**DOK6** promoter methylation serves as a potential biomarker affecting prognosis in de novo acute myeloid leukemia

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**Abstract**

**Background:** Downstream of tyrosine kinase 6 (**DOK6**), which is specifically expressed in the nervous system, was previously recognized as an adapter only in neurite outgrowth. Recent studies also demonstrated the potential role of **DOK6** in solid tumors such as gastric cancer and breast cancer. However, previous studies of **DOK6** have not dealt with its roles in myeloid malignancies. Herein, we verified the promoter methylation status of **DOK6** and further explored its clinical implication in de novo acute myeloid leukemia (AML).

**Methods:** A total of 100 newly diagnosed adult AML patients were involved in the current study. **DOK6** expression and methylation were detected by real-time qPCR and methylation-specific PCR (MSP), respectively. Bisulfite sequencing PCR (BSP) was performed to assess the methylation density of the **DOK6** promoter.

**Results:** Downstream of tyrosine kinase 6 promoter methylation was significantly increased in AML patients compared to controls (\(P = .037\)), whereas **DOK6** expression significantly decreased in AML patients (\(P < .001\)). The expression of **DOK6** was markedly up-regulated after treated by 5-aza-2'-deoxycytidine (5-aza-dC) in THP-1 cell lines. The methylation status of the **DOK6** promoter was associated with French-American-British classifications (\(P = .037\)). There was no significant correlation existed between **DOK6** expression and its promoter methylation (\(R = .077, P = .635\)). Interestingly, of whole-AML and non-APL AML patients, both have a tendency pertaining to the **DOK6** methylation group and a significantly longer overall survival (OS) than the **DOK6** unmethylation group (\(P = .042\) and .036, respectively).
1 | BACKGROUND

As a disease characterized by clonal hematopoietic stem cell disorders, acute myeloid leukemia (AML) has a cure rate of 35%-40% in those younger than 60 and a cure rate of 5%-15% in those over 60 years of age. However, there were only 5-10 months of median survival in older patients who could not tolerate the side effects of intensive chemotherapy. Despite the molecular diagnosis and chemotherapy improvements, the long-term survival rate for patients with advanced stage remains disappointing. Currently, the molecular evaluation that focused on a single consistent cancer pathway for intensive induction chemotherapy or complete remission in AML seems to be weak. Additionally, the cancer phenotype typically is kept by multiple oncogenic pathways or processes. Thus, newly integrated biomarkers which act as modulators for multiple oncogenic signaling pathways are urgently needed.

Downstream of tyrosine kinase (DOK) multigenic family consists of seven family members, which possess a similar structural topology and function as substrates of nonreceptor tyrosine kinases and multiple receptor tyrosine kinases. Some of them have been proved to play a key role in the negative regulation of immune cell signaling. For example, DOK1, DOK2, and DOK3 were identified as a tumor suppressor in lung tumor and aggressive histiocytic sarcoma (HS). Downstream of tyrosine kinase 4 and DOK5 is mainly expressed in the nervous system. However, DOK7 was mainly enriched in skeletal muscle and myocardium. Previously, DOK6 was found to be involved in neuronal development through Ret and neurotrophin-3 signaling. Leong et al showed that DOK6 is involved in a variety of oncogenic signaling pathways and functioned broadly in gastric cancer, and provided functional relevance of its binding to the epidermal growth factor receptor (EGFR). Tamara et al reported that DOK6 behaved as a tumor suppressor in human breast cancer. However, the research to date has tended to focus on solid tumors rather than the hematological tumor. The expression of DOK6 remains unknown. Furthermore, whether DOK6 expression is regulated by its promoter region in which a large CpG island is embedded is still unknown. This prompted us to investigate the methylation status of the DOK6 promoter and further explore its clinical significance in AML patients.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

In this study, the leukemia cell line THP-1 was cultured using RPMI 1640 medium with a serum concentration of 10% fetal calf. An environment having a temperature of 37°C and a carbon dioxide concentration of 5% was set as the cell culture condition. For demethylation experiments, cells were treated by a final concentration of 0, 0.1, 1 and 10 μmol/L 5-aza-dC (Sigma Aldrich) for 72 hours before harvest.

2.2 | Patients and tissue samples

Bone marrow (BM) specimens from 100 patients were collected for genomic DNA extraction. All patients had a confirmed diagnosis of previously untreated AML at the Affiliated People's Hospital of Jiangsu University, Jiangsu, China. Normal BM samples were picked up from 23 healthy donors. The diagnosis and clinical stages of AML were confirmed following the French-American-British (FAB) and the World Health Organization (WHO) criteria. All eligibility criteria and treatment protocols were consistent with our previous reports. Lymphocyte Separation Medium and gradient centrifugation were used to extract BM mononuclear cells (BMMNCs) from BM specimens. The study was approved by the Clinical Research Ethics Committee of the Affiliated People's Hospital of Jiangsu University and all patients signed informed consent for voluntary participation.

2.3 | RNA isolation, reverse transcription, and real-time qPCR

Trizol reagent (Invitrogen) was used to isolate total RNA from pre-extracted BMMNCs. Reverse transcription reaction with 40 μL volume was composed of 10 mmol/L of dNTPs (deoxyribonucleoside triphosphates), 5x buffer 10 mmol/L, 80 U of RNAsin, 10 μmol/L of random hexamers, and 200 U of MMLV

Conclusion: Our study suggested that DOK6 promoter hypermethylation was a common molecular event in de novo AML patients. Remarkably, DOK6 promoter methylation could serve as an independent and integrated prognostic biomarker not only in non-APL AML patients but also in AML patients who are less than 60 years old.

KEYWORDS
AML, biomarker, DOK6, methylation, prognosis
reverse transcriptase (Eppendorf). The reaction conditions were incubated for 10 minutes at 25°C, 60 minutes at 42°C, and then stored at −20°C. Analysis of DOK6 gene expression in AML and control specimens was performed by real-time qPCR with the primers shown in Table 1. The real-time qPCR reaction system with 20 μL volume composed of cDNA 20 ng, 0.8 μmol/L of primers, 0.4 μmol/L of ROX Reference Dye II (Takara), and 10 μmol/L of SYBR Premix TB Green. The real-time qPCR reaction conditions were 95°C for 5 minutes, followed by 40 cycles at 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 82°C for 30 seconds to collect fluorescence, finally followed by 95°C for 15 seconds, 60°C for 60 seconds, 95°C for 15 seconds, and 60°C for 15 seconds. Negative and positive controls were included to rule out false positives and false negatives, respectively. The relative expression levels of DOK6 were calculated by the $2^{-\Delta\Delta CT}$ method.

2.4 | DNA extraction, bisulfite modification and methylation-specific PCR

Genomic DNA from AML patients, AML cultured cells and healthy donors were isolated using genomic DNA purification kit (Gentra). The CpGenome DNA Modification Kit (Chemicon) was used to modify genomic DNA according to the manufacturer’s recommendations. Methylation-specific PCR (MSP) was used to detect DOK6 methylation status by the methylation primers (Table 1) with SYBR Premix Ex TaqII (Takara). The reaction conditions were 95°C for 30 seconds, 40 cycles for 5 seconds at 95°C, 30 seconds at 62°C, 30 seconds at 72°C, and 78°C for 32 seconds. DNA bisulfite modification was carried out using the CpGenome™ DNA Modification Kit (Chemicon). The quantification of DOK6 methylation was calculated with the same model as DOK6 expression.

2.5 | Bisulfite sequencing PCR

For bisulfite sequencing PCR (BSP), a 312-bp fragment was amplified from the DOK6 promoter region, using primers pair specific for bisulfite-modified sequences (Table 1). Bisulfite sequencing PCR reaction conditions were 98°C for 10 seconds, 40 cycles for 10 seconds at 98°C, 30 seconds at 59°C, 72°C for 30 seconds, and followed by a final 7 minutes extension step at 72°C. The reaction system of BSP was carried out as reported previously.26,27 AxyPrep DNA gel extraction kit (AxyGen) was used to purify BSP products, ligated into pMD19-T Vector (Takara), and then transfected into DH5α competent cells (Vazyme) for cloning. Finally, six independent clones of each sample were sequenced timely (BGI Tech Solutions Co.).

2.6 | Statistical analysis

All data were analyzed using IBM SPSS software package version 22.0 and GraphPad Prism 5.0. The Pearson Chi-square test or Fisher exact test was applied to compare two groups of categorical variables. Student’s t test was applied to compare two groups for normally distributed quantitative variables. Kaplan-Meier analysis and Cox regression model (univariate and multivariate analyses) were used to assess the effect of DOK6 methylation on the overall survival (OS). A two-sided P value of .05 or less was defined as statistically significant.

3 | RESULTS

3.1 | The methylation of DOK6 promoter in AML patients at diagnosis

To examine the promoter methylation status of DOK6 in AML patients and further analyze their clinical significance, the MSP and BSP primer sets and assays were designed at the CpG islands of the DOK6 gene promoter (Figure 1A). Firstly, DOK6 methylation status was examined by MSP, and the results showed that the DOK6 promoter methylation level of AML patients is significantly

| Primers | Sequence(5′-3′) | Product size (bp) |
|----------|-----------------|------------------|
| qPCR | DOK6-Forward | CAGGGCTACGTGAAAAATCCG | 200 |
| | DOK6-Reverse | TTCCTTGCTTCTCAGGGCCAG |  |
| MSP | DOK6-M-Forward | ATTAATTCTCGGTCCGTC | 128 |
| | DOK6-M-Reverse | AAAAAAACCAATCGTAGC |  |
| | DOK6-U-Forward | TAAATATCTTCTTGGTGGTT | 128 |
| | DOK6-U-Reverse | CACAAAAAAACAAATCATAAC |  |
| BSP | DOK6-B-Forward | TTATTGTGTTTTTATAATTAGGGGAGA | 312 |
| | DOK6-B-Reverse | CAAACCCCTTCTCTATACACACA |  |

Abbreviations: BSP, bisulfite sequencing PCR; DOK6, downstream of tyrosine kinase 6; M, methylation; MSP, real-time quantitative methylation-specific PCR; qPCR, real-time quantitative PCR; U, unmethylation.
higher than controls, with a median of 0.231 vs 0.060 (P = .037; Figure 1B). Secondly, two controls and a DOK6 methylated AML patient, as well as a DOK6 unmethylated AML patient, were selected randomly to verify the MSP results by BSP. Consistent with the result of MSP, both the DOK6 promoter of healthy donors and the unmethylated patient tend to present completely unmethylated, while the methylated AML patient demonstrated a high methylation density (Figure 1C). In addition, DOK6 promoter methylation was significantly decreased in MDS and CML patients compared to controls (P = .0002 and P < .0001, respectively; Additional file 1: Figure S1).

3.2 | Epigenetic mechanism regulating DOK6 expression in AML

To identify whether DOK6 expression is regulated by its promoter methylation in AML, 5-aza-dC, the DNMT inhibitor, was used to treat the THP-1 cell line. The expression of DOK6 was markedly up-regulated after 5-aza-dC treatment (Figure 2A). Meanwhile, the methylation level of the DOK6 promoter was significantly decreased in THP-1 cell lines which were treated by 5-aza-dC (Figure 2B). Additionally, a small quantity of AML samples was used to detect the expression of DOK6 in the current study. The results showed that DOK6 significantly decreased in de novo AML patients (P < .001; Figure 2C).

3.3 | Comparison of clinical characteristics between DOK6 unmethylated and DOK6 methylated group

To further analyze the clinical impact of DOK6 methylation, all patients of AML were divided into DOK6 unmethylated and DOK6 methylated groups according to the cutoff value. No significant differences were observed in variables including sex, age, white blood cell, platelets, hemoglobin, and BM blasts between the patients with and without DOK6 promoter methylation (P > .05; Table 2). Moreover, there was no significant difference in karyotypic classifications between the methylated and unmethylated patients (P > .05; Table 2). However, the gene mutation of nucleophosmin (NPM1) and isocitrate dehydrogenase (IDH1/2) was more frequently observed in unmethylated patients (P = .075, and .075, respectively; Table 2). Moreover, statistical analysis showed a significant difference in the distribution of FAB between the methylated and unmethylated patients (P = .037; Table 2).
3.4 | Prognostic significance of DOK6 promoter methylation in whole-AML and non-APL patients

To determine the prognostic value of DOK6 promoter methylation in AML, a total of 100 cases with follow-up data were used for survival analysis. No significant differences were observed in the complete remission (CR) rate between patients with and without DOK6 promoter methylation (52% vs 38%; \( P = .208 \)). However, in whole-AML cases, patients with DOK6 promoter methylated had a significantly longer OS than those without DOK6 promoter methylated (mean 23.10 vs 14.20 months; \( P = .042 \); Figure 3A). Furthermore, among non-APL patients, the patients with DOK6 promoter methylation also had significantly longer OS than those without DOK6 promoter methylation (mean 19.17 vs 9.96 months; \( P = .036 \); Figure 3B). To check out the independent prognostic factors on disease outcome in non-APL AML, a multivariate logistic analysis model was created (Table 3). Downstream of tyrosine kinase 6 promoter methylation was one of the independent factors which displayed an approximatively significant impact on OS (odds ratio [OR] = 0.577, 95% confidence interval [CI] [0.331-1.005], \( P = .052 \)) in non-APL patients, other factors associated with OS were age and karyotype risk (Table 3). In addition, DOK6 low-expression patients had a significantly longer OS (\( P = .011 \); Figure 3C).

3.5 | Prognostic significance of DOK6 promoter methylated in AML patients who are less than or equal 60 years old

Because age is usually treated as an important risk factor in cancer, patients who are less than 60 years old were separated in this study. Similarly, significant difference was found in OS between the patients with and without DOK6 promoter methylation (mean 29.77 vs 19.31 months; \( P = .031 \); Figure 3D). Multivariate Cox analysis identified DOK6 methylation as an independent prognostic factor (OR = 0.477, 95% CI [0.233-0.976] \( P = .043 \)) (Table 4).
**DISCUSSION**

Downstream of tyrosine kinase family, which acts as substrates of multiple receptor tyrosine kinases and nonreceptor tyrosine kinases, plays a unique role in different organs and tissues. All family members display a high degree of similarity over the regions, in which the Pleckstrin homology and phosphotyrosine-binding (PTB)
domains existed. Interestingly, despite the fact that all members of the DOK family share similar structure, they exert differently, or even opposite, roles based on the surrounding circumstances. As a sort of adapter with multiple docking sites for signaling proteins, DOK proteins act as both carcinogenic and tumor-suppressing proteins. Recently, He et al have proved that the expression of DOK1/2 was inactivated by their promoter methylation, and is associated with an adverse prognosis in AML. The study by Fu et al has shown that increased DOK4 and DOK5 expression were closely related to adverse prognosis, while increased DOK7 expression was associated with a favorable prognosis in AML. The above literature data demonstrated that different DOK protein exerts a different effect on OS and LFS in AML.

Downstream of tyrosine kinase 6, among them, was found to promote neurite outgrowth by the Ret-mediated signaling pathway in N2A-α1 cells. Wei et al demonstrated that DOK6 selectively combined with the NPQY motif of TrkC via its PTB domain in a kinase activity-dependent manner and is involved in NT-3-mediated neuronal development. Besides, Leong and his colleagues reported that DOK6 combined with various components in different steps of multiple signaling pathways, such as platelet-derived growth factor, nerve growth factor, EGFR, RAS, vascular endothelial growth factor and RAF/MAP kinase. Importantly, most of them had been proved as

![Figure 3](image-url)
Accumulating results imply that DOK6 enhances many oncogenic signaling pathways by interacting with a variety of different signaling proteins and receptors. Therefore, with the reduction of DOK6 expression, multiple carcinogenic signaling pathways would be inevitably affected.

As the most studied epigenetic alteration, DNA methylation has been involved in a variety of regulatory processes, such as genome integrity, loss of imprinting, genome integrity, transcriptional regulation, and chromatin structure. Therefore, cancer-specific promoter methylation contributes to the discovery of novel tumor suppressor genes and/or tumor-specific prognostic biomarkers, the development of novel treatment strategies, and treatment response prediction. Here, as far as we know, it is the first time to report that DOK6 promoter methylation was a common event in patients with newly diagnosed AML. Although we did not observe the significant impact of DOK6 promoter methylation on CR, our investigation revealed that the methylation status of the DOK6 promoter had a significant association with OS.
Interestingly, patients with DOK6 promoter methylation displayed a much longer OS in both whole-AML and non-APL patients. Notably, our results referring to the prognostic value of DOK6 expression were consistent with those reported in gastric cancer by Leong et al.21 Similar prognostic value of the other DOK family member such as DOK4/5 was also reported by Fu et al.17 A possible explanation for this was that decreased DOK6 expression affected multiple carcinogenic signaling pathways, which contributed to the favorable outcome of methylated AML patients. Further research should be taken to expand the molecular mechanisms involved in DOK6 adaptor protein’s function in multiple tyrosine kinases signaling pathways as well as their role in leukemogenesis.

As is well known, DNA methylation in promoter CpG islands played a crucial role in regulating gene expression. In this study, we also revealed that DOK6 was significantly decreased in de novo AML patients and decreased DOK6 expression was associated with a favorable outcome. Furthermore, the cell experiment indicated that 5-aza-dC increased DOK6 expression in leukemia cells THP-1 by inducing demethylation of the DOK6 promoter region.

5 | CONCLUSION

Taken together, our study identified that DOK6 promoter methylation is a common molecular event in de novo AML patients. Remarkably, DOK6 promoter methylation could serve as an independent and integrated prognostic biomarker not only in non-APL but also in AML patients who are less than or equal 60 years old.

DECLARATIONS

Ethics approval and consent to participate

The study was approved by the Clinical Research Ethics Committee of the Affiliated People's Hospital of Jiangsu University.

Consent for publication

Written informed consents were obtained from all enrolled voluntary individuals before their participation.

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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