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*Original Article*

# *MOK* overexpression is associated with promoter hypomethylation in patients with acute myeloid leukemia

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**Abstract:** Overexpression of MAPK/MAK/MRK overlapping kinase (*MOK*) has been found in various tumors. However, the mechanism underlying *MOK* upregulation remains unclear. A CpG island was identified in *MOK* promoter. In this study, we evaluated the expression and methylation status of *MOK* gene in acute myeloid leukemia (AML). Hypomethylation of *MOK* promoter was detected in 31.0% (45/145) of AML patients. The degree of *MOK* hypomethylation was significantly correlated with *MOK* expression in AML patients. *MOK*-hypomethylated patients had a trend towards lower WBCs. Receiver operating characteristic curve (ROC) analysis showed a good performance in distinguishing AML patients from controls with an area under the ROC curve (AUC) of 0.820 ($P < 0.001$). In summary, our results suggest *MOK* promoter hypomethylation is a common event and contributes to *MOK* overexpression in AML.

**Keywords:** *MOK*, hypomethylation, acute myeloid leukemia

## Introduction

Acute myeloid leukemia (AML) is an aggressive malignant disorder caused by uncontrolled proliferation of myeloid precursor cells and marked by genetic and epigenetic abnormalities leading to a block in differentiation and accumulation of leukemic blasts in blood and bone marrow. Genetic alterations are recognized as responsible for pathogenesis and progression of AML. In addition, those genetic abnormalities present in leukemic cells in the majority of AML patients have been already clinically applied as important prognostic factors in AML [1].

In addition to the genetic abnormalities, epigenetic lesions also play essential roles in the pathogenesis of AML. Compared with genetic alterations, epigenetic lesions appear to be more frequent and recurrent [2]. Aberrant DNA methylation including hypermethylation and hypomethylation is one of the most common and the most studied epigenetic feature in human cancers. It is now well known that molecular events such as global DNA hypomethylation, gene-specific hypermethylation occur during the transformation of malignant cells [3]. Aberrant promoter hypermethylation of a great many genes such as *p15*, *p73*, *ID4*, *E-cadherin*, *RARβ2* has been observed in AML [4, 5]. However, most researches have focused on the roles and mechanisms of hypermethylation of tumor suppressor genes (TSGs) in cancer so far, much less attention has been paid to the significance of the cancer-linked DNA hypomethylation.

MAPK/MAK/MRK overlapping kinase (*MOK*), also known as *RAGE-1* (renal tumor antigen-1) was firstly identified in a renal carcinoma cell line [6]. To date, *MOK* protein has been considered as a tumor-associated antigen (TAA) for its wide expression in various tumors including renal carcinoma, melanoma, head and neck cancer, mesothelioma, hepatocellular carcinoma and AML but absent in normal tissues other than retina, which was considered as an immu-

**Table 1.** Correlation of MOK promoter methylation with clinical features in AML patients

| Patient's parameters | The status of *MOK* methylation | | |
|---|---|---|---|
| | Hypomethylated (n = 45) | Methylated (n = 100) | *P* value |
| Sex (male/female) | 26/19 | 58/42 | 0.980 |
| Age (years)[a] | 53 (3-93) | 54 (10-87) | 0.801 |
| WBC (× $10^9$/L)[a] | 9.55 (1.50-249.30) | 20.60 (0.50-528.00) | 0.056 |
| Hemoglobin (g/L)[a] | 76.50 (32-147) | 74 (31-142) | 0.963 |
| Platelet counts (× $10^9$/L)[a] | 41 (3-447) | 42 (4-264) | 0.843 |
| FAB subtypes | | | 0.978 |
| M0 | 0 | 1 | |
| M1 | 4 | 7 | |
| M2 | 17 | 45 | |
| M3 | 6 | 12 | |
| M4 | 12 | 21 | |
| M5 | 4 | 10 | |
| M6 | 2 | 4 | |
| WHO | | | 0.964 |
| AML with t (8; 21) | 3 | 11 | |
| APL with t (15; 17) | 6 | 12 | |
| Minimally differentiated AML | 0 | 1 | |
| AML without maturation | 3 | 7 | |
| AML with maturation | 15 | 34 | |
| Acute myelomonocytic leukemia | 12 | 21 | |
| Acute monoblastic and monocytic leukemia | 4 | 10 | |
| Acute erythroleukemia | 2 | 4 | |
| Cytogenetics classification | | | 0.427 |
| Favorable | 7 | 22 | |
| Intermediate | 26 | 60 | |
| Poor | 5 | 11 | |
| No data | 7 | 7 | |
| Karyotype | | | 0.657 |
| normal | 20 | 46 | |
| T (8; 21) | 3 | 12 | |
| T (15; 17) | 4 | 9 | |
| 11q23 | 1 | 0 | |
| complex | 3 | 9 | |
| others | 7 | 17 | |
| Gene Mutation | | | |
| C-KIT (+/-) | 1/42 | 9/82 | 0.167 |
| C/EBPA (+/-) | 5/38 | 12/79 | 0.800 |
| NPM1 (+/-) | 2/41 | 10/81 | 0.337 |
| FLT3 ITD (+/-) | 2/41 | 1/90 | 0.241 |
| IDH1 (+/-) | 1/42 | 3/88 | 1.000 |
| IDH2 (+/-) | 3/40 | 4/87 | 0.680 |
| IDH1/IDH2 (+/-) | 4/39 | 7/84 | 0.745 |
| DNMT3A (+/-) | 2/41 | 8/83 | 0.500 |
| *MOK* transcript (%)[a] | 1.87 (0.00-16.34) | 0.30 (0.00-4.39) | 0.001 |

[a]Median (range); WBC, white blood cells; FAB, French-American-British classification; AML, acute myeloid leukaemia.

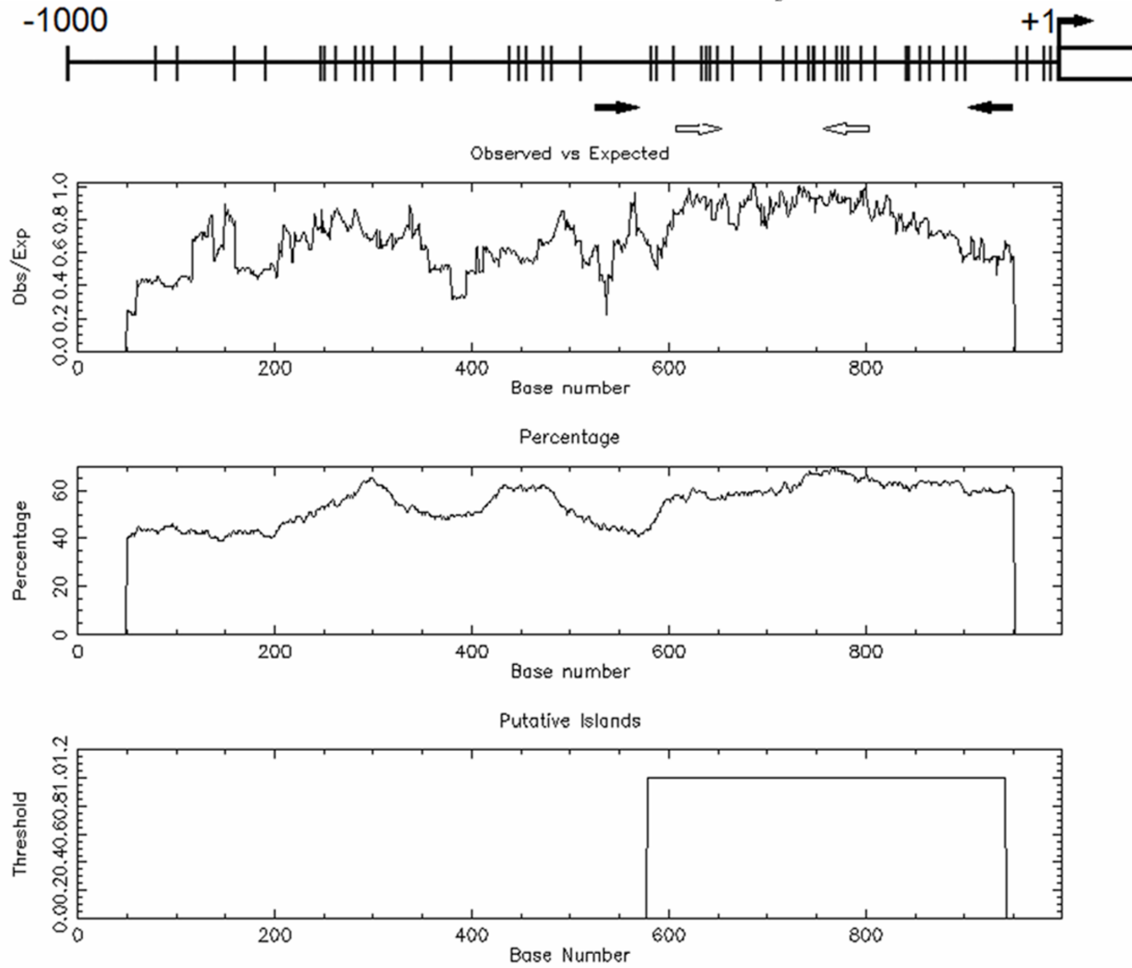


**Figure 1.** Bioinformatics analysis of *MOK* promoter on chromosome 14. The vertical lines on the top horizontal line indicate the cytosine resides of CpGs. Numbers in the top panel represent nucleotide positions from *MOK* translating initial codon. Turning black arrow indicates the translating initial codon of *MOK* gene; the straight black arrows indicate the locations of primers used for bisulfite sequencing analysis, and the blank arrows indicate the locations of primers used for RQ-MSP analysis. Second panel represents the distribution of observed/expected ratios of CpG dinucleotides; third panel plots the GC content as a percentage of the total; bottom panel represents the putative CpG island within the -1.0 kb of analyzed sequence.

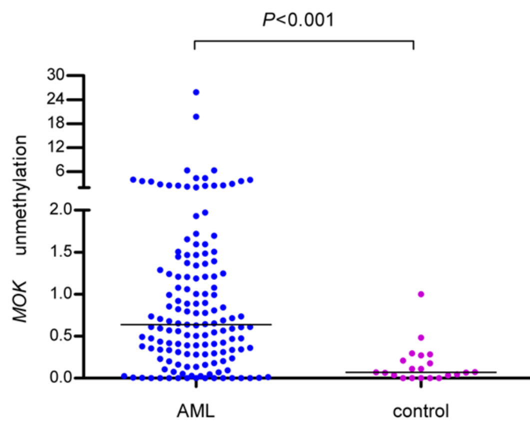


**Figure 2.** Levels of *MOK* hypomethylation in AML and control.

noprivileged site due to the lack of HLA expression [6-13]. *MOK* was likewise regarded as a potential target for cancer-specific immunotherapy because it could be recognized by cytotoxic T lymphocytes (CTLs) to induce immune response [6, 14]. However, the underlying mechanism of *MOK* overexpression in these tumors has not been understood. In reviewing the structure of *MOK* promoter, we found the existence of a large CpG island. Whether there is the aberrant methylation of *MOK* promoter in leukemia has not been reported until now. The primary aim of the present study is to explore the methylation status of *MOK* promoter and determine its clinical implications in AML patients.

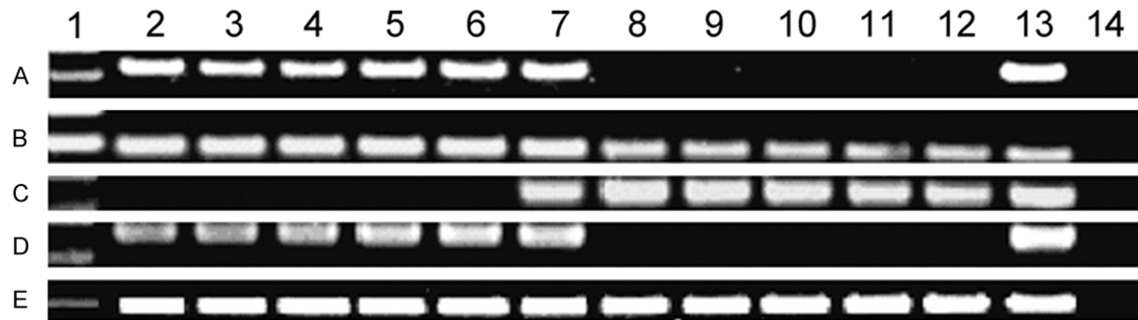


**Figure 3.** Electropheresis results of RQ-PCR and RQ-MSP products of *MOK* gene in AML patients. A: *MOK* expression; B: A*BL* expression; C: *MOK* methylation; D: *MOK* unmethylation; E: *ALU*. 1: 100 bp DNA Ladder; 2-11: AML; 12: normal control; 13: positive control; 14: negative control.

## Materials and methods

### *Patients and samples*

This study was authorized by the Ethics Committee Board of Affiliated People's Hospital of Jiangsu University. 145 primary AML patients in the present study were gathered based on the availability of obtained leukemic cells. The diagnosis and classification of AML patients was based on morphology and cytochemistry by French-America-British (FAB) and World Health Organization (WHO) criteria [15, 16]. Karyotype risk was classified according to reported previously [17]. The bone marrow (BM) samples from patients were obtained at the time of diagnosis after the written informed consent given. BM specimens collected from 21 patients with iron deficiency anemia (IDA) without the evidence of cancers were used as controls. The main clinical and laboratory features of AML patient cohort were summarized in **Table 1**.

### *RNA isolation, cDNA synthetics and real-time quantitative PCR*

BMNCs were separated using Ficoll solution. Total RNA was extracted from BMNCs and reverse transcribed into cDNA as described previously [18]. The primers of *MOK* expression (forward: 5'-GCTTTCGGGAGTGGTCAG-3'; reverse: 5'-TTCTTGCTCGCAGGGATG-3') were designed with the software Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR (RQ-PCR) was performed on a 7300 Thermo cycler (Applied Biosystems, CA, USA), using 50 ng of cDNA in a 25 μl reaction mixture with 0.2 mmol/L of dNTP, 0.4 μmol/L of each primer, 4 mmol/L of $MgCl_2$, 1.2 μl of EvaGreen, and 1.0 U of Taq DNA Polymerase (MBI Fermentas, Hanover, USA). RQ-PCR conditions were 5 min at 95°C for denaturation, followed by 45 cycles at 94°C for 30 s for denaturation, 60°C for 30 s for annealing, 72°C for 30 s for elongation, and 85°C for 30 s for collecting fluorescence data. The mRNA abundance of *MOK* gene was calculated relative to that of the housekeeping gene *ABL1*.

### *Real-time quantitative methylation-specific PCR*

DNA was isolated using Genomic DNA Purification Kit (Gentra, Minneapolis, MN, USA) following the manufacturer's standard method. 1 μg of genomic DNA was modified using the CpGenome$^{TM}$ DNA Modification Kit (Chemicon, Ternecula, CA, USA) according to the manufacturer's instruction.

Methylation status of *MOK* promoter was detected using real-time quantitative methylation-specific PCR (RQ-MSP). Primers for the methylated (M) *MOK* reaction were 5'-AAGATGTTTCGTTTATGTACGC-3' (forward) and 5'-ACGAAC CGAACGAAAATCG-3' (reverse), and primer sequences for the unmethylated (U) *MOK* reaction were 5'-TGTAAGATGTTTTGTTTATGTATG-3' (forward) and 5'-AACAAACCAAACAAAAATCA-3' (reverse). 25 μL of reaction volume contained 0.2 mmol/L of dNTP, 0.2 μmol/L of each primer, 2.0 mmol/L of $MgCl_2$, 1.2 μL of EvaGreen, and 1.0 U of Taq DNA Polymerase and 2 μL of modified DNA. RQ-MSP conditions consisted of an initial denaturation step of 95°C for 5 min, followed by an amplification program of 45 cycles for 30 s at 94°C, 30 s at

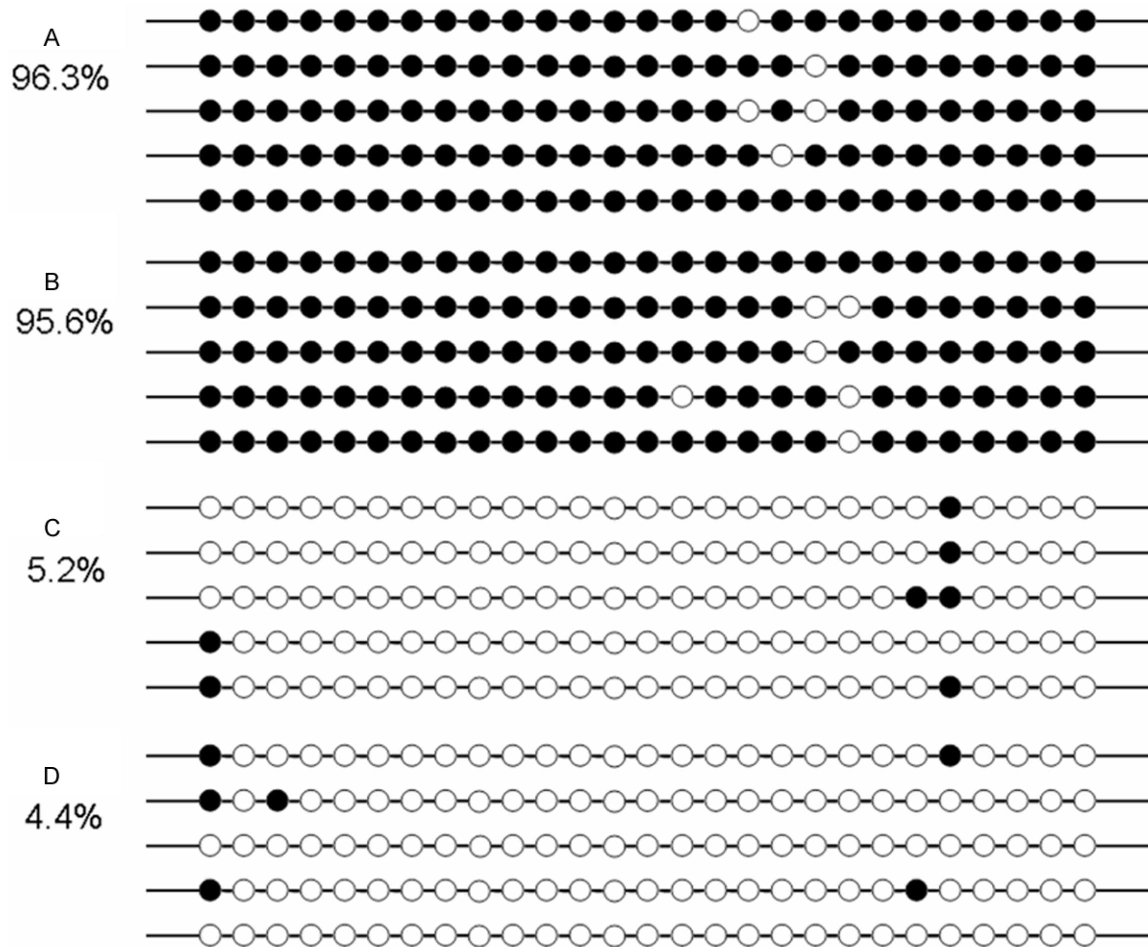


**Figure 4.** The results of bisulfite-sequencing in two *MOK*-hypomethylated and two *MOK*-methylated AML samples according to RQ-MSP. White cycle: unmethylated CpG dinucleotide; Black cycle: methylated CpG dinucleotide. A, B: Two cases with high methylated *MOK* promoter; C, D: Two cases with low methylated *MOK* promoter. Percentage was calculated by number of methylated CpG binucleotides divided by that of total CpG binucleotides of all sequenced clones in each sample.

59°C (M) or 61°C (U) 30 s at 72°C and 85°C for 30 s to collect data before a melting program of one cycle at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s and finally 60°C for 15 s. Negative (distilled water without DNA) and positive controls (recombined methylated and unmethylated *MOK* plasmids) were integrated in all PCR reactions. PCR products were run on 2% agarose gels and visualized after staining with ethidium bromide. The normalized ratio ($N_{unmethylation\text{-}MOK}$) calculated relative to the reference *ALU* was used to assess the methylation level of *MOK* promoter in samples. Positive products of M and U reaction from one AML patient were cloned and sequenced (Sangon, Shanghai, China).

### *Bisulfite sequencing analysis*

Bisulfite-modified DNA sequencing PCR (BSP) was conducted to verify the result of RQ-MSPPrimer sequences for BSP were 5'-TAGGAAGGTTGTTTTTTTGTTT-3' (forward) and 5'-CAAACCCAATTAAAACTCAAA-3' (reverse) with 382 bp products containing 27 CpG sites. PCR conditions were 94°C for 2 min, 40 cycles for 10 s at 98°C, 30 s at 58°C, 1 min at 68°C and a final extension for 7 min at 72°C. PCR products were then cloned into pMD®-19-T Vector (TaKaRa, Dalian, China) and E.coli DH5a (Life Technologies, Gaithersburg, MD) were transformed following the manufacturer's recommendations. 5 independent colonies of each sample were sequenced.

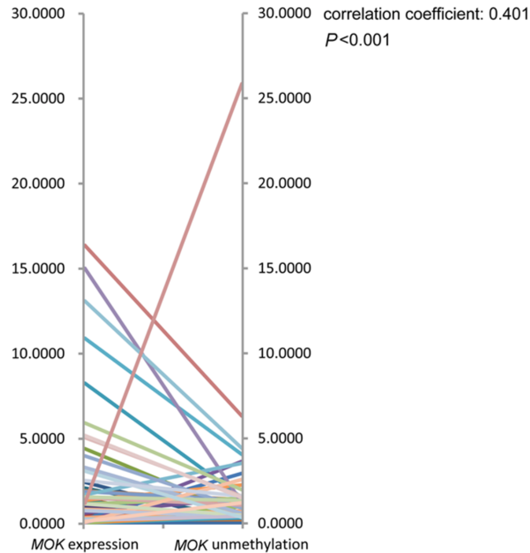


**Figure 5.** *MOK* expression correlates with promoter unmethylation levels. Spearman's rank correlation of *MOK* expression levels to *MOK* unmethylation levels shows a significant positive correlation (Spearman correlation coefficient = 0.401, $P < 0.001$).

*Mutations analysis*

*C/EBPA* mutations were detected by direct DNA sequencing. *FLT3* internal tandem duplication (ITD), *IDH1/IDH2*, *DNMT3A*, *NPM1* and *C-KIT* mutations were detected as reported previously [19-23]. All positive samples were confirmed by direct DNA sequencing.

*Statistical analysis*

Statistical analysis was performed using the SPSS 17.0 software package (SPSS, Chicago, IL, USA). Mann-Whitney's U-test was carried out to compare the difference of continuous variables between patient groups. Pearson chi-square analysis and Fisher exact test were carried out to compare the difference of categorical variables between patients group. The correlations between the frequency of *MOK* promoter hypomethylation and the clinical and hematologic parameters were analyzed with Spearman's rank correlation. Receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were used to assess the feasibility of using *MOK* unmethylation as a diagnostic tool in discriminating AML patients from controls. The *P* values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant for all analyses.

## Results

*CpG islands in MOK promoter*

The upstream 1000 bp region of *MOK* gene on chromosome 14 [strand (+), nucleotides (nt) 102,771,306-102,772,305] was surveyed for the presence of CpG islands using cpgplot software (http://emboss.bioinformatics.nl /cgi-bin/emboss/cpgplot). The criteria used were as follows: island size > 100 bp, GC percent > 50.0%, and ratio of observed (Obs) CpG sites to expected (Exp) CpG sites > 0.6). One CpG island was predicted spanning bp -421 to -58 (**Figure 1**). The University of California and Santa Cruz Genome Browser (UCSC Genome Browser) (http://genome.ucsc.edu/) and CpG Island Searcher (http://www.cpgislands.com/) also confirmed the presence of CpG island.

*Hypomethylation of MOK promoter in AML*

*MOK* promoter was significantly hypomethylated in AML patients (median 0.64, range 0.00-25.85) compared to controls (median 0.07, range 0.00-1.00) (**Figure 2**, $P < 0.001$). The representative electrophoresis results of RQ-MSP products were shown in **Figure 3**. $N_{unmethylation\text{-}MOK}$ ratio of all controls was 0-100% (15.87 ± 23.11%). AML patients were classified into two groups according to the value of mean plus 4SD obtained in normal controls: hypomethylated (> 108.31%) and methylated (≤ 108.31%).

In order to confirm the results of RQ-MSP, we assessed the methylation density of *MOK* promoter in two *MOK*-hypomethylated and two *MOK*-methylated AML samples according to the results of RQ-MSP (**Figure 4**). The results of bisulfite-sequencing and RQ-MSP were highly correlated ($R = -1.000$, $P < 0.01$).

*Association of MOK expression and hypomethylation*

*MOK* expression was examined in 68 AML patients with available mRNA. *MOK* expression was significantly up-regulated in AML patients (median 0.64, range 0.00-16.34) compared to controls (median 0.19, range 0.00-0.27) ($P < 0.001$). A significantly positive correlation was observed between the degree of *MOK* hypomethylation and the level of *MOK* expression ($R = 0.401$, $P < 0.001$, **Figure 5**). Patients with *MOK* hypomethylation (n = 23) had significantly

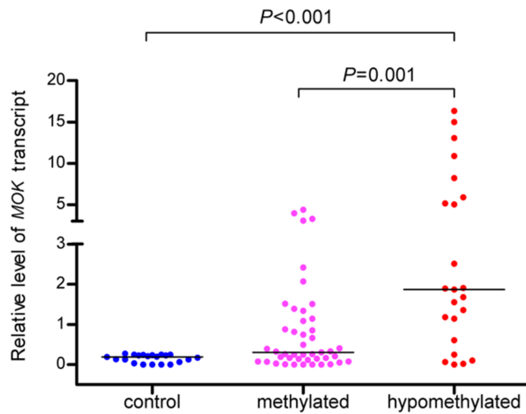


**Figure 6.** Levels of *MOK* expression in controls and in AML patients with hypomethylatin and methylation.

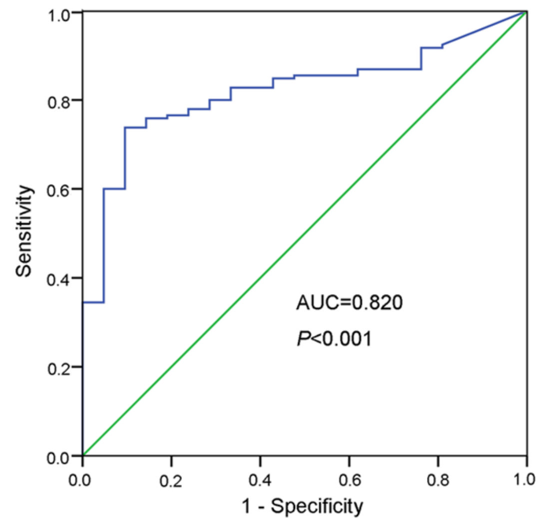


**Figure 7.** ROC curve of *MOK* unmethylation for distinguishing AML patients from controls.

higher level of *MOK* transcript (median 1.87, range 0.00-16.34) than those with *MOK* methylation (n = 45, median 0.30, range 0.00-4.39, *P* = 0.001) and controls (n = 21, median 0.19, range 0.00-0.27, *P* < 0.001) (**Figure 6**).

*Association between MOK hypomethylation and clinical characteristics in AML*

*MOK* hypomethylation was determined in 45 of 145 (31.0%) de novo AML patients. Our results indicated that the frequency of *MOK* hypomethylation was not associated with the sex, age, peripheral parameters, FAB subtypes, WHO classifications, cytogenetics, and gene mutations (**Table 1**).

*Evaluation of MOK unmethylation as a potential diagnostic marker*

ROC curve analysis was conducted to evaluate whether the *MOK* unmethylation can be used to be a potential marker for diagnosing AML. It was showed that the AUC of AML patients was 0.820 (95% confidence interval: 0.745-0.895) (*P* < 0.001) (**Figure 7**). At the cutoff value of 0.3032, the sensitivity and the specificity were 73.8% and 90.5%, respectively.

## Discussion

*MOK* has been demonstrated to be expressed in different cancers, however, the regulation mechanism of *MOK* expression is not well established. In the present study, we showed for the first time that the *MOK* overexpression was associated with hypomethylation of its promoter in AML. Firstly, *MOK* gene was hypomethylated with a high frequency of 31.0%. Secondly, *MOK* hypomethylation was correlated with *MOK* overexpression in AML patients. Similarly, several groups have also demonstrated that the *MOK* hypomethylation could induce or upregulate *MOK* expression by DNA demethylation in renal cell carcinoma cells and malignant mesothelioma cells [9, 24, 25].

Epigenetic changes are increasingly regarded as key events in the development of cancer. Aberrant DNA methylation profiles, histone modification landscapes and miRNA signatures are early characteristics of carcinogenesis occurring in precancerous lesions and in adjacent tissues [26]. Global DNA hypomethylation and gene-specific hypomethylation were common molecular alterations and play an important role in human cancers [27]. DNA hypomethylation may conduce to the initiation of a tumor cell through generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting [28, 29]. In the present study, we provided the first evidence that *MOK* was hypomethylated in AML patients but absent in controls, suggesting *MOK* hypomethylation may be associated with the pathogenesis of AML. To date, the function of *MOK* gene remains largely unknown. Cha et al found that *MOK* overexpression was associated with the invasion and poor prognosis of hepatocellular carcinoma, suggesting the role of *MOK* as a tumor-promoting gene [10]. Whether *MOK* upregulation induced by promoter hypomethyl-

aton contributes to leukemogenesis needs further explored.

*MOK* hypomethylation was found in each subtype of AML and we did not observe significant difference in *MOK* hypomethylation frequency either in FAB or WHO subtypes. In addition, there was no significant difference in *MOK* hypomethylation frequency among cytogenetics risk classification, or between in cytogenetically normal patients and abnormal patients. ROC curve was created to analyze the diagnostic value of *MOK* hypomethylation, the result clearly indicated that this molecular abnormality may be a helpful marker for distinguishing AML patients from control subjects.

Immunotherapy is an attractive approach to AML patients especially who are over 60 years but resistance to chemotherapy or not ineligible for hematopoietic stem cell transplants. However, the discrepancy of expression of the targeted antigens has been a potential difficulty in developing tumor vaccines [30]. In the present study, we demonstrated that *MOK* expression was regulated by its promoter hypomethylation in primary leukemic cells. Therefore, *MOK* may become an ideal target antigen for cancer-specific immunotherapy since it has been identified that MHC class II-binding peptides of *MOK* could activate T-helper cell response [31] in addition to induce the CTL-initiated immune response. Sequential *MOK*-targeted immunotherapy may be promising as a novel strategy to enhance the effect of DNA methylation inhibitors, especially in old patients who can not tolerate the toxic reaction of chemotherapy.

Taken together, overexpressed *MOK* is regulated by promoter hypomethylation in AML.

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**Disclosure of conflict of interest**

None.

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