Decitabine inhibits the proliferation of human T-cell acute lymphoblastic leukemia molt4 cells and promotes apoptosis partly by regulating the PI3K/AKT/mTOR pathway

GANG ZHANG[1], XIAOHUI GAO[2], XIAOYAN ZHAO[1], HAIBING WU[1], MINCHAO YAN[1], YUAN LI[1], HUI ZENG[1], ZHAONING JI[3] and XIAOJUN GUO[1]

Departments of [1]Hematology and [2]Pediatrics, First Affiliated Hospital of Jiaxing University, Jiaxing, Zhejiang 314000; [3]Department of Medical Oncology, The Cancer Center, Yijishan Hospital of Wannan Medical College, Wuhu, Anhui 241001, P.R. China

Received May 13, 2019; Accepted September 11, 2020

DOI: 10.3892/ol.2021.12601

Abstract. T cell acute lymphoblastic leukemia (T-ALL) is a highly aggressive hematological cancer; however, there is a lack of effective chemotherapeutic or targeted drugs for the treatment of T-ALL. Decitabine is a DNA demethylation agent but it has not been used for T-ALL treatment. Therefore, the present study aimed to assess the inhibitory effect of decitabine on T-ALL molt4 cells and determine its regulatory role in the PI3K/AKT/mTOR pathway. Molt4 cells were stimulated with decitabine *in vitro*, after which cell proliferation, apoptosis and cell cycle analyses were performed to assess cell viability. Subcellular morphology was observed using transmission electron microscopy. Expression levels of phosphate and tension homology (PTEN), genes involved in the PI3K/AKT/mTOR pathway and the corresponding downstream genes were analyzed using reverse transcription-quantitative PCR and western blotting. The results showed that decitabine induced apoptosis, inhibited proliferation and arrested molt4 cells in the G_2 phase. Following decitabine intervention, an increase in the number of lipid droplets, autophagosomes and mitochondrial damage was observed. At concentrations of 1 and 10 μM, decitabine downregulated the expression of PI3K, AKT, mTOR, P70S6 and eukaryotic initiating factor 4E-binding protein 1, which in turn upregulated PTEN expression; however, 50 μM decitabine downregulated PTEN levels. Overall, these results demonstrated that decitabine reduced the viability of molt4 cells partly by inhibiting the PI3K/AKT/mTOR pathway via PTEN, especially at low decitabine concentrations.

Correspondence to: Professor Xiaojun Guo, Department of Hematology, First Affiliated Hospital of Jiaxing University, 1882 Zhonghuan South Road, Jiaxing, Zhejiang 314000, P.R. China
E-mail: 921950782@qq.com

Key words: T cell acute lymphoblastic leukemia, decitabine, DNA methylation, phosphatase and tensin homolog, PI3K/AKT/mTOR pathway

Introduction

T cell acute lymphoblastic leukemia (T-ALL) is a highly aggressive hematological cancer caused by the malignant transformation of T cell progenitor cells. T-ALL accounts for 10-15% and 20-25% of pediatric and adult ALL cases, respectively (1). Compared with B cell ALL, T-ALL has lower event-free survival (EFS) and 5-year overall survival (OS) rate (2), as well as a higher recurrence rate, incidence of chemotherapeutic resistance and a poorer prognosis (3). At present, the therapeutic outcomes of T-ALL remain unsatisfactory, and ~20% of patients experience recurrence after chemotherapy (4). Therefore, novel combination chemotherapeutic or targeted drug regimens are required to improve therapeutic effects and reduce the rates of drug resistance (5).

The PI3K/AKT/mTOR pathway is closely associated with the occurrence, proliferation and metastasis of blood cancer and other malignant tumors (6,7). Abnormal activation of the PI3K/AKT/mTOR pathway is a common feature of cancer, characterizing T-ALL diseases and often leading to the malignant transformation of T cells (8). Phosphate and tension homology (PTEN) is a tumor suppressor gene that can prevent the proliferation of cancer cells and promotes apoptosis by dephosphorylating phosphatidylinositol (3-5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2). This thereby inhibits the activation of the PI3K/AKT/mTOR pathway (9). The PTEN gene is deleted or weakly expressed in leukemia, which was found to be associated with promoter methylation (9). Furthermore, downregulation of PTEN expression leads to continuous activation of the PI3K/AKT signaling pathway (10,11).

In the epigenetic regulation of ALL cells, key microRNAs (miRs), including miR-21, -22, -93-a and -221, activate the PI3K/AKT/mTOR signaling pathway through PTEN inhibition, thus affecting the migration activity of ALL cancer cells, drug resistance, and adverse outcomes in pediatric patients (12). Another common epigenetic change that mediates tumor occurrence is DNA methylation, which leads to the silencing of tumor suppressor genes (TSGs), thereby inducing the transformation of normal cells into cancer cells (13).

Therefore, inhibiting DNA methylation and activating silent TSGs could serve as a new approach for cancer therapy (14). Decitabine is a demethylation agent exerting antitumor effects that can reverse the methylation of CpG sites and restore methylation patterns in the silent TSGs (15).

Decitabine has been used for the treatment of myelodysplastic syndrome and acute/chronic myeloid leukemia (16-18). However, decitabine has not been used for T-ALL treatment, and basic research demonstrating its therapeutic effect for T-ALL is limited. The present study aimed to investigate the inhibitory effects of decitabine on the T-ALL molt4 cell line. In addition, decitabine-mediated regulation of PTEN expression, genes in the PI3K/AKT/mTOR pathway and downstream oncogenes were analyzed.

Materials and methods

Cell lines and cell culture. Contaminant-free molt4 cell line with short tandem repeat lineage certification was provided by Jiangsu KGI Biotechnology Co., Ltd.. The culture medium contained 90% RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (Shanghai ExCell Biology, Inc.). Cells were cultured at 37˚C with 5% CO_2 in a saturated humidity environment. Upon reaching 80-90% confluence, cells were centrifuged at 220 x g for 5 min at room temperature and passaged at a 1:3 ratio. Further experiments were carried out, when the cells reached the required density.

Cell proliferation assay. Molt4 cells were cultured in 96-well culture plates at a concentration of $5x10^4$ cells/ml and 100 μl cell culture medium per well. After 24 h of incubation at 37˚C, the medium was discarded, and the cells were washed with phosphate buffer saline (PBS). Culture medium containing varying concentrations of decitabine (0.00625, 0.0125, 0.025, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 μM) was added for the cell proliferation assay. A negative control (without decitabine) was also prepared and added to eight wells in each group. The 96-well plate cells were cultured for 24, 48, 72 and 96 h and treated with 10 μl Cell Counting kit (CCK)-8 (Dojindo Molecular Technologies, Inc.) for 3 h according to the manufacturer's protocol. After blending, optical density (OD) was measured at λ=450 nm. The half maximal inhibitory concentration (IC_{50}) was calculated using probability unit weighted regression (Bliss method), and the drug inhibition rate was calculated utilizing the following formula (19): Inhibition rate (%)=negative control group-experimental group/negative group x100. The data are based on three independent experiments with four replicates per sample in each experiment.

Apoptosis analysis. Annexin V-FITC/PI double staining kit (Jiangsu Kaiji Biotechnology Co., Ltd.) was used to detect cell apoptosis, both early (Annexin V-FITC+/PI-) and late stage (Annexin V-FITC+/PI+). Logarithmic phase cells were inoculated into 6-well plates. After cell adherence, the aforementioned culture medium was replaced with medium containing different concentrations of decitabine (1, 10 and 50 μM). A negative control without decitabine was also prepared. After 96 h of decitabine stimulation at 37˚C, the cells were collected, centrifuged at 300 x g at 37˚C for 5 min and washed with PBS. Next, the cell density was adjusted to $5x10^5$ cells in 500 μl binding buffer (Jiangsu KGI Biotechnology Co., Ltd.) using an Automated Cell Counter (Thermo Fisher Scientific, Inc.), and subsequently 5 μl Annexin V-FITC and 5 μl propidium iodide were added to the 500-μl cell suspension for incubation at 37˚C for 10 min in dark. Apoptotic cells were detected using flow cytometry with FACSCalibur (CellQuest Pro, version 6.0, BD Biosciences) after 5 to 15 min of reaction in the dark.

Cell cycle detection. Logarithmic growth phase cells were inoculated into six-well plates, and the culture medium was replaced with medium containing different concentrations of decitabine (1, 10 and 50 μM) after adherence. After 96 h in culture at 37˚C, cells were digested with 0.25% pancreatin (without EDTA) and washed with PBS. Subsequently, $5x10^5$ cells were collected and fixed with 70% ethanol overnight at 4˚C. After washing with 70% PBS and centrifugation at 220 x g for 5 min at room temperature, cells were treated with 100 ml RNase A and incubated at 37˚C for 30 min. Next, cells were incubated with 400 μl PI dye (Jiangsu Kaiji Biotechnology Co., Ltd.) and mixed at 4˚C for 30 min in the dark. Red fluorescence was measured (FACSCalibur; BD Biosciences) and analyzed (FACScan software; version 6.0; BD Biosciences) at the re-excitation wavelength of 488 nm. Three independent experiments were carried out.

Transmission electron microscope (TEM) analysis. Molt4 cells stimulated with varying concentrations of decitabine (1, 10 and 50 μM) and the negative control were analyzed after 96 h of incubation at 37˚C. The cells were washed with PBS, placed in fresh tubes, fixed with PBS containing 2.5% glutaraldehyde for 2 h at 4˚C, and washed with 0.1 M PBS. Next, cells were post-fixed with 1% osmium acid solution for 3 h at 4˚C, dehydrated sequentially in 50, 70, 90 and 100% ethanol for 15 min each (three times in 100% ethanol), embedded in epoxy resin and cut into 50-nm thick sections. After 3% uranium citrate staining for 30 min at room temperature, samples were observed under a TEM (magnification, x50) and images were captured.

Reverse transcription-quantitative (RT-q)PCR. Cells were collected after stimulation with varying concentrations of decitabine (0, 1, 10 and 50 μM). Cells were treated with 1 ml TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), incubated with chloroform for 3 min at room temperature, and then centrifuged at 12,000 x g at 4˚C for 15 min. The supernatant was collected, mixed with 0.6 ml of isopropanol for 10 min, and centrifuged at 12,000 g at 4˚C for 10 min. After discarding the supernatant, 70% ethanol was added to the cells, and they were centrifuged at 7,500 g at 4˚C for 10 min. RNA concentration was measured after discarding the supernatant and suspending the pellet in DNase/RNase-free ultrapure water (Table SI). Reverse transcription was performed to synthesize cDNA (catalog no. K1632; Fermentas; Thermo Fisher Scientific, Inc.). After PCR amplification, the reaction system contained 10 μl real-time PCR Master Mix (catalog no. EP0702; Fermentas; Thermo Fisher Scientific, Inc.), 1 μl DNA template, 2 μl each primer and 7 μl 0.1% DEPC water. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for

Table I. Primers of target genes for reverse transcription-quantitative PCR detection.

Target gene	Forward sequence, 5'-3'	Reverse sequence, 5'-3'
4EBP1	CAAGGGATCTGCCCACCATT	AACTGTGACTCTTCACCGCC
AKT	GGCTATTGTGAAGGAGGGTTG	TCCTTGTAGCCAATGAAGGTG
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG
mTOR	ATTTGATCAGGTGTGCCAGT	GCTTAGGACATGGTTCATGG
P70S6	CGGGTACTTGGTAAAGGGGG	TGCCTTTTTAAGCACCTTCATGG
PI3K	ATGCAAATTCAGTGCAAAGG	CGTGTAAACAGGTCAATGGC
PTEN	CAAGATGATGTTTGAAACTATTCCAATG	CCTTTAGCTGGCAGACCACAA

4EBP1, eukaryotic initiating factor 4E-binding protein 1; PTEN, phosphate and tension homology.

40 sec; extension at 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec, the fluorophore (cat no. DA7600; Zhongshan Bio Tech Co., Ltd.). The customized primers used for amplification are listed in Table I and GAPDH was used as the control (Jiangsu KeyGen Biotechnology Co., Ltd.). Three independent experiments were carried out. The relative expressions of the genes were calculated utilizing the $2^{-\Delta\Delta Cq}$ method (20).

Western blotting. Cells stimulated with varying concentrations of decitabine (0, 1, 10 and 50 μM) were collected. Total proteins were extracted after cell lysis (no. KGP701-100; Cell lysis buffer for Western; Jiangsu KeyGen Biotechnology Co., Ltd.). The protein concentration was determined using the bicinchoninic acid method. The OD value was measured at the wavelength of 520 nm (Jiangsu KeyGen Biotechnology Co., Ltd.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% separating gel and 5% layering gel. For sample preparation, 15 μl protein lysate was mixed with 5 μl SDS buffer and sampled. The membrane was transferred after electrophoresis at the voltage of 60 to 90 V. After cleaning and sealing the cellulose nitrate membrane containing protein, the corresponding antibodies against eukaryotic initiating factor 4E-binding protein (4EBP)1, AKT, MTOR, P70S6, PI3K, PTEN and GAPDH were added, and the membranes were incubated overnight at 4˚C. After washing with 0.05% Tween-20 (catalog no. TA-999-TT; Thermo Fisher Scientific, Inc.), goat anti-rabbit secondary antibodies were added. Antibody details are presented Table SII. An enhanced chemiluminescence kit (Jiangsu KGI Biotechnology Co., Ltd.) was used for coloration and to capture images for analysis. Signal intensities were quantified utilizing the Gel-Pro32 analyzer software (Media Cybernetics, Inc.). The relative expression ratio of target proteins was calculated as follows: Relative expression ratio of target protein integrated optical density (IOD)/GAPDH IOD. Three independent experiments were carried out and Gel-Proanalyzer version 4.0 software (Media Cybernetics) was used for densitometry.

Statistical analysis. Data are presented as mean ± standard deviation. Unpaired Student's t-tests and Mann-Whitney tests were used to compare significant differences between groups. Normality and homogeneity of variance were calculated. Then one-way ANOVA was performed to compare differences among the groups with Tukey's post hoc test. SPSS version 18 (SPSS Inc.) was used for statistical analysis. $P<0.05$ was considered statistically significant.

Results

Decitabine inhibits the proliferation of molt4 cells. The effects of different decitabine concentrations on molt4 cell proliferation at various time points are presented in Table II. Overall, after 24 h of incubation with different concentrations of decitabine, it was observed that 100 μM decitabine had the greatest inhibitory effect on cell proliferation at 18.59% ($P<0.01$). The inhibitory rate gradually decreased with decreasing decitabine concentrations, reaching-2.62% at 0.00625 μM ($P=0.035$) (Fig. 1A). The inhibition rate of cell proliferation was 32.56% at 100 μM decitabine after 48 h of treatment ($P<0.01$). Lowering the decitabine concentration decreased the inhibition rate to 1.15% at 0.00625 mM ($P=0.286$) (Fig. 1B). Treatment with 100 μM decitabine for 72 h led to an inhibition rate of 54.20% ($P<0.01$). Treatment with lower concentrations of decitabine reduced the inhibition rate to 2.81% at 0.00625 μM ($P=0.179$). In addition, the IC_{50} was 84.461 μM after 72 h of treatment (Fig. 1C). After 96 h of stimulation, the inhibition rate was 69.85% at 100 μM decitabine. Moreover, the inhibition rate gradually decreased to 18.48% at 0.00625 μM decitabine. Therefore, the IC_{50} was 10.113 μM after 96 h of treatment (Fig. 1D). These data demonstrated that decitabine inhibited the proliferation of molt 4 cells in a dose and time-dependent manner.

Decitabine induces molt4 apoptosis. Apoptosis was detected using Annexin V-FITC/PI double staining. Flow cytometry analysis showed that the percentage of apoptotic cells in the negative control group was 2.27% (Fig. 2A). Cells treated with 1, 10 and 50 μM decitabine exhibited apoptosis rates of 20.9, 43.7 and 62.38%, respectively (Fig. 2B-E). Decitabine induced molt4 cells apoptosis in a dose-dependent manner.

Effect of decitabine on the cell cycle. In cells that were not treated with decitabine, the percentage of cells in the G_1, S and G_2 phases were 51.09, 35.52 and 13.39%, respectively (Fig. 3A). The percentage of cells in the G_1, S and G_2 phases were 49.32, 28.26 and 22.42%, respectively, after 48 h

Table II. Effects of different concentrations of decitabine on the inhibitive rate of molt4 cells proliferation at various time points.

	Time after intervention, h							
	24		48		72		96	
Concentration, μM	OD±SD	Inhibition rate, %	OD±SD	Inhibition rate, %	OD±SD	Inhibition rate, %	OD±SD	Inhibition rate, %
NC	2.25±0.03	-	2.05±0.04	-	2.01±0.04	-	2.50±0.05	-
100	1.83±0.09[b]	18.59	1.38±0.06[b]	32.56	0.92±0.03[b]	54.20	0.75±0.04[b]	69.85
50	1.90±0.07[b]	15.69	1.47±0.05[b]	27.95	1.06±0.07[b]	47.15	0.84±0.08[b]	66.36
10	1.91±0.08[b]	15.38	1.69±0.06[b]	17.20	1.71±0.07[b]	14.96	1.39±0.07[b]	44.46
5	1.95±0.05[b]	13.31	1.71±0.07[b]	16.25	1.72±0.02[b]	14.56	1.48±0.06[b]	40.77
1	2.08±0.08[b]	7.51	1.84±0.05[b]	9.96	1.73±0.08[b]	13.98	1.80±0.10[b]	27.97
0.5	2.20±0.02[a]	2.34	1.88±0.07[b]	8.13	1.81±0.08[b]	10.00	1.81±0.10[b]	27.39
0.1	2.21±0.07	1.98	1.88±0.07[b]	7.90	1.82±0.08[b]	9.30	1.83±0.04[b]	26.73
0.05	2.30±0.04	-2.22	1.94±0.07[a]	5.39	1.85±0.06[b]	8.02	1.84±0.05[b]	26.36
0.025	2.27±0.04	-0.67	1.94±0.08[a]	5.22	1.86±0.05[b]	7.40	1.94±0.06[b]	22.24
0.0125	2.33±0.07	-3.27	2.00±0.03	2.08	1.95±0.04	2.85	2.00±0.10[b]	20.12
0.00625	2.31±0.04[a]	-2.62	2.02±0.03	1.15%	1.95±0.07	2.81	2.04±0.05[b]	18.48

[a]P<0.05 and [b]P<0.01 vs. NC. OD, optical density; NC, negative control.

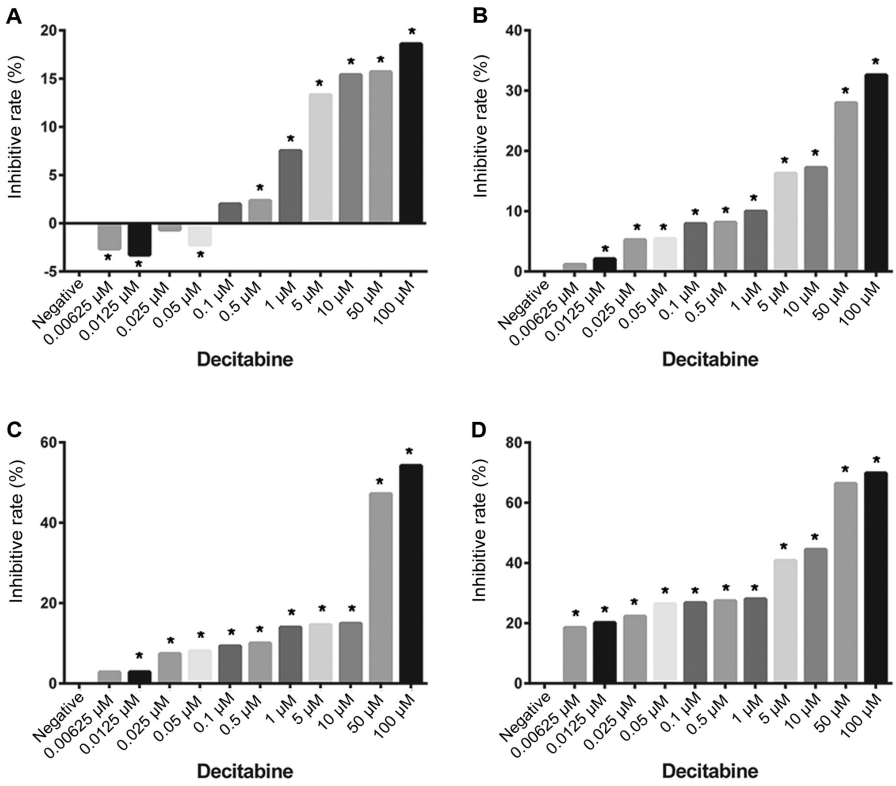

Figure 1. Effect of different decitabine concentrations on the inhibition of molt4 cells proliferation at (A) 24, (B) 48, (C) 72 and (D) 96 h. The presented data are based on three independent experiments with four replicates per treatment in each experiment. *P<0.05 vs. negative control.

treatment with 50 μM decitabine (Fig. 3B). After 72 h treatment with 50 μM decitabine, the percentage of cells in the G_1, S and G_2 phases were 46.00, 25.57 and 28.44% (Fig. 3C). The percentage of cells in the G_1, S and G_2 phases were 45.13, 19.56 and 35.31%, respectively, after 96 h treatment with 50 μM decitabine (Fig. 3D). Increasing the decitabine concentration decreased the proportion of cells in the S phase and gradually increased the proportion of cells in the G_2 phase (Fig. 3A-D). Three independent experiments were carried out and provided very similar results. These results indicated that the majority

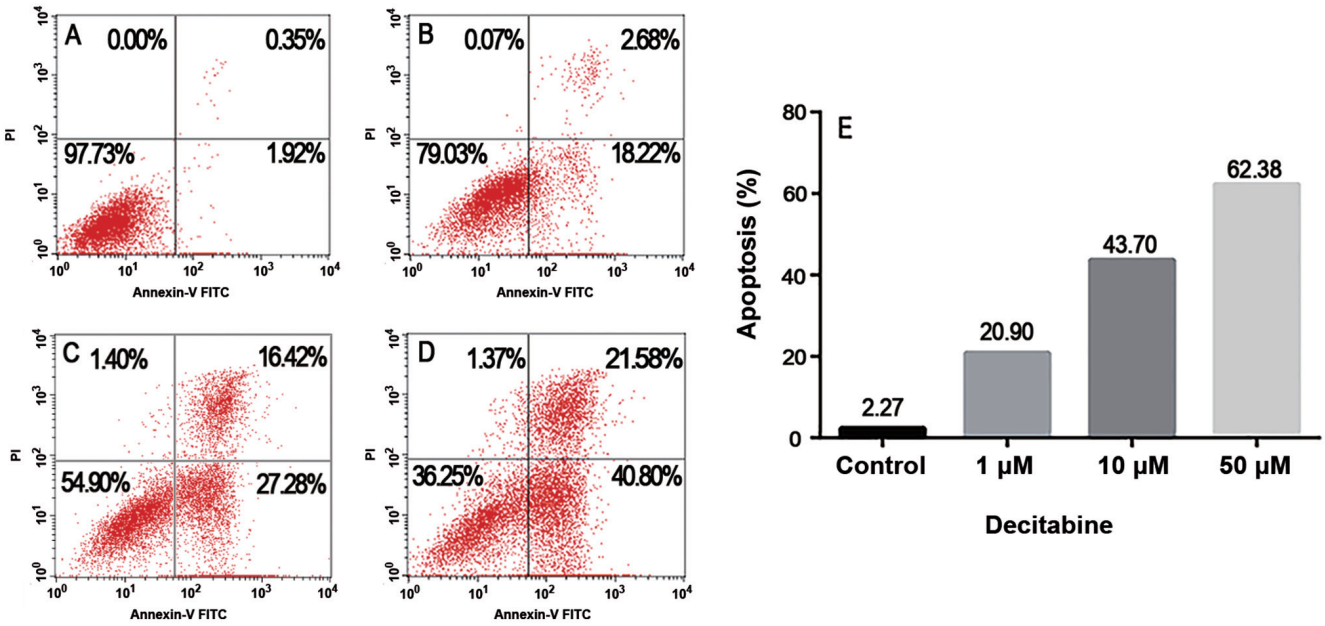

Figure 2. Effect of different decitabine concentrations on the apoptosis of molt4 cells at 96 h. (A) Negative control. (B) 1 μM. (C) 10 μM. (D) 50 μM. (E) Bar chart showing the percentage of apoptotic cells. Quadrant meaning: Upper left, Annexin V-/PI +; upper right, Annexin V+/PI+; lower left, Annexin V-/PI-; lower right, Annexin V+/PI-.

of molt4 cells were arrested in the G_2 phase after decitabine stimulation.

TEM detection. Molt4 cells that were not treated with decitabine showed normal cell morphology, and lipid droplets were observed in the cytoplasm of a subset of the cells (Fig. 4A and B). The number of lipid droplets (Fig. 4C and D) and damaged mitochondrial cells (Fig. 4C-H) increased gradually with higher decitabine concentrations. Autophagic body-like structures were observed in the cells after treatment with 50 μM decitabine (Fig. 4H). The effect of decitabine on molt4 was mainly manifested as mitochondrial damage and the autophagosomal-like structure formation, and the degree of damage was concentration-dependent.

Decitabine downregulates mRNA levels PI3K/AKT/mTOR pathway components partly by upregulating PTEN expression. The RT-qPCR results showed that PI3K mRNA levels gradually decreased at decitabine concentrations >10 μM compared with those in the negative control group after 96 h of intervention. In addition, the mRNA levels of AKT, mTOR, p70S6 and 4EBP1 significantly decreased, when the cells were treated with decitabine concentrations >1 μM. PTEN mRNA expression was upregulated in cells treated with 1 and 10 μM decitabine but was downregulated after treatment with 50 μM decitabine (Fig. 5 and Table III). Decitabine could downregulate mRNA levels PI3K/AKT/mTOR pathway with concentration increasing.

Decitabine downregulates levels of proteins involved in the PI3K/AKT/mTOR pathway. Western blotting results indicated that PTEN protein levels were slightly increased after 96 h of intervention with 1 to 50 μM decitabine. However, the protein expression of PI3K, AKT, mTOR, total P70S6 and 4EBP1 was downregulated (Fig. 6 and Table SIII). However, only at 50 μM decitabine intervention, protein levels of mTOR, P70S6 and 4EBP1 had statistical difference compared with 0 μM. No other statistical difference was found. So, Decitabine downregulated levels of proteins involved in the PI3K/AKT/mTOR pathway at 50 μM concentration.

Discussion

The present study demonstrated that decitabine can induce apoptosis and inhibit proliferation in molt4 cells. Cell cycle analysis showed that the majority of molt4 cells were arrested in the G_2 phase, indicating that the cells did not enter the mitotic phase, since they needed to repair damaged DNA after replication (21). In terms of affected subcellular structures, decitabine increased the production of lipid droplets and autophagosomes and promoted mitochondrial damage.

In the PI3K/AKT/mTOR signaling pathway, PI3K can phosphorylate PIP2 to PIP3. Moreover, PIP3 recruits and activates AKT (Ser 308 phosphorylation) as a secondary messenger. Following AKT activation, the mTOR complex is further activated to regulate protein synthesis (22,23). The primary downstream effectors include ribosomal p70S6 and 4EBP1, which further promote cell proliferation, and invasion (24). PTEN can block the phosphorylation of PIP2 to PIP3, thereby preventing the activation of this pathway (25,26). In the present study, analysis of PI3K/AKT/mTOR pathway showed that decitabine downregulated the mRNA expression of components involved in the pathway, including PI3K, AKT, mTOR, P70S6 and 4EBP1. In addition, PTEN mRNA expression was upregulated in cells treated with low decitabine concentrations of 1 and 10 μM. However, PTEN mRNA expression was downregulated at high decitabine concentrations of 50 μM. Higher decitabine concentrations caused a slight increase in PTEN protein levels and significantly downregulated the mRNA expression of PI3K, AKT and mTOR. Decreased 4EBP1 and

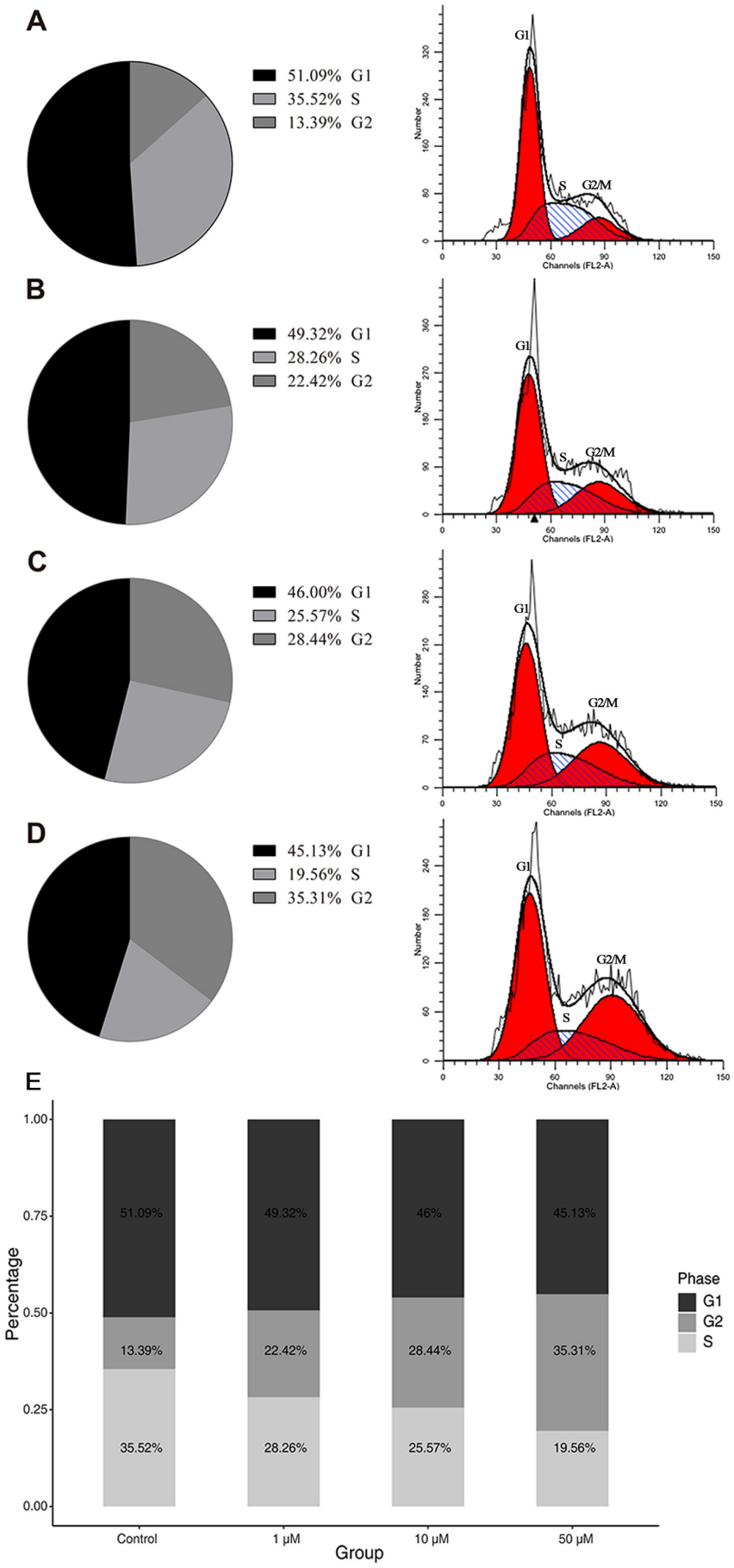

Figure 3. Effect of different decitabine concentrations on the cell cycle of molt4 cells at 96 h. (A) Negative control. (B) 1 μM. (C) 10 μM. (D) 50 μM. The pizza chart shows the proportion of cells in each phase of the cell cycle. (E) Cell cycle of molt4 cells treated with decitabine at different concentrations for 96 h and detected using flow cytometry.

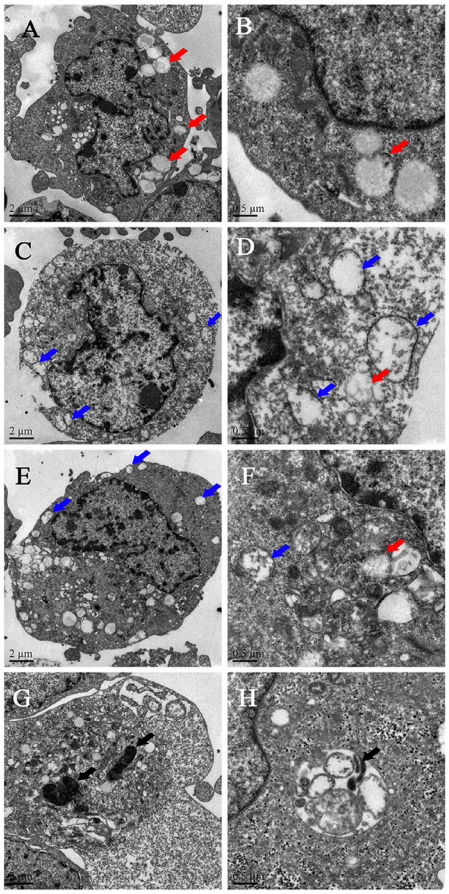

Figure 4. Subcellular morphology of molt4 cells after treatment with different decitabine concentrations for 96 h observed under a transmission electron microscope. (A and B) Negative control. (C and D) 1 μM. (E and F) 10 μM. (G and H) 50 μM. The red, blue and black arrows indicate lipid droplets, mitochondrial damage and an autophagic body-like structure, respectively. Scale bar, 2 or 0.5 μm.

P70S6 expression in mRNA and protein levels also indicated that decitabine indirectly inhibited the PI3K/AKT/mTOR pathway. Therefore, altered PTEN mRNA expression does not completely explain the observed inhibitory effect of decitabine on the viability of molt4 cells. Overall, low decitabine concentrations may have upregulated PTEN mRNA expression by suppressing the methylation of *PTEN* DNA to inhibit the activity of the PI3K/AKT/mTOR pathway in molt4 cells, turn affecting cell proliferation and promoting apoptosis. At high decitabine concentrations, other mechanisms may exist that can regulate the PI3K/AKT/mTOR pathway and further influence the proliferation of molt4 cells. However, the biological effects of the slightly increased PTEN protein levels should not be overlooked, although no significant expression changes were observed.

In a previous study, 5 μM decitabine treatment for 4 days increased the sensitivity of drug-resistant molt4 cells to daunorubicin and doxorubicin, and downregulated the expression of ABCB1/P-glycoprotein (27). In present results, it indicated that the proliferative inhibition IC_{50} of decitabine was 84.461 μM at 72 h, and the IC_{50} was 10.113 μM at 96 h of treatment, therefore prolonged treatment with decitabine requires a lower drug concentration to inhibit cell proliferation and promote apoptosis. Prolonging the duration of drug action (>96 h) or increasing the concentration of decitabine (>5 μM) could further inhibit cell activity. These results suggested that treatment with 5-10 μM decitabine for 4 days can inhibit the proliferation of molt4 cells and increased the sensitivity of these cells to decitabine. These effects are further enhanced with a prolonged treatment time and higher drug concentration. However, in clinical applications, toxicity still needs to be considered, and low concentrations administered for long periods may potentially diminish the toxic side effects in normal cells (28,29).

In another study, the proliferation inhibition rate of 0.5 μM decitabine on molt4 cells was 69.76±2.2% and the apoptotic rate was 37.75±3.87%. Under these conditions, the percentage of G_0/G_1 cells was significantly increased. The lactotransferrin (LTF) gene was analyzed after screening for differentially expressed genes in the transcriptome. The methylation rate of the CpG sites of LTF gene promoter decreased from 72.3 to 45.0% after 72 h treatment with 0.5 μM decitabine, which in turn upregulated LTF gene expression (30). The concentration of decitabine used in the present report was low with a short treatment period, but it effectively inhibited cell proliferation and promoted apoptosis. In the present study, the inhibition rate was ~10% after 72 h of 0.5 μM decitabine intervention, and the apoptotic rate ranged only between 2.27 and 20.90%. In addition, LTF acts as a tumor suppressor protein that inhibits the proliferation and metastasis of tumors and is known to exert antimicrobial, anti-viral and immune regulatory effects (31-33). LTF expression levels are low in molt4 cells without decitabine intervention but were observed to significantly increase after intervention (30). Therefore, these results suggested that LTF expression plays a major role in the inhibition of cancer cells under short-term treatment with low concentrations of decitabine. The present results confirmed that 1 and 10 μM decitabine can inhibit proliferation, promote apoptosis and induce G_2 cycle arrest by increasing PTEN expression and inhibiting the PI3K/AKT/mTOR pathway in molt4 cells. However, the downregulation of PTEN expression decreased at 50 μM decitabine, which suggested that the PI3K/AKT/mTOR pathway is not regulated via DNA methylation inhibition of the PTEN gene at relatively high decitabine concentrations. Other basic studies have showed that not only PTEN, but also Notch 1 (3) and RAS (34), can regulate the PI3K/AKT/mTOR pathway. Therefore, the upregulated expression of other TSGs could also be involved in the decitabine-induced decrease in the viability of tumor cells and regulation of the PI3K/AKT/mTOR pathway.

Various studies have indicated that different concentrations of decitabine are required to inhibit molt4 viability (27,30). Therefore, other potential factors may affect the results, such as culture conditions, cell passage, cell activity and gene expression levels. In addition, the findings of the aforementioned studies and the present report indicate that decitabine exerts inhibitory effects on molt4 cells in a time- and dose-dependent manner (30). The effect of decitabine on cells progresses over time and with increased concentrations. At low concentrations and short treatment times, decitabine preferentially acts on the more active DNA methylation genes that usually induce TSGs. At higher concentrations and prolonged treatment times, decitabine can inhibit a higher number of DNA methylation genes and consequently affect tumor cell viability (30).

Table III. Reverse transcription-quantitative PCR results for PI3K/AKT/mTOR pathway and related gene expression in molt4 cells after treatment with different decitabine concentrations.

Decitabine, μM	PI3K	AKT	mTOR	PTEN	p70s6	4EBP1
0	1.000±0.014	1.000±0.001	1.000±0.007	1.000±0.006	1.000±0.008	1.000±0.004
1	1.013±0.005	0.983±0.002[b]	0.968±0.002[a]	1.034±0.007[b]	0.981±0.006[a]	0.967±0.011[a]
10	0.932±0.003[a]	0.967±0.0004[b]	0.943±0.006[b]	1.001±0.007	0.979±0.003[a]	0.958±0.007[b]
50	0.929±0.001[a]	0.937±0.014[a]	0.928±0.001[b]	0.952±0.009[b]	0.952±0.010[b]	0.931±0.006[b]

[a]P<0.05 and [b]P<0.01 vs. 0 μM. 4EBP1, eukaryotic initiating factor 4E-binding protein 1; PTEN, phosphate and tension homology.

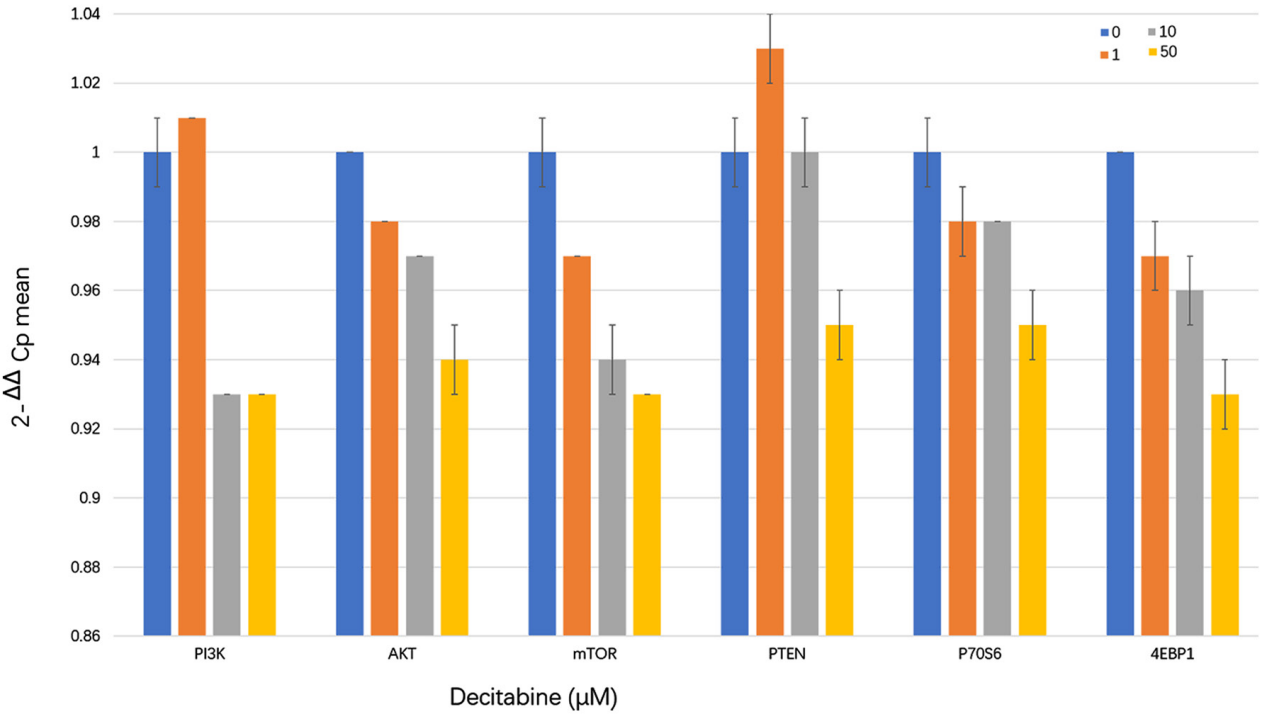

Figure 5. Reverse transcription-quantitative PCR detection of the PI3K/AKT/mTOR pathway and related gene expression in molt4 cells for different concentrations of decitabine. It indicated decitabine downregulates mRNA levels PI3K/AKT/mTOR pathway components partly by upregulating PTEN expression. Presented data are based on three independent experiments. Error bars indicate standard deviations.

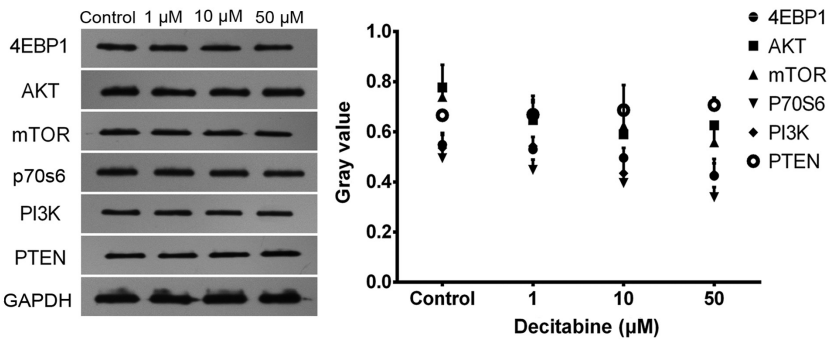

Figure 6. Effect of different decitabine concentrations on PI3K/AKT/mTOR pathway and related protein expression in molt4 cells. It indicated decitabine downregulates levels of proteins involved in the PI3K/AKT/mTOR pathway. Presented data are based on three independent experiments. Relative expression ratio of target proteins was calculated as follows: Relative expression ratio of target protein IOD/GAPDH IOD. IOD, integrated optical density.

Therefore, analyses based on epigenomics and transcriptome studies with a single concentration and at a single time point do not completely reflect the inhibitory mechanism of decitabine on tumor cells (30,35,36).

Previous findings demonstrated that decitabine exerts its effects on molt4 cells in a dose- and time-dependent manner (27,30). The present study only analyzed changes in the gene expression of PTEN and genes involved in the PI3K/AKT/mTOR pathway, and did not detect the methylation levels of CpG sites and the phosphorylation levels of AKT and mTOR. Furthermore, epigenomics and transcriptome analyses were not conducted. Decitabine can markedly inhibit DNA methyltransferase (27). In further studies, transcriptome analyses will be performed to investigate gene expression in molt4 cells at different concentrations of decitabine and at different time points. The dynamic inhibitory effects of decitabine should be evaluated to elucidate the underlying mechanisms of function in molt4 cells and other T-ALL cell lines.

In conclusion, decitabine can inhibit cell proliferation, induce cell cycle arrest in the G_2 phase and induce apoptosis in molt4 cells. Additionally, on the subcellular morphological level, decitabine can increase the production of lipid droplets and autophagosomes and induce mitochondrial damage. Decitabine intervention inhibited the activity of the PI3K/AKT/mTOR pathway, whereas PTEN levels were upregulated upon treatment with and 10 μM decitabine. However, PTEN levels were downregulated after treatment with 50 μM decitabine, which suggested the PI3K/AKT/mTOR pathway is not regulated via DNA methylation inhibition of the PTEN gene this concentration. The present results provided evidence to highlight the importance of investigating the dose and time-dependent effects of decitabine on molt4 cells.

Acknowledgements

The authors would like to thank Mr. Pang Shiwei for their comments and suggestions during the revision of the manuscript.

Funding

The study was funded by the Medical and Health Science and Technology Plan Project of Zhejiang Province (grant no. 2019KY693), the Jiaxing Key Discipline Fund (grant no. SY18-Z-06) and Hospital-Level Fund of the First Affiliated Hospital of Jiaxing University (grant no. 2016-YA-08).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GZ and XJG designed the study and wrote the manuscript. GZ, XHG and XZ collected the data and performed the experiments. HW, MY and YL analyzed the data. HZ and ZJ analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Akkapeddi P, Fragoso R, Hixon JA, Ramalho AS, Oliveira ML, Carvalho T, Gloger A, Matasci M, Corzana F, Durum SK, *et al*: A fully human anti-IL-7Rα antibody promotes antitumor activity against T-cell acute lymphoblastic leukemia. Leukemia 33: 2155-2168, 2019.
2. Teachey DT and Pui CH: Comparative features and outcomes between paediatric T-cell and B-cell acute lymphoblastic leukaemia. Lancet Oncol 20: e142-e154, 2019.
3. Hales EC, Taub JW and Matherly LH: New insights into Notch1 regulation of the PI3K-AKT-mTOR1 signaling axis: Targeted therapy of γ-secretase inhibitor resistant T-cell acute lymphoblastic leukemia. Cell Signal 26: 149-161, 2014.
4. Karrman K and Johansson B: Pediatric T-cell acute lymphoblastic leukemia. Genes Chromosomes Cancer 56: 89-116, 2017.
5. Rahmat LT, Nguyen A, Abdulhaq H, Prakash S, Logan AC and Mannis GN: Venetoclax in combination with decitabine for relapsed T-cell acute lymphoblastic leukemia after allogeneic hematopoietic cell transplant. Case Rep Hematol 2018: 6092646, 2018.
6. Fu JH, Yang S, Nan CJ, Zhou CC, Lu DQ, Li S and Mu HQ: MiR-182 affects renal cancer cell proliferation, apoptosis, and invasion by regulating PI3K/AKT/mTOR signaling pathway. Eur Rev Med Pharmacol Sci 22: 351-357, 2018.
7. Wang X, Han L, Zhang J and Xia Q: Down-regulated NRSN2 promotes cell proliferation and survival through PI3K/Akt/mTOR pathway in hepatocellular carcinoma. Dig Dis Sci 60: 3011-3018, 2015.
8. Silva A, Girio A, Cebola I, Santos CI, Antunes F and Barata JT: Intracellular reactive oxygen species are essential for PI3K/Akt/mTOR-dependent IL-7-mediated viability of T-cell acute lymphoblastic leukemia cells. Leukemia 25: 960-967, 2011.
9. Carnero A and Paramio JM: The PTEN/PI3K/AKT pathway in vivo, cancer mouse models. Front Oncol 4: 252, 2014.
10. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, Caparros E, Buteau J, Brown K, Perkins SL, *et al*: Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med 13: 1203-1210, 2007.
11. Medyouf H, Gao X, Armstrong F, Gusscott S, Liu Q, Gedman AL, Matherly LH, Schultz KR, Pflumio F, You MJ and Weng AP: Acute T-cell leukemias remain dependent on Notch signaling despite PTEN and INK4A/ARF loss. Blood 115: 1175-1184, 2010.
12. Ultimo S, Martelli AM, Zauli G, Vitale M, Calin GA and Neri LM: Roles and clinical implications of microRNAs in acute lymphoblastic leukemia. J Cell Physiol 233: 5642-5654, 2018.
13. Pan Y, Liu G, Zhou F, Su B and Li Y: DNA methylation profiles in cancer diagnosis and therapeutics. Clin Exp Med 18: 1-14, 2018.
14. Yang Y, Zhao L, Huang B, Hou G, Zhou B, Qian J, Yuan S, Xiao H, Li M and Zhou W: A new approach to evaluating aberrant DNA methylation profiles in hepatocellular carcinoma as potential biomarkers. Sci Rep 7: 46533, 2017.
15. Momparler RL, Côté S, Momparler LF and Idaghdour Y: Inhibition of DNA and histone methylation by 5-Aza-2'-deoxycytidine (Decitabine) and 3-deazaneplanocin-A on antineoplastic action and gene expression in myeloid leukemic cells. Front Oncol 7: 19, 2017.
16. Almasri J, Alkhateeb HB, Firwana B, Sonbol MB, Damlaj M, Wang Z, Murad MH and Al-Kali A: A systematic review and network meta-analysis comparing azacitidine and decitabine for the treatment of myelodysplastic syndrome. Syst Rev 7: 144, 2018.
17. He PF, Zhou JD, Yao DM, Ma JC, Wen XM, Zhang ZH, Lian XY, Xu ZJ, Qian J and Lin J: Efficacy and safety of decitabine in treatment of elderly patients with acute myeloid leukemia: A systematic review and meta-analysis. Oncotarget 8: 41498-41507, 2017.
18. Xie M, Jiang Q and Xie Y: Comparison between decitabine and azacitidine for the treatment of myelodysplastic syndrome: A meta-analysis with 1,392 participants. Clin Lymphoma Myeloma Leuk 15: 22-28, 2015.

19. Uitdehaag JC, de Roos JA, van Doornmalen AM, Prinsen MB, Spijkers-Hagelstein JA, de Vetter JR, de Man J, Buijsman RC and Zaman GJ: Selective targeting of CTNBB1-, KRAS- or MYC-driven cell growth by combinations of existing drugs. PLoS One 10: e0125021, 2015.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Shi P, Zhang L, Chen K, Jiang Z, Deng M, Zha J, Guo X, Li P and Xu B: Low-dose decitabine enhances chidamide-induced apoptosis in adult acute lymphoblast leukemia, especially for p16-deleted patients through DNA damage. Pharmacogenomics 18: 1259-1270, 2017.
22. Simioni C, Martelli AM, Zauli G, Melloni E and Neri LM: Targeting mTOR in acute lymphoblastic leukemia. Cells 8: 190, 2019.
23. Bertacchini J, Heidari N, Mediani L, Capitani S, Shahjahani M, Ahmadzadeh A and Saki N: Targeting PI3K/AKT/mTOR network for treatment of leukemia. Cell Mol Life Sci 72: 2337-2347, 2015.
24. Fransecky L, Mochmann LH and Baldus CD: Outlook on PI3K/AKT/mTOR inhibition in acute leukemia. Mol Cell Ther 3: 2, 2015.
25. Wang X, Huang H and Young KH: The PTEN tumor suppressor gene and its role in lymphoma pathogenesis. Aging (Albany NY) 7: 1032-1049, 2015.
26. Pulido R: PTEN inhibition in human disease therapy. Molecules 23: 285, 2018.
27. Onda K, Suzuki R, Tanaka S, Oga H, Oka K and Hirano T: Decitabine, a DNA methyltransferase inhibitor, reduces P-glycoprotein mRNA and protein expressions and increases drug sensitivity in drug-resistant MOLT4 and Jurkat cell lines. Anticancer Res 32: 4439-4444, 2012.
28. Atallah E, Kantarjian H and Garcia-Manero G: The role of decitabine in the treatment of myelodysplastic syndromes. Expert Opin Pharmacother 8: 65-73, 2007.
29. Bohl SR, Bullinger L and Rücker FG: Epigenetic therapy: Azacytidine and decitabine in acute myeloid leukemia. Expert Rev Hematol 11: 361-371, 2018.
30. Liu J, Huang C, Cheng H, Tang G, Hu X, Zhou H, Wang J and Yang J: Effects of decitabine against acute T lymphoblastic leukemia cell line Molt4. Zhonghua Xue Ye Xue Za Zhi 36: 230-234, 2015 (In Chinese).
31. Deng M, Zhang W, Tang H, Ye Q, Liao Q, Zhou Y, Wu M, Xiong W, Zheng Y, Guo X, *et al*: Lactotransferrin acts as a tumor suppressor in nasopharyngeal carcinoma by repressing AKT through multiple mechanisms. Oncogene 32: 4273-4283, 2013.
32. Giansanti F, Panella G, Leboffe L and Antonini G: Lactoferrin from milk: Nutraceutical and pharmacological properties. Pharmaceuticals (Basel) 9: 61, 2016.
33. Drago-Serrano ME, Campos-Rodriguez R, Carrero JC and de la Garza M: Lactoferrin: Balancing ups and downs of inflammation due to microbial infections. Int J Mol Sci 18: 501, 2017.
34. Ksionda O, Melton AA, Bache J, Tenhagen M, Bakker J, Harvey R, Winter SS, Rubio I and Roose JP: RasGRP1 overexpression in T-ALL increases basal nucleotide exchange on Ras rendering the Ras/PI3K/Akt pathway responsive to protumorigenic cytokines. Oncogene 35: 3658-3668, 2016.
35. Kim YI, Park SW, Kwon HS, Yang HS, Cho SY, Kim YJ and Lee HJ: Inhibin-α gene mutations and mRNA levels in human lymphoid and myeloid leukemia cells. Int J Oncol 50: 1403-1412, 2017.
36. Davies C, Hogarth LA, Dietrich PA, Bachmann PS, Mackenzie KL, Hall AG and Lock RB: p53-independent epigenetic repression of the p21(WAF1) gene in T-cell acute lymphoblastic leukemia. J Biol Chem 286: 37639-37650, 2011.