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*Original Article*

# *GPX3* hypermethylation serves as an independent prognostic biomarker in non-M3 acute myeloid leukemia

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**Abstract:** Hypermethylation of *GPX3* (glutathione peroxidase 3) promoter has been identified in various solid tumors. However, the pattern of *GPX3* promoter methylation in acute myeloid leukemia (AML) remains poorly known. The current study was intended to investigate the clinical significance of *GPX3* promoter methylation in de novo AML patients and further determine its role in regulating *GPX3* expression. *GPX3* promoter methylation status in 181 de novo AML patients and 44 normal controls was detected by real-time quantitative methylation-specific PCR and bisulfite sequencing PCR. Real-time quantitative PCR was carried out to assess *GPX3* expression. *GPX3* promoter was significantly methylated in 181 AML patients compared with normal controls ($P$=0.022). The patients with *GPX3* methylation presented significantly older age than those with *GPX3* unmethylation ($P$=0.011). *GPX3* methylated patients had significantly lower frequency of *C/EBPA* mutation and higher incidence of *FLT3*-ITD mutation ($P$=0.037 and 0.030). The non-M3 patients with *GPX3* methylation had significantly lower overall survival than thoes with *GPX3* unmethylation ($P$=0.036). No significant correlation was observed between *GPX3* expression and its promoter methylation (R=0.110, $P$=0.284). However, *GPX3* mRNA level was significantly increased after 5-aza-2'-deoxycytidine treatment in leukemic cell line THP1. *GPX3* methylation predicts adverse clinical outcome in non-M3 AML patients. Moreover, *GPX3* expression is regulated by its promoter methylation in leukemic cell line THP1.

**Keywords:** *GPX3*, methylation, prognosis, regulation, acute myeloid leukemia

## Introduction

Acute myeloid leukemia (AML) is a clonal hematological malignancy with diverse clinical outcome, characterized by a block in differentiation of hematopoiesis and growth of a clonal population of neoplastic cells or blasts [1, 2]. Genetic alterations play crucial roles not only in the pathogenesis but also in the prognosis of AML [3-5]. Recently, epigenetic modifications such as aberrant DNA methylation have been identified to contribute to the pathogenesis of AML [3]. Moreover, abnormal methylation of numerous oncogenes/tumor suppress genes (TSGs) has been found as potential biomarker for the prognosis of AML [6, 7]. These give new insights into disease pathogenesis and provide opportunities for therapeutic advances.

*GPX* (glutathione peroxidase) family is composed of 8 members (*GPX1-GPX8*) with their role in reducing redundant reactive oxygen species (ROS) against oxidative damages to host cells [8]. *GPX3*, locates on chromosome 5q23, accounts for nearly all of the *GPX* activity in plasma [8]. Tumor suppressor role of *GPX3* has been identified in quite a few tumors [9-11]. Accumulating studies have revealed the pattern of *GPX3* promoter hypermethylation in a variety of cancers [12-19]. Moreover, the prognostic value of *GPX3* promoter hypermethylation has also been revealed in several cancers [17-19]. However, little is known about the pattern of *GPX3* promoter methylation and its clinical relevance in AML by far. The present study was aimed to investigate the clinical significance of *GPX3* promoter methylation in de novo AML

**Table 1.** Association between *GPX3* promoter methylation and clinical parameters in AML patients

| Patient's parameters | Status of *GPX3* promoter methylation | | |
|---|---|---|---|
| | Unmethylated (n=134) | Methylated (n=47) | *P* value |
| Sex, male/female | 77/57 | 31/16 | 0.388 |
| Median age, years (range) | 48 (3-93) | 59 (15-87) | 0.011 |
| Median WBC, $\times 10^9$/L (range) | 16.4 (0.8-528.0) | 16.3 (0.9-185.4) | 0.742 |
| Median hemoglobin, g/L (range) | 74 (32-131) | 74.5 (33-138) | 0.834 |
| Median platelets, $\times 10^9$/L (range) | 40 (3-264) | 40.5 (6-119) | 0.859 |
| BM blasts, % (range) | 43.5 (5.0-97.5) | 51.5 (3.0-94.5) | 0.259 |
| FAB | | | 0.541 |
| M0 | 1 (1%) | 0 (0%) | |
| M1 | 13 (10%) | 7 (15%) | |
| M2 | 49 (37%) | 17 (36%) | |
| M3 | 21 (16%) | 7 (15%) | |
| M4 | 27 (21%) | 12 (26%) | |
| M5 | 14 (10%) | 4 (8%) | |
| M6 | 9 (7%) | 0 (0%) | |
| WHO | | | 0.294 |
| AML with t (8; 21) | 15 (11%) | 4 (9%) | |
| APL with t (15; 17) | 21 (16%) | 7 (15%) | |
| AML with 11q23 | 1 (1%) | 1 (2%) | |
| AML without maturation | 10 (7%) | 7 (15%) | |
| AML with maturation | 36 (27%) | 13 (28%) | |
| Acute myelomonocytic leukemia | 26 (19%) | 13 (28%) | |
| Acute monoblastic and monocytic leukemia | 14 (10%) | 2 (4%) | |
| Acute erythroid leukemia | 9 (7%) | 0 (0%) | |
| No data | 2 (1%) | 0 (0%) | |
| Karyotype classification | | | 0.563 |
| Favorable | 36 (27%) | 11 (23%) | |
| Intermediate | 76 (57%) | 24 (57%) | |
| Poor | 15 (11%) | 8 (17%) | |
| No data | 7 (5%) | 4 (9%) | |
| Karyotype | | | 0.834 |
| normal | 59 (44%) | 18 (38%) | |
| t(8;21) | 15 (11%) | 4 (9%) | |
| t(15;17) | 20 (15%) | 7 (15%) | |
| 11q23 | 1 (1%) | 1 (2%) | |
| complex | 12 (9%) | 6 (13%) | |
| others | 20 (15%) | 7 (15%) | |
| No data | 7 (5%) | 4 (9%) | |
| Gene Mutation | | | |
| *C/EBPA* (+/-) | 24/97 | 3/43 | 0.037 |
| *NPM1* (+/-) | 15/106 | 5/41 | 1.000 |
| *FLT3*-ITD (+/-) | 10/111 | 10/36 | 0.030 |
| *c-KIT* (+/-) | 6/115 | 2/44 | 1.000 |
| *N/K RAS* (+/-) | 12/109 | 6/40 | 0.581 |
| *IDH1/2* (+/-) | 8/113 | 1/45 | 0.447 |
| *DNMT3A* (+/-) | 8/113 | 4/42 | 0.738 |
| *U2AF1* (+/-) | 3/118 | 2/44 | 0.616 |
| CR (+/-) | 42/43 | 20/22 | 1.000 |

WBC, white blood cells; FAB, French-American-British classification; AML, acute myeloid leukemia; CR, complete remission.

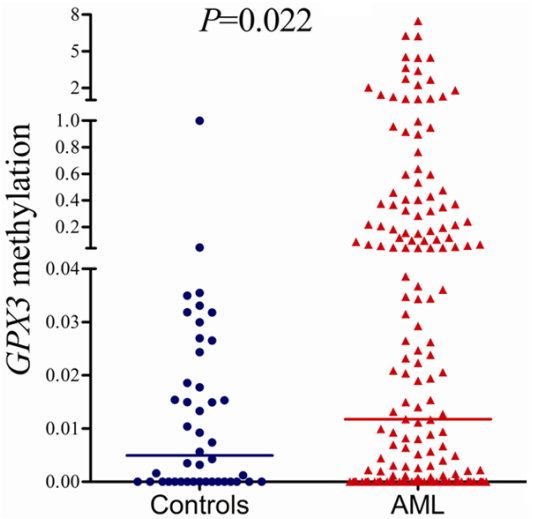


**Figure 1.** Relative methylation levels of *GPX3* in normal controls and AML patients.

patients and further determine its role in regulating *GPX3* expression.

## Materials and methods

### *Patients*

A total of 181 patients with a diagnosis of AML as well as 44 healthy donors were included 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 French-American-British (FAB) classification and the 2008 World Health Organization (WHO) criteria [20, 21]. Treatment protocol for AML patients was described previously [22]. The parameters of AML patients were summarized in **Table 1**. Bone marrow (BM) specimens were collected form all the patients and healthy donors after written informed consents were obtained. BM mononuclear cells were extracted from BM specimens by gradient centrifugation using Lymphocyte Separation Medium (TBD sciences, Tianjin, China).

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

Human leukemic cell line THP1 cells were cultured in IMDM medium containing 10% fetal calf serum and grown at 37°C in 5% $CO_2$ humidified atmosphere. For demethylation studies, cells were incubated with a final concentration of 0 μM, 0.1 μM, 1 μM, 10 μM, and 50 μM

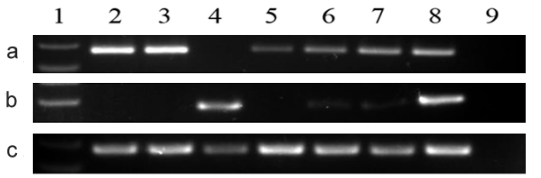


**Figure 2.** Electrophoresis results of RQ-PCR and RQ-MSP products in normal controls and AML patients. 1: Gene Ruler™ 100bp DNA ladder; 2, 3: controls; 4-7: AML patients; 8: positive control; 9: negative control. a: *GPX3* expression; b: *GPX3* methylation; c: *GPX3* unmethylation.

5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, Steinheim, USA) for 72 h. All cells were cultured until harvested for extraction of RNA and DNA.

### *RNA isolation, reverse transcription and RQ-PCR*

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction with 40 μL volume was composed of 5×buffer 10 mM, 10 mM of dNTPs, 10 μM of random hexamers, 80 U of RNAsin, and 200 U of MMLV reverse transcriptase (MBI Fermentas, Hanover, USA). The reaction conditions were incubated for 10 min at 25°C, 60 min at 42°C, and then stored at -20°C.

Real-time quantitative PCR (RQ-PCR) was performed on a 7300 Thermo cycler (Applied Biosystems, CA, USA). The primer sequences for *GPX3* expression were 5'-GCCGGGGACAAGAGAAGT-3' (forward) and 5'-GAGGACGTATTTGCCAGCAT-3' (reverse) [17]. The reaction system with 20 μL volume consisted of cDNA 20 ng, 0.8 μM of primers, 10 μM of AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA), and 0.4 μM of ROX Reference Dye 1 (Invitrogen, Carlsbad, CA, USA). The RQ-PCR reaction conditions were 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, 63°C for 30 s, 72°C for 30 s, and 80°C for 30 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 *GPX3* transcript levels were calculated by the formulas $N_{GPX3}=(E_{GPX3})^{\Delta CT\ GPX3\ (control\text{-}sample)} \div (E_{ABL})^{\Delta CT\ ABL\ (control\text{-}sample)}$ and $E=10^{(-1/slope)}$ (the slope referred to CT versus cDNA concentration plot).

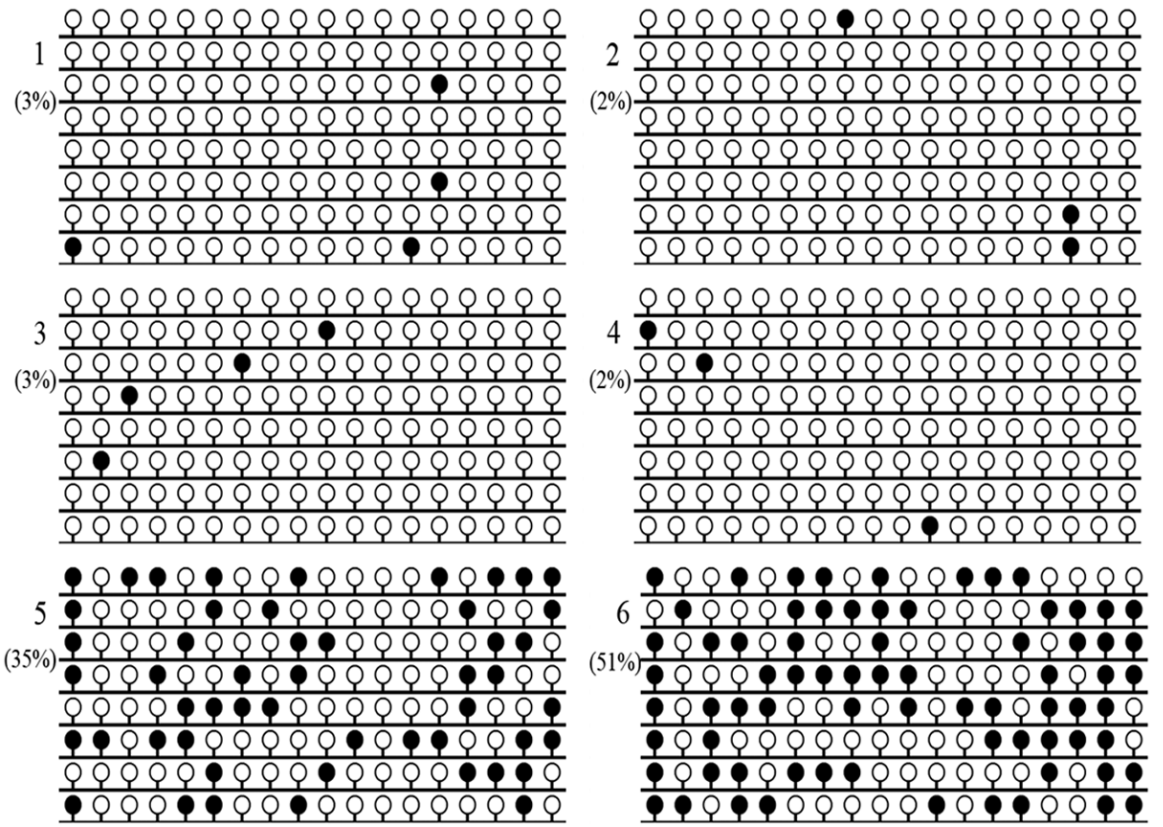


**Figure 3.** Methylation density of *GPX3* promoter in normal controls and AML patients. White cycle: unmethylated CpG dinucleotide; Black cycle: methylated CpG dinucleotide. 1, 2: controls; 3, 4: unmethylated AML patients; 5, 6: methylated AML patients.

*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 recommendations. The primer sequences for the methylated (M) *GPX3* promoter were 5'-TATGTTATTGTCG-TTTCGGGAC-3' (forward) and 5'-GTCCGTCTAA-AATATCCGACG-3' (reverse), and for the unmethylated (U) *GPX3* promoter were 5'-TTTATGTTA-TTGTTGTTTTGGGATG-3' (forward) and 5'-ATC-CATCTAAAATATCCAACACTCC-3' (reverse) [15]. Real-time quantitative methyaltion-specific PCR (RQ-MSP) was performed for M-MSP reaction composed of primers 0.8 μM, 10 μM of AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA), 0.4 μM of ROX Reference Dye 1 (Invitrogen, Carlsbad, CA, USA), and 20 ng of modified DNA. The program for amplification was 95°C for 5 min, 40 cycles for 10 s at 95°C, 30 s at 64°C, 72°C for 30 s, and 80°C for 30 s, finally 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. While, U-MSP reaction using the same reagent was incubated for 95°C for 5 min, 40 cycles for 10 s at 95°C, 30 s at 58°C , and 30 s at 72°C followed by a final 7 min extension step at 72°C. Both positive and negative controls were included in each assay. The normalized ratio ($N_{M\text{-}GPX3}$) calculated relative to the reference *ALU* was used to assess the level of *GPX3* promoter methylation in samples. $N_{M\text{-}GPX3}$ was calculated using the equation: $N_{M\text{-}GPX3}=(E_{M\text{-}GPX3})^{\Delta CT\ M\text{-}GPX3\ (control\text{-}sample)} \div (E_{ALU})^{\Delta CT\ ALU\ (control\text{-}sample)}$.

*Bisulfite sequencing*

The primer sequences for bisulfite modified *GPX3* promoter were 5'-ATTTTGGAGTTAAAA-GAGGAAG-3' (forward) and 5'-CTACCTAATCCC-TAACCACC-3' (reverse). Bisulfite sequencing PCR (BSP) reaction system contained 10×PCR

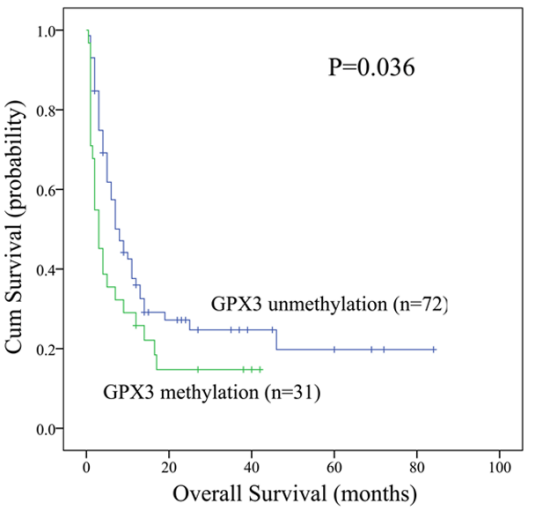


**Figure 4.** The impact of *GPX3* methylation on overall survival of non-M3 patients.

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 BSP condition was carried out at 98°C for 10 s, 40 cycles for 10 s at 98°C, 30 s at 56°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. The PCR products were purified and cloned into pMD19-T Vector (Takara, Tokyo, Japan), then transfected into DH5A competent cells (Vazyme, Carlsbad, CA, USA). Eight clones from each sample were sequenced (BGI Tech Solutions Co., Shanghai, China).

*Gene mutation detection*

The detection of *N/K-RAS*, *DNMT3A*, *U2AF1*, *IDH1/2*, *c-KIT*, and *NPM1* mutations were performed for PCR products using HRMA with the LightScanner platform (Idaho Technology Inc., Salt Lake City, Utah) [23-26]. All positive samples were confirmed by DNA direct sequencing. *FLT3*-ITD and *C/EBPA* mutations were detected by direct DNA sequencing [27].

*Statistical analysis*

SPSS 18.0 software package (SPSS, Chicago, IL) was applied to perform statistical analyses. Mann-Whitney's U test was carried to compare the difference of continuous variables in two groups. Pearson Chi-square analysis or Fisher exact test was employed to compare the difference of categorical variables. Correlation analysis between *GPX3* expression and its promoter methylation was performed by spearman rank correlation test. Kaplan-Meier analysis and multivariate analysis were used to analyze the impact of *GPX3* expression on survival respectively. For all analyses, a two-tailed $P<0.05$ was defined as statistically significant.

## Results

*GPX3 methylation in normal controls and AML patients*

According to RQ-MSP, *GPX3* promoter was significantly methylated in AML patients (median 0.012, range 0.000-7.493) compared with normal controls (median 0.005, range 0.000-1.000) ($P=0.022$, **Figure 1**). The representative electrophoresis results of RQ-MSP products were shown in **Figure 2**.

Two controls and two *GPX3* unmethylated AML patients as well as two *GPX3* methylated AML patients were selected randomly to further investigate the *GPX3* methylation density by BSP. Both controls and unmethylated AML patients presented almost fully unmethylated *GPX3* promoter (**Figure 3**). While the two methylated AML patients presented higher density of *GPX3* methylation (**Figure 3**).

*Association between GPX3 expression and its promoter methylation*

*GPX3* expression was detected in 97 AML patients with available mRNA. *GPX3* mRNA level in AML patients ranged from 0.000 to 9.407 with a median level of 0.035. No significant correlation was observed between *GPX3* expression and its promoter methylation ($R=0.110$, $P=0.284$).

*Association between GPX3 methylation and clinical characteristics of AML patients*

The level of methylated *GPX3* promoter in controls was 0.034±0.150 (range 0.000-1.000). $N_{M\text{-}GPX3}$ above the value of 0.184 (defined as the mean + SD) was set to define *GPX3* promoter methylation in AML patients. Only 1 of 44 (2%) controls presented methylated *GPX3* promoter. However, *GPX3* promoter methylation was identified in 26% (47/181) of AML patients. According to the cutoff value, the whole AML

**Table 2.** Multivariate analysis of prognostic factors for overall survival in non-M3 AML patients

| | hazard ratio (95% CI) | *P* value |
|---|---|---|
| Age | 2.344 (1.396-3.936) | 0.001 |
| WBC | 2.063 (1.242-3.425) | 0.005 |
| Karyotypic classification | 1.425 (1.003-2.025) | 0.048 |
| *GPX3* methylation | 1.851 (1.051-3.262) | 0.033 |
| *FLT3* mutation | 0.460 (0.209-1.013) | 0.054 |
| *NPM1* mutation | 1.192 (0.505-2.811) | 0.688 |
| *C/EBPA* mutation | 0.999 (0.486-2.054) | 0.997 |
| *c-KIT* mutation | 0.362 (0.048-2.738) | 0.325 |
| *N/K RAS* mutation | 1.397 (0.647-3.021) | 0.395 |
| *IDH1/2* mutation | 1.061 (0.406-2.770) | 0.904 |
| *DNMT3A* mutation | 1.112 (0.399-3.094) | 0.839 |
| *U2AF1* mutation | 3.372 (1.269-8.961) | 0.015 |

patients were divided into two groups: *GPX3* methylated and *GPX3* unmethylated. There were no significant differences in sex, white blood cell, hemoglobin, platelets, and BM blasts between the methylated and unmethylated patients (*P*>0.05, **Table 1**). No significant difference was observed in the distribution of both FAB and WHO as well as karyotypic classifications between the patients with and without *GPX3* methylation (*P*>0.05, **Table 1**). However, *GPX3* methylated cases showed significantly older age than *GPX3* unmethylated cases (*P*=0.011, **Table 1**). Significant differences were observed in the frequencies of both *C/EBPA* and *FLT3*-ITD mutations between *GPX3* methylated and unmethylated cases. The methylated patients had significantly lower frequency of *C/EBPA* mutation and higher incidence of *FLT3*-ITD mutation (*P*=0.037 and 0.030, **Table 1**). Due to the *GPX3* gene locates at the chromosome 5, we further analyzed *GPX3* methylation pattern in the patients with and without -5/5q-. No significant difference was found in the level of *GPX3* methylation between the -5/5q- and non-(-5/5q-) cases (median 0.060 vs 0.010, *P*=0.211).

*Association between GPX3 expression and clinical outcome*

127 patients with available follow-up data were obtained. *GPX3* methylated and unmethylated patients showed similar complete remission (CR) rate in whole AML (48% vs 49%, *P*=1.000, **Table 1**). Moreover, there were also no significant differences in CR rate between *GPX3* methylated and unmethylated patients among both non-M3 AML [37% (13/35) vs 45% (34/75), *P*=0.535] and cytogenetically normal AML (CN-AML) [47% (8/17) vs 42% (23/55), *P*=1.000]. Survival analyses were performed in 121 patients with survival data ranging from 1 to 92 months with a median of 8 months. No significant differences were observed in overall survival (OS) between the methylated and unmethylated cases in both whole AML and CN-AML (median 4 vs 9 months, *P*=0.439 and median 3 vs 11 months, *P*=0.179). However, among non-M3 patients, *GPX3* methylated patients had significantly lower OS than *GPX3* unmethylated patients (median 3 vs 8 months, *P*=0.036, **Figure 4**). Moreover, multivariate analysis also confirmed the prognostic significance of *GPX3* methylation in non-M3 patients (**Table 2**) but not in whole AML as well as CN-AML patients (data not shown).

*Epigenetic mechanism regulating GPX3 expression in leukemic cell line*

To determine the role of *GPX3* promoter methylation in regulating *GPX3* expression in AML, THP1 cell line was treated by 5-aza-dC. THP1 showed extremely low *GPX3* mRNA level and fully methylated *GPX3* promoter before 5-aza-dC treatment (**Figure 5**). *GPX3* mRNA level was significantly increased after 5-aza-dC treatment in a dose-dependent manner, meanwhile, *GPX3* promoter methylation level was decreased (**Figure 5**).

## Discussion

Alterations in DNA methylation are frequent, early events in carcinogenesis [28]. Hypermethylation of TSGs in promoter-associated CpG islands is correlated with gene silencing, whereas hypomethylation in other regions is associated with genomic instability [29]. Moreover, DNA methylation is of various TSGs has been identified as potential biomarkers for early detection, diagnosis, prognosis, therapeutic stratification, and post-therapeutic monitoring in a host of cancers [28]. *GPX3* is one of these TSGs having been identified. Li et al demonstrated that *GPX3* promoter methylation could serve as the potential biomarker for the early diagnosis in esophageal squamous cell carcinoma [30]. Peng et al disclosed the association between *GPX3* promoter methylation and lymph node metastasis in gastric carcino-

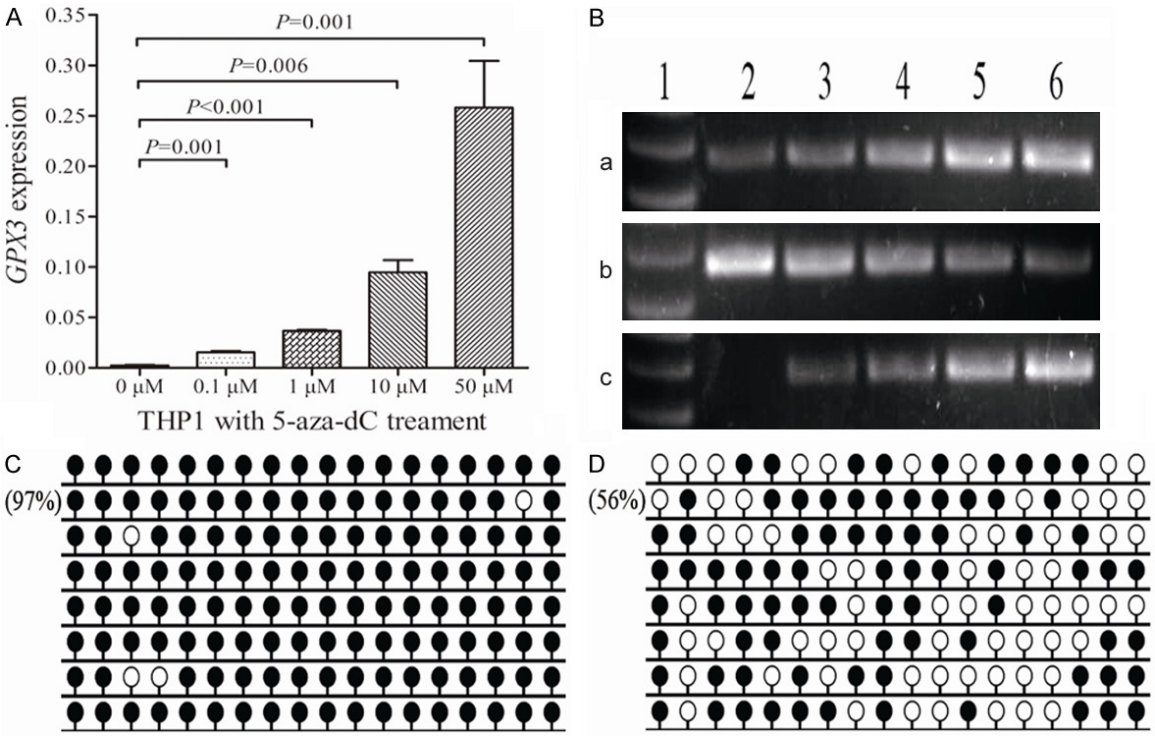


**Figure 5.** GPX3 expression and methylation in THP1 cell line before and after 5-aza-dC treatment. A: GPX3 relative expression levels. B: Electrophoresis results of RQ-PCR and RQ-MSP products. 1: Gene Ruler™ 100bp DNA ladder; 2: 0 μM; 3: 0.1 μM; 4: 1 μM; 5: 10 μM; 6: 50 μM. a: GPX3 expression; b: GPX3 methylation; c: GPX3 unmethylation. C: GPX3 methylation density before treatment. D: GPX3 methylation density after treatment (50 μM).

mas [17]. Chen et al revealed that *GPX3* promoter hypermethylation was associated with head and neck cancer (HNC) chemoresistance and acted as a potentially prognostic indicator for HNC patients treated with cisplatin-based chemotherapy [18]. Furthermore, Kaiser et al also indicated the prognostic significance of *GPX3* promoter methylation in multiple myeloma [19].

In the current study, we investigated the status of *GPX3* promoter methylation and indicated that *GPX3* promoter hypermethylation was a frequent event in de novo AML patients. Although we did not observe the adverse impact of *GPX3* methylation on CR in AML patients, our study by both Kaplan-Meier and multivariate analyses revealed the prognostic value of *GPX3* methylation among non-M3 AML patients. To the best of our knowledge, our investigation for the first time reported that *GPX3* promoter methylation serving as a new potential biomarker could provide helpful prognostic information in de novo AML patients. Recently, several gene mutations including *IDH1/2*, *TET2*, *JAK2*-V617F, and *PML* contributed to epigenetic modifications having been identified in myeloid malignancies [31]. However, our study did not observe the significant association between *GPX3* promoter methylation and these gene mutations. Interestingly, we observed the significantly increased incidence of *C/EBPA* wild type and *FLT3*-ITD mutation in the methylated AML patients. Further studies are required to determine the underlying role of *C/EBPA* and *FLT3*-ITD mutations during the process of leukemogenesis caused by *GPX3* promoter methylation.

Accumulating studies have revealed the association between *GPX3* expression and its promoter methylation in a host of cancers [13-19]. Moreover, *GPX3* expression could be up-regulated after 5-aza-dC treatment in different cancer cell lines including human esophageal squamous cell carcinoma cell lines, cervical cancer cell lines, gastric carcinoma cell lines, and multiple myeloma cell lines [15, 17, 19, 32]. Our investigation further confirmed the

epigenetic mechanism in the regulation of *GPX3* expression in leukemic cell line THP1. However, our study did not observe the significant association between *GPX3* expression and its promoter methylation in the AML patients. These results suggested that other mechanism might be involved in the regulation of *GPX3* expression in de novo AML patients. Further studies are needed to explore the specific mechanism regulating *GPX3* expression in de novo AML patients.

Taken together, our study indicates that *GPX3* methylation correlates with *C/EBPA* wild type and *FLT3*-ITD mutation in de novo AML patients. In spite of the correlation, *GPX3* methylation also acts as an independent prognostic biomarker in non-M3 AML patients. Moreover, *GPX3* expression is regulated by its promoter methylation in leukemic cell line THP1.

## Acknowledgements

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## References

[1] Estey E, Döhner H. Acute myeloid leukaemia. Lancet 2006; 368: 1894-907.

[2] Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W. Adult acute myeloid leukaemia. Crit Rev Oncol Hematol 2004; 50: 197-222.

[3] Chen J, Odenike O, Rowley JD. Leukaemogenesis: more than mutant genes. Nat Rev Cancer 2010; 10: 23-36.

[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, Carroll AJ, Edwards CG, Arthur DC, Pettenati MJ, Patil SR, Rao KW, Watson MS, Koduru PR, Moore JO, Stone RM, Mayer RJ, Feldman EJ, Davey FR, Schiffer CA, Larson RA, Bloomfield CD; Cancer and Leukemia Group B (CALGB 8461). 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] Deneberg S. Epigenetics in myeloid malignancies. Methods Mol Biol 2012; 863: 119-37.

[7] Marcucci G, Yan P, Maharry K, Frankhouser D, Nicolet D, Metzeler KH, Kohlschmidt J, Mrózek K, Wu YZ, Bucci D, Curfman JP, Whitman SP, Eisfeld AK, Mendler JH, Schwind S, Becker H, Bär C, Carroll AJ, Baer MR, Wetzler M, Carter TH, Powell BL, Kolitz JE, Byrd JC, Plass C, Garzon R, Caligiuri MA, Stone RM, Volinia S, Bundschuh R, Bloomfield CD. Epigenetics meets genetics in acute myeloid leukemia: clinical impact of a novel seven-gene score. J Clin Oncol 2014; 32: 548-56.

[8] Brigelius-Flohé R, Maiorino M. Glutathione peroxidases. Biochim Biophys Acta 2013; 1830: 3289-303.

[9] Yu YP, Yu G, Tseng G, Cieply K, Nelson J, Defrances M, Zarnegar R, Michalopoulos G, Luo JH. Glutathione peroxidase 3, deleted or methylated in prostate cancer, suppresses prostate cancer growth and metastasis. Cancer Res 2007; 67: 8043-50.

[10] Barrett CW, Ning W, Chen X, Smith JJ, Washington MK, Hill KE, Coburn LA, Peek RM, Chaturvedi R, Wilson KT, Burk RF, Williams CS. Tumor suppressor function of the plasma glutathione peroxidase GPX3 in colitis-associated carcinoma. Cancer Res 2013; 73: 1245-55.

[11] Qi X, Ng KT, Lian QZ, Liu XB, Li CX, Geng W, Ling CC, Ma YY, Yeung WH, Tu WW, Fan ST, Lo CM, Man K. Clinical significance and therapeutic value of glutathione peroxidase 3 (GPx3) in hepatocellular carcinoma. Oncotarget 2014; 5: 11103-20.

[12] Mohamed MM, Sabet S, Peng DF, Nouh MA, El-Shinawi M, El-Rifai W. Promoter hypermethylation and suppression of glutathione peroxidase 3 are associated with inflammatory breast carcinogenesis. Oxid Med Cell Longev 2014; 2014: 787195.

[13] Min SY, Kim HS, Jung EJ, Jung EJ, Jee CD, Kim WH. Prognostic significance of glutathione peroxidase 1 (GPX1) down-regulation and correlation with aberrant promoter methylation in human gastric cancer. Anticancer Res 2012; 32: 3169-75.

[14] Falck E, Karlsson S, Carlsson J, Helenius G, Karlsson M, Klinga-Levan K. Loss of glutathi-

one peroxidase 3 expression is correlated with epigenetic mechanisms in endometrial adenocarcinoma. Cancer Cell Int 2010; 10: 46.
[15] He Y, Wang Y, Li P, Zhu S, Wang J, Zhang S. Identification of GPX3 epigenetically silenced by CpG methylation in human esophageal squamous cell carcinoma. Dig Dis Sci 2011; 56: 681-8.
[16] Lee OJ, Schneider-Stock R, McChesney PA, Kuester D, Roessner A, Vieth M, Moskaluk CA, El-Rifai W. Hypermethylation and loss of expression of glutathione peroxidase-3 in Barrett's tumorigenesis. Neoplasia 2005; 7: 854-61.
[17] Peng DF, Hu TL, Schneider BG, Chen Z, Xu ZK, El-Rifai W. Silencing of glutathione peroxidase 3 through DNA hypermethylation is associated with lymph node metastasis in gastric carcinomas. PLoS One 2012; 7: e46214.
[18] Chen B, Rao X, House MG, Nephew KP, Cullen KJ, Guo Z. GPX3 promoter hypermethylation is a frequent event in human cancer and is associated with tumorigenesis and chemotherapy response. Cancer Lett 2011; 309: 37-45.
[19] Kaiser MF, Johnson DC, Wu P, Walker BA, Brioli A, Mirabella F, Wardell CP, Melchor L, Davies FE, Morgan GJ. Global methylation analysis identifies prognostically important epigenetically inactivated tumor suppressor genes in multiple myeloma. Blood 2013; 122: 219-26.
[20] Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. France: IARC Press; 2008.
[21] Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposed revised criteria for the classification of acute myeloid leukaemia. A report of the French-American-British Cooperative Group. Ann Intern Med 1985; 103: 620-5.
[22] Li Y, Lin J, Yang J, Qian J, Qian W, Yao DM, Deng ZQ, Liu Q, Chen XX, Xie D, An C, Tang CY. Overexpressed let-7a-3 is associated with poor outcome in acute myeloid leukemia. Leuk Res 2013; 37: 1642-7.
[23] Lin J, Yao DM, Qian J, Chen Q, Qian W, Li Y, Yang J, Wang CZ, Chai HY, Qian Z, Xiao GF, Xu WR. Recurrent DNMT3A R882 muta-tions in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. PLoS One 2011; 6: e26906.
[24] Lin J, Yao DM, Qian J, Chen Q, Qian W, Li Y, Yang J, Wang CZ, Chai HY, Qian Z, Xiao GF, Xu WR. IDH1 and IDH2 mutation analysis in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. Ann Hematol 2012; 91: 519-25.
[25] Yang X, Qian J, Sun A, Lin J, Xiao G, Yin J, Chen S, Wu D. RAS mutation analysis in alarge cohort of Chinese patients with acute myeloid leukemia. Clin Biochem 2013; 46: 579-83.
[26] Qian J, Yao DM, Lin J, Qian W, Wang CZ, Chai HY, Yang J, Li Y, Deng ZQ, Ma JC, Chen XX. U2AF1 Mutations in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. PLoS one 2012; 7: e45760.
[27] Wen XM, Lin J, Yang J, Yao DM, Deng ZQ, Tang CY, Xiao GF, Yang L, Ma JC, Hu JB, Qian W, Qian J. 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.
[28] Taby R, Issa JP. Cancer epigenetics. CA Cancer J Clin 2010; 60: 376-92.
[29] Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene 2002; 21: 5400-13.
[30] Li X, Zhou F, Jiang C, Wang Y, Lu Y, Yang F, Wang N, Yang H, Zheng Y, Zhang J. Identification of a DNA methylome profile of esophageal squamous cell carcinoma and potential plasma epigenetic biomarkers for early diagnosis. PLoS One 2014; 9: e103162.
[31] Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer 2012; 12: 599-612.
[32] Zhang X, Zheng Z, Yingji S, Kim H, Jin R, Renshu L, Lee DY, Roh MR, Yang S. Downregulation of glutathione peroxidase 3 is associated with lymph node metastasis and prognosis in cervical cancer. Oncol Rep 2014; 31: 2587-92.