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Original Article

Down-regulation of *GPX3* is associated with favorable/intermediate karyotypes in de novo acute myeloid leukemia

Jing-Dong Zhou[1*], Xiang-Mei Wen[2*], Ying-Ying Zhang[1], Lei Yang[1], Yu-Juan Ma[1], Ji-Chun Ma[2], Jing Yang[1], Hong Guo[2], Dong-Ming Yao[2], Jiang Lin[2], Jun Qian[1]

[1]*Department of Hematology, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, People's Republic of China;* [2]*Laboratory Center, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, People's Republic of China.* [*]*Equal contributors.*

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Abstract: Decreased glutathione peroxidase 3 (*GPX3*) expression has been identified in numerous solid tumors. However, *GPX3* expression pattern in acute myeloid leukemia (AML) remains poorly known. Our study was intended to explore *GPX3* expression status and further analyze the clinical relevance of *GPX3* expression in AML. *GPX3* mRNA level was detected by real-time quantitative PCR in 122 de novo AML patients and 44 normal controls. *GPX3* transcript level was significantly decreased compared with normal controls ($P < 0.001$). The patients with low *GPX3* expression had significantly higher hemoglobin and platelets than those with high *GPX3* expression ($P = 0.049$ and 0.020). The frequency of low *GPX3* expression in favorable karyotype (66%, 23/35) and intermediate karyotype (65%, 45/69) was higher than in poor karyotype (29%, 4/14) ($P = 0.017$). No significant differences were observed in both complete remission and overall survival between the *GPX3* low-expressed and high-expressed patients ($P > 0.05$). Reduced *GPX3* expression is associated with favorable/intermediate karyotypes but not with survival in de novo AML patients.

Keywords: *GPX3*, expression, karyotype, acute myeliod leukemia

Introduction

Acute myeloid leukemia (AML), the most common adult leukemia, is a group of heterogeneous disorders characterized by a clone of leukemic stem cells (LSCs), and resulted from a failure of hematopoiesis [1, 2]. The specific pathogenesis and progression of AML are not well understood. Morphology, immunology, cytogenetics, and molecular biology (MICM) features comprise the main points for the diagnosis of AML. Recent advances in genetic, cellular and molecular methodologies provide new insights into disease pathogenesis and opportunities for therapeutic advances. Many different cytogenetic and molecular abnormalities have been demonstrated to be involved in leukemogenesis [3-5]. Moreover, the genetic or molecular alterations implicated in pathogenesis of the disease are also important to predict response to therapy and survival [6].

Glutathione peroxidase 3 (*GPX3*), a member of glutathione peroxidase (*GPX*) family, locates on chromosome 5q23. *GPX* family plays crucial roles in protecting cell from oxidative damages by reducing hydrogen peroxide or organic hydroperoxides [7]. Recently, *GPX* inhibition has been identified to be associated with cancer progression by regulating cancer cells proliferation, invasion, migration, angiogenesis and apoptosis [8]. Down-regulation of *GPX3* expression has been found in various solid tumors [9-18]. Moreover, increasing studies have revealed the prognostic significance of *GPX3* expression in several cancers [10-12]. However, *GPX3* expression pattern in AML is rarely known. The present study was aimed to investigate the *GPX3* expression pattern and further analyze the clinical significance in de novo AML patients.

Table 1. Association between *GPX3* expression as well as *GPX3* promoter methylation and clinical parameters of AML patients

Patient's parameters	Status of *GPX3* expression		
	Low (n = 73)	High (n = 49)	*P* value
Sex, male/female	44/29	30/19	1.000
Median age, years (range)	54 (10-93)	58 (21-81)	0.496
Median WBC, × 10^9/L (range)	12.2 (0.8-528.0)	6.6 (0.3-185.4)	0.331
Median hemoglobin, g/L (range)	78 (34-131)	68.5 (32-138)	0.049
Median platelets, × 10^9/L (range)	50 (3-264)	31 (4-447)	0.020
BM blasts, % (range)	52.0 (3.0-109.0)	42.0 (1.0-94.5)	0.396
FAB			0.336
M0	0 (0%)	1 (2%)	
M1	4 (5%)	4 (8%)	
M2	24 (33%)	21 (43%)	
M3	20 (27%)	7 (14%)	
M4	16 (22%)	12 (24%)	
M5	6 (8%)	4 (8%)	
M6	3 (4%)	0 (0%)	
WHO			0.232
AML with t(8;21)	3 (4%)	5 (10%)	
APL with t(15;17)	20 (27%)	7 (14%)	
AML with 11q23	0 (0%)	1 (2%)	
AML without maturation	4 (5%)	4 (8%)	
AML with maturation	21 (29%)	16 (33%)	
Acute myelomonocytic leukemia	16 (22%)	13 (26%)	
Acute monoblastic and monocytic leukemia	6 (8%)	2 (4%)	
Acute erythroid leukemia	3 (4%)	0 (0%)	
No data	0 (0%)	1 (2%)	
Karyotype classification			0.026
Favorable	23 (32%)	12 (24%)	
Intermediate	45 (62%)	24 (49%)	
Poor	4 (5%)	10 (20%)	
No data	1 (1%)	3 (6%)	
Karyotype			0.006
Normal	38 (52%)	18 (37%)	
T(8;21)	3 (4%)	5 (10%)	
T(15;17)	20 (27%)	7 (14%)	
11q23	0 (0%)	1 (2%)	
Complex	2 (3%)	9 (18%)	
Others	9 (12%)	6 (12%)	
No data	1 (1%)	3 (6%)	
Gene Mutation			
C/EBPA (+/-)	5/60	6/37	0.340
NPM1 (+/-)	3/62	6/37	0.151
FLT3-ITD (+/-)	7/58	7/36	0.560
C-KIT (+/-)	1/64	0/43	1.000
N/K RAS (+/-)	7/58	2/41	0.312
IDH1/2 (+/-)	4/61	2/41	1.000
DNMT3A (+/-)	6/59	2/41	0.473
U2AF1 (+/-)	2/63	4/39	0.213
CR (+/-)	33/30	20/27	0.339

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

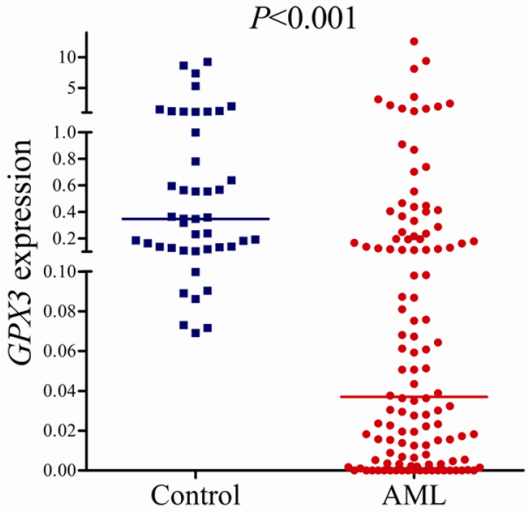

Figure 1. Relative expression levels of *GPX3* in AML patients and controls.

Materials and methods

Patients, cell lines and cell culture

After obtaining the written informed consent, BM was collected from 122 patients who had a diagnosis of de novo AML according to the French-America-British (FAB) and the World Health Organization (WHO) criteria [19, 20]. All patients were treated at the Affiliated People's Hospital of Jiangsu University from October 2005 to March 2014. Treatment protocol was described as reported previously [21]. The characteristics of the patients were summarized in **Table 1**. 44 healthy donors were collected as normal controls. Seven human leukemic cell lines including HL-60, NB4, U937, THP-1, HEL, SHI-1, and K562 cultured in IMDM medium containing 10% fetal calf serum and grown at 37°C in 5% CO_2 humidified atmosphere were also included.

BMMNCs separation, RNA isolation and reverse transcription

BM mononuclear cells (BMMNCs) were extracted from BM specimens by Ficoll solution and washed twice with PBS. Total RNA was isolated from the BMMNCs and the leukemic cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction with 40 μL volume contained 5 × buffer 10 mM, dNTPs 10 mM, random hexamers 10 μM, RNAsin 80 U, and MMLV reverse transcriptase 200 U (MBI Fermentas, Hanover, USA) was performed on

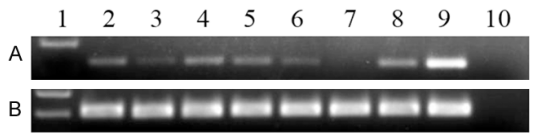

Figure 2. Electrophoresis results of RQ-PCR products in leukemic cell lines. 1: Gene Ruler™ 100bp DNA ladder; 2: K562; 3: SHI-1; 4: U937; 5: THP1; 6: NB4; 7: HL60; 8: HEL; 9: positive control; 10: negative control. A: *GPX3*; B: *ABL*.

iCycler Thermal Cycler (Eppendorf, Hamburg, Germany). The system of reverse transcription was incubated for 10 min at 25°C, 60 min at 42°C, and then stored at -20°C.

GPX3 mRNA level detection

Real-time quantitative PCR (RQ-PCR) was performed on a 7300 Thermo cycler (Applied Biosystems, CA, USA). The primers of *GPX3* expression were 5'-GCCGGGGACAAGAGAAGT-3' (forward) and 5'-GAGGACGTATTTGCCAGCAT-3' (reverse) as reported previously [12]. The RQ-PCR reaction with a volume of 20 μL was composed 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 were carried out at 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 (GPX3-plasmid) and negative (water) controls were included in each assay. *ABL* was used to calculate the abundance of *GPX3* mRNA. Relative *GPX3* transcript levels were calculated by the following equation: $N_{GPX3} = (E_{GPX3})^{\Delta CT\ GPX3\ (control\text{-}sample)} \div (E_{ABL})^{\Delta CT\ ABL\ (control\text{-}sample)}$. The parameter efficiency (E) was counted by the formula $E = 10^{(-1/slope)}$ (the slope referred to CT versus cDNA concentration plot).

Gene mutation detection

DNMT3A, *U2AF1*, *IDH1/2*, *N/K-RAS,* and *C/EBPA* mutations were detected as reported previously [22-26]. *NPM1* and *c-KIT* mutations were detected by PCR and high-resolution melting analysis (HRMA). All positive samples were confirmed by DNA direct sequencing. *FLT3-ITD* mutation was detected by PCR and direct DNA sequencing.

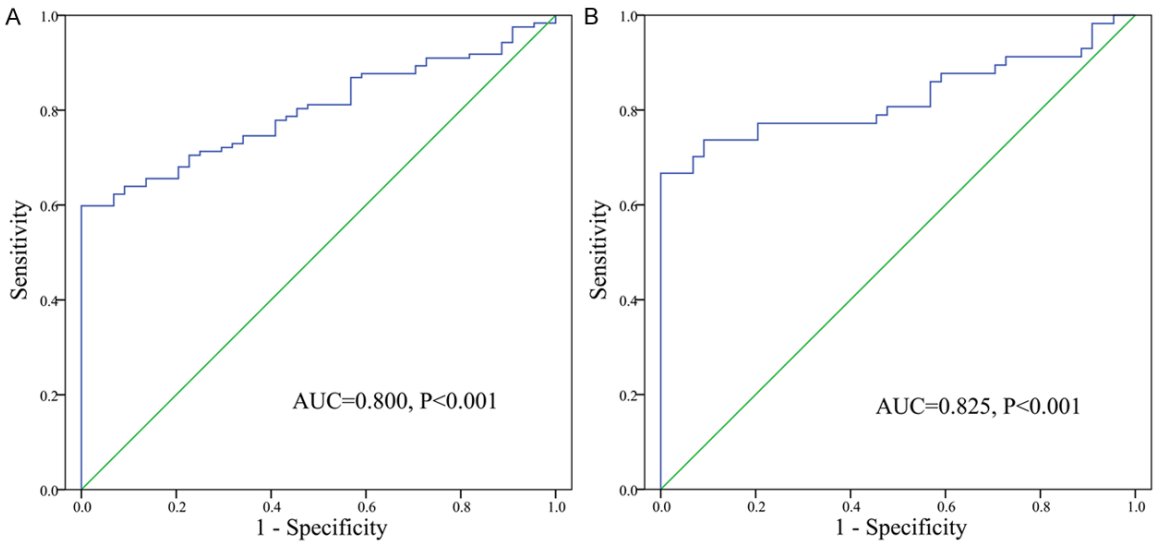

Figure 3. ROC curve analysis using *GPX3* for discriminating AML patients. A: All patients; B: Cytogenetically normal patients.

Statistical analysis

Statistical analyses were performed on SPSS 18.0 software package (SPSS, Chicago, IL). Pearson Chi-square analysis or Fisher exact test were employed to compare the difference of categorical variables. Mann-Whitney's U test was carried out to compare the difference of continuous variables between two groups. Receiver operating characteristic curve (ROC) and area under the ROC curve (AUC) were conducted to assess the value of *GPX3* expression in distinguishing AML patients from normal controls. Kaplan-Meier analysis and multivariate analysis were performed to analyze the impact of *GPX3* expression on survival respectively. For all analyses, a two-tailed *P* value of 0.05 or less was determined as statistically significant.

Results

GPX3 expression in normal controls, AML patients and cell lines

GPX3 mRNA level in normal controls ranged from 0.069 to 9.258 with a median level of 0.348. However, the level of *GPX3* transcript in de novo AML patients (0.000-12.600, median 0.037) was significantly down-regulated as compared with normal controls ($P < 0.001$, **Figure 1**). In addition, all the seven leukemic cell lines presented decreased *GPX3* expression ranging from 0.000 to 0.047 (**Figure 2**).

Differentiating value of GPX3 expression

ROC curve indicated that *GPX3* expression could serve as a promising biomarker for distinguishing whole AML patients from normal controls (AUC = 0.800, 95% CI: 0.735-0.864, $P < 0.001$) (**Figure 3A**). ROC curve analysis disclosed that at the cut-off value less than 0.067, the sensitivity and the specificity were 60% and 100%, respectively. Moreover, significant differentiating value was also revealed in cytogenetically normal AML (CN-AML) patients (AUC = 0.825, 95% CI: 0.741-0.909, $P < 0.001$) (**Figure 3B**).

Association between GPX3 expression and clinical parameters of AML patients

The whole AML patients were divided into two groups: *GPX3* low-expressed ($GPX3^{low}$) (≤ 0.067) and *GPX3* high-expressed ($GPX3^{high}$) (> 0.067) according to the cut-off value of 0.067. There were no significant differences in age, white blood cell (WBC), BM blasts, and sex distribution between the two group patients ($P > 0.05$, **Table 1**). However, $GPX3^{low}$ cases had significantly higher hemoglobin (HB) and platelets (PLT) than $GPX3^{high}$ cases ($P = 0.049$ and 0.020). There were no significant differences in the distribution of FAB as well as WHO classifications between the two groups ($P > 0.05$). However, significant differences were observed between $GPX3^{low}$ and $GPX3^{high}$ cases in the distribution of both karyotype and karyotype clas-

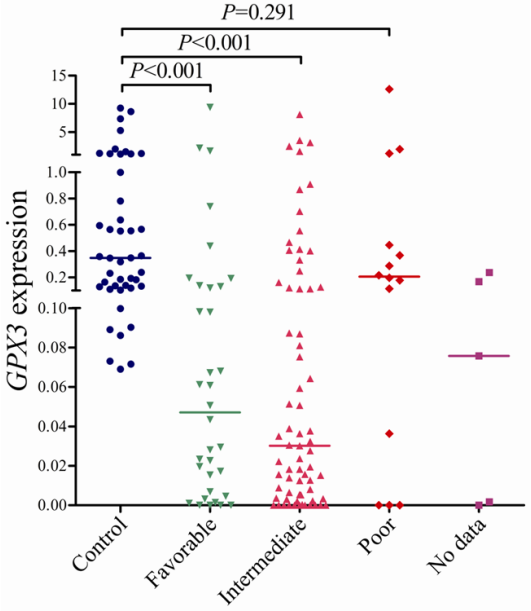

Figure 4. Relative levels of *GPX3* expression in AML according to karyotypic risking.

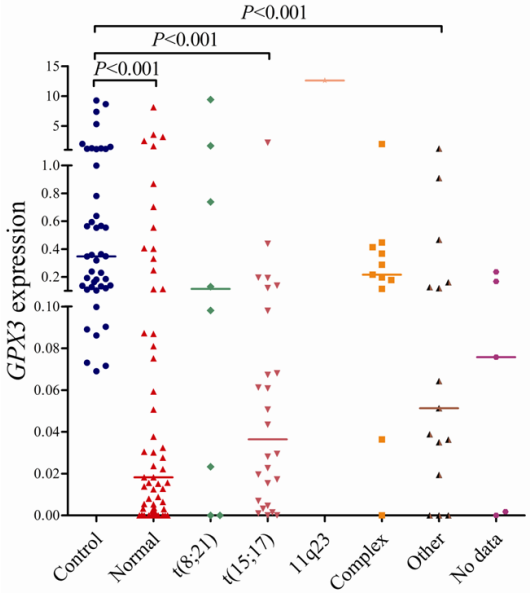

Figure 5. Relative levels of *GPX3* expression in AML with different karyotypes.

sification (P = 0.006 and 0.026). The frequency of low *GPX3* expression in favorable karyotype (66%, 23/35) and intermediate karyotype (65%, 45/69) was higher than in poor karyotype (29%, 4/14) (P = 0.017). The level of *GPX3* transcript in the patients with favorable (median 0.043) and intermediate karyotypes (median 0.030) was significantly lower than in controls (P < 0.001), whereas those with poor karyotypes (median 0.207) had similar level of *GPX3* mRNA as controls (P = 0.291) (**Figure 4**). The level of *GPX3* expression among different karyotypes was shown in **Figure 5**. Due to the *GPX3* gene locates on chromosome 5, we further analyzed *GPX3* expression in the patients with and without -5/5q-. Interestingly, patients with -5/5q- (20%, 1/5) trended to have higher incidence of low *GPX3* expression than those without -5/5q- (62%, 68/109) (P = 0.078). Additionally, no significant correlations were observed between *GPX3* expression and ten gene mutations (P > 0.05, **Table 1**).

Association between GPX3 expression and prognosis in AML patients

110 patients with available follow-up data were included in the complete remission (CR) analysis. *GPX3*^{low} patients had higher CR rate than *GPX3*^{high} patients in whole AML (52% vs. 43%, P = 0.339, **Table 1**). Moreover, *GPX3*^{low} and *GPX3*^{high} patients showed similar CR rate in both non-M3 and CN-AML [44% (22/50) vs. 39% (16/41) and 51% (18/35) vs. 47% (9/19); P = 0.674 and 1.000]. Survival analyses were performed in 107 patients with survival data ranging from 1 to 92 months with a median of 10 months. *GPX3*^{low} patients had a little longer overall survival (OS) than *GPX3*^{high} patients (median 11 vs. 9 months, P = 0.256, **Figure 6**). Among non-M3 and CN-AML patients, there were also no significant difference in OS between the *GPX3*^{low} and *GPX3*^{high} groups (median 6 vs. 7 months and 12 vs. 5 months; P = 0.774 and 0.555). Furthermore, multivariate analysis also failed to disclose the prognostic impact of *GPX3* expression in both whole AML and non-M3 as well as CM-AML patients (data not shown).

Discussion

GPX3, the most studied *GPX* family member, accounts for almost all of the *GPX* activity in plasma [7]. Recently, tumor suppressive roles of the *GPX3* have been observed in numerous solid cancers including prostate cancer, colitis-associated carcinoma and hepatocellular carcinoma [27-29]. Interestingly, a few studies revealed the up-regulated status of *GPX3* expression in clear cell epithelial ovarian carcinoma tissues as well as in ovarian serous

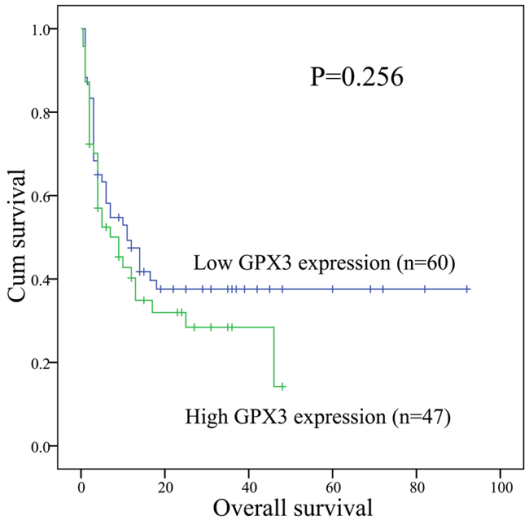

Figure 6. Overall survival of whole AML patients.

carcinoma effusions [30, 31]. These results suggest that *GPX3* expression may be tumor-specific in the process of tumorigenesis. However, *GPX3* expression pattern is rarely studied in hematological malignancies. A previous study has revealed the role of *GPX3* in LSCs as well as normal hematopoietic stem cells (HSCs) and indicated that *GPX3* is critical for the competitiveness of LSCs and self-renewal activity of HSCs [32]. Moreover, Kaiser et al. [33] disclosed the hypermethylation status of *GPX3* promoter is associated with shorter OS in multiple myeloma. These results suggested that *GPX3* also played a vital role in hematological malignancies.

In the current study, we investigated the *GPX3* expression pattern and further analyzed the clinical significance of *GPX3* expression in de novo AML patients. Our study revealed that *GPX3* was down-regulated in both leukemic cell lines and de novo AML patients. Moreover, our investigation by ROC curve analysis disclosed that *GPX3* expression could be a promising biomarker for discriminating AML as well as CN-AML from normal controls.

When analyzed the *GPX3* expression status in different karyotypes and karyotypic classifications, our data demonstrated that *GPX3* expression was significantly decreased in patients with favorable/intermediate karyotypes especially in t(15;17) and normal karyotypes but not in those with poor karyotypes. Similarly, the previous study also observed the increased level of *GPX3* expression with the rising risk of karyotype [32]. These results suggested that the role of *GPX3* in the process of leukemogenesis may be dependent on the context of different cytogenetics.

Accumulating studies have revealed the prognostic value of *GPX3* expression in several solid cancers. In gastric carcinomas, decreased *GPX3* expression was associated with lymph node metastasis [12]. Zhang et al. [10] indicated that down-regulation of *GPX3* was a predictive marker not only for the lymph node metastasis but also for the poor prognosis in cervical cancer. Yang et al. [11] demonstrated that positive *ALDH1A3* and negative *GPX3* expressions were associated with adverse prognosis in gallbladder cancer. Moreover, the negative impact of low *GPX3* expression on prognosis has also been revealed in hepatocellular carcinoma [29]. However, our investigation by both Kaplan-Meier analysis and multivariate analysis failed to indicate that *GPX3* expression could be a potential biomarker for the prognosis of AML.

The underlying mechanism regulating *GPX3* expression has been abundantly investigated. The association between *GPX3* expression and its promoter methylation has been found in various cancers [9, 12, 13, 15-18, 33]. Moreover, hypermethylation of *GPX3* promoter has been identified to be helpful not only for the early diagnosis but also for the prognosis in a few cancers [15, 33-35]. A recent study demonstrated the epigenetic mechanism regulating *GPX3* expression in multiple myeloma [33]. Further studies are needed to explore *GPX3* promoter methylation pattern in AML patients and determine its role in regulating *GPX3* expression.

In conclusion, our study indicates that decreased GPX3 expression is associated with favorable/intermediate karyotypes. Moreover, down-regulation of GPX3 expression has no impact on the clinical outcome of de novo AML patients.

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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 Rd., Zhenjiang 212002, People's Republic of China. Fax: +86. 511.85234387; E-mail: qianjun0007@hotmail.com; Dr. Jiang Lin, Laboratory Center, Affiliated People's Hospital of Jiangsu University, 8 Dianli Rd., Zhenjiang 212002, People's Republic of China. Fax: +86. 511.85234387; E-mail: linjiangmail@sina.com

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