GPAA1 promotes progression of childhood acute lymphoblastic leukemia through regulating c-myc

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Abstract. – OBJECTIVE: Previous studies have shown that glycosylphosphatidylinositol Anchor Attachment Protein 1 (GPAA1) is a cancer-promoting gene; however, the role of GPAA1 in childhood acute lymphoblastic leukemia (ALL) has not been reported. This study aims to illustrate the role of GPAA1 in promoting the metastasis of ALL by targeting c-myc and the potential mechanism.

PATIENTS AND METHODS: Quantitative real time-polymerase chain reaction (qRT-PCR) was performed to examine serum levels of GPAA1 and c-myc in 42 childhood ALL patients and healthy volunteers. The interaction between GPAA1 expression and prognosis of childhood ALL was analyzed. Meanwhile, expressions of GPAA1 and c-myc in ALL cell lines were determined by qRT-PCR. Furthermore, after GPAA1 knockdown model was constructed by lentivirus transfection in MOLT-4 and SUP-B15 cells, cell counting kit-8 (CCK-8), transwell invasion, and cell wound healing assays were conducted to analyze the effect of GPAA1 on the biological functions of ALL cells. Potential mechanism was further explored through Luciferase reporter gene assay and cell recovery experiments.

RESULTS: QRT-PCR results indicated that serum level of GPAA1 in childhood ALL patients was remarkably higher than that of healthy volunteers, and the difference was statistically significant. Childhood ALL patients with high expression of GPAA1 had lower overall survival rate compared with those expressing low expression of GPAA1. Proliferation and metastasis abilities of pediatric ALL cells with GPAA1 knockdown remarkably decreased. Subsequently, c-myc expression was also found remarkably upregulated in ALL cell lines and serum samples of childhood ALL patients and it was positively correlated with GPAA1 level. In addition, Luciferase reporter gene assay demonstrated that overexpression of c-myc remarkably attenuated the Luciferase activity of the wild-type GPAA1 vector without attenuating that of the mutant vector or empty vector, further demonstrating that GPAA1 can be targeted by c-myc. At the same time, cell recovery experiment found that the interaction between GPAA1 and c-myc together regulated the malignant progression of ALL.

CONCLUSIONS: GPAA1 was up-regulated in serum of childhood ALL patients, which was remarkably associated with the prognosis. In addition, GPAA1 may contribute to the malignant progression of childhood ALL via activating c-myc.

Key Words:
GPAA1, C-myc, Childhood ALL, Progression.

Introduction

Leukemia is a malignant disease of hematopoietic system, which is characterized by the clonal proliferation of hematopoietic cells that stagnates in the primitive low differentiation stage[1-3]. Proliferating leukemia cells are dysfunctional, which will infiltrate into tissues and organs throughout the body after entering the blood circulation, resulting in a progressive decrease in the number of normal blood cells. Acute lymphoblastic leukemia (ALL) patients may have symptoms such as anemia, hemorrhage, infection, etc. In addition, liver, spleen, and lymph nodes of the whole body may be enlarged to varying degrees, accompanied by a series of clinical symptoms such as bone pain[4,5]. At present, the pathogenesis and mechanism of leukemia have not been fully clarified, and the causes may be related to viral infection, physical and chemical stimulation, genetic defects, etc[6,7].

In China, as a malignant tumor threatening people's life and health, the incidence of leuke-

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mia increases year by year[8,9]. The main affected population of leukemia is under 35 years old, especially among adolescents and children under the age of 18[9]. According to statistical data, 15,000 new cases of leukemia have been found in China every year, among which childhood ALL accounts for 70-80%. With the continuous advancement of modern immunology, molecular biology, and genetic technology, we have a deeper understanding of the etiology and pathogenesis of leukemia. At the same time, with the continuous improvement of clinical diagnosis, treatment technology and individualized treatment scheme, the successful treatment rate of childhood leukemia has increased year by year after receiving multi-drug and multi-course combined chemotherapy[10,11]. Since the 1980s, the overall cure rate of childhood ALL has reached about 80%, and the prognosis has also been greatly improved. Nevertheless, about 30% of patients relapse in bone marrow, central nervous system or testis[12,13].

Some foreign scholars have reported that the phospholipid inositol on the hepatocyte membrane combines with the Anchor Attachment protein to form a glycosylphosphatidylinositol Anchor Attachment Protein 1 (GPAA1) complex, which is overexpressed and related to the development of liver cancer[14]. In this study, the serum level of GPAA1 in childhood ALL patients was detected. Since GPAA1 and c-myc genes are located in the same chromosome region and both highly expressed in tumors, and c-myc gene is the target gene of Wnt signaling pathway, we believed that GPAA1 may regulate the expression level of c-myc through the Wnt signaling pathway[15,16]. This will provide new ideas and methods for gene therapy in childhood ALL. However, the exact mechanism of GPAA1 in regulating the proliferation and invasion abilities of ALL cells remains to be further explored.

Patients and Methods

Childhood ALL Patients and Serum Samples

42 cases with newly diagnosed childhood ALL were collected from the pediatric department. All cases were diagnosed according to the Hematological Diagnosis and Efficacy Criteria, and 42 serum samples were obtained from healthy volunteers without any disease. All patients and healthy volunteers in this study have been fully informed that their specimens would be used for scientific research, and informed consent had been signed. This investigation was approved by the Ethics Oversight Committee. This study was conducted in accordance with the Declaration of Helsinki.

Cell Lines and Reagents

Acute lymphoblastic leukemia cell lines (MOLT-3, MOLT-4, SUP-B15) and human primary peripheral blood mononuclear cell line (PBMC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from American Life Technologies (Gaithersburg, MD, USA). Cells were cultured with DMEM containing 10% FBS in a 37°C, 5% CO_2 incubator.

Transfection

sh-NC and sh-GPAA1 were purchased from GenePharma (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 30-40%, and lentiviral transfection was performed according to the manufacturer's instructions. After 48 h, cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and cell function experiments.

Cell counting kit-8 (CCK-8) Assay

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. Cells were cultured for 24 h, 48 h, 72 h, and 96 h, respectively, followed by CCK-8 (Dojindo Laboratories, Kumamoto, Japan) solution incubation for 2 h. Optical density (OD) of each well was measured in the microplate reader at 490 nm absorption wavelength.

Transwell Migration and Invasion Assay

After 48 h of transfection, ALL cells were trypsinized and resuspended in serum-free medium. Suspension was prepared to 2.0×10^5/ml. Transwell chambers either pre-coated with Matrigel or not were placed in a 24-well plate, where 200 μL of the cell suspension was added in the upper chamber, and 500 μL of medium containing 10% FBS to the bottom chamber. After 48 h incubation, chambers were removed, fixed with 4% paraformaldehyde for 30 min, and stained with crystal violet for 15 min. Next, cells were washed with phosphate-buffered saline (PBS), and inner layer cells of the chamber were carefully cleared. The perforated cells stained in the

outer layer were observed under the microscope. Cells in 5 randomly selected fields of view were calculated.

QRT-PCR

Total RNA was extracted from tissue samples using the TRIzol (Invitrogen, Carlsbad, CA, USA) method. The primers were synthesized by Aoke Biotechnology Co., LTD., (Chengdu, China). The following primers were used for qRT-PCR: GPAA1 (forward: 5'-TCTCAAGGCTCTGGAACTG-3', reverse: 5'-GCCCCACACCCTGTGATG-3'), c-myc (forward: 5'-TCTCAAGGCTCTGGAACTG-3', reverse: 5'-GCCCCACACCCTGTGATG-3'), β-actin (forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference gene. The reaction system was 25 μL, and the reaction conditions were as followed: denaturation at 95°C for 5 min, followed by 41 cycles for denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 70°C for 30 s at 82°C. Relative levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was conducted in triplicate.

Western Blot

Bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China) was used to detect protein concentrations, with all protein samples separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Thereafter, the separated protein was transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), followed by blockage in 5% skim milk with Tris-Buffered Saline and Tween-20 (TBST). GAPDH was used as an internal reference, and primary anti-GPAA1 and anti-c-myc (dilution 1:1000, CST, Danvers, MA, USA) were added to incubate overnight in a 4°C refrigerator. After washing three times with TBST (5 min/time), the secondary antibody (diluted 1:1000, CST, Danvers, MA, USA) was added to the membrane to incubate for 2 h at room temperature, and then the membrane was washed three times with TBST (10 Minutes/time). Enhanced chemiluminescence (ECL) solution was added to band exposure.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA). The *t*-test was used to compare the measurement data, and the categorical variables were analyzed by χ^2-test or Fisher's exact probability method. Survival analysis was performed using the Kaplan-Meier method. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and $p<0.05$ was considered to be statistically significant.

Results

GPAA1 Was Up-Regulated in Serum of Childhood ALL Patients and ALL Cell Lines

Serum level of GPAA1 in 42 childhood ALL patients and 42 healthy volunteers, as well as in the ALL cell lines was detected by qRT-PCR. The results indicated that compared with healthy volunteers, serum level of GPAA1 was higher in childhood ALL patients (Figure 1A). Meanwhile, compared with PBMC cells, GPAA1 was also highly expressed in ALL cell lines (Figure 1B), especially MOLT-4 and SUP-B15 cells, which were therefore selected for subsequent experiments.

GPAA1 Expression Was Correlated with Overall Survival in Childhood ALL Patients

According to the median serum level of GPAA1, childhood ALL patients were divided into high expression and low expression groups to further explore the relationship between GPAA1 level and the prognosis. Follow-up data were collected, and Kaplan-Meier survival curves revealed that high expression of GPAA1 was remarkably associated with poor prognosis of childhood ALL ($p<0.05$; Figure 1C). It is suggested that GPAA1 may be a new biological indicator for predicting the prognosis of childhood ALL.

Down-Regulation of GPAA1 Inhibited Cell Growth and Metastasis in ALL

To explore the influence of GPAA1 on the proliferation and metastasis of ALL cells, GPAA1 knockdown model was first constructed. After transfecting the GPAA1 lentiviral knockdown vector in MOLT-4 and SUP-B15 cell lines, qRT-PCR assay was performed to verify the interference efficiency (Figure 2A). In addition, CCK-8 results showed that the proliferative ability of ALL cells was remarkably reduced after silence of GPAA1 (Figure 2B). At the same time, the results of transwell invasion and migration assay

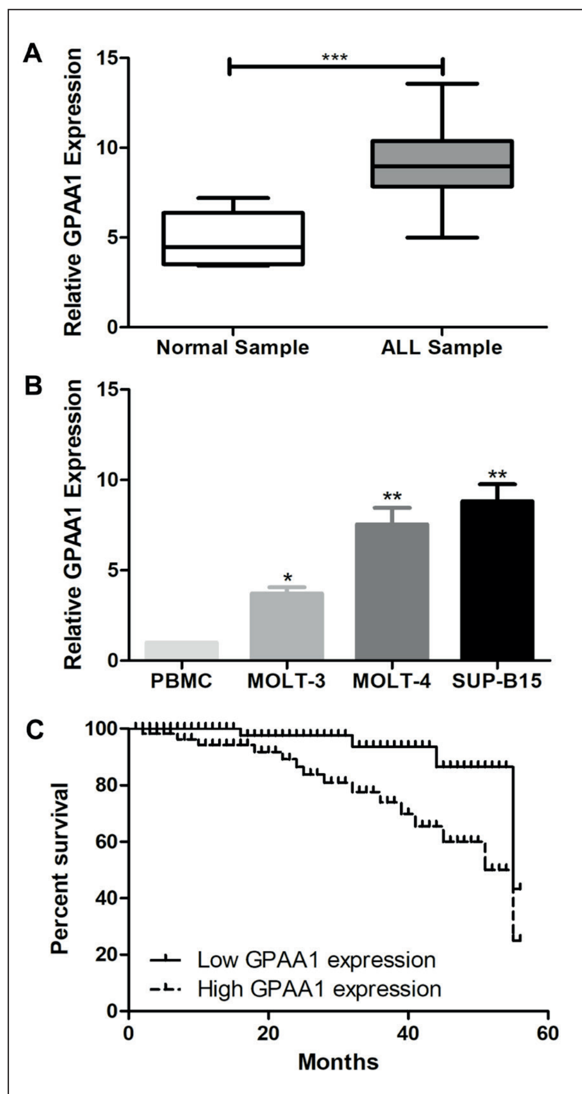

Figure 1. GPAA1 is highly expressed in serum of childhood acute lymphoblastic leukemia patients and cell lines. **A,** QRT-PCR was used to detect serum levels of GPAA1 in childhood acute lymphoblastic leukemia patients and healthy volunteers; **B,** QRT-PCR was used to detect the expression level of GPAA1 in pediatric acute lymphoblastic leukemia cell lines; **C,** Kaplan-Meier survival curves of childhood acute lymphoblastic leukemia patients based on GPAA1 expression were plotted. Data are expressed as mean ± SD, $*p<0.05$, $**p<0.01$, $***p<0.001$.

indicated that knockdown of GPAA1 inhibited invasive and migratory abilities in ALL cells (Figure 2C).

C-myc Was the Direct Target of GPAA1

To further validate the targeting of c-myc to GPAA1, GPAA1 sequences were cloned into the Luciferase reporter plasmid pmirGLO to construct pmirGLO-GPAA1-WT and pmirGLO-GPAA1-mut. Overexpression of c-myc remarkably attenuated the Luciferase activity of the wild-type GPAA1 vector ($p<0.05$) without attenuating that of the mutant vector ($p>0.05$) or the empty vector ($p>0.05$). It is demonstrated that GPAA1 could be targeted by c-myc (Figure 3A and 3B). In addition, Western blot and qRT-PCR results revealed that knockdown of GPAA1 simultaneously down-regulated protein and mRNA levels of c-myc (Figure 3C and 3D).

C-myc Was Up-Regulated in Serum of Childhood ALL Patients and ALL Cell Lines

QRT-PCR data revealed that compared with healthy volunteers, c-myc expression in serum of childhood ALL patients was higher (Figure 4A). Meanwhile, c-myc was also highly expressed in ALL cell lines when compared with PBMC cells (Figure 4B). In addition, the Kaplan-Meier survival curve revealed that high expression of c-myc was remarkably associated with poor prognosis in childhood ALL (Figure 4C). Expression levels of GPAA1 and c-myc were found to be positively correlated in serum samples of childhood ALL (Figure 4D).

GPAA1 Modulated C-myc Expression in ALL Cells

The c-myc knockdown vector was constructed and Western blot and qRT-PCR were performed to confirm the transfection efficiency (Figure 4E and 4F). To further explore the interaction between GPAA1 and c-myc in ALL cells, GPAA1 and c-myc were co-silenced, and transfection efficiency was verified by qRT-PCR (Figure 5A). After that, CCK-8 and transwell invasion experiments demonstrated that downregulation of c-myc could partially restore the decreased proliferative and invasive abilities of ALL cells induced by knockdown of GPAA1 (Figure 5B and 5C).

Discussion

Leukemia, commonly known as "blood cancer", is a highly malignant proliferative disease of hematopoietic system. It is characterized by abnormal proliferation of leukemia cells in bone marrow or other hematopoietic tissues or organs, resulting in quantity and quality changes of surrounding blood cells[1,2]. Leukemia is one of the major malignant tumors that causes death of

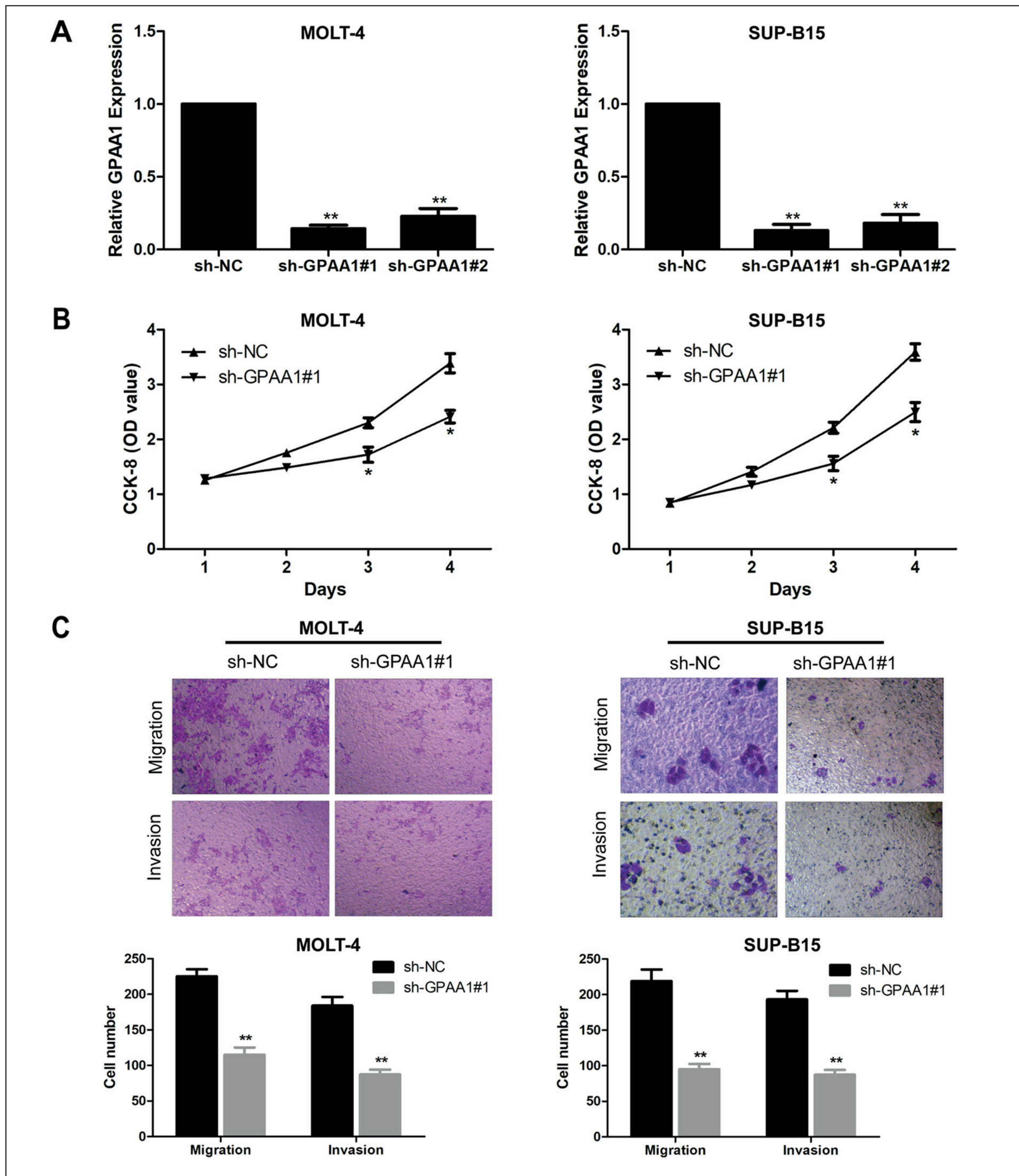

Figure 2. Knockdown of GPAA1 inhibits proliferation and metastasis of childhood acute lymphoblastic leukemia cells. **A,** QRT-PCR verified the interference efficiency of GPAA1 after transfection of GPAA1 knockdown vector in acute lymphoblastic leukemia MOLT-4 and SUP-B15 cell lines; **B,** CCK-8 assay was used to detect the effect of GPAA1 knockdown on the proliferation of MOLT-4 and SUP-B15 cell lines; **C,** Transwell assay was used to detect the effect of GPAA1 knockdown on the invasion and migration abilities of MOLT-4 and SUP-B15 cells (magnification: 40×). Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$.

children worldwide. More than 90% of them are acute leukemia, while the proportion of chronic leukemia is only 3-5%[2-4]. At present, the known factors causing leukemia include ionizing radiation, viral infection, long-term exposure to harmful chemicals, and patients' own abnormalities or

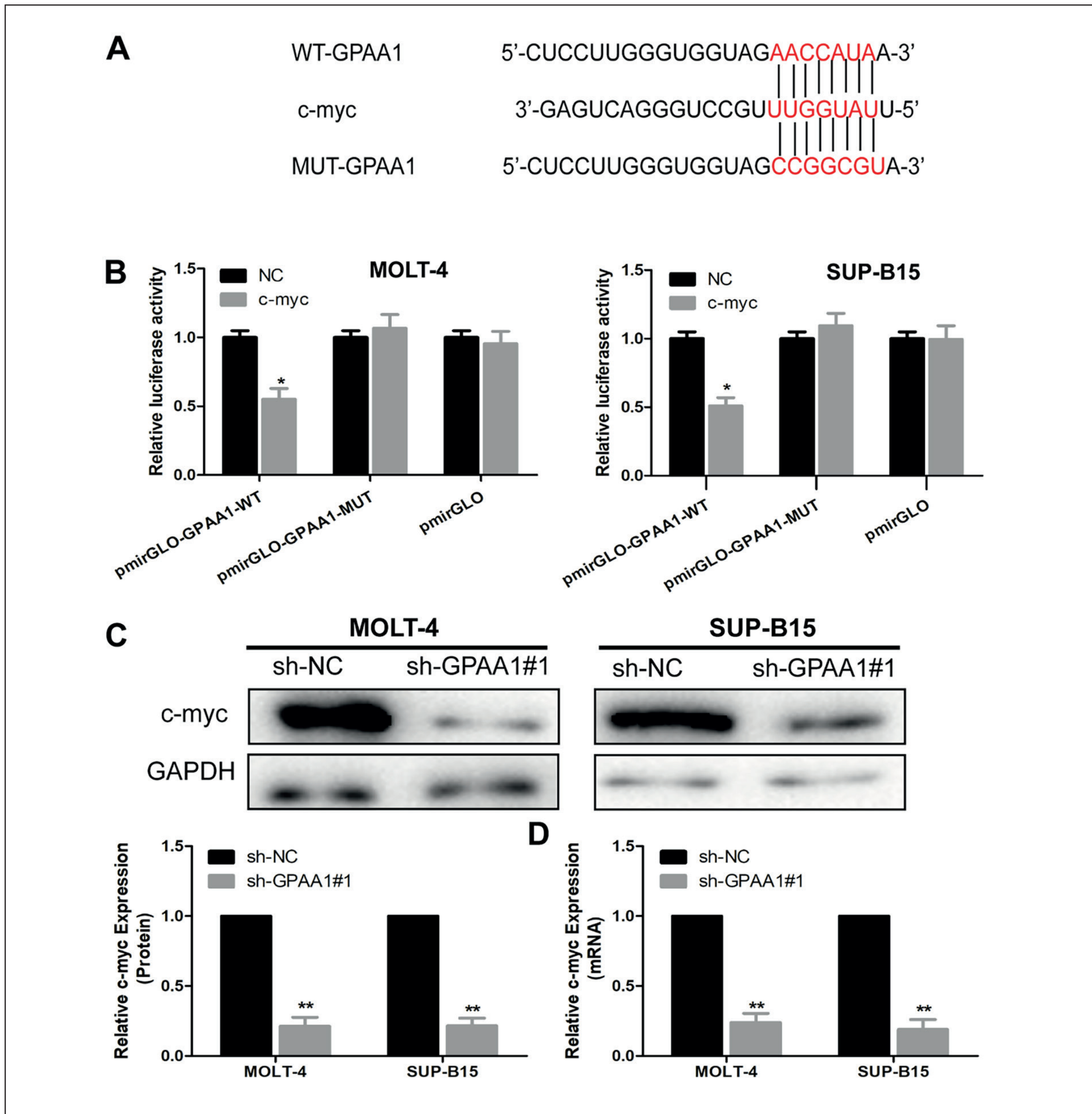

Figure 3. Direct targeting of c-myc by GPAA1. **A,** The predicted binding site of GPAA1 and c-myc; **B,** Luciferase reporter gene assay validated the direct targeting of GPAA1 and c-myc; **C,** Western Blot verified the difference in protein expression of c-myc after transfection of the GPAA1 knockdown vector in the MOLT-4 and SUP-B15 cell lines; **D,** QRT-PCR verified the difference in mRNA expression of c-myc after transfection of the GPAA1 knockdown vector in the MOLT-4 and SUP-B15 cell lines. Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$.

abnormal immune functions[5-7]. ALL is the most common type of leukemia, and up to 75% of children suffer from this type of leukemia[8-10]. Along with the advancement of molecular biology technology, the clinical cure rate of childhood ALL has been remarkably improved. However, there is still a part of patients suffering from a poor prognosis. It is of great importance to seek for reliable prognostic molecular markers in individualized treatment of different types of childhood ALL[11-13].

Glycosylated phosphatidylinositol anchoring protein (GPI-AP) is a kind of protein that anchors the eukaryotic membrane surface through its fusiform terminal structure, which plays a pivotal role in immune recognition, complement regulation, and transmembrane signal transduction[14,15].

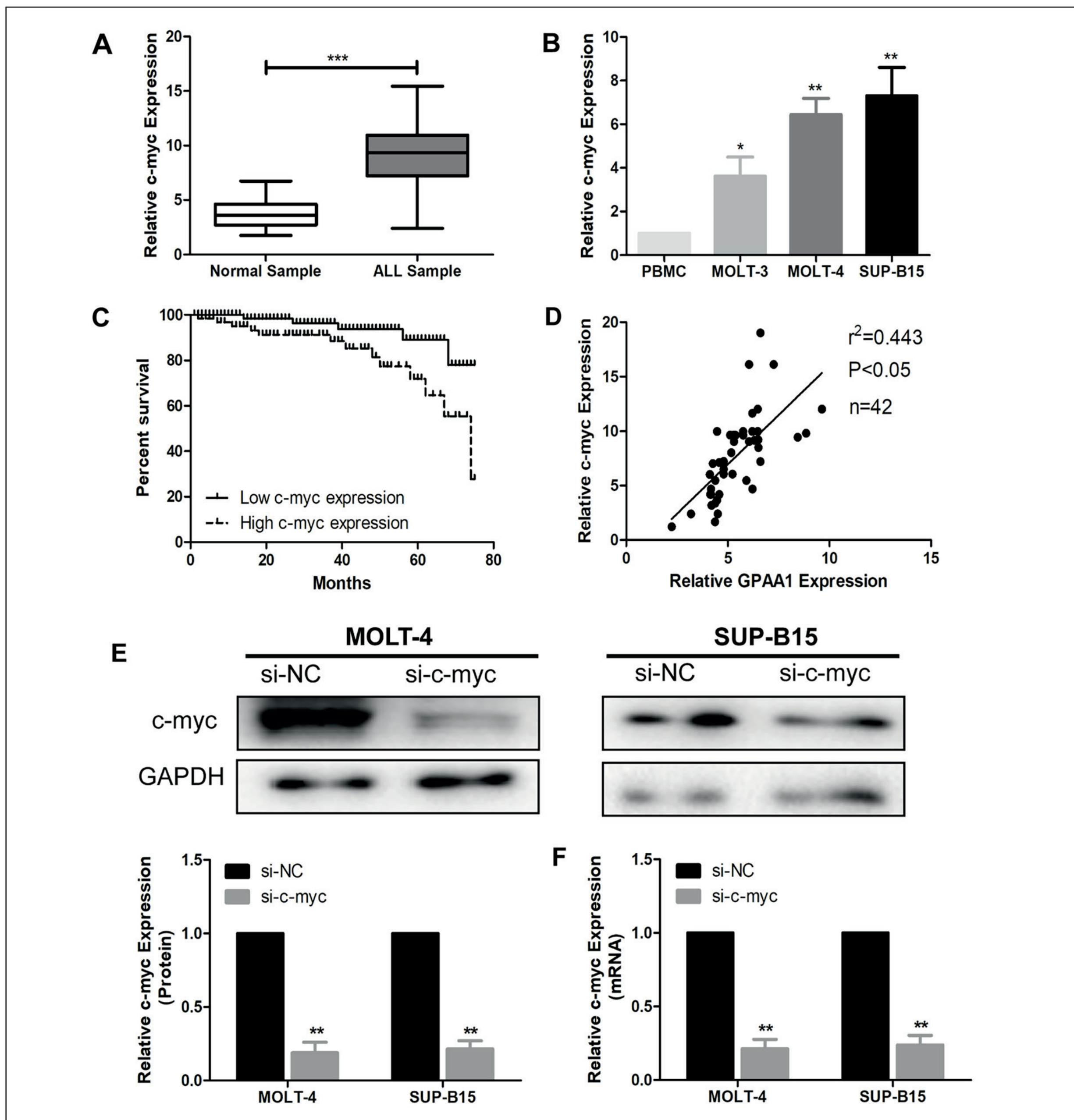

Figure 4. c-myc is highly expressed in serum of childhood ALL patients and ALL cell lines. **A,** qRT-PCR was used to detect the difference in serum level of c-myc in childhood acute lymphoblastic leukemia patients and healthy volunteers; **B,** QRT-PCR was used to detect the expression level of c-myc in childhood acute lymphoblastic leukemia cell lines; **C,** Kaplan-Meier survival curves were plotted in childhood acute lymphoblastic leukemia patients based on c-myc expression; **D,** There was a significant positive correlation between the expression of GPAA1 and c-myc in serum of childhood acute lymphoblastic leukemia patients; **E,** Western blot verified the c-myc protein level after transfection of the c-myc knockdown vector in the MOLT-4 and SUP-B15 cell lines; **F,** qRT-PCR verified the c-myc mRNA level after transfection of the c-myc knockdown vector in the MOLT-4 and SUP-B15 cell lines. Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The Anchor Attachment protein is an important component of the glycosylphosphatidylinositol transamidase complex (GPIT) by forming a GPAA1 complex with phospholipid creatinine[15,16]. In this study, serum level of GPAA1 was found to be remarkably up-regulated in 42 childhood ALL patients compared with healthy volunteers, suggesting that GPAA1 may exert a role in promoting the development of childhood ALL to further explore the effects of GPAA1 on the cytology

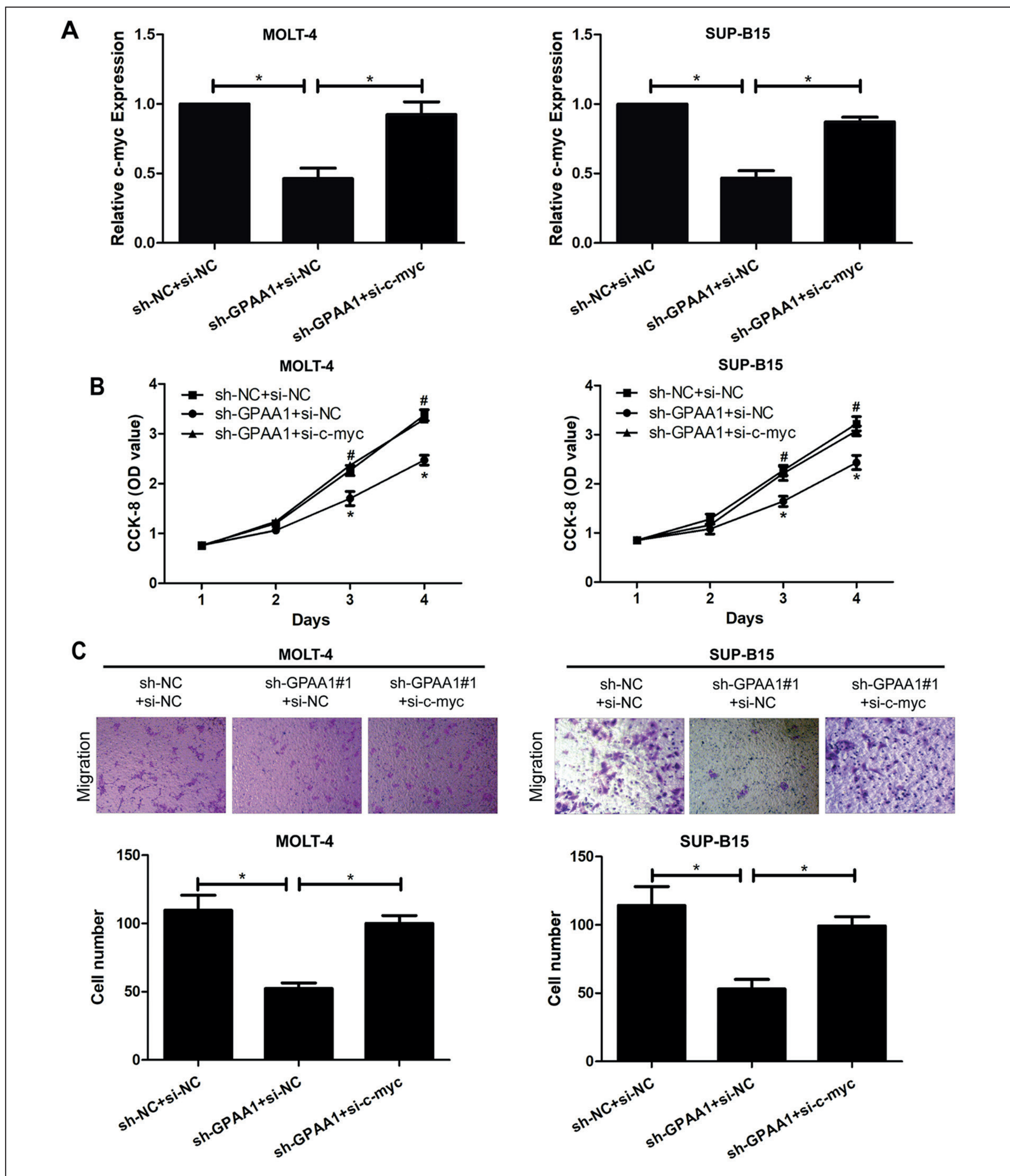

Figure 5. GPAA1 regulates the expression of c-myc in serum of childhood ALL patients and ALL cell lines. **A,** QRT-PCR was used to detect c-myc expression level in MOLT-4 and SUP-B15 cell lines after co-transfection of GPAA1 and c-myc; **B,** CCK-8 assay was used to detect the proliferation of MOLT-4 and SUP-B15 cell lines after co-transfection of GPAA1 and c-myc; **C,** Transwell invasion assay was used to detect the invasion and migration abilities of MOLT-4 and SUP-B15 cells after co-transfection of GPAA1 and c-myc (magnification: 40×). Data are expressed as mean ± SD, $*^{\#}p<0.05$.

of childhood ALL, GPAA1 knockdown model was constructed by lentivirus transfection. Cell function experiments suggested that GPAA1 can promote the proliferation and metastasis of ALL cells. However, the specific molecular mechanism still remains elusive.

Through bioinformatics analysis, it was found that GPAA1 and c-myc were on the same chromosome, and there may be some mutual regulatory relationship between them. C-myc gene is activated mainly through amplification and chromosomal translocation rearrangement, which has the ability to transform cells and the characteristic of combining with chromosome DNA. It is capable of regulating cell growth, differentiation, and malignant transformation[17,18]. Our findings showed that c-myc was highly expressed in serum of childhood ALL patients, and it could promote the malignant phenotypes of ALL cells. In addition, c-myc level was positively regulated by GPAA1 in ALL cells, suggesting that GPAA1 may promote the metastasis of ALL in children by activating c-myc. The novelty of this study is that we demonstrated for the first time that GPAA1 and c-myc expressions were upregulated and associated with a poor prognosis of childhood ALL patients. Moreover, C-myc was a direct target of GPAA1 and it was involved in GPAA1-modulated ALL cell phenotypes.

Conclusions

In summary, serum level of GPAA1 was upregulated in childhood ALL patients and closely relevant to the poor prognosis. In addition, GPAA1 may promote the malignant progression of childhood ALL by regulating c-myc.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Meng J, Liu GJ, Song JY, Chen L, Wang AH, Gao XX, Wang ZJ. Preliminary results indicate resveratrol affects proliferation and apoptosis of leukemia cells by regulating PTEN/PI3K/AKT pathway. Eur Rev Med Pharmacol Sci 2019; 23: 4285-4292.
2) Juliusson G, Hough R. Leukemia. Prog Tumor Res 2016; 43: 87-100.
3) Liersch R, Muller-Tidow C, Berdel WE, Krug U. Prognostic factors for acute myeloid leukaemia in adults--biological significance and clinical use. Br J Haematol 2014; 165: 17-38.
4) Haydu JE, Ferrando AA. Early T-cell precursor acute lymphoblastic leukaemia. Curr Opin Hematol 2013; 20: 369-373.
5) van der Linden MH, Creemers S, Pieters R. Diagnosis and management of neonatal leukaemia. Semin Fetal Neonatal Med 2012; 17: 192-195.
6) Berg S, Nand S. Neurological complications of the leukemias across the ages. Curr Neurol Neurosci Rep 2017; 17: 13.
7) Giles FJ, Krawczyk J, O'Dwyer M, Swords R, Freeman C. The role of inflammation in leukaemia. Adv Exp Med Biol 2014; 816: 335-360.
8) Yang D, Zhang X, Zhang X, Xu Y. The progress and current status of immunotherapy in acute myeloid leukemia. Ann Hematol 2017; 96: 1965-1982.
9) Jin MW, Xu SM, An Q, Wang P. A review of risk factors for childhood leukemia. Eur Rev Med Pharmacol Sci 2016; 20: 3760-3764.
10) Yu MG, Zheng HY. Acute myeloid leukemia: advancements in diagnosis and treatment. Chin Med J (Engl) 2017; 130: 211-218.
11) Xu H, Shu Y. Insights of acute lymphoblastic leukemia with development of genomic investigation. Methods Mol Biol 2018; 1754: 387-413.
12) Hunger SP, Mullighan CG. Acute lymphoblastic leukemia in children. N Engl J Med 2015; 373: 1541-1552.
13) Larson RA. Managing CNS disease in adults with acute lymphoblastic leukemia. Leuk Lymphoma 2018; 59: 3-13.
14) Ho JC, Cheung ST, Patil M, Chen X, Fan ST. Increased expression of glycosyl-phosphatidylinositol anchor attachment protein 1 (GPAA1) is associated with gene amplification in hepatocellular carcinoma. Int J Cancer 2006; 119: 1330-1337.
15) Wang Q, Zhou Y, Rychahou P, Harris JW, Zaytseva YY, Liu J, Wang C, Weiss HL, Liu C, Lee EY, Evers BM. Deptor is a novel target of Wnt/beta-catenin/c-Myc and contributes to colorectal cancer cell growth. Cancer Res 2018; 78: 3163-3175.
16) Posternak V, Ung MH, Cheng C, Cole MD. MYC Mediates mRNA Cap methylation of canonical Wnt/beta-catenin signaling transcripts by recruiting CDK7 and RNA methyltransferase. Mol Cancer Res 2017; 15: 213-224.
17) Sadeghi S, Hojati Z, Tabatabaeian H. Cooverexpression of EpCAM and c-myc genes in malignant breast tumours. J Genet 2017; 96: 109-118.
18) Niknezhad Z, Hassani L, Norouzi D. Investigating actinomycin D binding to G-quadruplex, i-motif and double-stranded DNA in 27-nt segment of c-MYC gene promoter. Mater Sci Eng C Mater Biol Appl 2016; 58: 1188-1193.