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Original Article

Promoter methylation of the candidate tumor suppressor gene TCF21 in myelodysplastic syndrome and acute myeloid leukemia

Yu Gu[1,3,4], Jing-Dong Zhou[1,3,4], Zi-Jun Xu[2,3,4], Ting-Juan Zhang[1,3,4], Xiang-Mei Wen[2,3,4], Ji-Chun Ma[2,3,4], Ren-Bi Ji[3], Qian Yuan[2,3,4], Wei Zhang[1,3,4], Qin Chen[2,3,4], Jiang Lin[2,3,4], Jun Qian[1,3,4]

[1]Department of Hematology, [2]Laboratory Center, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, People's Republic of China; [3]Zhenjiang Clinical Research Center of Hematology, [4]The Key Lab of Precision Diagnosis and Treatment of Zhenjiang City, Zhenjiang, Jiangsu, People's Republic of China

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Abstract: Transcription factor 21 (*TCF21*) has been identified as a candidate tumor suppressor gene which was epigenetically inactivated in a variety of human cancers. However, *TCF21* methylation pattern remains unknown in hematologic malignancies. The aim of this study was to investigate *TCF21* methylation and its clinical relevance in myelodysplastic syndrome (MDS) and non-M3 acute myeloid leukemia (AML). A total cohort of 33 MDS patients, 100 non-M3 AML patients and 25 healthy donors were enrolled in the study. Targeted bisulfite sequencing assay was performed to identify the methylation pattern of CpG islands within the promoter of *TCF21* gene. The bioinformatics analyses were based on The Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO). The results showed that there were significant differences in the methylation levels of *TCF21* between MDS, non-M3 AML and controls (P = 0.003 and < 0.001, respectively). *TCF21* hypermethylation might be served as a promising biomarker which could distinguish MDS/AML from normal controls (P < 0.001 and = 0.003, respectively). There was a significant difference in cytogenetic risk categories between *TCF21* hypermethylation and non-hypermethylation AML patients (P = 0.032). Notably, *TCF21* hypermethylation occurred frequently in AML patients with adverse risk category, compared with those with favorable and intermediate categories, respectively (67% vs 44% and 29%). *TCF21* non-hypermethylation AML patients showed a higher probability of normal karyotype than abnormal karyotype (P = 0.003). The rate of *DNMT3A* gene mutation was significantly higher in the non-hypermethylation AML patients than that in the hypermethylation (8/44 vs 0/34, P = 0.020). These results suggested that aberrant DNA promoter methylation of *TCF21* was frequent event in MDS and non-M3 AML, and *TCF21* hypermathylation was associated with adverse risk karyotype in AML.

Keywords: Acute myeloid leukemia, myelodysplastic syndrome, TCF21, methylation

Introduction

MDS is a heterogeneous group of malignant myeloid disorders that usually manifests as peripheral blood (PB) cytopenia, bone marrow (BM) hyperplasia accompanied by dysplasia and has high risk of development into AML. AML is the most common adult acute leukemia, characterized by a clonal proliferation of immature myeloid precursor cells in the BM, PB and/or other tissues. It is traditionally accepted that AML is result of various genetic alterations, leading to irreversible pathologic changes of pivotal gene functions, including proliferation, apoptosis, differentiation and gene transcription related to leukemogenesis [1].

During tumor initiation and progression, the epigenome goes through multiple alterations, including a genome-wide loss of DNA methylation (hypomethylation), frequent increase in promoter methylation of CpG islands, changes in histone modification profiles [2]. Recent studies have demonstrated that aberrant DNA methylation was one of the most important epigenetic modifications especially in the leukemogenesis [3]. Methylation patterns, or its dynamic change during treatment, may also be

used as biomarkers for patient stratification, disease prognosis, and response to treatment [4]. The transcription factor 21 (*TCF21*) is located on chromosome 6q23-q24 and encodes a cell type-specific class II basic helix-loop-helix transcription factor, which is known to regulate mesenchymal cell transition into epithelial cells. Smith et al. for the first time identified *TCF21* as a candidate tumor suppressor which was epigenetically inactivated in lung cancer and head and neck cancer [5]. In recent years, a growing number of studies have confirmed that hypermethylation-mediated silenced expression of *TCF21* were tumor-specific and common in many kinds of human cancers [6-10], but has not been reported in malignancies of hematopoietic and lymphoid tissues.

Our study aimed to examine the methylation pattern of *TCF21* promoter region by targeted bisulfite sequencing assay in MDS and non-M3 AML patients. We evaluated the correlation between *TCF21* methylation pattern and various clinical parameters. Here, we found that *TCF21* was very frequently hypermethylated in AML, and its methylation pattern was relevant to prognostic risk categories based on cytogenetics, as well as *DNMT3A* mutation.

Materials and methods

Patients and samples

This study was approved by the Ethics Committee of Affiliated People's Hospital of Jiangsu University. After obtaining informed consent, we analyzed a total cohort of 33 MDS and 100 non-M3 AML patients who were diagnosed according to the French-American-British (FAB) and World Health Organzation (WHO) classifications [11, 12]. AML patients ranged in age from 18 to 85 year with a median of 53 year, whereas MDS patients ranged in age from 28 to 86 year with a median of 65 year. BM specimens were enriched for mononuclear cells by density gradient at the time of diagnosis. The BM specimens from 25 healthy volunteers served as controls.

Cytogenetic analysis and gene mutation detection

Cytogenetics for MDS and AML patients were detected at the time of initial diagnosis by conventional R-banding method. Prognostic risk based on cytogenetic classification was classified according to the report published [13]. Gene mutations including *CEBPA*, *NPM1*, *FLT3-ITD*, *c-KIT*, *N/K-RAS*, *IDH1/2*, *DNMT3A*, *U2AF1*, *SRSF2* and *SETBP1* were detected by high-resolution melting analysis (HRMA) using the Light Scanner platform (Idaho Technology Inc., Salt Lake City, Utah) and verified by direct DNA sequencing as reported previously [14-17].

DNA isolation, bisulfate modification and targeted bisulfite sequencing assay

Genomic DNA was isolated by using Puregene Blood Core Kit B (Qiagen, Hamburg, Germany) and bisulfite conversion was performed as reported previously [18]. Primer sequences of the methylated *TCF21* were 5'-TYGGGGTTGTAGTTGTAGTTTAGG-3' (forward) and 5'-CTCTATACCAACTCAACACACTTACAAAC-3' (reverse). The DNA fragments were sequenced by Illumina MiSeq Benchtop Sequencer (Illumina, San Diego, CA, USA). Methylation level at each CpG site was calculated as the percentage of the methylated cytosines over the total tested cytosines. The average methylation level was calculated using methylation levels of all measured CpG sites within the gene as described [19].

TCGA and microarray validation data

The human disease methylation database DiseaseMeth version 2.0 (http://www.bio-bigdata.com/diseasemeth/analyze.html), based on The Cancer Genome Atlas (TCGA) database [20] and Gene Expression Omnibus (GEO) was used for differential methylation analysis [20, 21]. Level 3 Illumina HumanMethylation450k (HM-450) *TCF21* gene promoter methylation array data for 194 AML patients from TCGA were obtained via cBioPortal (http://www.cbioportal.org). The raw data were pre-processed using functional normalization in the R package "limma" [22, 23]. The Genomicscape Survival Analysis (http://genomicscape.com/microarray/survival.php) was applied to determine the impact of *TCF21* expression on survival of cytogenetically normal AML (CN-AML) patients.

Statistical analyses

SPSS 20.0 software package (IBM Corp, Armonk, NY, USA) and GraphPad Prism 5.0 were applied to statistical analyses. Mann-Whitney U-test was performed to compare the

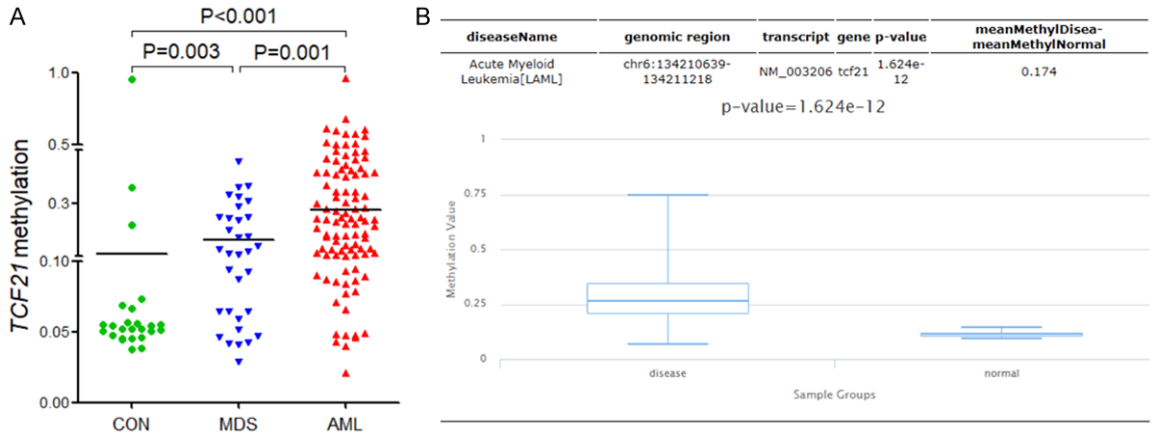

Figure 1. Relative methylation levels of *TCF21* promoter in controls, MDS and AML patients. A. *TCF21* methylation was examined by targeted bisulfite sequencing assay. The distributions of the *TCF21* methylation in controls, MDS patients and non-M3 AML patients were presented with scatter plots. The median level of *TCF21* methylation in each group was shown with horizontal line. B. *TCF21* methylation information of AML patients and normal controls (DiseaseMeth version 2.0) was analyzed through bioinformatics. (http://www.bio-bigdata.com/diseasemeth/analyze.html).

differences of continuous variables between two groups, while the difference of categorical variables between two groups was analyzed by Pearson χ^2-analysis/Fisher's exact test. The receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were carried out to assess the discriminative capacity of *TCF21* methylation level between patients and controls. Overall survival (OS) was surveyed from first diagnosis to last follow-up or death from any cause. Leukemia-free survival (LFS) was defined as time from complete remission (CR) to either relapse or death from any cause. The prognostic value of *TCF21* methylation for OS and LFS was analyzed by Kaplan-Meier analysis. Statistical significance was set at $P < 0.05$, and all tests were two sided.

Results

Methylation patterns of TCF21 in MDS and AML patients

The relative methylation levels of *TCF21* promoter in controls, MDS and AML patients are visually shown in **Figure 1A**. *TCF21* promoter methylation level showed significantly increased in MDS patients ($P = 0.003$) and AML patients ($P < 0.001$) compared with healthy controls respectively. We also observed a higher level of *TCF21* promoter methylation in AML patients compared with MDS patients ($P = 0.001$). For further verification, we applied the online database to analysis *TCF21* methylation pattern. According to DiseaseMeth version 2.0, the level of *TCF21* gene promoter methylation was significantly higher in AML patients (median level of 0.265) compared with controls (median level of 0.114) ($P < 0.001$, **Figure 1B**).

Differentiating value of TCF21 methylation in MDS and AML

ROC curve analysis showed that the level of *TCF21* promoter methylation could discriminate MDS and AML from controls with an AUC of 0.728 [95% confidence interval (CI): 0.588-0.869; $P = 0.003$] (**Figure 2A**) and 0.858 [95% CI: 0.761-0.954; $P < 0.001$] (**Figure 2B**) respectively. It can also distinguish AML from MDS with an AUC of 0.687 [95% CI: 0.587-0.787; $P = 0.001$] (**Figure 2C**).

According to the empirical cut-off value of 0.298 based on the methylation level of mean plus standard deviation of controls', patients were divided into two groups: *TCF21* non-hypermethylation (< 0.298) and *TCF21* hypermethylation (> 0.298). In MDS and AML, hypermethylation rates of *TCF21* gene promoter were 18.2% and 39%, while the rate in controls was 8% (**Table 1**). The distribution of *TCF21* hypomethylated and hypermethylated patterns in controls, MDS and AML were significantly different ($P = 0.002$).

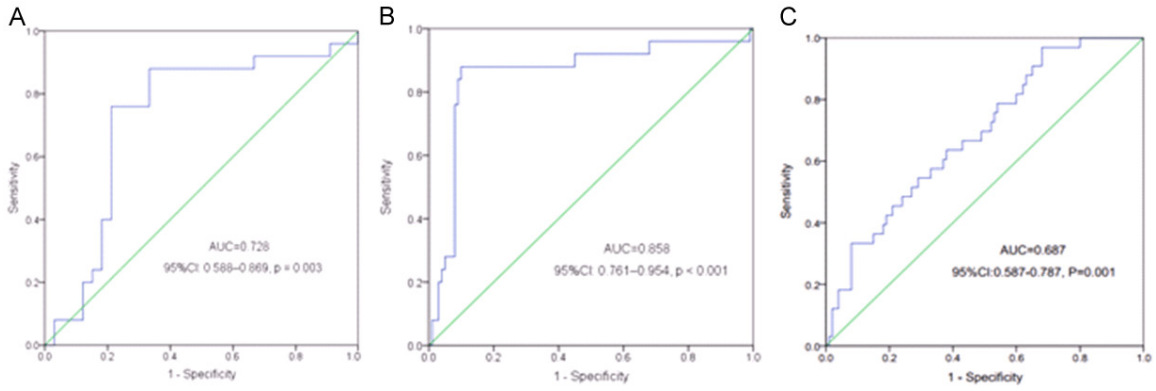

Figure 2. ROC curve analysis of *TCF21* methylation among controls, MDS and AML patients. AUC and *P* value reflected differentiating value of *TCF21* methylation among controls, MDS and AML. Discriminating AML from controls (A), discriminating MDS from controls (B), and discriminating AML from MDS (C).

Table 1. Comparison of *TCF21* non-hypermethylated and hypermethylated in AML and MDS patients

Cases	*TCF21* methylation		*P* value
	Non-hypermethylated	Hypermethylated	
Controls	23 (92%)	2 (8%)	0.002
MDS	27 (81.8%)	6 (18.2%)	
AML	61 (61%)	39 (39%)	

Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome.

Clinical and laboratory features of MDS and AML

We further compared the clinical correlation in MDS patients between *TCF21* non-hypermethylation and hypermethylation groups. There was no significant difference in age, gender, white blood cell (WBC) count, platelet count and BM blast percentage (P > 0.05, **Table 2**). We also did not find any significant difference in WHO and FAB classification, as well as the distributions of cytogenetic classification and IPSS. However, there was a significant difference in the hemoglobin level between *TCF21* non-hypermethylation and hypermethylation MDS patients (P = 0.009).

Among AML patients, there was no significant difference in age, gender, WBC count, hemoglobin level, BM blast percentage and FAB classification between *TCF21* non-hypermethylation and hypermethylation AML patients (**Table 3**). However, the platelet count was significantly lower in *TCF21* hypermethylation AML patients compared with *TCF21* non-hypermethylation (P = 0.010). Notably, there was a significant difference in the distribution of three cytogenetics prognostic risk categories between *TCF21* non-hypermethylated and hypermethylated patients (P = 0.032, **Table 3**). Meaningfully, we found that patients with adverse cytogenetic category had higher hypermethylation rates compared with those with favorable and intermediate cytogenetic categories, respectively (67% vs 44% and 29%). In addition, distribution of karyotypes between two groups of AML patients had a significant difference (P = 0.046). We also found that *TCF21* non-hypermethylated patients showed a higher probability of normal karyotype (P = 0.003, **Figure 3**).

Correlation of TCF21 methylation and gene mutation in MDS and AML

We investigated the differences of *IDH1/2*, *DNMT3A*, *U2AF1* and other gene mutations associated with MDS and AML between *TCF21* non-hypermethylation and hypermethylation groups (**Tables 2**, **4**). Except the higher incidence of *SF3B1* gene mutation in *TCF21* hypermethylation patients (P = 0.088), no significant distributional difference has been found in MDS patients between two groups. Although some mutations occurred in both or only one group of AML patients, the rate of *DNMT3A* gene mutation was significantly higher in the non-hypermethylation group than in the hypermethylation group (8/44 vs 0/34, P = 0.020), including R882H and R882P. Further analysis also verified that AML patients with *DNMT3A*

Table 2. Comparison of clinical manifestations and laboratory features between *TCF21* non-hypermethylated and hypermethylated MDS patients

Patient's parameter	Non-hypermethylated (n = 27)	Hypermethylated (n = 6)	*P* value
Sex (male/female)	13/14	3/3	1.000
Age (years)	67 (28-86)	51.5 (28-83)	0.183
WBC ($\times 10^9$/L)	3.7 (1.3-44.4)	2.7 (1.7-6)	0.599
HB (g/L)	68 (41-115)	45.5 (42-71)	0.009
PLT ($\times 10^9$/L)	71 (1-754)	43.5 (10-323)	0.500
BM blasts (%)	5 (0-18)	5 (1-11)	0.980
FAB			1.000
RA/RARS	15	3	
RAEB	11	3	
WHO			0.500
RA/RARS	4	0	
RCMD/RS	10	2	
RAEB-1	4	3	
RAEB-2	8	1	
MDS-U	1	0	
Cytogenetic classification			1.000
Favorable	17 (63%)	5 (83%)	
Intermediate	6 (22%)	1 (17%)	
Adverse	2 (7%)	0 (0%)	
No data	2 (7%)	0 (0%)	
IPSS			1.000
Low	3 (11%)	0 (0%)	
Int-1	12 (45%)	4 (67%)	
Int-2	7 (26%)	2 (33%)	
High	3 (11%)	0 (0%)	
No data	2 (7%)	0 (0%)	
Gene mutations			
IDH1/2 (+/-)	2/23	0/6	1.000
DNMT3A (+/-)	1/24	0/6	1.000
U2AF1 (+/-)	1/24	1/5	0.355
SF3B1 (+/-)	1/24	2/4	0.088
SRSF2 (+/-)	1/24	1/5	0.355

Abbreviations: WBC, white blood cells; HB, Hemoglobin; PLT, Platelet count; BM, bone marrow; IPSS, International Prognostic Scoring System; WHO, World Health Organization; RA, refractory anemia; RARS, RA with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, RCMD with ringed sideroblasts; RAEB, RA with excess of blasts. MDS-U, MDS with isolated del (5q).

gene mutation had a trend of appearing *TCF21* hypomethylation (P = 0.085, **Figure 4**).

The relationship between TCF21 methylation and prognosis in MDS and AML

According to Kaplan-Meier survival analysis, there was no significant correlation between *TCF21* methylation pattern and prognosis in MDS patients (P = 0.388, **Figure 5A**), as well as in AML patients (P = 0.536, **Figure 5B**). In order to eliminate the inaccuracies caused by methodology and sample size, we further investigate the prognostic value by utilizing TCGA data through HM450 BeadChip analysis. We divided the AML patients into two groups by the median methylation level and did not observe the prognostic impact of *TCF21* methylation on OS and LFS (P > 0.05, **Figure 5C**, **5D**).

Although our results showed that the methylation of *TCF21* promoter had no significant effect on the survival of MDS and AML, we wanted to know the relationship between the expression level of *TCF21* and the survival of MDS and AML patients. Next, we analyzed the relationship between *TCF21* mRNA expression and the prognosis of AML. We analyzed the association between *TCF21* mRNA expression and clinical outcomes of 163 CNAML and MDS patients in Metzeler dataset by GenomicScape survival analysis. CN-AML patients with lower *TCF21* mRNA expression level had a shorter OS time than those with higher *TCF21* mRNA level (P = 0.019, **Figure 6**) [24].

Discussion

Aberrant promoter hypermethylation represents a major mechanism leading to silencing of tumor suppressor genes in many kinds of human cancers [25, 26]. During the onset and progression of hematological malignancies, many changes can occur in the cellular epigenome, such as hypomethylation or increases in the methylation of CpG islands in promoter regions of key genes [27]. *TCF21* is a candidate

Table 3. Comparison of clinical manifestations and laboratory features between *TCF21* non-hypermethylated and hypermethylated AML patients

Patient's parameters	Non-hypermethylated (n = 61)	Hypermethylated (n = 39)	*P* value
Sex, male/female	39/22	25/14	1
Median age, years (range)	55 (18-85)	50 (18-81)	0.178
Median WBC, $\times 10^9$/L (range)	31.1 (0.8-300.0)	30.15 (1.1-160)	0.854
Median hemoglobin, g/L (range)	77 (32-147)	75 (33-138)	0.931
Median platelets, $\times 10^9$/L (range)	46.5 (3-415)	29.5 (5-136)	0.010
BM blasts, % (range)	43 (21.5-99.0)	58 (20.0-94.5)	0.721
FAB			0.725
M0	0 (0%)	1 (3%)	
M1	5 (8%)	4 (10%)	
M2	26 (43%)	17 (44%)	
M4	17 (28%)	11 (28%)	
M5	10 (16%)	6 (15%)	
M6	3 (5%)	0 (0%)	
Cytogenetic classification			0.032
Favorable	10 (16%)	8 (21%)	
Intermediate	42 (69%)	17 (44%)	
Adverse	6 (10%)	12 (31%)	
No data	3 (5%)	2 (5%)	
Karyotype			0.046
Normal	33 (54%)	13 (33%)	
Abnormal	24 (39%)	18 (46%)	
No data	4 (7%)	8 (21%)	

Abbreviations: WBC, white blood cells; HB, Hemoglobin; PLT, Platelet count; BM, bone marrow; FAB, French-American-British classification.

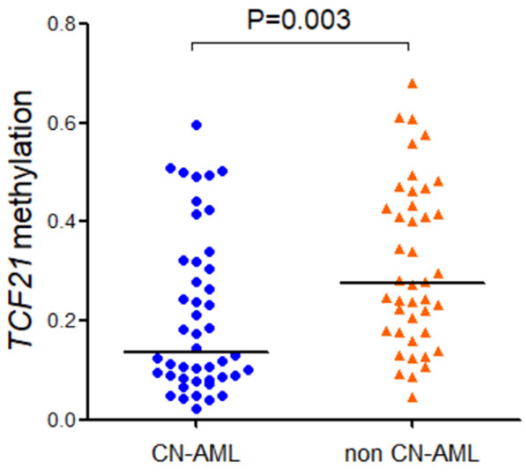

Figure 3. Relative methylation levels of *TCF21* in CN-AML and non-CN-AML patients. The distributions of the *TCF21* methylation in CN-AML and non-CN-AML patients were presented with scatter plots. The median level of *TCF21* methylation in each group was shown with horizontal line.

tumor suppressor gene with promoter hypermethylation has been demonstrated in different cancers [28]. To identify whether differences in *TCF21* promoter methylation be implicated in AML and MDS, We studied *TCF21* promoter methylation levels and their association with clinical and genetic characteristics in novo non-M3 AML and MDS patients. The *TCF21* promoter methylation level of AML and MDS patients was significantly higher than that of the control group, and the former is higher than the latter, suggesting that *TCF21* may involve the development and progression of myeloid tumor. *TCF21* methylation was significantly correlated with platelet count in AML patients, and hemoglobin count in MDS patients, while no significant correlation was found in other clinical parameters. The significance of these findings needs to be further explored. ROC curve analysis of *TCF21* promoter methylation was helpful to distinguish between MDS, AML and normal control, respectively. Many groups have confirmed that aberrant methylation patterns of multiple genes can be used for biomarker of AML and MDS for disease onset and progression, patient stratification, disease prognosis, and response to treatment. MDS have preleukemic features and frequently evolve to AML. *TCF21* methylation detection may be a potential predictor of MDS progress towards AML [29].

We found that *TCF21* non-hypermethylated AML patients had a higher probability of normal karyotype. The nonrandom chromosomal abnormalities detected at diagnosis are important predictors of prognosis and risk of relapse in patients with AML [30]. Here, we demonstrated an association between *TCF21* hypermethylation and genetics adverse stratification in AML

Table 4. Comparison of Gene mutation between AML patients with TCF21 non-hypermethylation and hypermethylation

Gene mutation	TCF21 methylation		*P* value
	Non-hypermethylated (n = 52)	Hypermethylated (n = 34)	
CEBPA (+/-)	10/42	5/29	0.773
NPM1 (+/-)	7/45	2/32	0.309
FLT3-ITD (+/-)	7/45	2/32	0.309
c-KIT (+/-)	5/47	2/32	0.699
N/K-RAS (+/-)	6/46	8/26	0.231
IDH1/2 (+/-)	4/48	0/34	0.149
DNMT3A (+/-)	8/44	0/34	0.020
U2AF1 (+/-)	0/52	2/32	0.153
SRSF2 (+/-)	1/51	0/34	1.000
SETBP1 (+/-)	0/52	2/32	0.153

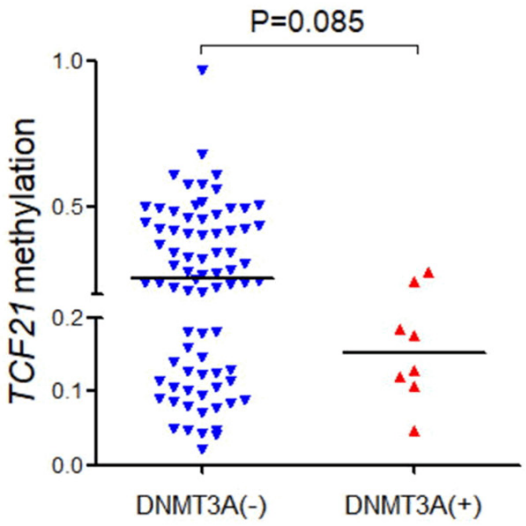

Figure 4. Relative methylation levels of *TCF21* in AML patients without *DNMT3A* gene mutation and with *DNMT3A* gene mutation. The distributions of the *TCF21* methylation in AML patients without DNMT3A gene mutation and with DNMT3A gene mutation were presented with scatter plots. The median level of *TCF21* methylation in each group was shown with horizontal line.

patients. In addition, we detected a variety of mutations in AML patients, including mutations in *CEBPA*, *NPM1*, *c-KIT*, *DNMT3A*, and etc. Interestingly, we found that *DNMT3A* mutation is related to *TCF21* methylation pattern. *DNMT3A* gene mutation occurred frequently in *TCF21* non-hypermethylation AML patients than in hypermethylation patients. *DNMT3A* is an S-adenosyl methionine (SAM)-dependent DNA methyltransferases in mammalian [31]. *DNMT3A* mutation at amino acid position (R882) dramatically reduced cellular DNA methyltransferase activity [32, 33]. We deduced that loss of function of DNA methyltransferases caused by DNMT3A mutation may lead to the *TCF21* hypomethylation during leukemogenesis. Studies indicate that AML patients with *DNMT3A* mutations suffer poor prognoses [34-37], but they are sensitive to hypomethylating agent decitabine (5-aza-2'-deoxycytidine) [38]. Decitabine treatment can also resulted in *TCF21* gene reactivation in lung cancer cells and head and neck squamous cell carcinoma cells, however, it had little effect in cells where *TCF21* is expressed [5]. The relationship between *TCF21* hypermethylation and *DNMT3A* mutations needs to be further explored. We want to know whether the hypomethylation of *TCF21* is caused by *DNMT3A* mutation, and whether patients with *DNMT3A* mutation and *TCF21* hypomethylation have better effect on decitabine.

Although the promoter methylation of *TCF21* had no significant correlation with the survival of AML, further studies showed the association of *TCF21* expression with prognosis in AML. Moreover, some studies proved that *TCF21* expression or methylation was related to prognosis in solid tumors, which further indicated that it can be used as a prognosis marker, as well as in AML. Hereby, we can speculate that AML prognosis may have some links with *TCF21* expression.

In summary, based on the results of our study, we believe that TCF21 methylation patterns plays a potential role in the pathophysiology of AML and MDS.

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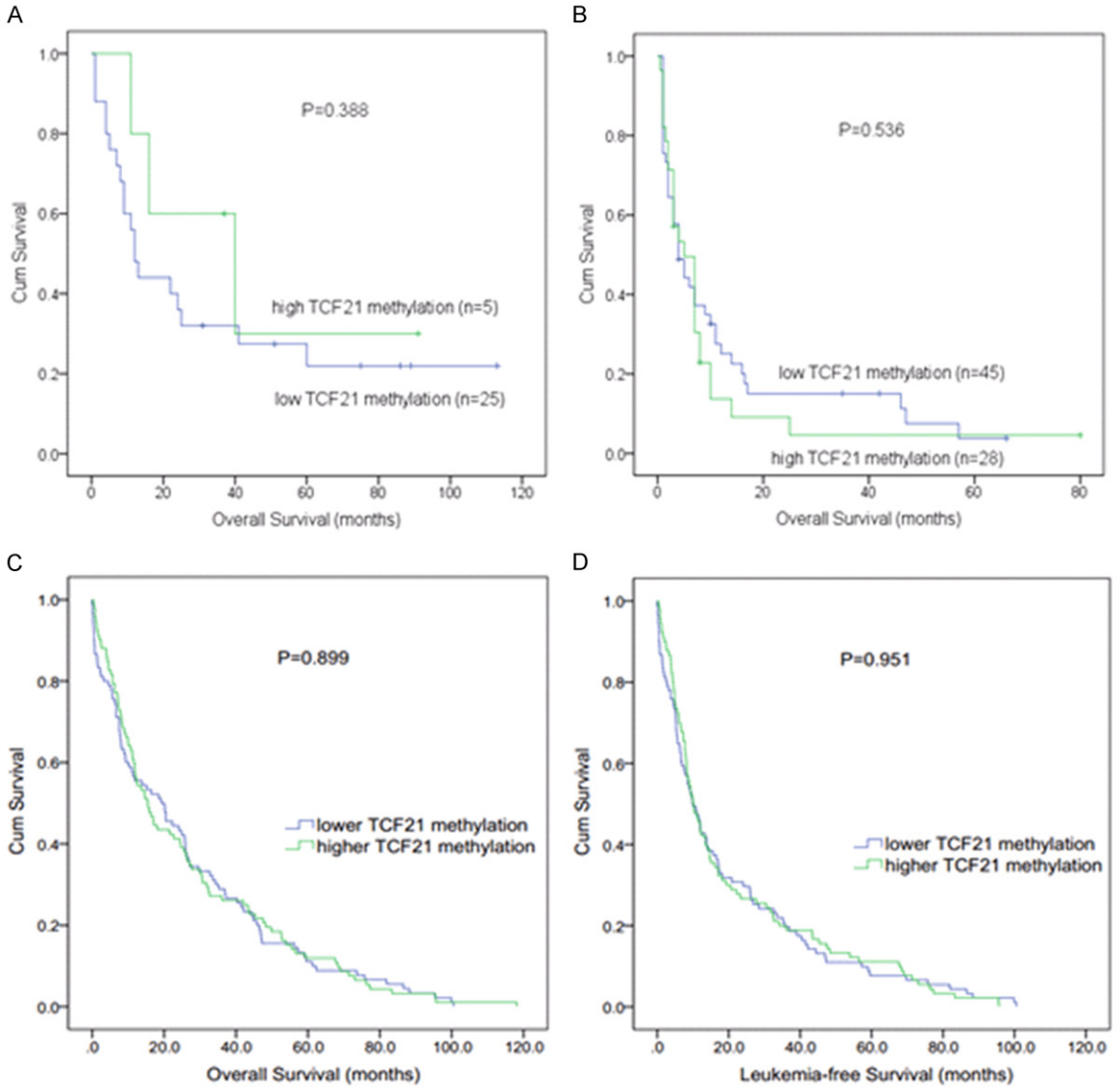

Figure 5. Survival analysis of *TCF21* methylation in MDS and AML patients. OS analysis of MDS patients (A) and AML patients (B) from our cohort. OS (C) and LFS analysis (D) of AML patients from TCGA datasets. TCGA AML (HM450) data was obtained via cBioPortal (http://www.cbioportal.org). AML patients were divided into two groups by the median methylation level of *TCF21*.

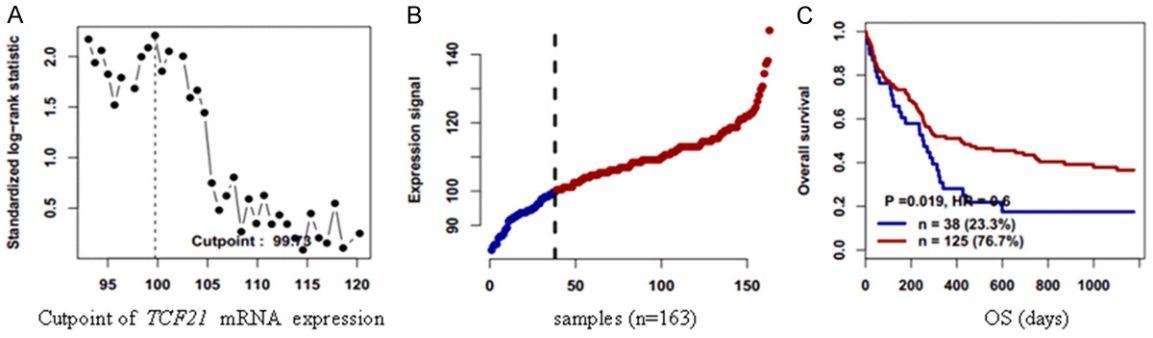

Figure 6. Survival analysis Gene-expression profiling was performed using Affy metrix U133 A microarrays (probe 204931_at *TCF21*). Survival analysis of 163 CN-AML and MDS patients associated with *TCF21* mRNA expression

in Metzeler ataset (Metzeler, Blood, 2008) analyzed by the Genomicscape Survival Analysis. (http://genomicscape.com/microarray/survival.php): cut point of *TCF21* mRNA expression in CN-AML patients (A), *TCF21* mRNA expression signal of 163 CN-AML patients (B) and OS analysis (C).

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Qian, Department of Hematology, Affiliated People's Hospital of Jiangsu University, 8 Dianli Road, Zhenjiang 212002, Jiangsu, People's Republic of China. Tel: 13852912838; Fax: +86.511.85234387; E-mail: qianjun0007@hotmail.com; Dr. Jiang Lin, Laboratory Center, Affiliated People's Hospital of Jiangsu University, 8 Dianli Road, Zhenjiang 212002, Jiangsu, People's Republic of China. Tel: 18952819839; Fax: +86.511.85234387; E-mail: 2651329493@qq.com

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