

GATA4 is highly expressed in childhood acute lymphoblastic leukemia, promotes cell proliferation and inhibits apoptosis by activating *BCL2* and *MDM2*

QIUGUO HAN¹, XIN XU¹, JING LI¹, JINGGANG WANG¹, LI BAI¹,
AIHONG WANG¹, WEI WANG¹ and BO ZHANG²

¹Department of Pediatrics, Daqing Oilfield General Hospital, Daqing, Heilongjiang 163000;

²Department of Pediatric Neurology, The First Hospital of Jilin University, Changchun, Jilin 130000, P.R. China

Received October 30, 2016; Accepted June 16, 2017

DOI: 10.3892/mmr.2017.7369

Abstract. Members of the GATA-binding factor protein family, including GATA1, GATA2 and GATA3, serve an inhibiting role in leukemia. The present study demonstrated that GATA4 was upregulated in children with acute lymphoblastic leukemia (ALL). Results from a number of functional experiments, including cell proliferation analysis, cell cycle analysis, cell apoptosis assay and Transwell migration and invasion analyses, have suggested that high expression of GATA4 may facilitate proliferation and metastasis, and suppress apoptosis in ALL cells. Chromatin immunoprecipitation assay and luciferase reporter assay revealed that GATA4 was a transcription factor that activated mouse double minute 2 homolog (*MDM2*) and B cell lymphoma 2 (*BCL2*) expression in ALL cells. *BCL2* is a key anti-apoptosis protein that was demonstrated to suppress cell apoptosis. In addition, GATA4 was revealed to regulate p53 through the transcriptional activation of *MDM2*, subsequently influencing cell cycle and apoptosis. Results from the present study suggested that GATA4 may be a key marker in ALL diagnosis and a potential target of molecular therapy.

Introduction

Acute lymphoblastic leukemia (ALL) predominantly occurs in children between the ages of 2 and 5 years, but may also occur in adults (1-4). Over the past decade, under different treatments, the survival rate of childhood ALL has approached ~90% (1,5); however, the treatment of adult ALL still needs improvement (2,6). The activation or inhibition of various genes, including

lysine methyltransferase 2A, IKARS family zinc-finger 1, AT-rich interaction domain 5B, CCAAT/enhancer-binding protein and cyclin dependent kinase inhibitor 2A, may lead to the development of ALL (7,8).

The GATA-binding protein family of zinc-finger transcription factors comprises six members, including GATA1, GATA2, GATA3, GATA4, GATA5 and GATA6 (9), which bind to GATA sequences in the DNA with the consensus 5'-WGATAR-3', where W is either T or A nucleotides and R is either G or A (10,11). GATA4 was originally revealed to serve an important role in cardiac development (12,13); subsequent studies have reported that GATA4 mediated apoptosis (14,15) and regulated the mRNA expression of B cell lymphoma 2 (*BCL2*) (16) and *BCL-X_L* (17), and serves as a survival factor in carcinoma. A number of GATA family proteins have been revealed to serve inhibitory roles in leukemia, including GATA1, GATA2 and GATA3 (18-21). However, the expression and function of GATA4 in ALL remains unknown.

The present study demonstrated that GATA4 expression was upregulated in ALL and binds to the promoter region of *BCL2* and mouse double minute 2 homolog (*Mdm2*), activating their transcription. *MDM2* protein expression was revealed to negatively regulate the expression of p53, thereby inhibiting apoptosis and promoting cell cycle. In conclusion, the present results suggested that GATA4 may be a marker for ALL diagnosis and a target of molecular therapy.

Materials and methods

Cell culture and transfection. MOLT-4 adult ALL, Jurkat childhood ALL cell lines and H9 normal human T lymphocyte cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) that contained 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. Then ~4x10⁵ cells were transfected with pCMV-Tag 2B empty vector (Youbio, Hunan, China) FLAG-GATA4 (pCMV-Tag 2B-GATA4) or small interfering RNA (siRNA) against scrambled (SCR), GATA4 siRNA (siGATA4) or siGATA4 and sip21 simultaneously

Correspondence to: Dr Bo Zhang, Department of Pediatric Neurology, The First Hospital of Jilin University, 71 Xinmin Street, Changchun, Jilin 130000, P.R. China
E-mail: bozhanghh@sina.com

Key words: GATA-binding factor 4, acute lymphoblastic leukemia, apoptosis, B cell lymphoma 2, mouse double minute 2 homolog

using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following 48 h transfection, cells were collected by 800 x g centrifugation at 4°C for 5 min and used for the further experiments. siRNA sequences were as follows: siGATA4#1, AAC CGGCCGCTCATCAAGCCT; siGATA4#2, AATCTCGTA GATATGTTTGAC; sip21, AACTTCGACTTTGTACC GAG.

Blood samples. A total of 42 blood samples from patients with ALL and 42 samples from healthy controls were collected from patients at the First Hospital of Jilin University (Changchun, China) between 2010 and 2016. All patients had been informed about the nature of the experiments and provided written informed consent. The study was approved by the Ethics Committee of the First Hospital of Jilin University.

Western blot analysis. Cells (~4x10⁶) were lysed with radio-immunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a protease inhibitor cocktail (Beijing Transgen Biotech Co., Ltd., Beijing, China) at 4°C for 30 min. Protein concentrations were measured using the Bradford Reagent (Beyotime Institute of Biotechnology). A total of 40 µg protein was loaded in each well and resolved by 8% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes, which were subsequently blocked with 5% non-fat milk at 37°C for 1 h. The membranes were incubated with the following primary antibodies at 4°C overnight: anti-GATA4 (ab124265; 1:600) anti GADD45 (ab105060; 1:3,000), anti-p21 (ab109520; 1:2,000), anti-14-3-3σ (ab14123; 1:2,000) and anti-β-actin (1:1,000) from Abcam (Cambridge, MA, USA); and anti-FLAG (F1804 1:5,000), anti-MDM2 (SAB4300601; 1:2,000), anti-BCL2 (SAB4300340; 1:500) and anti-p53 (P9249; 1:1,000) from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Membranes were washed three times with TBS + 1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary goat anti-mouse immunoglobulin (Ig)G (ab6728; 1:5,000; Abcam) or goat anti-rabbit IgG (ab6721; 1:5,000; Abcam) at room temperature for 1 h. Immunoreactive protein bands were visualized using the Enhanced Chemiluminescence kit (Beyotime Institute of Biotechnology). Each experiment was performed at least 3 times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 3x10⁶ cells using the RNeasy Pure kit (Qiagen Biotech Co., Ltd., Beijing, China), following the manufacturer's protocol. Total RNA (2 µg) was reverse transcribed into cDNA using the FastKing RT kit (Tiangen Biotech Co., Ltd.), according to the manufacturer's protocol. qPCR was performed with the RealMaster mix SYBR-Green (Tiangen Biotech Co., Ltd.) and an ABI PRISM 7700 Real Time Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RT-qPCR conditions were as follows: 5 min at 95°C, denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 60 sec, performed for 34 cycles. The primers were as follows: GATA4, 5'-CCCAATCTCGTAGATATGTTTGAC-3' and 5'-CCGTTC ATCTTGTTGGTAGAG-3'; BCL2, 5'-GCTATAACTGGAGAG TGCTG-3' and 5'-ACTTGATTCTGGTGTTCCTCC-3'; MDM2,

5'-TCAAACCTCCTGACCTCAAGTG-3' and 5'-TTACCATCA TAAGCCTACAGACC-3'; p53, 5'-GACCTATGGAACTA CTTCCTG-3' and 5'-CATTCTGGGAGCTTCATCTG-3'; p21, GCATGACAGATTTCTACCAC-3' and 5'-GACTAAGGC AGAAGATGTAGAG; GADD45, 5'-CGAGAACGACATCAA CATCC-3' and 5'-ATGAATGTGGATTTCGTCACC-3'; 14-3-3σ, 5'-AGACAACCTAACACTTTGGAC-3' and 5'-AGGAAA GAAGGATGACACCC-3'; GAPDH, 5'-CATTTCTCTGGTA TGACAACGA-3' and 5'-TACATGGCAACTGTGAGGAG-3'. Relative mRNA expression levels of indicated genes were calculated by the 2^{-ΔΔC_q} method (22). GAPDH was used as internal control and for normalization. Each experiment was performed at least 3 times.

Cell Counting Kit-8 (CCK-8) proliferation assay. Following 48 h transfection, cells (4x10³ cells/well in 100 µl RPMI-1640 medium) were cultured in 96-well plates at 37°C. CCK-8 solution (10 µl; Beyotime Institute of Biotechnology) was added to each well and incubated at 37°C for 3 h. A microplate reader was used to measure the absorbance at 450 nm; the absorbance indicated cells proliferation. Each experiment was performed at least 3 times.

Apoptosis assay. Adriamycin was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in phosphate-buffered saline (PBS). Following 48 h transfection, Adriamycin (5 µg/ml) was added to the culture medium and incubated for 24 h at 37°C to induce apoptosis. Subsequently, apoptotic rates were examined with the Annexin V/fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Abcam), according to the manufacturer's protocol. Briefly, approximately 4x10⁵ cells were collected by 800 x g centrifugation at 4°C for 5 min, washed with PBS and stained with Annexin V and propidium iodide. Attune™ NxT Software (version 1.0; Thermo Fisher Scientific, Inc.) was used to analyze apoptotic cells using a flow cytometer. Each experiment was performed at least 3 times.

Transwell migration and invasion assay. Costar Transwell chambers (Corning Inc., Corning, NY, USA) were used to detect the cell migration and invasion ability. Uncoated Transwell inserts were used to assess cell migration ability, whereas Transwell inserts precoated with Matrigel (BD Biosciences, San Jose, CA, USA) were used to determine cell invasion ability. Jurkat cells were transfected for 48 h, resuspended in RPMI-1640 without FBS, and plated (2x10⁴ cells) into the upper chamber; the lower chamber was filled with 500 µl RPMI-1640 supplemented with 10% FBS. Cells were incubated at 37°C with 5% CO₂ in a humidified air atmosphere for 24 h. Following incubation, cells in the upper chamber were removed with cotton swabs, and cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Stained cells were counted (6 fields were counted for each membrane) under a light microscope. Each experiment was performed at least 3 times.

Chromatin immunoprecipitation (ChIP). ChIP analysis was performed with the Chromatin Immunoprecipitation Assay kit (Beyotime Institute of Biotechnology), according to manufacturer's protocol. Briefly, cells were grown to 85-95%

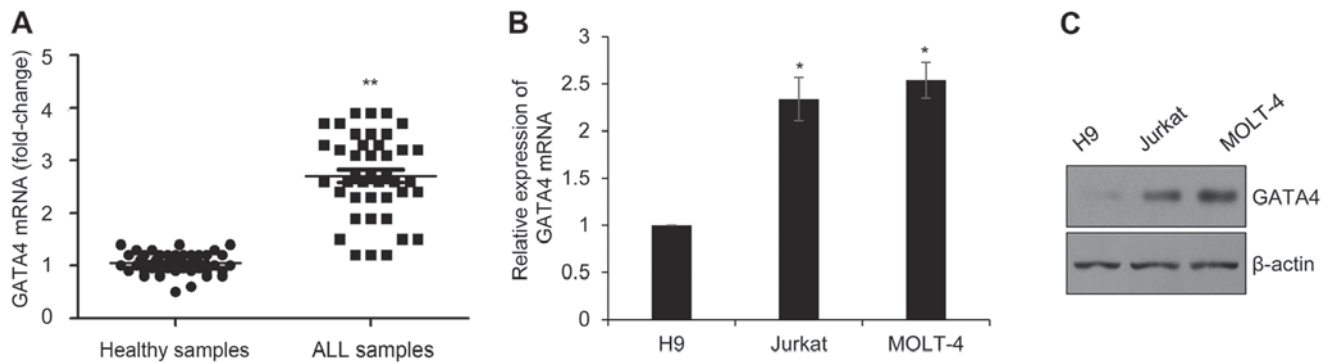


Figure 1. GATA4 is highly expressed in childhood ALL. (A) RT-qPCR was performed to measure the mRNA expression level of GATA4 in 42 childhood ALL blood samples and 42 blood samples from healthy patients. Fold-change was determined by evaluating the relative mRNA level of GATA4 and results were analyzed by Student's t-test. ** $P < 0.01$ the expression of GATA4 in ALL samples vs. health samples. (B) RT-qPCR analysis of GATA4 mRNA expression levels in H9 normal human T lymphocyte (control), Jurkat childhood ALL and MOLT-4 adult ALL cells. * $P < 0.05$ the expression of GATA4 in Jurkat and MOLT-4 vs. H9. (C) GATA4 protein expression levels in H9, Jurkat and MOLT-4 cells. ALL, acute lymphoblastic leukemia; GATA4, GATA-binding factor 4; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

confluence, washed cells three times with PBS and chemically cross-linked with 1% formaldehyde for 20–30 min at 37°C. Subsequently, cells were lysed with lysis buffer at 4°C for 30 min and sonicated 3 cycles at 4°C, each cycle of 15 times. Following lysis, 3 μ g GATA4 antibody was added to the lysis solution and incubated at 4°C overnight. Anti-rabbit IgG was used as control. Protein A beads were used to isolate antibody-interacted DNA fragments. The binding chromatin was purified by Qiagen PCR Purification kit (Qiagen China Co., Ltd., Shanghai, China). Chromatin fragments was analyzed by PCR and detected by gel electrophoresis. The PCR assay was performed using 2XEasyTaq PCR SuperMix (Transgene, Beijing, China) according to manuscript's protocol. The PCR conditions were as follows: 5 min at 95°C, denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, performed for 32 cycles. The primers were as follows: BCL2, 5'-GGACTTCTGCGAATACCG-3' and 5'-GTC CCTGAGGGCTTCATT-3'; MDM2, 5'-CGGGTTCACAGG ATTGTC-3' and 5'-GATGCTGGTTACCGTTGG-3'. Each experiment was performed at least 3 times.

Wound-healing assay. Cells (3×10^5) were transfected with vector, GATA4 or SCR, siGATA4, when the density of cells was almost 90–100%, cells were scratched with a 20 μ l pipette and photographed at 0 h or 48 h under a light microscope. The distance of cells migration indicated the migration ability of cells. Each experiment was performed at least 3 times.

Dual-luciferase reporter assay. The promoter sequence of MDM2 or BCL2 was cloned into a pGL3 firefly luciferase reporter plasmid vector. Jurkat cells (3×10^6) were co-transfected with 0.2, 0.5, 1 and 2 μ g FLAG-GATA4, Renilla and pGL3-MDM2-promoter or pGL3-BCL2-promoter using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The Renilla luciferase plasmid vector was used as an internal control. Following 24 h transfection at 37°C, firefly and Renilla luciferase activities were measured through the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). Firefly luciferase activity was normalized to Renilla luciferase activity. Each experiment was performed at least 3 times.

Statistical analysis. All data were expressed as the mean \pm standard deviation; and each experiment was performed at least three times. The data were analyzed by SSPS 19.0 (IBM Corp., Armonk, NY, USA). Comparisons between groups were made by one-way analysis of variance followed by Tukey's test, and comparisons between two groups were made with Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GATA4 is highly expressed in childhood ALL. To determine the expression of GATA4 in childhood ALL, blood samples from 42 patients with ALL and 42 healthy controls were collected. RT-qPCR was used to assess GATA4 mRNA expression (Fig. 1A). Compared with healthy control group, the expression of GATA4 mRNA was significantly higher in the childhood ALL samples. In addition, GATA4 mRNA and protein expression was examined in Jurkat and MOLT-4 ALL cell lines, using the H9 normal human T lymphocyte cell lines as a control. The results indicated that the mRNA and protein levels of GATA4 expression were upregulated in ALL cell lines compared with H9 (Fig. 1B and C, respectively).

GATA4 promotes cell proliferation by inhibiting apoptosis and facilitating G₁/S phase transition. To further analyze the function of GATA4 in ALL, GATA4 was either overexpressed or silenced in Jurkat and MOLT-4 cell lines, and subsequent mRNA and protein expression levels were determined by RT-qPCR and western blotting, respectively. The results indicated that the expression of GATA4 was upregulated in FLAG-GATA4-transfected cells, but downregulated in siGATA4-transfected cells (Fig. 2A and B); transfection with siGATA4#2 appeared to be more efficient compared with siGATA4#1, therefore siGATA4#2 was used for the further experiments.

To elucidate the function of GATA4 on the progression of ALL, CCK-8 cell viability analysis was conducted. As shown in Fig. 2C, the proliferation of Jurkat cells that ectopically expressed GATA4 was significantly increased compared with the Vector control group. Conversely, siGATA4 knockdown of

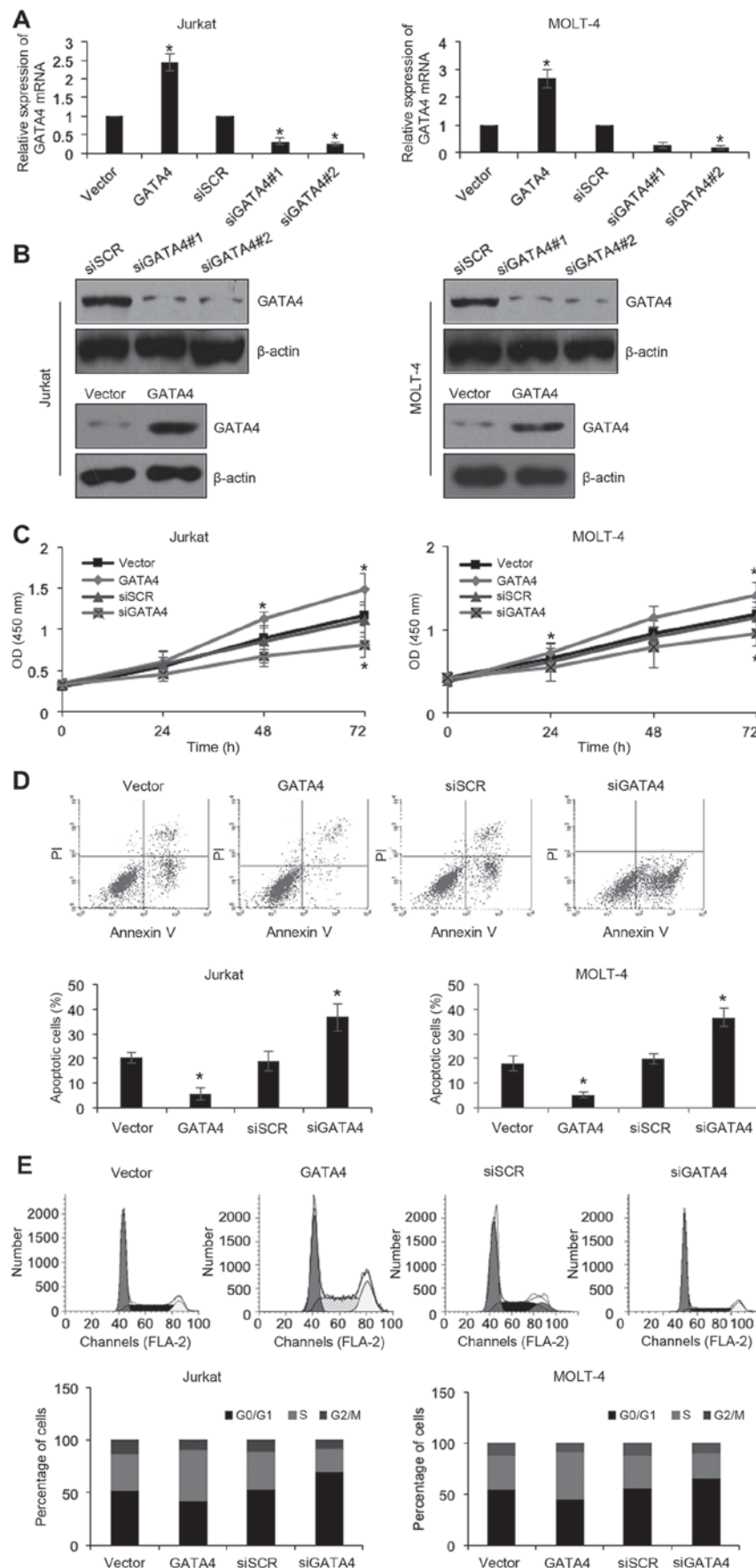


Figure 2. GATA4 promotes cell proliferation by inhibiting apoptosis and facilitating G_1/S phase transition. Jurkat and MOLT-4 ALL cells were transfected with Vector control, FLAG-GATA4, SCR, siGATA4#1 or siGATA4#2 for 48 h. (A) GATA4 mRNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction. * $P < 0.05$; GATA4 vs. vector, siGATA4 vs. SCR. (B) GATA4 protein expression was examined by western blotting. (C) Transfected cells proliferation was examined by Cell Counting Kit-8 assay. * $P < 0.05$ GATA4 vs. vector, siGATA4 vs. SCR. (D) Apoptosis rates and (E) cell cycle analysis were detected by flow cytometry. * $P < 0.05$ GATA4 vs. vector, siGATA4 vs. SCR. GATA4, GATA-binding factor 4; OD, optical density; SCR, scramble; si, small interfering RNA.

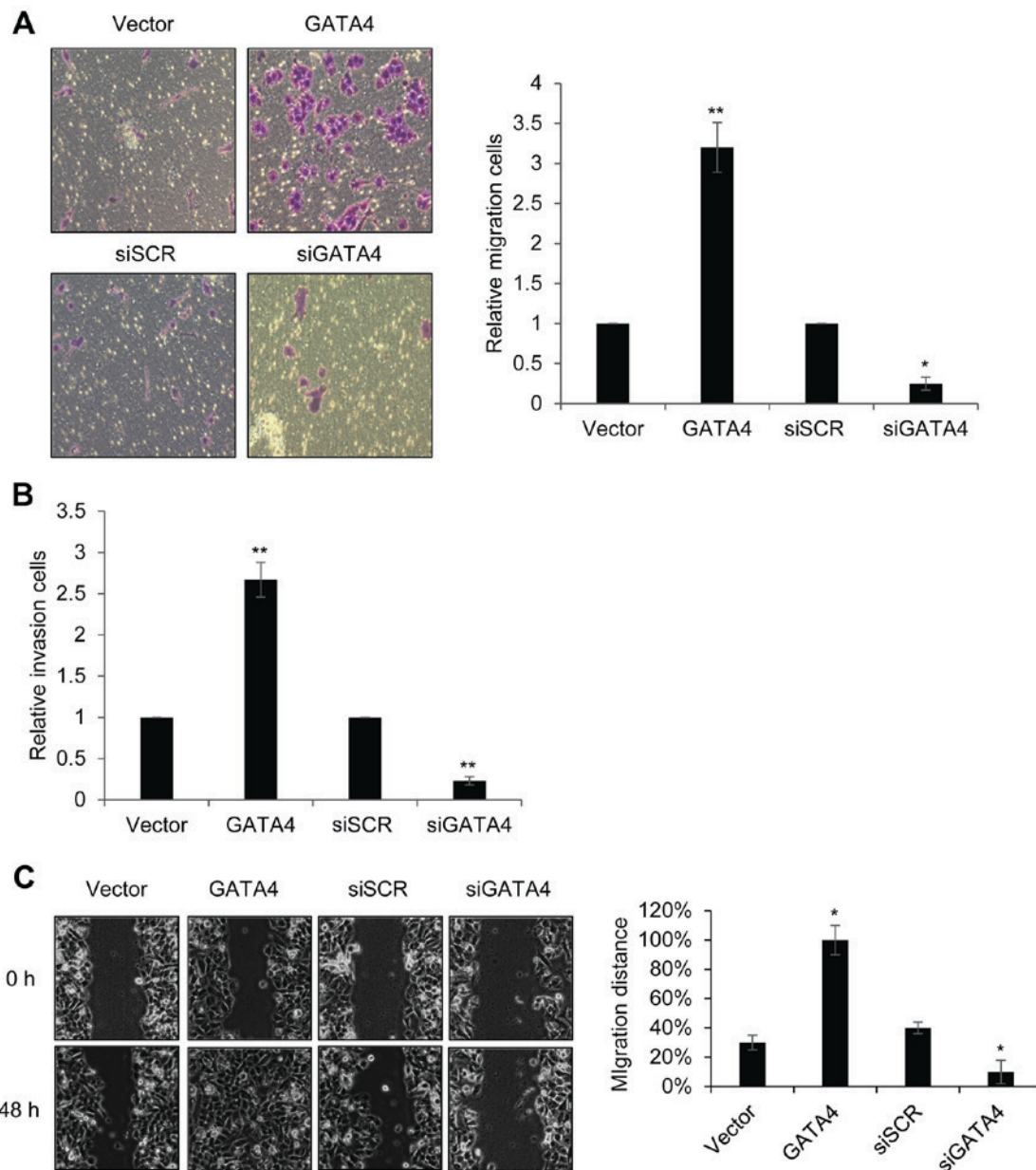


Figure 3. GATA4 facilitates migration and invasion of acute lymphoblastic leukemia cells. (A) Transwell chamber was used to assess cell migration. Following 48 h transfection, cells (2×10^4) were plated into the upper chamber and incubated for 24 h; cells in the lower chamber were fixed and stained with 0.5% crystal violet. Fold-change $P < 0.05$ siGATA4 vs. SCR; $**P < 0.01$ GATA4 vs. vector. (B) Matrigel-coated Transwell inserts were used to determine cell invasion. Following 48 h transfection, cells (2×10^4) were plated into the upper chamber and incubated cells for 24 h; cells in the lower chamber with fixed and stained cells with 0.5% crystal violet. Fold-change $**P < 0.01$ GATA4 vs. vector, siGATA4 vs. SCR. (C) Following 48 h transfection, a scratch was made in the cell monolayer with a 20 μ l pipette tip. $*P < 0.05$ siGATA4 vs. SCR; GATA4 vs. vector. Images of migrating cells were captured at 0 h and 48 h, and the cell migration distance was measured. GATA4, GATA-binding factor 4.

GATA4 expression significantly suppressed cell proliferation compared with expression in the SCR control (Fig. 2C); similar results were observed in MOLT-4 cells (Fig. 2C). To determine the mechanism of GATA4 in proliferation, the role of GATA4 in apoptosis was examined using flow cytometry. Compared with the control groups, the rate of apoptosis was decreased in the cells that overexpressed GATA4; however, the apoptotic rate was increased in the cells transfected with siGATA4 (Fig. 2D). In addition, cell cycle analysis revealed that overexpression of GATA4 promoted G_1/S phase transition; however, inhibition of GATA4 arrested cell cycle at G_0/G_1 phase (Fig. 2E). These results suggested that GATA4 may facilitate cell proliferation by inhibiting cell apoptosis and promoting cell G_1/S phase transition.

GATA4 facilitates migration and invasion in ALL cells. To further investigate the roles of GATA4 on ALL progression, Transwell analysis was performed to explore the migratory and invasive abilities of ALL cells under different experimental conditions. The number of migrating cells in the GATA overexpression group was as significantly higher compared with the Vector control group (Fig. 3A). By contrast the number of migrating cells in the siGATA4 was significantly decreased compared with the SCR control group (Fig. 3A). Invasion analysis results revealed similar results (Fig. 3B). Results from the wound-healing assay confirmed that GATA4 expression promoted cell migration: Overexpression of GATA4 significantly increased cell migration distance following 48 h

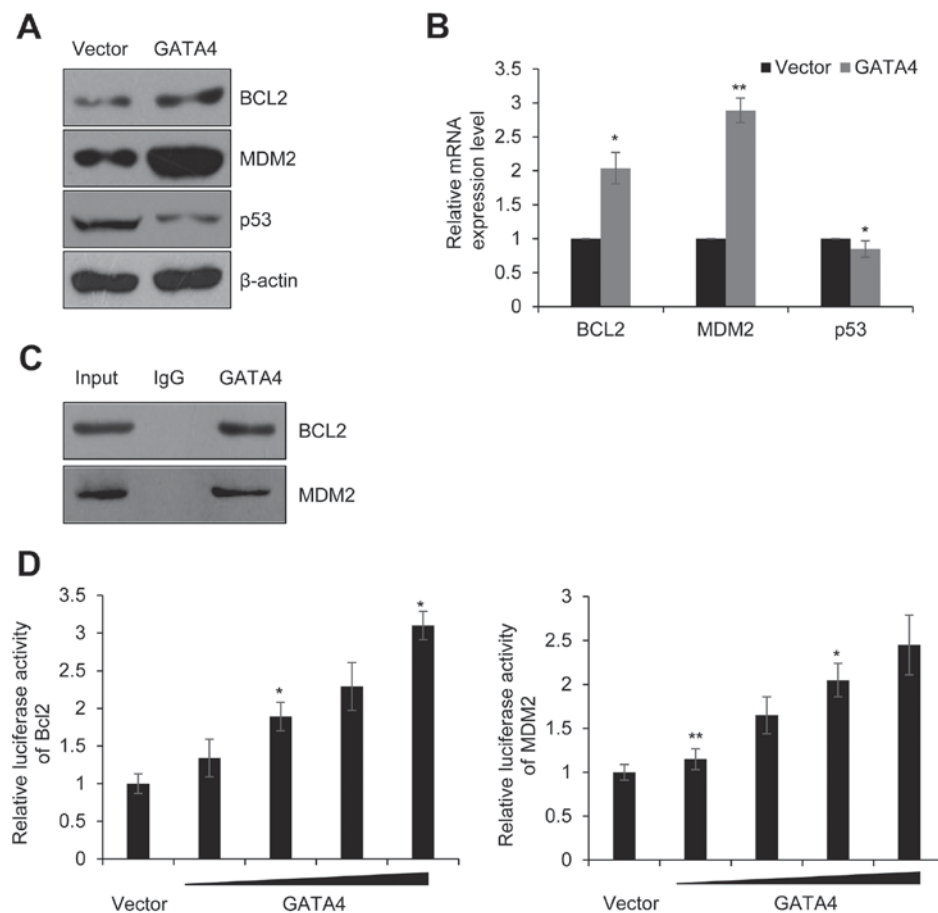


Figure 4. GATA4 inhibits apoptosis by activating the transcriptional of *BCL2* and *MDM2*. (A) Protein and (B) mRNA expression levels of *BCL2*, *MDM2* and *p53* were detected in Jurkat cells that ectopically expressed GATA4 by western blotting and reverse transcription-quantitative polymerase chain reaction, respectively. * $P < 0.05$ siGATA4 vs. SCR, ** $P < 0.01$ GATA4 vs. vector. (C) Chromatin immunoprecipitation assay was used to detect whether GATA4 interacted with the *BCL2* promoter region or the *MDM2* promoter region. (D) Transcriptional activity of *BCL2* and *MDM2* in Jurkat cells transfected with vector or GATA4 was examined by dual-luciferase assay. * $P < 0.05$, ** $P < 0.01$ GATA4 vs. vector. GATA4, GATA-binding factor 4; *BCL2*, B cell lymphoma 2; *MDM2*, mouse double minute 2 homolog.

culture; however, knockdown of GATA4 expression lead to a decreased in cell migration distance (Fig. 3C). These data suggested that GATA4 may serve a role in promoting cell migration and invasion.

GATA4 inhibits apoptosis by regulating the transcriptional activity BCL2 and MDM2 in ALL. GATA4 has been previously reported to regulate the apoptotic process by regulating the transcription of *BCL2* in embryonic stem cells or ovarian follicles (14,15,23,24). However, whether GATA4 mediated apoptosis in this manner in ALL was unknown. To explore the mechanism of GATA4 on apoptosis in ALL, the expression of *BCL2* protein and mRNA in cells in which GATA4 is overexpressed or silenced was examined. The results revealed that both the protein and mRNA levels of expression of *BCL2* were notably increased in GATA4-overexpression cells (Fig. 4A and B, respectively). Cell apoptosis was also previously demonstrated to be regulated by the p53-MDM2 pathway (25); therefore, the expression of p53 and MDM2 was examined in GATA4-overexpressing Jurkat cells. The expression level of MDM2 protein was increased, whereas the expression of p53 protein was decreased in cells that ectopically expressed GATA4 (Fig. 4A). Similarly, the mRNA expression level of MDM2 was significantly increased and the

mRNA expression level of p53 was slightly decreased in cells overexpressing GATA4 (Fig. 4 and B). The results suggested that GATA4 may regulate apoptosis in ALL through *BCL2* and the p53-MDM2 pathway.

As GATA4 is a zinc-finger-containing transcription factor, the present study hypothesized that GATA4 activated the transcription of *BCL2* and *MDM2*. To verify this hypothesis, ChIP analysis was performed with anti-GATA4 in Jurkat cells (Fig. 4C). The results suggested that GATA4 interacted with the promoter region of *BCL2* and *MDM2* in Jurkat cells. In addition, results from the dual-luciferase reporter assay demonstrated that GATA4 activated the transcription of *BCL2* and *MDM2* (Fig. 4D). These results demonstrated that GATA4 may target the promoter of *BCL2* and *MDM2* to change the expression levels of *BCL2* and *MDM2*.

GATA4 promotes cell cycle through inhibition of p53-regulated p21 in ALL. It was found that GATA4 negatively regulated p53 through transcriptional activating *MDM2*. The p53 pathway has been found to regulate cell cycle through transcriptional activating of *p21*, *GADD45* and *14-3-3σ*. p21 interacted with Cyclin-CDK complex and inhibited it phosphorylating Rb and resulted in cells arresting at G₁ phase. To investigate the mechanism of GATA4 facilitation of G₁/S phase transition, the

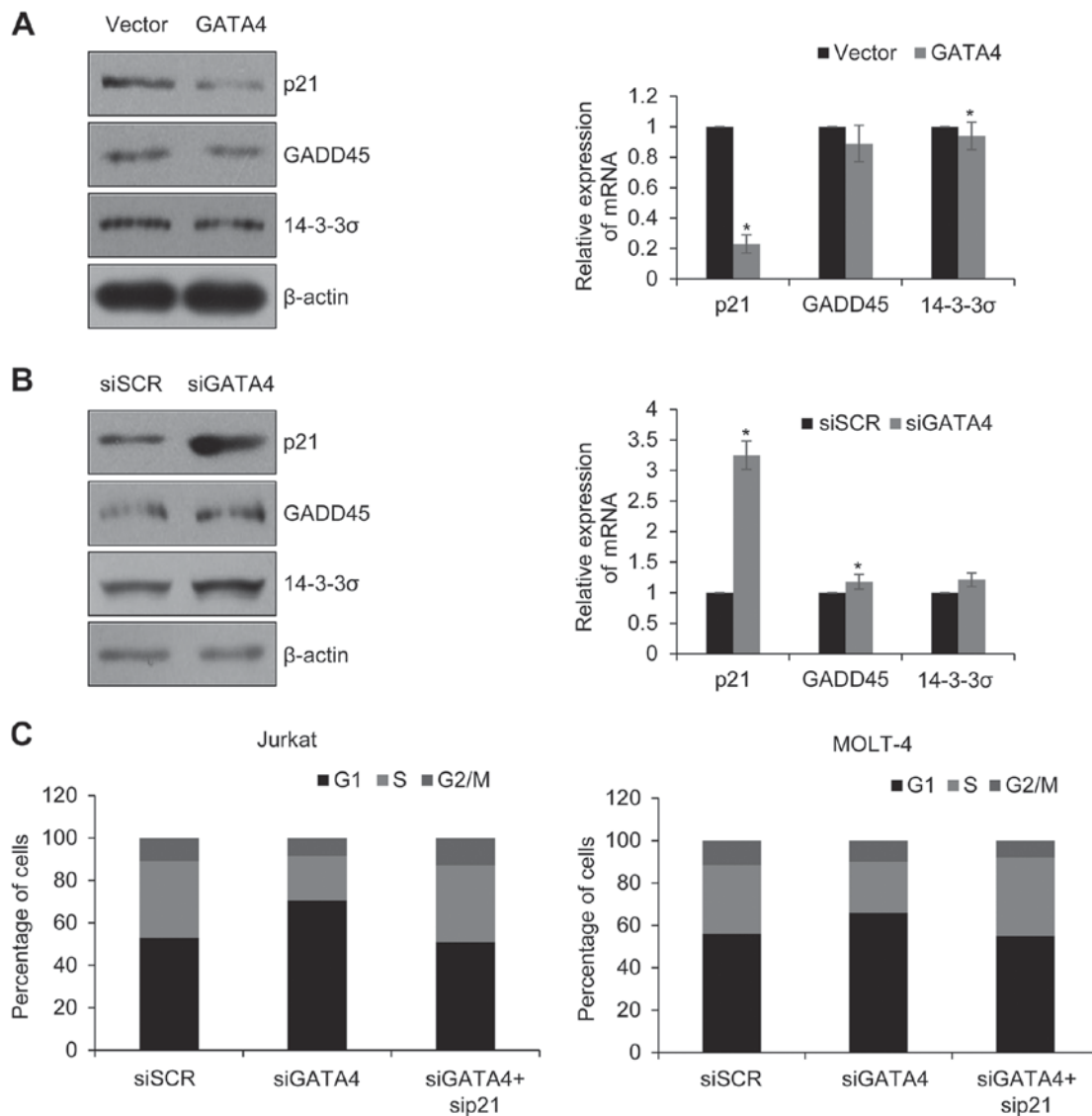


Figure 5. GATA4 promotes cell cycle through inhibition of p53-regulated p21 in ALL. (A) GATA4 was overexpressed or knocked down in Jurkat cells, after transfection 48 h, the protein level of p21, GADD45 and 14-3-3 σ were detected by western blotting. * $P < 0.05$ GATA4 vs. vector. (B) GATA4 was overexpressed or knocked down in Jurkat cells, after transfection 48 h, the mRNA level of p21, GADD45 and 14-3-3 σ were determined by RT-qPCR. * $P < 0.05$ siGATA4 vs. SCR. (C) GATA4 was knocked down or GATA4 and p21 were silenced simultaneously in Jurkat cells. The effect of GATA4 and p21 on cell cycle were detected by flow cytometry. GATA4, GATA-binding factor 4; ALL, acute lymphoblastic leukemia.

expression of p21, GADD45 and 14-3-3 σ were examined in Jurkat cells in which GATA4 was overexpressed or silenced (Fig. 5A and B, respectively). Consistent with our hypothesis, the expression of p21 was decreased in GATA4-overexpressing cells, whereas no notable changes in the protein expression levels of GADD45 and 14-3-3 σ were observed (Fig. 5A). However, knockdown of GATA4 increased the expression of p21 (Fig. 5B). Next, GATA4 and p21 were knocked down together, and the cell cycle determined. It was found that when GATA4 and p21 were silenced together, the percentage of cells at S was increased compared with the group which only silenced GATA4; similar results were observed in MOLT-4 cells (Fig. 5C). Together, GATA4 facilitated G₁/S transition through p53-p21 pathway.

Discussion

ALL is a malignant hematologic cancer that arises from the hematopoietic precursors of lymphoid blood cell lineage. ALL

predominantly occurs in children. T-ALL represents 25% of adult ALL cases and 10-15% of pediatric cases (26), resulting from the malignant transformation of T cell progenitors. Although there are several treatments for ALL, patients still have poor prognosis (4,27,28).

The GATA family of transcription factors serve various roles in the process of cell proliferation and differentiation (29,30). The expression of GATA4 has been extensively studied in a number of carcinomas (31-34). Previous studies have reported that the expression of GATA4 was low in several cancers, including colorectal cancer, adrenocortical tumors, epithelial ovarian cancer and hepatocellular carcinoma, which suggested that GATA4 may serve as a tumor suppressor (35). By contrast, high expression levels of GATA4 were previously demonstrated in the Huh6 pediatric liver tumor cell line (36). In addition, it was reported that GATA family members, including GATA1, GATA2 and GATA3, served inhibiting functions in leukemia (18-21), although the function of GATA4 in ALL remained unknown.

The present study demonstrated that GATA4 mRNA expression was significantly upregulated in child patients with ALL compared with healthy children. CCK-8 analysis revealed that high expression of GATA4 promoted proliferation in ALL cells. Cell cycle analysis demonstrated that the inhibition of GATA4 arrested cells at G₀/G₁ phase, whereas ectopic expression of GATA4 promoted G₁/S phase transition. Previous studies have demonstrated that GATA1 regulated cell cycle arrest through activation of p21 and GATA3 regulated hematopoietic stem cell maintenance and cell-cycle entry (37-39). The above evidence indicated that the GATA family serve different functions in the cell cycle and the reason why they do so requires further investigation. Results from the present study also indicated that GATA4 suppressed apoptosis in ALL cells. In addition, the Transwell assays revealed that GATA4 may regulate ALL cell invasive ability. Cell apoptosis is an important mechanism that prevents cell overgrowth and DNA damage. Inhibition cell apoptosis promotes cancer cell proliferation. GATA4 was also demonstrated to activate the transcription of BCL2, thereby suppressing cell apoptosis, and GATA4 may also regulate apoptosis through regulation of the p53-MDM2 pathway.

A recent report suggested that GATA4 may facilitate hepatoblastoma cell proliferation by regulating the expression of DKK3 and microRNA (miRNA) miR125b (40). A number of other studies have also demonstrated that miRNAs and transcription factors are closely correlated in gene regulatory networks (41), and bioinformatics analyses suggested that the promoter sequences of most mammalian miRNA genes included at least one GATA box, which indicated that GATA4 may transcriptionally regulate certain miRNAs. A detailed molecular mechanism of GATA4 remains to be investigated, and miRNAs may be an important factor.

In conclusion, the present study revealed GATA4 served a key function in ALL, and high expression of GATA4 may serve an important function in the development of ALL. GATA4 was demonstrated to regulate the cell cycle and apoptosis through the regulation of the transcriptional activity *MDM2* and *BCL2*. GATA4 may be a potential target of ALL molecular therapy.

References

- Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, Reaman GH and Carroll WL: Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: A report from the children's oncology group. *J Clin Oncol* 30: 1663-1669, 2012.
- Bassan R and Hoelzer D: Modern therapy of acute lymphoblastic leukemia. *J Clin Oncol* 29: 532-543, 2011.
- Stanulla M and Schrappe M: Treatment of childhood acute lymphoblastic leukemia. *Semin Hematol* 46: 52-63, 2009.
- Pui CH, Robison LL and Look AT: Acute lymphoblastic leukaemia. *Lancet* 371: 1030-1043, 2008.
- Pui CH, Campana D, Pei D, Bowman WP, Sandlund JT, Kaste SC, Ribeiro RC, Rubnitz JE, Raimondi SC, Onciu M, *et al*: Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med* 360: 2730-2741, 2009.
- Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, Hovi L, LeBlanc T, Szczepanski T, Ferster A, *et al*: A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): An observational study and a multicentre randomised trial. *Lancet* 370: 240-250, 2007.
- Papaemmanuil E, Hosking FJ, Vijaykrishnan J, Price A, Olver B, Sheridan E, Kinsey SE, Lightfoot T, Roman E, Irving JA, *et al*: Loci on 7p12.2, 10q21.2 and 14q11.2 are associated with risk of childhood acute lymphoblastic leukemia. *Nat Genet* 41: 1006-1010, 2009.
- Trevino LR, Yang W, French D, Hunger SP, Carroll WL, Devidas M, Willman C, Neale G, Downing J, Raimondi SC, *et al*: Germline genomic variants associated with childhood acute lymphoblastic leukemia. *Nat Genet* 41: 1001-1005, 2009.
- Molkentin JD: The zinc finger-containing transcription factors GATA-4, -5 and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem* 275: 38949-38952, 2000.
- Evans T, Reitman M and Felsenfeld G: An erythrocyte-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. *Proc Natl Acad Sci USA* 85: 5976-5980, 1988.
- Orkin SH: GATA-binding transcription factors in hematopoietic cells. *Blood* 80: 575-581, 1992.
- Arceci RJ, King AA, Simon MC, Orkin SH and Wilson DB: Mouse GATA-4: A retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol* 13: 2235-2246, 1993.
- Kelley C, Blumberg H, Zon LI and Evans T: GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* 118: 817-827, 1993.
- Heikinheimo M, Ermolaeva M, Bielinska M, Rahman NA, Narita N, Huhtaniemi IT, Tapanainen JS and Wilson DB: Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* 138: 3505-3514, 1997.
- Vaskivuo TE, Anttonen M, Herva R, Billig H, Dorland M, te Velde ER, Stenbäck F, Heikinheimo M and Tapanainen JS: Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins and transcription factor GATA-4. *J Clin Endocrinol Metab* 86: 3421-3429, 2001.
- Kyrölähti A, Rämö M, Tamminen M, Unkila-Kallio L, Butzow R, Leminen A, Nemer M, Rahman N, Huhtaniemi I, Heikinheimo M and Anttonen M: GATA-4 regulates Bcl-2 expression in ovarian granulosa cell tumors. *Endocrinology* 149: 5635-5642, 2008.
- Jääskeläinen M, Nieminen A, Pöykkö RM, Kauppinen M, Liakka A, Heikinheimo M, Vaskivuo TE, Klefström J and Tapanainen JS: Regulation of cell death in human fetal and adult ovaries-role of Bok and Bcl-X (L). *Mol Cell Endocrinol* 330: 17-24, 2010.
- Bergman M and Ringertz N: Gene expression pattern of chicken erythrocyte nuclei in heterokaryons. *J Cell Sci* 97: 167-175, 1990.
- Shimamoto T, Ohyashiki K, Ohyashiki JH, Kawakubo K, Fujimura T, Iwama H, Nakazawa S and Toyama K: The expression pattern of erythrocyte/megakaryocyte-related transcription factors GATA-1 and the stem cell leukemia gene correlates with hematopoietic differentiation and is associated with outcome of acute myeloid leukemia. *Blood* 86: 3173-3180, 1995.
- Ono Y, Fukuhara N and Yoshie O: TAL1 and LIM-only proteins synergistically induce retinaldehyde dehydrogenase 2 expression in T-cell acute lymphoblastic leukemia by acting as cofactors for GATA3. *Mol Cell Biol* 18: 6939-6950, 1998.
- Wang L, Dong L, Liu G, Lin Y, Zhang J, Jia S, Lu S, Pian H, Yao B and Chen H: GATA-2 gene expression in leukemia patients and its significance. *Zhonghua Xue Ye Xue Za Zhi* 22: 27-29, 2001 (In Chinese).
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* 25: 402-408, 2001.
- Kobayashi S, Lackey T, Huang Y, Bisping E, Pu WT, Boxer LM and Liang Q: Transcription factor gata4 regulates cardiac BCL2 gene expression in vitro and in vivo. *FASEB J* 20: 800-802, 2006.
- Zhang SJ, Shi JY and Li JY: GATA-2 L359 V mutation is exclusively associated with CML progression but not other hematological malignancies and GATA-2 P250A is a novel single nucleotide polymorphism. *Leuk Res* 33: 1141-1143, 2009.
- Haupt Y, Barak Y and Oren M: Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J* 15: 1596-1606, 1996.
- Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, Behm FG, Pui CH, Downing JR, Gilliland DG, *et al*: Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer cell* 1: 75-87, 2002.
- Goldberg JM, Silverman LB, Levy DE, Dalton VK, Gelber RD, Lehmann L, Cohen HJ, Sallan SE and Asselin BL: Childhood T-cell acute lymphoblastic leukemia: The Dana-Farber Cancer Institute acute lymphoblastic leukemia consortium experience. *J Clin Oncol* 21: 3616-3622, 2003.

28. Oudot C, Auclerc MF, Levy V, Porcher R, Piguet C, Perel Y, Gandemer V, Debre M, Vermylen C, Pautard B, *et al*: Prognostic factors for leukemic induction failure in children with acute lymphoblastic leukemia and outcome after salvage therapy: The FRALLE 93 study. *J Clin Oncol* 26: 1496-1503, 2008.
29. Wang Z, Kang J, Deng X, Guo B, Wu B and Fan Y: Knockdown of GATAD2A suppresses cell proliferation in thyroid cancer in vitro. *Oncol Rep* 37: 2147-2152, 2017.
30. Walker EM, Thompson CA and Battle MA: GATA4 and GATA6 regulate intestinal epithelial cytodifferentiation during development. *Dev Biol* 392: 283-294, 2014.
31. Castro IC, Breiling A, Luetkenhaus K, Ceteci F, Hausmann S, Kress S, Lyko F, Rudel T and Rapp UR: MYC-induced epigenetic activation of GATA4 in lung adenocarcinoma. *Mol Cancer Res* 11: 161-172, 2013.
32. Anttonen M, Pihlajoki M, Andersson N, Georges A, L'hôte D, Vattulainen S, Färkkilä A, Unkila-Kallio L, Veitia RA and Heikinheimo M: FOXL2, GATA4 and SMAD3 co-operatively modulate gene expression, cell viability and apoptosis in ovarian granulosa cell tumor cells. *PLoS One* 9: e85545, 2014.
33. Färkkilä A, Andersson N, Bützow R, Leminen A, Heikinheimo M, Anttonen M and Unkila-Kallio L: HER2 and GATA4 are new prognostic factors for early-stage ovarian granulosa cell tumor-a long-term follow-up study. *Cancer Med* 3: 526-536, 2014.
34. Mehta G, Kumarasamy S, Wu J, Walsh A, Liu L, Williams K, Joe B and de la Serna IL: MITF interacts with the SWI/SNF subunit, BRG1, to promote GATA4 expression in cardiac hypertrophy. *J Mol Cell Cardiol* 88: 101-110, 2015.
35. Agnihotri S, Wolf A, Munoz DM, Smith CJ, Gajadhar A, Restrepo A, Clarke ID, Fuller GN, Kesari S and Dirks PB: A GATA4-regulated tumor suppressor network represses formation of malignant human astrocytomas. *J Exp Med* 208: 689-702, 2011.
36. Soini T, Haveri H, Elo JM, Kauppinen M, Kyrönlähti A, Salo MK, Lohi J, Andersson LC, Wilson DB and Heikinheimo M: Transcription factor GATA-4 is abundantly expressed in childhood but not in adult liver tumors. *J Pediatr Gastroenterol Nutr* 54: 101-108, 2012.
37. Goldfarb AN: Coordinating red cell differentiation with cell cycle arrest: GATA-1 activation of p21. *Cell Cycle* 9: 2061, 2010.
38. Papetti M, Wontakal SN, Stopka T and Skoultschi AI: GATA-1 directly regulates p21 gene expression during erythroid differentiation. *Cell Cycle* 9: 1972-1980, 2010.
39. Ku CJ, Hosoya T, Maillard I and Engel JD: GATA-3 regulates hematopoietic stem cell maintenance and cell-cycle entry. *Blood* 119: 2242-2251, 2012.
40. Pei Y, Yao Q, Yuan S, Xie B, Liu Y, Ye C and Zhuo H: GATA4 promotes hepatoblastoma cell proliferation by altering expression of miR125b and DKK3. *Oncotarget* 7: 77890-77901, 2016.
41. Safe S: MicroRNA-specificity protein (sp) transcription factor interactions and significance in carcinogenesis. *Curr Pharmacol Rep* 1: 73-78, 2015.