Clinical Significance of Decreased GPX1 Expression in Patients with Acute Myeloid Leukaemia (Non-M3)

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ABSTRACT

Objective: To determine the expression levels of *GPX1*, *SOCS5* and *IL7* and their clinical significance in patients with acute myeloid leukaemia (AML).

Study Design: A case-control study.

Place and Duration of Study: Department of Hematology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China, from January 2013 to November 2020.

Methodology: Based on the bioinformatics analysis, the expression levels of *GPX1, SOCS5* and *IL7* in the bone marrow of 64 AML patients (non-M3) and 32 healthy individuals were evaluated by real-time PCR. Correlation between *GPX1* expression and the clinical characteristics, response to induced chemotherapy, and survival time of AML patients were analysed as the outcome measure.

Results: *GPX1* was significantly downregulated in AML patients, which helped in distinguishing AML patients from normal controls. The area under the curve (AUC) of the receiver operator characteristic (ROC) was 0.741 ($p < 0.001$). Additionally, *GPX1* expression was correlated with gender ($r = -0.250$, $p = 0.045$), FAB classification ($r = -0.332$, $p = 0.004$), and chemotherapy response ($r = 0.366$, $p = 0.003$). AML patients with high *GPX1* expression levels had a lower rate of remission ($p = 0.021$) and poor long-term survival ($p = 0.036$) than those with low *GPX1* expression levels.

Conclusion: Low *GPX1* expression in AML patients may be closely associated with the pathogenesis and chemoresistance of AML.

Key Words: *Acute myeloid leukaemia, Clinical outcome, Gene expression, GPX1.*

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INTRODUCTION

AML is a malignant clonal disease of the hematopoietic system that originates from myeloid hematopoietic stem/progenitor cells and presents enormous molecular heterogeneity.[1] Recently, through the application of high-throughput microarray analysis, the role of AML in diagnosis, classification, outcome prediction and recognition of subsets has been understood.[2] Gene expression profiling (GEP), which identifies differentially expressed genes (DEGs), has been widely applied. Some genes may provide prognostic forecasts and contribute to resistance to therapy, while acting as credible diagnostic biomarkers.[1] Here, this study investigated novel potential biomarkers for the treatment and prognosis of AML.

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Authors preparatory work identified several DEGs. Until now, the effects of *GPX1* or *SOCS5* on the treatment and prognosis of AML patients has not been extensively studied. *IL7* and *SOCS5* are the activator and the profiling of the *JAK/STAT* pathway, respectively; however, the connection between these genes in the pathogenesis of AML remains unclear.

Glutathione peroxidase-1 (*GPX1*) is a member of the glutathione peroxidase (GPX) system that plays an important role of cellular oxidative metabolism. When cells are stimulated by oxidation, the transcription factor *Nrf1/2*, upregulates *GPX1* expression, to reduce reactive oxygen species (ROS) generation.[3] *GPX1* influences the progression of several tumours. Based on bioinformatics analysis, *GPX1* expression is upregulated in AML and myelodysplastic syndrome (MDS),[4] and the high level of *GPX1* expression is associated with poor clinical outcomes in AML.[5,6] However, the role and mechanism underlying *GPX1* in AML remain to be elucidated.

Suppressor of cytokine signalling-5 (*SOCS5*) plays an important role in regulating gene, controlling bioprocesses such as growth and differentiation of cells and haematopoiesis, and immune function, mainly by suppressing the activation of the *JAK/STAT* signalling pathway.[7] Abnormal expression and regulation of *SOCS5* are involved in tumour genesis and progress.[8] Interleuk-

in-7 (*IL7*), an activator of the *JAK/STAT* pathway, is a growth factor for multi-effects that stimulates the development, proliferation and differentiation of B and T cells.[9] A recent study demonstrated that *IL7* is expressed at low levels in the peripheral blood of AML patients,[10] whose level and function in the bone marrow (BM) of AML are unclear.

To solve this problem, the aim of the present study was to explore these genes *GPX1*, *SOCS5*, and *IL7* in the development, treatment, and prognosis of AML, which may provide a foundation for the development of novel therapeutic targets for AML treatment.

METHODOLOGY

In this study, 64 BM samples were obtained from patients with newly diagnosed AML before any chemotherapy between 2013–2016 in the Department of Hematology, The First Affiliated Hospital of Guangxi Medical University, China. Patients with other haematological diseases or malignant tumours were excluded, and all patients with AML were followed up regularly until November 2020. The diagnosis and subtypes of AML were established according to the WHO recommended criteria and FAB classification. The study was approved by the Human Ethics Committee of The First Affiliated Hospital of Guangxi Medical University, and all participants provided written informed consent according to the Declaration of Helsinki.

Identification of DEGs in AML and their prognostic value were performed using the ONCOMINE database. The protein-protein interaction (PPI) networks and hub genes were analysed using the Ssearch Tool for the Retrieval of Interacting Genes/Proteins (STRING) database to describe the biological relationship between genes, especially the PPI information. To analyse the DEGs at the functional level, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the Database for Annotation, Visualisation, and Integrated Discovery (DAVID). The results were considered statistically significant, when the p-values were <0.05.

BM samples (2 ml) were extracted from each subject, and BM mononuclear cells (BM-MNCs) were separated from the BM samples by density gradient centrifugation. According to the manufacturer's protocol, TRIzol reagent (Invitrogen, USA) was added to the samples for conservation in a -80°C refrigerator until required for total RNA extraction. RNA concentration and purity were measured by ultraviolet spectrophotometric examination and agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA. Sense and antisense primers for target genes were designed using Primer 5.0. The primers used for each gene were as follows: *GPX1* forward 5'-CGCTGGCTTCTTGGACAAT-3' and reverse 5'-AGAAGGCATACACCGACTGG-3', *IL7* forward 5'-AGGGTCCTGGGAGTGACTATGG-3' and reverse 5'-TGATGCTACTGGCAACAGAACAA-3', *SOCS5* forward 5'-CTCCCTCTACCCTCAATGTTAC-3' and reverse 5'-TTACTTTGCCTTGACTGGTTCT-3'. The housekeeping gene β-actin was used in PCR amplification as the internal reference to normalise the data, with the following primers: forward 5'-GTGGCCGAGGACTTTGATTG-3' and reverse 5'-CCTGTAACAACG CATCTCATATT-3'.

Real-time PCR was performed on RNA samples using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, USA). The Real-time PCR was carried out under the following conditions: 10 min at 95°C, followed by 40 cycles of 10 s at 95°C and 60 s at 60°C. Finally, melting was carried out between 60–99°C (with 0.5°C increments) for 5 s at each step. Besides, PCR product specificity was verified using a melting curve. The relative expression levels of *GPX1*, *IL7*, and *SOCS5* were calculated using the comparative $2^{-\Delta\Delta ct}$ method.

All statistical analyses were performed using SPSS (version 23.0; SPSS Inc., Chicago, IL). Data were depicted by GraphPad Prism (version 8.0, Inc, La Jolla, CA, USA). Because the data did not meet the normal distribution, Mann-Whitney's U-test, Pearson Chi-square test, and Fisher's Exact probabilities were used to evaluate the difference in gene expression among different groups and the relationship between *GPX1* expression and clinical parameters. Quantitative variables are expressed as median (IQR) and qualitative variables are expressed as number and percentages. Pearson's correlation analysis was performed to evaluate the correlation between the two variables. Receiver operator characteristic (ROC) analysis was used to assess the sensitivity, specificity, and the area under the curve (AUC) of *GPX1* expression. The Kaplan-Meier survival curve was used to examine the relationship between gene expression levels and clinical outcomes. Cox proportional hazards regression model analysis was applied to analyse the risk factors for AML patients with statistical significance in clinical prognosis. In the study, all comparisons were considered statistically significant at $P<0.05$.

RESULTS

First, DEGs of AML were analysed in the ONCOMINE database and identified a total of 15 genes (*CUL5, IL7, IL7r, JAK2, STAT5A, STAT5b, ADAM28, TCEB1, SOCS5, IL2RG, GPX1, SOD1, SOD2,* and *GSR*) that were most related to the clinical prognosis of AML, and then two PPI networks and 14 hub genes were identified (Figure 1). KEGG enrichment analysis demonstrated that these DEGs were significantly enriched in the *JAK/STAT* signalling pathway (Table I). Considering the value of *GPX1* expression levels and that the relationship between *SOCS5* and *IL7* in AML remains undefined, the authors investigated the expression levels of these three genes in BM-MNCs and their clinical significance in AML.

The clinical characteristics of the patients are summarised in Table II. The control group consisted of 32 healthy volunteers and donors without cancer: 15 men and 17 women, with a median age of 32 years (range 24–47). The relative expression levels of *GPX1*, *IL7* and *SOCS5* in AML patients and normal controls were analysed using real-time PCR arrays, as shown in Figure 2A. *GPX1* expression was significantly downregulated in AML patients compared to that in healthy individuals ($p<0.001$; Figure 2B). The ROC curve showed that *GPX1* is a potential indicator which could discriminate AML patients from normal subjects with an AUC of 0.741 ($p<0.001$, 95% confidence interval (CI): 0.636-0.845, Figure 3A). There was no significant difference in

the expression levels of *IL7* and *SOCS5* between patients and normal controls; therefore, authors herein focus on analysis of *GPX1*. Furthermore, significant differences were observed in the relative expression of *GPX1* between different FAB classifications, specifically in M1 and M5 (p = 0.039), M2 and M4 (p = 0.007), M2 and M5 (p = 0.018; Figure 2C). With the median expression of *GPX1* as the cut-off value, the patients were divided into a low expression group (n = 32) and a high expression group (n = 32). The relationship between *GPX1* expression and clinical characteristics is shown in Table I. *GPX1* expression was associated with gender (r = -0.250, p = 0.045), FAB classification (r = -0.332, p = 0.004), and chemotherapy response (r = 0.366, p = 0.003) in AML patients.

Table I: KEGG pathway analysis of DEGs associated with AML.

KEGG Term	Counts	p	Genes
aml04630: JAK/STAT signaling pathway	7	9.42E-09	IL7, STAT5A, STAT5B, JAK2, IL2RG, SOCS5, IL7R
aml04917: Prolactin signaling pathway	4	1.54E-04	STAT5A, STAT5B, JAK2, SOCS5
aml05162: Measles	4	6.40E-04	STAT5A, STAT5B, JAK2, IL2RG
aml04151: PI3K-Akt signaling pathway	4	0.011893	IL7, JAK2, IL2RG, IL7R
aml04060: Cytokine-cytokine receptor interaction	3	0.03085	IL7, IL2RG, IL7R
aml05340: Primary immunodeficiency	2	0.046449	IL2RG, IL7R
aml05166: HTLV-I infection	3	0.048462	STAT5A, STAT5B, IL2RG

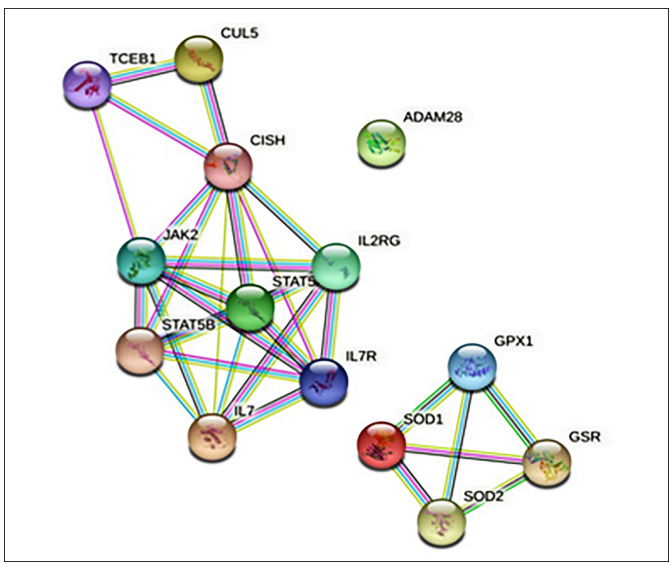

Figure 1: The protein-protein interaction networks and hub genes of DEGs.

According to the response to initial remission-induction chemotherapy, all patients were divided into two groups: those with complete remission (CR group, 43 cases) and those with no response (NR group, 21 cases). There was a significant difference in the relative expression level of *GPX1* between the two groups: patients in the CR group had a lower *GPX1* expression than those in the NR group without remission (p = 0.021; Figure 2D). Furthermore, to verify the effect on prognosis of *GPX1* in AML patients, the relationship between *GPX1* expression and overall survival(OS) was assessed. The results showed that the high expression group was significantly different from the low expression group in OS (p = 0.036; Figure 3B), which suggested that *GPX1* expression was associated with the survival of AML patients. However, *GPX1* expression status did not persist as an OS predictor in the multivariate analysis (Table III).

Table II: Comparison of clinical and molecular characteristics with the expression of GPX1 in AML patients.

Clinical characteristics	GPX1 mRNA level		p-value
	Low(n=32)	High(n=32)	
Age, years, median(IQR)	32.50(25.25-47.00)	34.0(25.00-42.25)	0.835
Age group, years, n (%)			0.599
<40	20(31.3)	22(34.4)	
>40	12(18.8)	10(15.6)	
Gender, n(%)			**0.045**
Male	11(17.2)	19(29.7)	
Female	21(32.8)	13(20.3)	
Hemoglobin, g/L, median(IQR)	75.6(65.0-88.7)	72.5(63.4-82.7)	0.489
Leukocyte, $\times 10^9$/L, median(IQR)	11.5(5.25-28.77)	17.6(5.72-99.55)	0.240
Platelet, $\times 10^9$/L, median(IQR)	49.8(23.58-78.40)	33.0(18.08-87.45)	0.277
BM blasts ,%, median(IQR)	52.2(39.75-67.25)	59.0(37.25-72.75)	0.428
FAB type, n(%)			**0.004**
M1	1(1.6)	4(6.3)	
M2	3(4.7)	12(18.8)	
M4	17(26.6)	9(14.1)	
M5	11(17.2)	5(7.8)	
M6	0(0)	2(3.1)	
Chromosome karyotype, n (%)			0.777
Normal	24(37.5)	23(35.9)	
Abnormal	8(12.5)	9(14.1)	
Mutation status, n (%)			
NPM1			0.491
Mutation	6(9.4)	4(6.3)	
Wild type	26(40.6)	28(43.8)	
FLT3-IDT			0.474
Mutation	3(4.7)	6(9.4)	
Wild type	29(45.3)	26(40.6)	
C-kit			0.613
Mutation	1(1.6)	3(4.7)	
Wild type	31(48.4)	29(45.3)	
DNMT3			0.355
Mutation	4(6.3)	1(1.6)	
Wild type	28(43.8)	31(48.4)	
CEBPA			0.491
Mutation	4(6.3)	6(9.4)	
Wild type	28(43.8)	26(40.6)	
Hematopoietic stem cell transplantation (HSCT), n (%)			0.226
Yes	9(14.1)	5(7.8)	
No	23(35.9)	27(42.2)	
Complete remission, n(%)			**0.003**
Yes	27(42.2)	16(25.0)	
No	5(7.8)	16(25.0)	

Table III: Univariate and multivariate analyses in AML patients.

Variables	Univariate		Multivariate	
	HR(95% CI)	p	HR(95% CI)	p
GPX1 (high vs. low)	2.062 (1.023-4.154)	**0.043**	1.394 (0.674-2.883)	0.371
Gender (male vs. female)	0.591(0.297-1.176)	0.134		

Age (<40 vs. ≥40)	1.231(0.605-2.506)	0.566		
Leukocyte (<20 vs. ≥20×10^9/L)	0.898(0.441-1.829)	0.768		
NPM1 (mutated vs. wild)	2.327(0.709-7.637)	0.164		
FLT3–ITD (mutated vs. wild)	0.788(0.304-2.044)	0.624		
C-kit (mutated vs. wild)	0.989(0.236-4.149)	0.987		
DNMT3 (mutated vs. wild)	1.484(0.355-6.203)	0.589		
CEBPA (mutated vs. wild)	0.965(0.372-2.502)	0.942		
BM blasts (<60 vs. ≥60%)	1.652(0.833-3.277)	0.151		
Complete remission (yes vs. no)	0.170(0.083-0.348)	**<0.001**	0.278 (0.131-0.589)	**0.001**
HSCT (yes vs. no)	0.070(0.010-0.513)	**0.009**	0.120 (0.016-0.921)	**0.041**

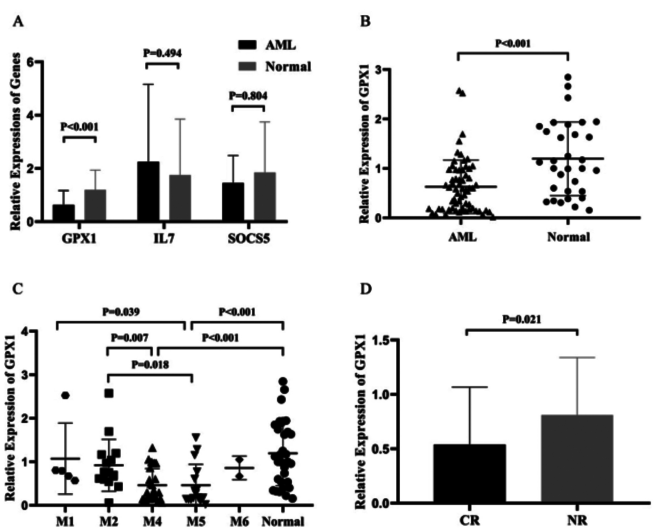

Figure 2: A. The relative expression levels of *GPX1*, *IL7* and *SOCS5* in AML patients and normal controls. B. The relative expression of *GPX1* in AML group and normal controls. C. The relative expression of *GPX1* in each FAB subgroup of AML patients and normal controls. D. Correlation between *GPX1* relative expression and response to induction chemotherapy.

DISCUSSION

GPX1 is closely connected with the oxidative metabolism of cells, which degrades ROS levels under oxidative stress. ROS, which is tightly associated with the emergence, expansion, metastasis and survival of cancer patients, is mostly higher in cancer cells than in normal cells.[11]

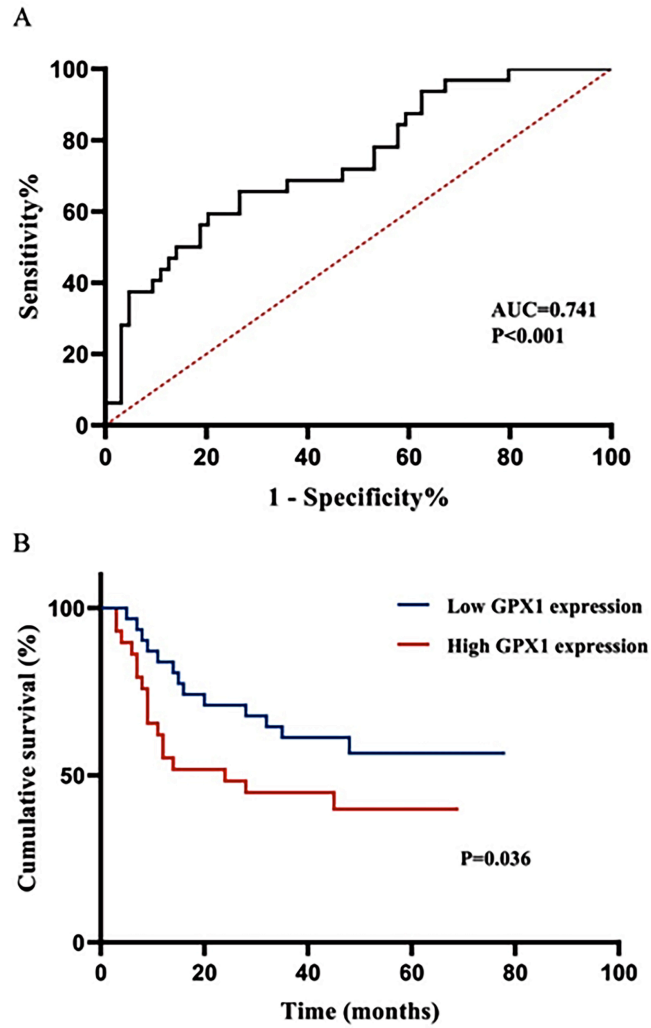

Figure 3: (A) ROC analysis using *GPX1* for separating AML cases from normal controls. (B) Kaplan-Meier survival curves for AML patients which were divided into two groups based on the median value of *GPX1* expression level.

Accumulated evidence suggests that the expression levels of *GPX1* vary among different tumors. Cancers with high levels of *GPX1* expression includes mainly squamous cell carcinoma, such as laryngeal squamous cell carcinoma and esophageal squamous cell carcinoma,[12,13] while cancers with low *GPX1* levels includes mainly glandular cell carcinoma, such as pancreatic cancer, breast cancer and gastric cancer.[14-16]

Bioinformatics analysis has previously shown that *GPX1* is upregulated in AML; however, large-scale clinical studies are needed to verify these conclusions.[6] It is thought-provoking that authors observed a decrease in *GPX1* mRNA expression in patients with AML, opposing the previous study as well as the result based on the ONCOMINE database, the authors analysed before. However, bioinformatics analysis only lays the foundation of this project, and the results may differ for each race, as well as the experimental results shall prevail. The authors speculate that AML cells generate less *GPX1* mRNA than normal cells due to promoter methylation of *GPX1*, as in gastric cancer, DNA promoter methylation correlates with the loss of *GPX1* expression.[16] Furthermore, treatment with a methylation inhibitor increased the mRNA and

protein levels of *GPX1* and enhanced the cell's antioxidant capacity,[17] indicating that promoter methylation influences *GPX1* expression.

There is abnormal DNA methylation in AML revealed by whole-genome DNA methylation sequencing,[18] indicating a possible explanation for *GPX1* down-regulation. Further experiments are required for the detection of *GPX1* promoter methylation levels in AML, to confirm the above conjecture. In summary, *GPX1* had a relatively low expression in AML, which is partly consistent with a previous report.[19] Rasool *et al.* suggested that *GPXs* were reduced in patients suffering from AML, and low expression of *GPX1* participated in the occurrence and development of AML. However, over-expression of *GPX1* under induction to determine whether it can suppress the multiplication and promote apoptosis of AML cells is the next step the authors plan to do. Additionally, abnormal expression of *GPX1* is associated with clinical features of cancers, including TNM staging and lymphatic metastasis.[13,16] This study indicates that *GPX1* expression has a connection with FAB classification. The authors speculate that different FAB types may have diverse degrees of BM-MNC differentiation. Thus, the authors need more clinical specimens and cell experiments to verify the above thoughts, especially the samples of M1 and M6.

GPX1 plays a crucial role in tumour growth and chemoresistance. Some scholars believe that oxidative metabolic stress is involved in the relapse and drug resistance of AML.[20] Over 60% of AML cells could result in high levels of NOX-derived ROS, accelerating primary AML blast multiplication.[21] In multidrug resistance of Burkitt lymphoma cells, the production of ROS induces the phosphorylation of *STAT3*, which improves the expression of *GPX1* and *SOD2* to protect cells from oxidative stress.[22] *GPX1* is also upregulated in resistant B-cell lymphoma cells treated with cisplatin and methotrexate.[23] The results showed that *GPX1* expression in the NR group was significantly higher than that in the CR group. It is likely that AML patients in the CR group with a low expression of *GPX1* may have increased ROS levels induced by chemotherapy, which exert synergistic combination with chemotherapeutic drugs to kill tumour cells together. Similarly, one study suggested that in the treatment of acute promyelocytic leukaemia, arsenic trioxide combined with ROS can accelerate the degradation of the *PML-RARA* oncoprotein.[24] Despite evidence manifests that a high level of ROS stimulates the proliferation of tumour cells, a study verified that cytarabine and daunorubicin, the first-line therapy in the induction chemotherapy of AML, could promote ROS acumulation and induce apoptosis in AML cells.[25] Abnormal *GPX1* expression in AML likely affects the efficacy of chemotherapy; hence, regulating its expression may contribute to the treatment of relapsed or refractory AML and become a novel therapeutic strategy for AML. Nevertheless, the relative expression of *GPX1* in AML patients with refractory/relapse before and after standard treatment has not been reported. Further investigations on the role of *GPX1* in AML resistance and its exact mechanisms are essential.

GPX1 is involved in the pathogenesis of many diseases, and its function varies with tumour type in the occurrence and progression of diseases. Authors preliminary work revealed that high expression of *GPX1* suggested a poor prognosis, as analysed by the GEPIA database ($p = 0.013$), was consistent with current study. Similarly, Wei *et al*. revealed that high *GPX1* levels are related to adverse clinical outcomes and poor survival.[6] A possible explanation could be that the reduced *GPX1* expression and the accumulation of ROS led to leukaemia cell deaths, and the patients reached remission and had a better survival. On the other hand, chemotherapy-resistant AML patients tend to have increased *GPX1*, which leads to a shorter OS. Considering this findings, further studies in this area are necessary to gain insight into the role of *GPX1* mRNA expression in the clinical outcome and chemotherapy resistance of haematological malignancies. Authors aimed to detect the level of *GPX1* in BM and measure the activity of glutathione peroxidase and ROS levels in the peripheral blood of AML patients after chemotherapy to consummate above investigation. Since *GPX1* expression is related to prognosis in some tumours, controlling its level may be a strategy to prolong the survival time of patients, which can be seen as a potential target for therapy in the future.

CONCLUSION

GPX1 expression in the bone marrow of AML patients is significantly downregulated compared to that in normal controls, and may play an important role in the diagnosis, pathogenesis, and chemoresistance of AML.

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ETHICAL APPROVAL:
This study was approved by the Human Ethics Committee of The First Affiliated Hospital of Guangxi Medical University, China. (2020(KY-E-147).

PATIENTS' CONSENT:
Informed consents were obtained from patients to publish the data concerning this case.

CONFLICT OF INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
XL, YY, ZL: Designed the project.
XL, YY, HL, JW, CW: Performed the experiment and analysed the data.
XL, HL, JW: Drafted the manuscript.
YY, CW, ZL: Approved the final version to be published.

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