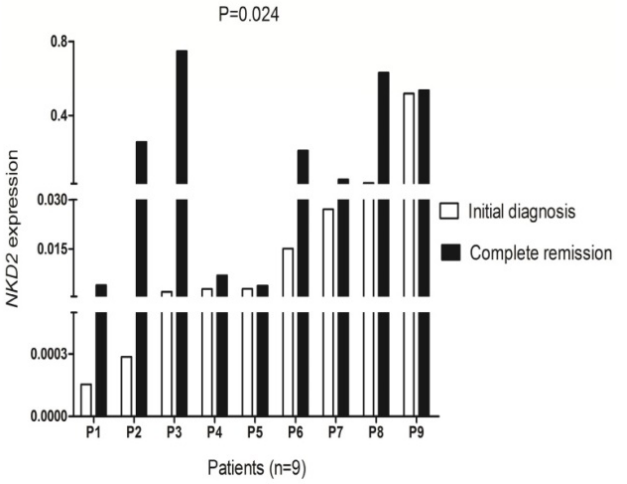

Figure 9. Changes of *NKD2* expression in follow-up AML patients (n=9) from the initial diagnosis to complete remission. White represents AML patients before therapy, black indicates complete remission after therapy.

Table 3. Univariate and multivariate analyses of prognostic factors for leukemia-free survival in CN-AML patients.

prognostic factors	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
age	2.019 (1.111-3.671)	0.021	2.086 (0.926-4.700)	0.076
NKD2 expression	0.565 (0.264-1.209)	0.141	0.404 (0.168-0.973)	0.043
WBC	1.562 (0.879-2.778)	0.129	2.280 (1.022-5.086)	0.044
FLT3-ITD mutation	0.726 (0.288-1.829)	0.497	-	-
NPM1 mutation	0.820 (0.368-1.827)	0.628	-	-
CEBPA mutation	1.035 (0.465-2.303)	0.933	-	-
c-KIT mutation	0.386 (0.053-2.800)	0.346	-	-
NRAS or *KRAS* mutation	0.994 (0.394-2.506)	0.990	-	-
IDH1 or *IDH2* mutation	1.265 (0.613-2.609)	0.525	-	-
DNMT3A mutation	0.821 (0.326-2.069)	0.676	-	-

Discussion

The activity of the Wnt pathway is controlled by extracellular and intracellular inhibitors mainly including secreted frizzled-related proteins (*SFRPs*), dickkopfs (*DKK*), SRY-box containing gene (*SOX*), and *NKD* family [19]. Frequent promoter hypermethylation of Wnt pathway inhibitor genes and their effect on gene expression have been revealed in malignant tumors [19]. Recently, accumulating evidence has revealed the direct role of *NKD2* in tumorigenesis. Reduced *NKD2* expression is found in osteosarcoma and has a significant role in driving tumor growth and metastasis [33]. *NKD2* suppresses breast cancer cell proliferation both in vitro and in vivo and induced G1/S arrest and inhibits Wnt signaling in breast cancer cells [20]. In human gastric cancer, *NKD2* restoration suppresses cell proliferation, colony formation, cell invasion and

migration, induced G2/M phase arrest, and sensitizes cancer cells to docetaxel [34]. In addition, curcumin significantly upregulated the expression of *NKD2* in SW620 colorectal cancer cells and inhibited the proliferation of colorectal cancer cells, resulting in the down-regulation of crucial markers in the Wnt signaling [35]. Zhi Li et al suggested that *NKD2* is a direct target of miR-130b, and miR-130b regulated proliferation and apoptosis of osteosarcoma cells by targeting *NKD2* and regulating the Wnt signaling [36]. Moreover, long noncoding RNA *ZFAS1* repressed underlying targets *KLF2* and *NKD2* transcription to promote gastric cancer cells proliferation by interacting with *EZH2* and *LSD1/CoREST* [37]. Our previous studies have disclosed that *SOX17* and *SRFP1/2* expression was down-regulated in AML and low *SOX17* expression was associated with adverse prognosis in CN-AML patients [38-40]. Additionally, decreased *SFRP1* expression was found more frequently in the less well differentiated subgroups of AML and is associated with *NPM1* mutation in AML [39], whereas reduced *SFRP2* expression was correlated with intermediate/poor karyotypes in AML [40]. As far as we know, this is the first report about *NKD2* expression and its clinical significance in patients with AML.

In this study, we observed that down-regulation of *NKD2* expression was a frequent event in AML patients, which indicates that *NKD2* might act as tumor suppressor role as well in leukemogenesis. Obviously, function studies of *NKD2* in leukemia are needed to support our results. Although no significant associations were observed between *NKD2* expression and laboratory features as well as common gene mutations, survival analyses revealed that low *NKD2* expression were associated with shorter OS and LFS among CN-AML patients. Notably, dynamic monitoring the level of *NKD2* in 9 cases of patients illustrated that *NKD2* were significantly increased from the initial diagnosis to CR by mentioned therapeutic protocols. In recent years, clinical significance of *NKD2* expression has been increasingly investigated in various human cancers. Zhao et al demonstrated that decreased expression of *NKD2* was associated with a significantly worse OS in human osteosarcoma tumors [33]. These results together indicated that *NKD2* expression was a valuable predictor in assessing treatment outcome as well as status, and might serve as a criterion for the therapeutic evaluation in AML.

The regulation of *NKD2* expression is associated with the methylation of its promoter [21]. We found that *NKD2* methylation was a common event in AML patients and its role in silencing *NKD2* expression was confirmed in leukemic cell lines THP1. In addition, our study further observed that *NKD2* expression was negatively correlated with *NKD2* expression in clinical samples. Recently, the clinical significance of *NKD2* methylation has been also identified in several cancers [20,21,34]. In human breast cancer, *NKD2* methylation is associated with higher tumor stages [20]. Moreover, in gastric cancer, methylation of *NKD2* is significantly associated with the degree of cell differentiation, TNM stage, and distant metastasis and may serve as a poor prognostic predictor [34].

All in all, decreased *NKD2* expression inactivated by promotor hypermethylation is a common event in AML and is associated with adverse outcome in CN-AML patients.

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Estey E, Döhner H. Acute myeloid leukaemia. Lancet. 2006;368:1894-907.
2. Smith M, Barnett M, Bassan R, et al. Adult acute myeloid leukaemia. Crit Rev Oncol Hematol. 2004;50:197-222.
3. Zhou JD, Yang L, Zhu XW, et al. Clinical significance of up-regulated ID1 expression in Chinese de novo acute myeloid leukemia. Int J Clin Exp Pathol. 2015;8:5336-44.
4. Grimwade D. The clinical significance of cytogenetic abnormalities in acute myeloid leukaemia. Best Pract Res Clin Haematol. 2001;14:497-529.
5. Byrd JC, Mrózek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100: 4325-36.
6. Rowley JD. Chromosomal translocations: revisited yet again. Blood 2008; 112: 2183-9.
7. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev 2004; 18: 115-36.
8. Gregory TK, Wald D, Chen Y, et al. Molecular prognostic markers for adult acute myeloid leukemia with normal cytogenetics. J Hematol Oncol 2009; 2: 23.
9. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937-51.
10. Gulley ML, Shea TC, Fedoriw Y. Genetic tests to evaluate prognosis and predict therapeutic response in acute myeloid leukemia. J Mol Diagn. 2010;12:3-16.
11. Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. Nat Rev Cancer. 2008;8:387-98.

12. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781-810.
13. Ysebaert L, Chicanne G, Demur C, et al. Expression of beta-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. Leukemia. 2006;20:1211-6.
14. Wang Y, Krivtsov AV, Sinha AU, et al. The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. Science. 2010;327:1650-3.
15. Metzeler KH, Heilmeier B, Edmaier KE, et al. High expression of lymphoid enhancer-binding factor-1 (LEF1) is a novel favorable prognostic factor in cytogenetically normal acute myeloid leukemia. Blood. 2012;120: 2118-26.
16. Fu Y, Zhu H, Wu W, et al. Clinical significance of lymphoid enhancer-binding factor 1 expression in acute myeloid leukemia. Leuk Lymphoma. 2014;55:371-7.
17. Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov. 2006;5:997-1014.
18. Hu T, Krezel AM, Li C, et al. Structural studies of human Naked2: a biologically active intrinsically unstructured protein. Biochemical and biophysical research communications. 2006; 350:911-5.
19. Götze S, Wolter M, Reifenberger G, et al. Frequent promoter hypermethylation of Wnt pathway inhibitor genes in malignant astrocytic gliomas. Int J Cancer. 2010;126:2584-93.
20. Dong Y, Cao B, Zhang M, et al. Epigenetic silencing of *NKD2*, a major component of Wnt signaling, promotes breast cancer growth. Oncotarget. 2015;6:22126-38.
21. Jia Y, Cao B, Yang Y, et al. Silencing *NKD2* by promoter region hypermethylation promotes gastric cancer invasion and metastasis by up-regulating SOX18 in human gastric cancer. Oncotarget. 2015;6:33470-85.
22. Feng W, Shen L, Wen S, et al. Correlation between CpG methylation profiles and hormone receptor status in breast cancers. Breast Cancer Res. 2007;9:R57.
23. Swerdlow SH CE, Harris NL, Jaffe ES, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. France: IARC Press; 2008.
24. Li Y, Lin J, Yang J, et al. Overexpressed let-7a-3 is associated with poor outcome in acute myeloid leukemia. Leuk Res. 2013; 37: 1642-7.
25. Zhou JD, Yang L, Zhang YY, et al. Overexpression of BAALC: clinical significance in Chinese de novo acute myeloid leukemia. Med Oncol. 2015;32:386.
26. Zhou JD, Yao DM, Zhang YY, et al. GPX3 hypermethylation serves as an independent prognostic biomarker in non-M3 acute myeloid leukemia. Am J Cancer Res. 2015;5:1786-94.
27. Lin J, Yao DM, Qian J, et al. Recurrent DNMT3A R882 muta-tions in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. PLoS One. 2011;6:e26906.
28. Lin J, Yao DM, Qian J, et al. IDH1 and IDH2 mutationanalysis in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. Ann Hematol. 2012;91:519-25.
29. Yang X, Qian J, Sun A, et al. RAS mutation analysis in alarge cohort of Chinese patients with acute myeloid leukemia. Clin Biochem. 2013;46:579-83.
30. Qian J, Yao DM, Lin J, et al. U2AF1 Mutations in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. PloS One. 2012;7:e45760.
31. Wen XM, Lin J, Yang J, et al. Double CEBPA mutations are prognostically favorable in non-M3 acute myeloid leukemia patients with wild-type NPM1 and FLT3-ITD. Int J Clin Exp Pathol. 2014; 7: 6832-40.
32. Metzeler KH, Hummel M, Bloomfield CD, et al. An 86-probe-set gene-expression signature predicts survival in cytogenetically normal acute myeloid leukemia. Blood. 2008;112:4193-201.
33. Zhao S, Kurenbekova L, Gao Y, et al. *NKD2*, a negative regulator of Wnt signaling, suppresses tumor growth and metastasis in osteosarcoma. Oncogene. 2015;34:5069-79.
34. Jia Y, Cao B, Yang Y, et al. Silencing *NKD2* by promoter region hypermethylation promotes gastric cancer invasion and metastasis by up-regulating SOX18 in human gastric cancer. Oncotarget. 2015;6:33470-85.
35. Zhang Z, Chen H, Xu C, et al. Curcumin inhibits tumor epithelial-mesenchymal transition by downregulating the Wnt signaling pathway and upregulating *NKD2* expression in colon cancer cells. Oncol Rep. 2016 May;35(5):2615-23.
36. Li Z, Li Y, Wang N, et al. miR-130b targets *NKD2* and regulates the Wnt signaling to promote proliferation and inhibit apoptosis in osteosarcoma cells. Biochem Biophys Res Commun. 2016 Mar 18;471(4):479-85.
37. Nie F, Yu X, Huang M, et al. Long noncoding RNA ZFAS1 promotes gastric cancer cells proliferation by epigenetically repressing KLF2 and *NKD2* expression. Oncotarget. 2016 May 26.
38. Tang CY, Lin J, Qian W, et al. Low SOX17 expression: prognostic significance in de novo acute myeloid leukemia with normal cytogenetics. Clin Chem Lab Med. 2014;52:1843-50.
39. An C, Guo H, Wen XM, et al. Clinical significance of reduced SFRP1 expression in acute myeloid leukemia. Leuk Lymphoma. 2015;56:2056-60.
40. Guo H, Lin J, Wen XM, et al. Decreased SFRP2 expression is associated with intermediate and poor karyotypes in de novo acute myeloid leukemia. Int J Clin Exp Pathol. 2014;7:4695-703.