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Original Research Article

Upregulation of AKAP12 by demethylation inhibits proliferation and increases chemosensitivity to adriamycin in leukemic cells

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Abstract

Purpose: *To elucidate the role of AKAP12 in leukemia cells.*
Methods: *Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting (WB) were employed to determine the expression of AKAP12 in leukocyte cell lines, while 5-azacytidine was used to treat the cells, followed by assessment of the expression of AKAP12. After constructing the overexpressing vector pc-AKAP12 and transfecting it into cells or treating the cells with 5-azacytidine, cell counting kit-8 assay (CCK-8) was used to determine cell proliferation. Cloning ability of the cells was evaluated by colony formation assay. Furthermore, flow cytometry was employed to measure the degrees of cell cycle and cell apoptosis. The effect of AKAP12 on PI3K/AKT were determined by western blot.*
Results: *The results showed that AKAP12 was lowly expressed in lymphocytic leukemia cell lines ($p < 0.001$), but was reversed by 5-azacytidine. Transfection of AKAP12 or 5-azacytidine treatment increased the expression of AKAP12 in the cells ($p > 0.001$), inhibited leukemia cell proliferation and clonality, and arrested cell cycle in G1 phase as well as induced apoptosis. In addition, PI3K/AKT signaling pathway was inhibited by AKAP12.*
Conclusion: *AKAP12 is lowly expressed in leukemia cells, and may also play a role in inhibiting leukemia progression by suppressing the activity of PI3K/AKT pathway.Thus, targeting AKAP12 mght be a potential strategy in the management of lukemia.*

Keywords: *Leukemia, AKAP12, PI3K/Akt, 5-Azacytidine, Chemosensitivity, Adriamycin*

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INTRODUCTION

Leukemia is a malignant Crohn's disease of hematopoietic stem cells. The onset of leukemia is often accompanied by malignant proliferation of blood cells, and the apoptosis of leukemia cells is not inhibited [1]. Clinically, leukemia is divided into two categories, and acute leukemia is more common with a considerable number of children diagnosed as such. Another type is chronic leukemia [2]. However, the therapeutic outcomes for leukemia is still limited, and this highly affects the health of patients.

The pathogenesis of leukemia is complicated. However, the treatment of leukemia has now entered the era of precision medicine [2], which can be adjusted according to the algorithm's precise treatment strategy for each patient [3].

PI3K/Akt and its related signaling pathways are frequently activated in leukemia [4]. Several studies have shown that PI3K/Akt plays a crucial role in tumorigenesis [5]. A-kinase-anchored protein 12 (AKAP12) is a key regulator of protein kinase A and C signaling, and belongs to the kinase-anchored protein (AKAP) family, which is associated with the cAMP-dependent protein kinase (PKA) holoenzyme type I or Type II regulatory subunits, to maintain the signal transduction scaffold [6]. The tumor suppressor effect of AKAP12 is disabled in many human tumors, and molecular biology studies have shown that the 6q24-25.2 locus of the gene promoter is inactive, resulting in the progression of malignancy and metastasis [7].

The downregulation of microRNA-144 inhibits myelodysplastic syndrome cell proliferation and promotes apoptosis by activating AKAP12-dependent ERK1/2 signaling pathway [8]. AKAP12 upregulation inhibits colorectal cancer progression and migration [9]. In addition to the inactivation of the promoter region leading to the deterioration of myeloid, the successive inactivation of the genes that it affects also exacerbates the leukemia progression [10]. The epigenetic inhibition of AKAP12 has been reported in various childhood myeloid leukemias [11]. Demethylation is expected to restore the AKAP12 expression, which can inhibit the progression of childhood leukemia.

This study aimed to elucidate the role and mechanism of AKAP12 in the development of leukemia.

METHODS

Cells and reagents

Human peripheral blood B lymphocyte IM-9, human B lymphocytic acute leukemia cell BALL-1, and human T lymphocytic leukemia cell HuT 78 cell lines were obtained from National Infrastructure of Cell Line Resource (NICR; Beijing, China). The cells were cultured in RPMI-1640 medium supplemented with 10 % FBS (Gibco, CA, USA) and 1 % penicillin-streptomycin (Arizona, USA) at 37 °C in a cell incubator containing 5 % CO_2. 5-Azacytidine (Cat no. HY-10586) and Adriamycin (ADM, Cat no. HY-15142A) were purchased from drug manufacture (MedChemExpress, Shanghai, China). Diluted sterile water and RPMI-1640 medium (Arizona, USA) were used to dissolve and dilute 5-azacytidine. The grouping scheme, based on the three cell lines, were IM-9 group, HuT 78 group and BALL-1 group. Subsequently, when evaluating the effect of 5-azacytidine, the grouping scheme was 5-azacytidine group and control group.

Western blot

Protein lysates were isolated from cells by using RIPA buffer (Cat no. 89901; Thermo Fisher, MA, USA) followed by centrifugation at 16,000 × g at 4 °C for 15 min. To quantify the protein concentration, a bicinchoninic acid (BCA) protein assay (Cat no. 23225; Thermo Fisher, MA, USA) was performed. Denaturation of the total protein was processed in a metal bath at 95 °C for 5 min, and then separated on SDS-PAGE (7.5 %) with 30 μg of protein per sample. After that, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. 5 % skimmed milk was dissolved in Tris Buffered Saline with Tween (TBST) to block the PVDF membrane for 2 h at room temperature. The membranes were washed and incubated with the primary antibodies overnight.

The primary antibodies include AKAP12 (1:1,500) (Cat no. 25199-1-AP; Proteintech, Wuhan, Hubei, China), Akt (1:1,500) (Cat no. 60203-2-Ig; Proteintech, Wuhan, Hubei, China), p-Akt(1:1,500) (Cat no. 80455-1-RR; Proteintech, Wuhan, Hubei, China), PI3K (1:1,500) (Cat no. 20584-1-AP; Proteintech, Wuhan, Hubei, China), and p-PI3K (Ser32) (1:1000) (Cat no. 60225-1-Ig; Proteintech, Wuhan, Hubei, China) antibodies. Then, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) (Cat. no. 7074; Cell Signaling Technology, Inc) for 2 h at 37 °C. The Easysee Western Blot Kit (Beijing TransGen Biotech, Beijing, China) was utilized to measure the chemiluminescence signals. ImageJ version 1.53 software was used to quantify the proteins.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

A SteadyPure Plant RNA Extraction Kit (Hunan, China) was used to lysate cells to extract total RNA. Then cDNA was synthesized by a HiScript II Q Select RT SuperMix (Vazyme, Nanjing, China). SYBR Green Master Mix (BioRad, USA) was employed for the qRT-PCR, and GAPDH was used as an internal control. The primers used are listed in Table 1.

Table 1: Primer sequences used in PCR

Gene	Sense	Antisense
AKAP12	AGC CAC ATC GCT CAG ACA C	GCC CAA TAC GAC CAA ATC C
GAPDH	GTG AAG GTC GGT GTG AAC GGA TTT	CAC AGT CTT CTG AGT GGC AGT GAT

Overexpression of AKAP12 in cells

AKAP12-specific plasmids were purchased from Addgene. To overexpress AKAP12 in BALL-1 and HuT 78 cell lines, the cells were seeded in six-well plates, and 1.5 μL per well of Lipofectamine 2000 (Invitrogen) and a plasmid expressing AKAP12 (100 ng) were used to transfect them. An equal amount of the empty plasmid vector pcDNA3.1 was used as a control. 48 h after transfection, the cells were harvested and used for subsequent experiments. BALL-1 and HuT 78 cell lines were divided into four groups: 1) Control group; 2) 5-azacytidine group; 3) NC group; and 4) pc-AKAP12 group.

CCK-8 assay

CCK-8 assay kit (Solarbio, Beijing, China) was employed to measure the cell viability of cells which were previously transfected. Cell proliferation rates were measured on days 1, 2, 3, 4 and 5. The absorbance value were immediately read at 490 nm by a microplate reader (SpectraMax i3X, Molecular Devices, CA). The experiments were repeated three times, followed by data analysis.

Colony formation assay

When cells had grown to a logarithmic growth stage, they were treated with 5-Azacytidin. Anchorage-independent growth was assessed by measuring colony formation in soft agar. 2 × RPMI-1640 medium supplemented with 30 % FBS was prepared, mixed with an equal volume of 1 % soft agar at 40 °C, and then made up to 0.5 % agar as the base agar in six-well plates. 0.1 mL of cell suspension containing 2.0 × 10^5/mL was suspended in the above medium in 3 mL of 0.7 % agar, and followed by adding 1.5 mL of cell suspension to each well of a six-well plate to form 0.35 % top agar, and covered with medium, with the final cell density of 5 × 10^3 cells per well. Colonies were formed and observed, and the culture was terminated and the supernatant was discarded, followed by immersion twice with PBS. 4 % paraformaldehyde was added to fix cells for 15 min, and then fixing solution were removed, followed by dyeing with proper amount of Giemsa solution for 10-30 min. Then the dyeing solution was slowly washed off the cells using running water, and the cells were air dried. The absorbance of each well was determined at 490 nm in a microplate reader.

Flow cytometry

To evaluate the proportions of the various phases of the cell cycle and the apoptosis rates, a BD Accuri™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was utilized. Cell Cycle Assay Kit Plus (Yuhengbio, Suzhou, Jiangsu, China) and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Solarbio, Beijing, China) were applied for detecting the proportions of the cell cycle and cell apoptosis, and then the results were analyzed according to the manufacturer's protocols. The ratio of cell apoptosis was computed and calculated using FACS scan software (BD, San Jose, USA).

Statistical analysis

All data were processed and shown as mean ± standard deviation (SD) for experiments performed in triplicate. Unpaired or paired Student's t-test or one-way analysis of variance (ANOVA) was utilized to analyze results followed by Tukey's test to compare each group. Data were analyzed and graphs were plotted using GraphPad Prism version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ indicated significant difference.

RESULTS

5-Azacytidine treatment increases AKAP12 expression in leukemia cells

The expression of AKAP12 was detected in leukemia cells, and it was found that IM-9 cells expressed the highest level of AKAP12, BALL-1 expressed a relatively low level of AKAP12, and HuT 78 expressed the lowest level of AKAP12 (Figure 1 A). IM-9, BALL-1 and HuT 78 cells were treated with 5-azacytidine, and the mRNA level of AKAP12 and the protein expression of AKAP12 level were tested. Considering the effect of 5-azacytidine on the expression of AKAP12, the three cell lines were divided into two groups, 5-azacytidine-treated group and control group. PCR and WB results showed that 5-azacytidine treatment did not change the expression level of AKAP12 in IM-9 (Figure 1 B). Notably, 5-azacytidine treatment increased the expression

levels of AKAP12 in BALL-1 and HuT78 (Figure 1 C and D).

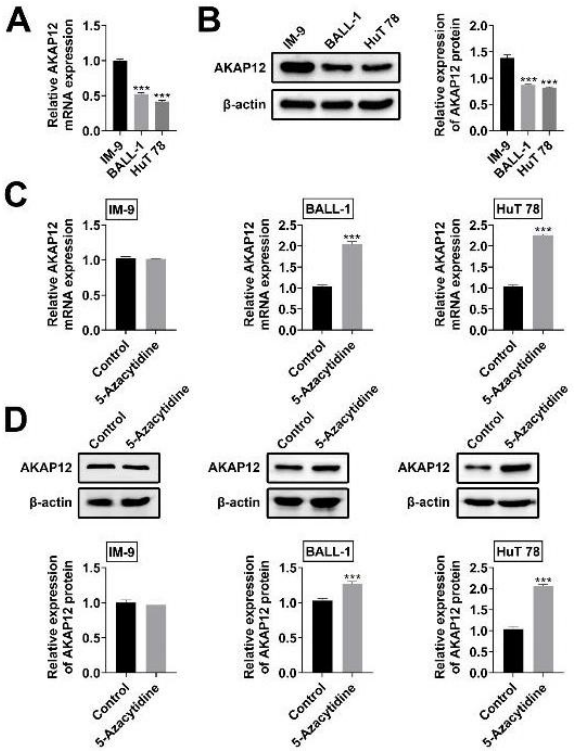

Figure 1: Gene and protein expression of AKAP12 in IM-9, BALL-1 and HuT 78 cell lines. (A) Relative gene expression of AKAP12 (B) AKAP12 protein expression (C) The effect of 5-azacytidine treatment on AKAP12 gene expression (D) The effect of 5-azacytidine treatment on AKAP12 protein expression. $^{***}P < 0.001$ vs. the control group (n = 3)

AKAP12 inhibits the proliferation of leukemia cells

AKAP12 was overexpressed in BALL-1 and HuT-78, so as to observe its effect on cell proliferation. The two cell lines were divided into four groups: control group, 5-azacytidine group, NC group (empty vector group) and pc-AKAP12 transfection group. The results of the WB test showed that the expression levels of AKAP12 in the NC group and the control group were the same, while the expression levels of AKAP12 in the pc-AKAP12 group and the 5-Azacytidine group were slightly the same, but greater than that in the NC group and the control group (Figure 2 A). The results indicated that the overexpression is successful. The cell proliferation and cloning ability was determined by CCK8 and colony formation assay separately, and the results showed that the cell proliferation ability of the NC group and the control group was higher (Figure 2 B). However, the proliferation rate of cells in pc-AKAP12 group and 5-Azacytidine group was lower than that in the NC group and the control group (Figure 2 C). These results indicated that AKAP12 inhibits the proliferation of leukemia cells.

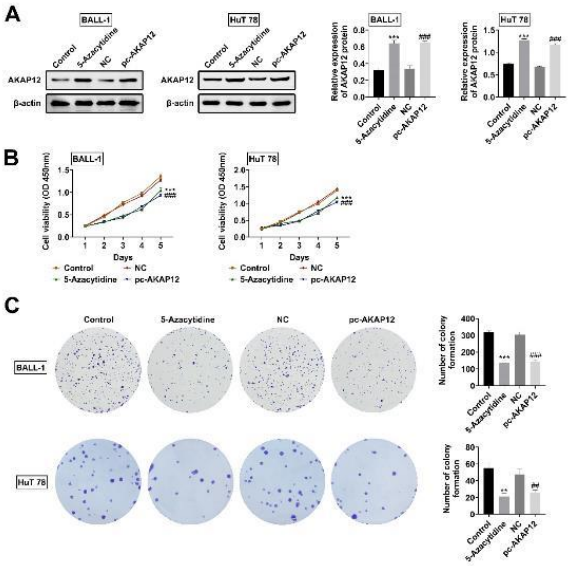

Figure 2: Effects of AKAP12 overexpression and 5-azacytidine treatment on proliferation of BALL-1 and HuT 78 cell lines. (A) The relative protein expression of AKAP12 in different treatment groups; (B) the cell proliferation rate at 1, 2, 3, 4 and 5 days; (C) the clone formation rate in different treatment groups. $^{**}P < 0.01$ vs. the control group; $^{***}p < 0.001$ vs. the control group; $^{\#\#}p < 0.01$ vs. the NC group; $^{\#\#\#}p < 0.001$ vs. the NC group (n = 3)

AKAP12 regulates cell cycle and apoptosis

The two cell lines were divided into four groups, control group, 5-azacytidine group, NC group (empty vector group) and pc-AKAP12 transfection group. Cell numbers and apoptosis at each stage of G0/G1, S, G2/M were determined by flow cytometry. The results showed that the number of cells in G0/G1, S, G2/M stages in the control group and NC group did not significantly change, while the 5-azacytidine group and the pc-AKAP12 transfection group had the most cells in the G0/G1 phase, less cells in the S phase and few cells in the G2/M phase (Figure 3). The apoptotic rates of two cell lines in the four groups were detected, and the results showed that the 5-Azacytidine treatment and the pc-AKAP12 transfection significantly increased the apoptotic rates of the cells compared with the NC and control groups (Figure 4).

AKAP12 increases chemosensitivity of leukemia cells to adriamycin

BALL-1 and HuT78 cells were exposed to 3 μM adriamycin (ADM) for 24 h after 5-azacytidine treatment or pc-AKAP12 transfection. The

experimental grouping scheme is as follows: control group, ADM group, ADM plus 5-azacytidine group, ADM plus NC group and ADM plus pc-AKAP12 group.

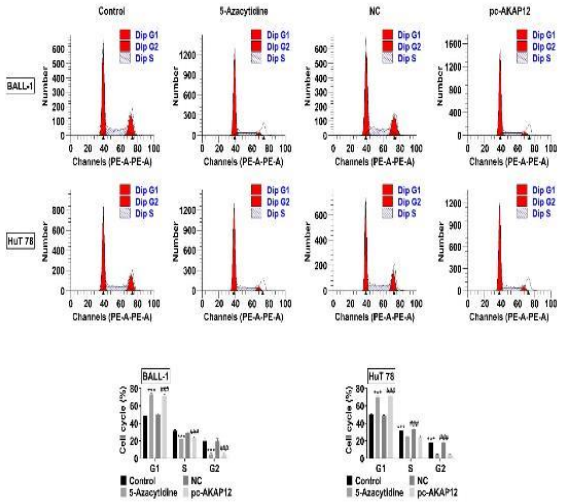

Figure 3: Overexpression of AKAP12 and 5-azacytidine treatment on BALL-1 and HuT 78 cell lines at different stages of cell cycle, G0/G1, S, G2/M cell ratios at different stages were calculated among different treatment groups. $^{***}P < 0.001$ vs. the control group; $^{###}p < 0.001$ vs. the NC group (n = 3)

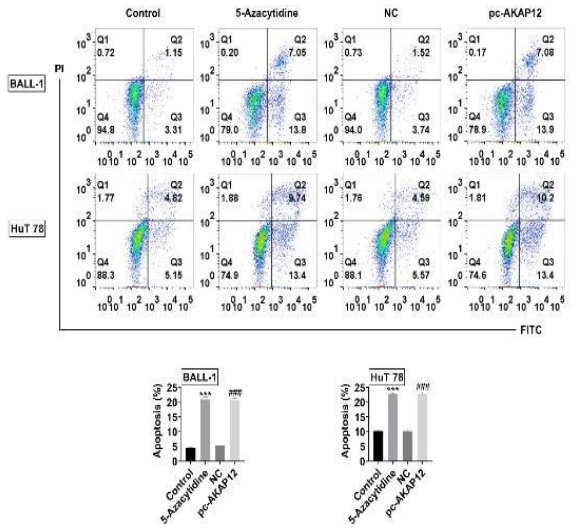

Figure 4: Effect of overexpression of AKAP12 and 5-azacytidine treatment on the apoptosis of BALL-1 and HuT 78 cell lines among different treatment groups. $^{***}P < 0.001$ vs. the control group; $^{###}p < 0.001$ vs. the NC group (n = 3)

Cell clonality was tested using colony formation assay. The results showed that in the two cell lines, more purple crystals could be observed in the control group, indicating good cell migration ability. Compared with the control group, ADM significantly reduced the number of purple crystals, indicating that the cell migration ability was weakened by ADM. Notably, ADM plus 5-azacytidine group had fewer purple crystals than the ADM alone group, indicating a stronger impairment of cell migration. There was no significant difference in cell migration ability between ADM plus NC group and ADM alone group, but ADM plus pc-AKAP12 group significantly reduced the cell migration ability (Figure 5). These results suggest that AKAP12 may play a role similar to 5-azacytidine in reducing cell migration. Further, the results of flow cytometry showed that the apoptosis rate of the control group was lower. Relative to the control group, ADM significantly increased apoptosis, and 5-azacytidine further increased apoptosis. The addition of Pc-AKAP12 also resulted in more apoptosis compared with the ADM plus NC group. These two experiments showed that AKAP12 could increase the chemosensitivity of leukemia cells to doxorubicin (Figure 6).

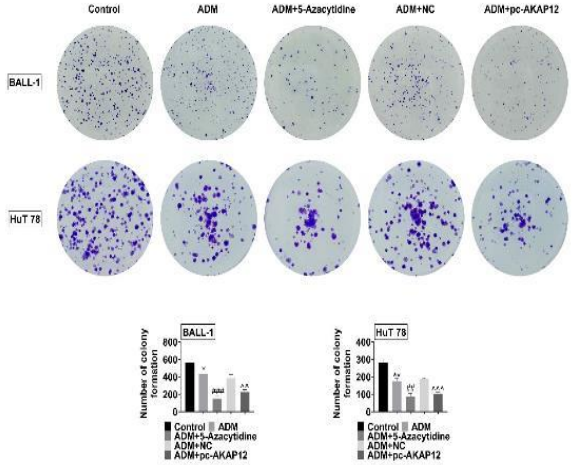

Figure 5: AKAP12 enhances the chemosensitivity of cells to ADM and reduces cell migration ability in BALL-1 and HuT78 cells among different groups. $^{*}P < 0.05$ vs. the control group; $^{**}p < 0.01$ vs. the control group; $^{##}p < 0.01$ vs. the NC group; $^{###}p < 0.001$ vs. the NC group; $^{\wedge\wedge}p < 0.01$ vs. the NC group; $^{\wedge\wedge\wedge}p < 0.001$ vs. the NC group (n = 3)

AKAP12 inhibits PI3K/Akt pathway activity

In order to study the signaling pathway specifically regulated by AKAP12, the expression levels of Akt, p-Akt, PI3K and p-PI3K in the two cell lines of the above four groups were detected. The results showed that the expression of Akt and PI3K did not significantly change in the two cell lines. While, compared with the NC group and the control group, p-Akt and p-PI3K were increased in the 5-azacytidine group and the pc-AKAP12 group, which indicated that the PI3K/Ark signaling pathway was activated (Figure 7 A and B).

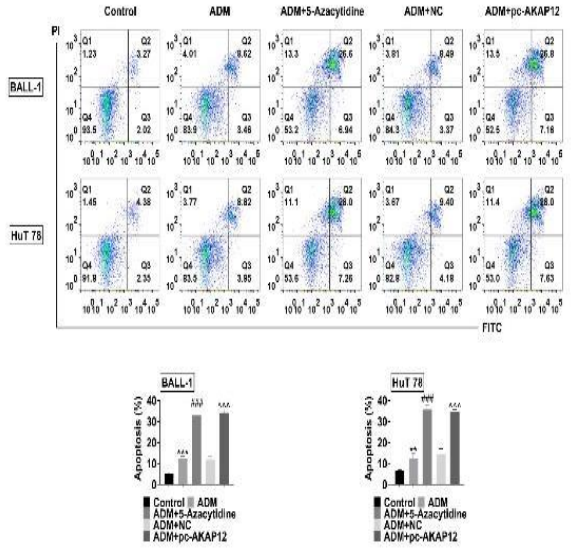

Figure 6: AKAP12 enhances the chemosensitivity of cells to ADM and thereby increasing apoptosis in BALL-1 and HuT78 cells. $^{**}p < 0.01$ vs. the control group; $^{***}p < 0.001$ vs. the control group; $^{\#\#}p < 0.01$ vs. the NC group; $^{\#\#\#}p < 0.001$ vs. the NC group; $^{\wedge\wedge}p < 0.01$ vs. the NC group; $^{\wedge\wedge\wedge}p < 0.001$ vs. the NC group (n = 3)

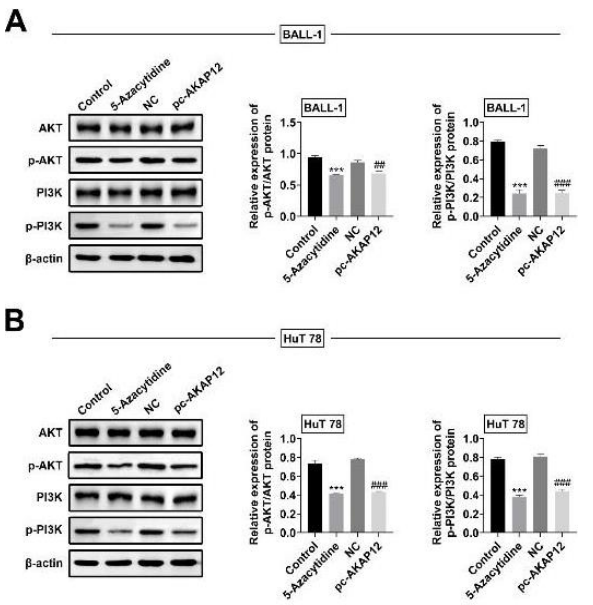

Figure 7: Overexpression of AKAP12 and 5-Azacytidine treatment affected the protein expression of Akt, p-Akt, PI3K and p-PI3K in BALL-1 and HuT 78 cell lines. (A) Protein bands and phosphorylation levels of Akt, p-Akt, PI3K and p-PI3K in different treatment groups in BALL-1 (B) Akt, p-Akt, PI3K and p- Protein bands and phosphorylation levels of PI3K. $^{***}P < 0.001$ vs. the control group; $^{\#\#}p < 0.01$ vs. the NC group; $^{\#\#\#}p < 0.001$ vs. the NC group (n = 3)

DISCUSSION

Leukemia is a devastating blood cancer. Despite the fact that survival and quality of life have significantly improved over the past five decades in leukemia patients, the prognosis for many people with this disease remains bleak. The pathogenesis of leukemia has not been fully elucidated. Studies have shown that genetic and environmental factors often work together to contribute to the development of leukemia [12]. Studies have shown that AKAP12 is downregulated in many cancers, including leukemia. The mechanism may be that the 5' CpG in the AKAP12 promoter region is hypermethylated, resulting in the inhibition of AKAP12 expression. In addition, studies have shown that AKAP12 plays an important role in intracellular signal transduction, such as remodeling of the cytoskeleton, cell cycle regulation, etc. Moreover, G protein-coupled receptor-activated signal transduction and apoptosis are also included. Other studies have shown that AKAP12 can alter cellular processes by regulating cytokines.

Downregulation of AKAP12 expression directly or indirectly affected cell survival [13]. At present, the main method for the treatment of leukemia is still chemotherapy, but the efficacy of chemotherapy alone is still limited [14]. Leukemia occurs when a large number of abnormal blood cells, which are neonatal [15], are usually produced. The three cell lines used in this study, IM-9, BALL-1 and HuT 78, correspond to the three disease states of the patients and can be used to study the mechanism of AKAP12 in leukemia.

The high expression of AKAP12 in IM-9 cells and the lower expression in BALL-1 and HuT 78 cells were detected, and 5-azacytidine treatment increased AKAP12 expression in leukemia cells, which indicates that 5-azacytidine can promote AKAP12 expression. No other studies have found this phenomenon, but it has been reported in the literature that the low expression of AKAP12 in leukemia cells may be related to the methylation of the promoter region of AKAP12, which inhibits its activity [11].

To further investigate the effect of AKAP12, an AKAP12 overexpression plasmid was constructed and 5-azacytidine was used to treat leukemia cells BALL-1 and HuT 78. Cell proliferation experiments showed that the proliferation of leukemia cells was inhibited by the overexpression of AKAP12 and 5-azacytidine treatment. The 5-azacytidine treatment may promote the expression of AKAP12 in order to exert its inhibitory effect. It has long been reported that AKAP12 is a gene with tumor suppression properties, and it is frequently methylated and inactivated in malignant tumors

[16]. To verify whether AKAP12 has an effect on the cell cycle and apoptosis of BALL-1 and HuT 78, flow cytometry was applied. The results showed that AKAP12 was able to regulate the cell cycle, and a large number of cells were arrested in G0/G1 phase, followed by S phase, and fewer cells entered in G2/M phase. Furthermore, AKAP12 promotes apoptosis. Again, the question as to whether AKAP12 increased the drug sensitivity of leukemia cells was investigated.

Doxorubicin is a widely used antibiotic. Results show that AKAP12 plays a role similar to 5-azacytidine in increasing the chemosensitivity of leukemia cells to doxorubicin. A study supports the conclusion of this study that some drugs for example, increase drug sensitivity by enhancing receptor activity through pathways involving AKAP. Whether AKAP12 increases the drug sensitivity of leukemia cells is less studied. Hence, it deserves further study.

Multiple signaling pathways may be involved in the development of leukemia [17]. It has been reported that the PI3K/Akt/mTOR signaling pathway is a key activated pathway, and it is also closely related to the WNT/β-catenin pathway, Notch pathway and TGF-β pathway[18]. In this study, Akt and PI3K were activated by phosphorylation, and the activity of PI3K/Akt pathway was increased to promote the progression of leukemia. However, overexpression of AKAP12 or 5-azacytidine treatment inhibited the above process. Ecker *et al* found that targeting PI3K/Akt hyperactivation induced chronic lymphocytic leukemia cell death [19]. Evangelisti *et al* also found that targeting PI3K/Akt/mTOR pathways in leukemia hindered leukemia progression [20]. These studies supported the finding of this study, showing that AKAP12 inhibited PI3K/Akt activity.

This study remains limited to preliminary *in vitro* studies. The effects of 5-azacytidine in leukemia needs further research, including *in vivo* studies and clinical trials, to determine whether the effect of 5-azacytidine on leukemia is directly through the PI3K/Akt signaling pathway. In subsequent studies, pathway inhibitors will be employed, the animal leukemia models will be established, and the toxicity of 5-azacytidine to normal cells will be determined. Methylation of the promoter region of AKAP12 has been observed in many cancers, but currently there are a few detailed studies on the methylation of the promoter of AKAP12 in leukemia, but according to several existing studies, the expression of AKAP12 in leukemia is at a lower level. The BALL-1 and HuT78 cells used in this study are cells with low expression of AKAP12, one of the possible reasons is the methylation of the promoter region of AKAP12.

However, one of the limitations of this study is that the mechanism is not clear enough. In the follow-up, further studies as to whether the methylation of the promoter region of AKAP12 leads to the decrease of AKAP12 expression and its impact on cell processes will be carried out.

CONCLUSION

The findings of this study show that AKAP12 is lowly expressed in leukemia cells, and that the expression of AKAP12 is increased after treatment with methyltransferase inhibitor, azacytidine. Overexpression of AKAP12 inhibits the proliferation of leukemia cells, regulates cell cycle, and promotes apoptosis. Thus, AKAP12 inhibits PI3K/Akt signaling pathway.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Senjun Liu and Shuping Liu designed the study and carried them out; Yebo Zhong supervised the data collection, analyzed the data, interpreted the data; Qi You prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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