HSH2D contributes to methotrexate resistance in human T-cell acute lymphoblastic leukaemia

JING WANG and YIYING XIONG

Department of Hematology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P.R. China

Received February 10, 2020; Accepted July 27, 2020

DOI: 10.3892/or.2020.7772

Abstract. Acute lymphoblastic leukaemia (ALL) is a malignant proliferative disease that originates from B-lineage or T-lineage lymphoid progenitor cells. Resistance to chemotherapy remains an important factor for treatment failure. The aim of the present study was to investigate drug resistance in T-cell ALL (T-ALL). Bioinformatics analysis of Oncomine and Gene Expression Omnibus data was performed to evaluate the expression of haematopoietic SH2 domain containing (HSH2D) in various lymphomas. HuT-78 cells with HSH2D overexpression and or knockdown were constructed, and the effect on related downstream signalling molecules was detected. To study the effect of HSH2D on methotrexate (MTX) resistance, cell cycle and apoptosis analyses were conducted using flow cytometry, and MTT and EdU assays were used to detect the effect of MTX resistance and HSH2D gene expression on the biological function of HuT-78 cells. Via the analysis of the data sets, it was identified that the expression of HSH2D was downregulated in T-ALL compared with B-cell ALL. Western blotting and reverse transcription-quantitative PCR demonstrated that the overexpression of HSH2 resulted in the inhibition of CD28-mediated IL-2 activation. In related experiments with drug-resistant cell lines, it was determined that HSH2D expression is necessary for HuT-78 cells to be resistant to MTX. In conclusion, the results suggested that HSH2D serves an important role in the resistance of T-ALL to MTX, which provides a potential research target for the study of drug resistance of T-ALL.

Introduction

Acute lymphoblastic leukaemia (ALL) is a type of malignant proliferative disease that originates from B-lineage or T-lineage lymphoid progenitor cells (1). ALL is mainly composed of primitive and immature lymphocyte clones with high specificity (2). Patients with ALL, both adults and paediatrics, face unique clinical challenges. In adults, ALL is a rare disease with poor prognosis, and the 5-year overall survival rate is 30-40% for patients <60 years of age and 15% for patients >60 years of age (3,4). The prognosis of recurrent disease is very poor, and the median survival time is only 6 months (5). Moreover, ALL is the most common malignant tumour in paediatrics, and thus it is an important cause of cancer-related mortality in children.

The treatment strategies for leukaemia include systemic chemotherapy, local radiotherapy and surgical treatment, among which chemotherapy is the most important clinical method (6). In recent years, single drug plus small dose chemotherapy has been improved to which greatly improves the therapeutic effect in patients with ALL (7). Currently, the chemotherapy drugs for T-cell Acute lymphoblastic leukaemia (T-ALL) include etoposide and methotrexate (MTX) (8,9). Although most patients with T-ALL achieve remission after treatment, the presence of resistant leukaemia cells means ~20% of patients do not accomplish remission, which results in pain to patients and their families (10). Therefore, in-depth study of the mechanism of T-ALL drug resistance has an important social and medical value.

Early T-cell precursor (ETP) of ALL (ETP-ALL) was identified to be a subtype of T-ALL in 2009, and this subtype accounts for up to 15% of T-ALL and has a high risk of becoming refractory (11,12). To understand and overcome ETP-ALL, researchers have analysed the differences between ETP-ALL and classic T-ALL at the genomic level, and with the publication of the ETP-ALL-related gene expression profile, numerous differentially expressed genes have been identified; for example, whole-exome sequencing in adult ETP-ALL revealed a high rate of DNA methyltransferase 3 α mutations (13,14). However, the specific functions of a large number of differentially expressed genes in ETP-ALL, and whether these have potential effects on treatment requires further research and verification. Furthermore, to the best of our knowledge, the specific role of HSH2D in T-ALL has not been previously reported.

Haematopoietic SH2 domain containing (HSH2D) is an important signalling molecule that affects T-cell activation (15,16). It has been revealed that HSH2D can inhibit the transcriptional activation of the IL-2 promoter, especially at the RE/AP element of IL-2, which is mediated by CD28 (17,18). Considering that the expression of HSH2D in ETP-ALL is

Correspondence to: Dr Yiying Xiong, Department of Hematology, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Yuzhong, Chongqing 400016, P.R. China
E-mail: xiongyiying@foxmail.com

Key words: haematopoietic SH2 domain containing, T-cell acute lymphoblastic leukaemia, methotrexate, resistance

higher compared with that in classical T-ALL, it was hypothesized that HSH2D may serve a role in IL-2-dependent human T-lymphoma cells and affect the chemotherapy response.

Materials and methods

Database analysis. The Haferlach leukaemia data set, which included patients of ALL, from the Oncomine database (https://www.oncomine.org/) was analysed (19). HSH2D expression was assessed in leukaemia tissue compared with healthy tissue, and differences of $P=1x10^{-4}$ were considered to be significant. To further assess the expression of HSH2D in T-ALL, the results of a differential microarray based on GEO data were analysed (NCBI GEO database ETP-ALL; dataset record GDS4299; P=0.0268) (13,20).

Cell culture. HuT-78 cells (BeNa Cell Culture Collection) were maintained in DMEM (Thermo Fisher Scientific, Inc.) supplementing with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

For knockdown of HSH2D, a small interfering (si)RNA targeting HSH2D (150 nM; siHSH2D, 5'-UGGUUAUUC UGUUCAUCUCUGTT-3' and 5'-CAGAGAUGAACAGAA UAACCATT-3'; siHSH2D-2, 5'-AGCTGGAGTGGAATG GCACAGTCTATT-3' and 5'-TAGACTGTGCCATTCCAC TCCAGCTTT-3') and the corresponding negative control (50 nM; NC; 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3') were designed by Shanghai GenePharma Co. Ltd. For HSH2D overexpression, HSH2D overexpression plasmid (pcDNA3.1-HSH2D) was also purchased from Shanghai GenePharma Co., Ltd. Cells were transfected using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. Cells were harvested 48 h after transfection for cell cycle analysis, reverse transcription-quantitative PCR (RT-qPCR), EdU assay and western blotting.

According to the manufacturer's instructions, 50 μg/ml recombinant human IL-2 (Beyotime Institute of Biotechnology; cat. no. P5115) was prepared via dissolution in room temperature. Briefly, HSH2D overexpressing cells ($2x10^5$ cells/well) were seeded in 12-well plates and incubated at 37˚C with 5% CO_2 for 24 h. After carefully removing the medium, 1 μl IL-2 was added to 999 μl medium [50 ng per ml (100 U)] and the cells were cultured at 37˚C for 48 h.

Cell counting Kit (CCK)-8 assay for IC_{50} analyses. The IC_{50} of HuT-78 cell line to MTX was evaluated via CCK-8 assay. Briefly, the cells were seeded into 96-well plates at a density of $5x10^3$ cells per well and left overnight in 37˚C. The cells were then treated with different concentrations (0, 1, 2, 4, 8, 16 and 32 nmol) of MTX (Selleck Chemicals; cat. no. S1210) at 37˚C for 24 h. Then, 10 μl CCK-8 solution (Wuhan Boster Biological Technology, Ltd.) was added into each well and incubated for 4 h at 37˚C. Subsequently, the absorbance of each well was determined using a microplate reader (BioTek ELx800; BioTek China) at 450 nm. Each sample was designed with five repeats and each experiment was performed ≥3 times. The cytotoxic effect of the treatment was determined as percentage of viability as compared to untreated cells, with the following equation (1): Cell viability (%) = Absorbance of sample cells/Absorbance of untreated cells x100. Moreover, the IC_{50} is 50% inhibition concentration, that is, the concentration corresponding to Absorbance of sample cells/Absorbance of untreated cells = 50%.

RT-qPCR. Total RNA was extracted using an RNA Sample Total RNA kit (Qiagen GmbH) according to the manufacturer's instructions. GAPDH served as a reference gene. cDNA templates were constructed via reverse transcription of RNA performed using a BestarTM qPCR RT kit (Shanghai Xinghan Biological Technology Co., Ltd.) under the following conditions: 37˚C for 15 min and 98˚C for 5 min to synthesize the first strand of cDNA. The final RT-qPCR reaction mixture had a volume of 20 μl and contained 10 μl Bestar® SybrGreen qPCR master mix (Shanghai Xinghan Biological Technology Co., Ltd.), 1 μl each primer (10 μM; IL-2 forward: 5'-TACAAG AACCCGAAACTGACTCG-3' and reverse, 5'-ACATGAAGG TAGTCTCACTGCC-3'; and CD28 forward, 5'-CTATTTCCC GGACCTTCTAAGCC-3' and reverse, 5'-GCGGGGAGTCAT GTTCATGTA-3'), 1 μl cDNA template and 8 μl RNase-free H_2O. The thermocycling conditions used for RT-qPCR were as follows: Activation at 50˚C for 2 min, Initial denaturation at 95˚C for 2 min, followed by 40 cycles of 94˚C for 20 sec and 58˚C for 20 sec. Relative expression levels of the targeted genes were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Western blot analysis. Total proteins were extracted using a Total Protein Extraction kit (Wanleibio Co., Ltd.) according to the manufacturer's instructions. The protein concentration was quantified using BCA protein concentration determination kit (Beyotime Institute of Biotechnology; cat. no. P0012). A 20 μg sample of total protein was subjected to 10% SDS-PAGE, and the separated protein bands were transferred onto PVDF membranes, which were subsequently blocked with 5% skim milk at room temperature for 1 h. After washing, the membranes were incubated with the primary antibodies against HSH2D (cat. no. ab169172, 1:1,000; Abcam), IL-2 (cat. no. ab92381, 1:1,000; Abcam), CD28 (cat. no. ab243228, 1:1,000; Abcam), β-actin (cat. no. ab8226; 1:5,000; Abcam) or GAPDH (cat. no. ab8245, 1:2,000; Abcam) at 4˚C overnight. Next, horseradish peroxidase-conjugated secondary antibodies (cat. no. ab205719, 1:5,000; Abcam) were added and incubated with the membranes for 45 min at 37˚C. The immunostained protein blots were developed using Beyo ECL Plus reagent (Beyotime Institute of Biotechnology; cat. no. P0018FS) and detected with a Gel Imaging system (Thermo Fisher Scientific, Inc.). The relative expression levels of the targeted proteins were calculated using a Gel-Pro-Analyzer Plus 4.0 (Media Cybernetics, Inc.).

Flow cytometry analysis. According to the manufacturer's instructions, the cell cycle was evaluated using Cell Cycle and Apoptosis Analysis kit (Beyotime Institute of Biotechnology; cat. no. C1052). The treated HuT-78 cells ($2x10^6$ cells/ml) with MTX were harvested and incubated with 5 μl PI and 10 μg/ml RNase (Beyotime Institute of Biotechnology; cat. no. ST577) for 15 min at room temperature in dark.

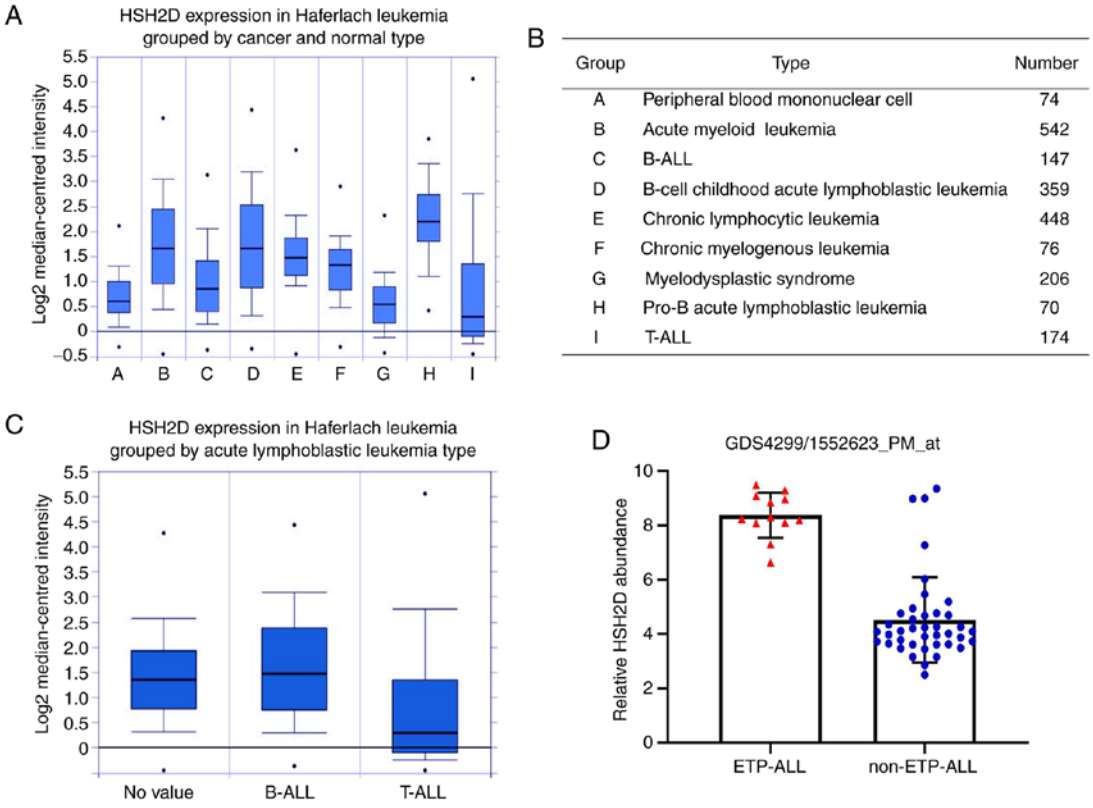

Figure 1. HSH2D expression in the ALL database of Oncomine and GEO databases. (A) HSH2D expression in the Haferlach leukaemia database. (B) Haferlach leukaemia grouping and sample size. (C) Expression of HSH2D in B-ALL and T-ALL groups in the Haferlach leukaemia database. (D) Expression of HSH2D of GDS4299/1552623 in the GEO database ($N_{ETP-ALL}$=12, $N_{non-ETP-ALL}$=40). Data are presented as the mean ± SD, which were compared using a two-tailed Student's t-test. ETP-ALL, early T-cell precursor of acute lymphocytic leukaemia; non-ETP-ALL, non-early T-cell precursor of acute lymphocytic leukaemia; HSH2D, haematopoietic SH2 domain containing.

Cell apoptosis was evaluated using Annexin V FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology; cat. no. C1062). The treated HuT-78 cells (2x10^6 cells/ml) with siRNA were resuspended and incubated with 5 μl Annexin V-FITC and 10 μl PI for 15 min, further incubated at 20-25˚C in the dark for 10-20 min and then place in an ice bath. Cell cycle and apoptosis were examined using BD FACSCalibur flow cytometer (Becton Dickinson and Company) and CFLOW plus (Becton Dickinson and Company).

EdU proliferation assay. The proliferative ability of the siRNA transfected Hut-78 cells was detected using a Cell-LightTM EdU kit (Guangzhou RiboBio Co., Ltd.). Transfected HuT-78 cells (1x10^4 cells/well) were placed into 96-well plates (Costar; Corning, Inc.) and maintained at 37˚C with 5% CO_2 for 48 h. Cellular medium was discarded and HuT-78 cells were then fixed with 4% paraformaldehyde at room temperature for 30 min (Beyotime Institute of Biotechnology). The proliferation assay was conducted following the manufacturer's instructions of the EdU kit. The images were captured using a digital fluorescent microscope (BX51 Olympus; Olympus Corporation; magnification, x200). Each experiment was repeated for three times, and five images were captured each time.

Statistical analysis. Data are presented as the mean ± SD, with n=3/group. Differences between groups were analysed using unpaired Student's t-test, and differences among ≥3 groups were detected using one-way ANOVA analysis, followed by a post hoc Tukey's test. Statistical analysis was conducted using GraphPad Prism 6.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Result

HSH2D is expressed at low levels in T-ALL. To study the expression of HSH2D in leukaemia, the Haferlach leukaemia data set from the Oncomine database was analysed. In the comparison of healthy tissue and cancer tissue, pro-B ALL had the highest expression of HSH2D (P=1x10^{-4}; Fig. 1A and B), while the expression in T-ALL was lower compared with that in peripheral blood mononuclear cells. Moreover, the expression of HSH2D in B-cell Acute lymphoblastic leukaemia (B-ALL) was higher compared with T-ALL, which was also confirmed in a group of ALL cells (Fig. 1C).

To further assess the expression of HSH2D in T-ALL, the results of a differential microarray based on GEO data was evaluated and it was found that the expression of HSH2D in patients with ETP-ALL was markedly higher compared with patients without ETP T-ALL (NCBI GEO database ETP-ALL; dataset record GDS4299) (Fig. 1D). The aforementioned results suggested that the expression of HSH2D was significantly higher in T-ALL compared with all other patients (NCBI GEO database ETP-ALL; dataset record GDS4299) (Fig. 1D), and expression in the T-ALL subtype was lower compared with that in the B-ALL subtype.

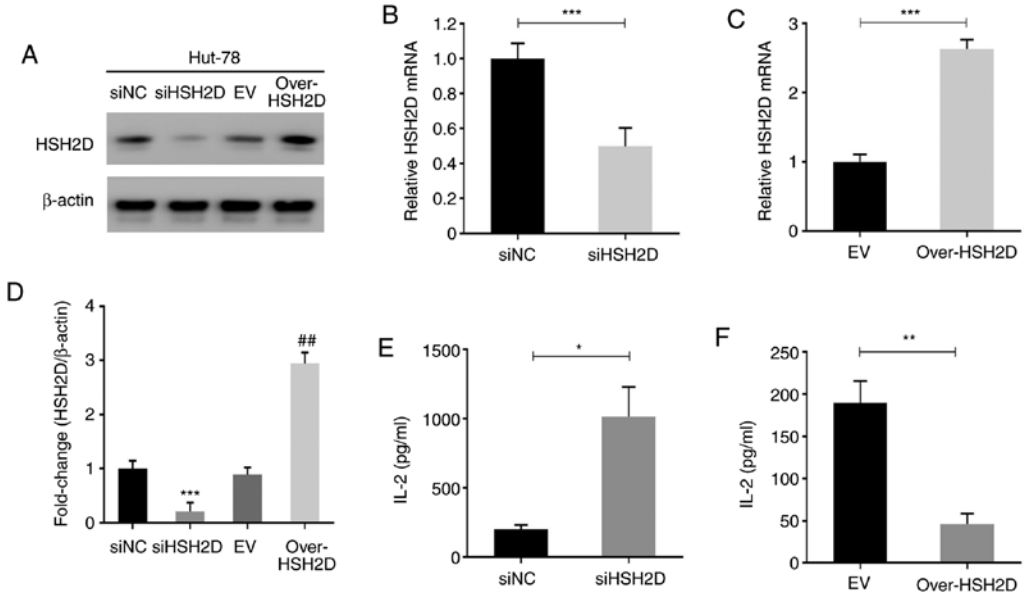

Figure 2. HUT-78 cells that overexpress and interfere with HSH2. (A) Western blotting results of siRNA interference and overexpression of HSH2D in HuT-78 cells, (D) which were semi-quantified. Reverse transcription-quantitative was conducted to assess HSH2D (B) knockdown and (C) overexpression. ELISA to detect the expression of IL-2 in cell supernatants after HSH2D (E) knockdown and (F) overexpression. Data are presented as the mean ± SD, n=3. Data were analysed using a two-tailed Student's t-test. *P<0.05, **P<0.01, ***P<0.005 vs. siNC or EV; $^{\#\#}$P<0.01 vs. EV. EV, empty vector; siNC, siRNA negative control; siHSH2, HSH2D knockdown; HSH2D, haematopoietic SH2 domain containing.

HSH2D inhibits CD28-mediated activation of IL-2. To investigate the role of HSH2D in T-ALL, an expression plasmid for HSH2D was constructed and HuT-78 cells were transfected with this plasmid and with siRNA technology to construct HSH2D overexpression and knockdown cell lines. Then, the cells were assessed via western blotting, and the effects of overexpression and knockdown were confirmed (Fig. 2A). Grey scale analysis was performed on three repeated experiments, and statistical analysis was performed, which identified that there were significant differences between the overexpression and knockdown cell lines compared with the corresponding NC (Fig. 2D). RT-qPCR was conducted to measure the mRNA expression levels of the transfected HuT-78 cells, and the results indicated that the constructed plasmid and interference sequences were effective (Fig. 2B and C).

As previous studies have reported that HSH2D can inhibit the transcriptional activation of the IL-2 promoter, the present study detected the expression and secretion of IL-2 using RT-qPCR and ELISA. The results demonstrated that overexpression of HSH2D inhibited IL-2 transcription and that HSH2D knockdown promoted exocrine IL-2 secretion (Fig. 2E and F). It has been revealed that CD28 serves a key role in the transcriptional expression of IL-2, and thus in the current study human recombinant IL-2 [50 ng per ml (100 U)] was added to HUT-78 cells transfected with the HSH2D expression vector. Western blotting results identified that the addition of exogenous IL-2 could restore HSH2D expression (Fig. 3A). Overexpression of HSH2D inhibitsCD28 and IL-2 protein expression (Fig. 3A and B), and the RT-qPCR analysis demonstrated the same results (Fig. 3C and D).

Anticancer drug methotrexate inhibits the proliferation and promotes the apoptosis of HuT-78 cells. MTX (Fig. 4A) is a chemical drug commonly used in children with ALL, and it serves a major role in the treatment of T-ALL. Cells were treated with different concentrations of MTX (0, 1, 2, 4, 8, 16 and 32 nmol), and it was identified that the survival rate of the cells was first-order concentration-dependent: The higher the concentration, the lower the survival rate (Fig. 4B). The flow cytometry results demonstrated that as the concentration of MTX increased, the apoptotic rate of the cells increased (Fig. 4C and D). Thus, the results indicated that MTX may inhibit the proliferation of tumour cells by promoting apoptosis.

HSH2D is associated with the resistance of MTX. As drug resistance to MTX is a key factor in the success of T-ALL treatment, MTX was used to culture resistant cell lines (22). The drug-resistant cell lines and drug-sensitive cell lines were compared via the CCK-8 method, and it was found that the IC_{50} value of the resistant cell lines was $1.773x10^{-8}$-$3.367x10^{-8}$ M, and the IC_{50} of the sensitive strain was $3.847x10^{-9}$-$4.802x10^{-9}$ M (Fig. 5A). Compared with the experimental results from the sensitive strain group, the resistant strain relieved the cytotoxicity induced by MTX (20 nmol) (Fig. 5C).

To determine the association between MTX resistance and HSH2D, the protein expression of HSH2D was detected via western blotting. The results demonstrated that the expression of HSH2D was markedly higher in the resistant cell lines compared with the sensitive cell lines (Fig. 5B). In addition, the experimental results of the RT-qPCR analysis indicated the same conclusion (Fig. 5D).

HSH2D is a prerequisite for the resistance of HuT-78 cells to MTX. To investigate whether HSH2D is important for MTX resistance in HuT-78 cells, a loss-of-function assay was performed. Synthetic siRNA was transfected into HuT-78 cells and interfered with HSH2D expression. It was identified

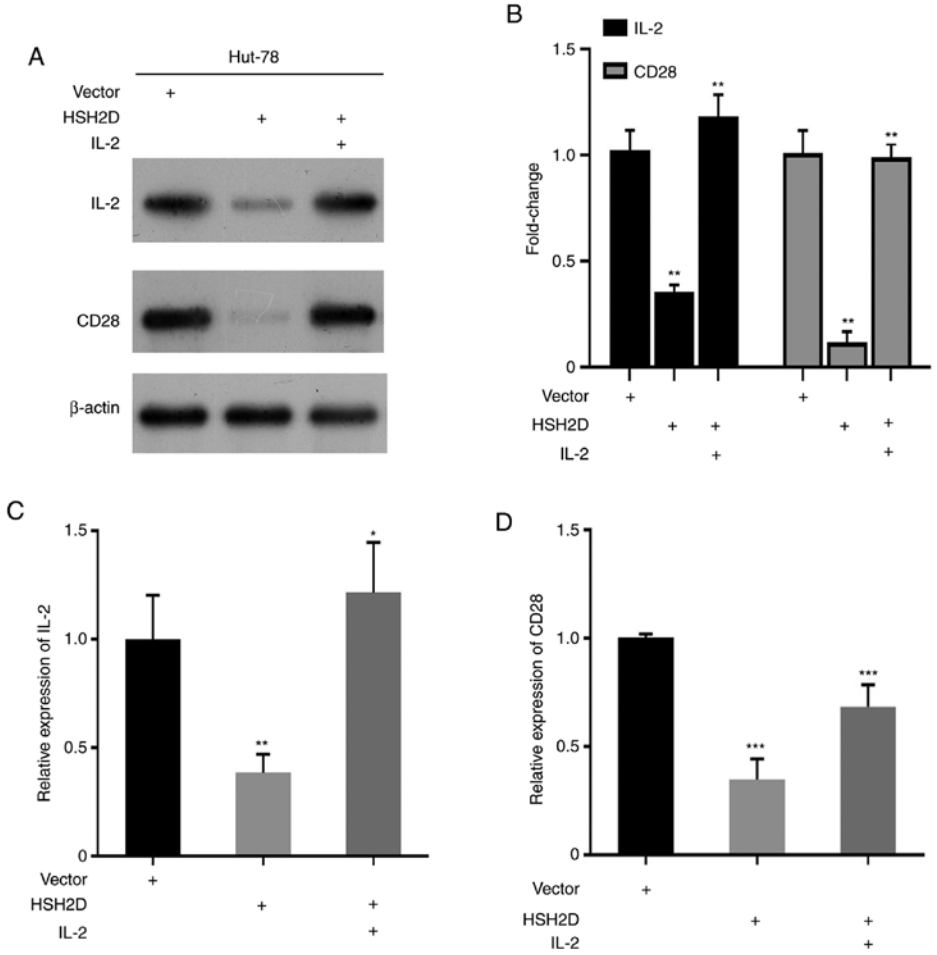

Figure 3. HSH2D regulates IL-2 and CD28. (A) Western blotting results were (B) semi-quantified to measure IL-2 and CD28 expression levels. Reverse transcription-quantitative was conducted to detect (C) IL-2 and (D) CD28 mRNA expression levels. Data are presented as the mean ± SD, n=3. Data were analysed using a one-way ANOVA with Tukey's test. *P<0.05, **P<0.01, ***P<0.005 vs. vector. HSH2D, haematopoietic SH2 domain containing.

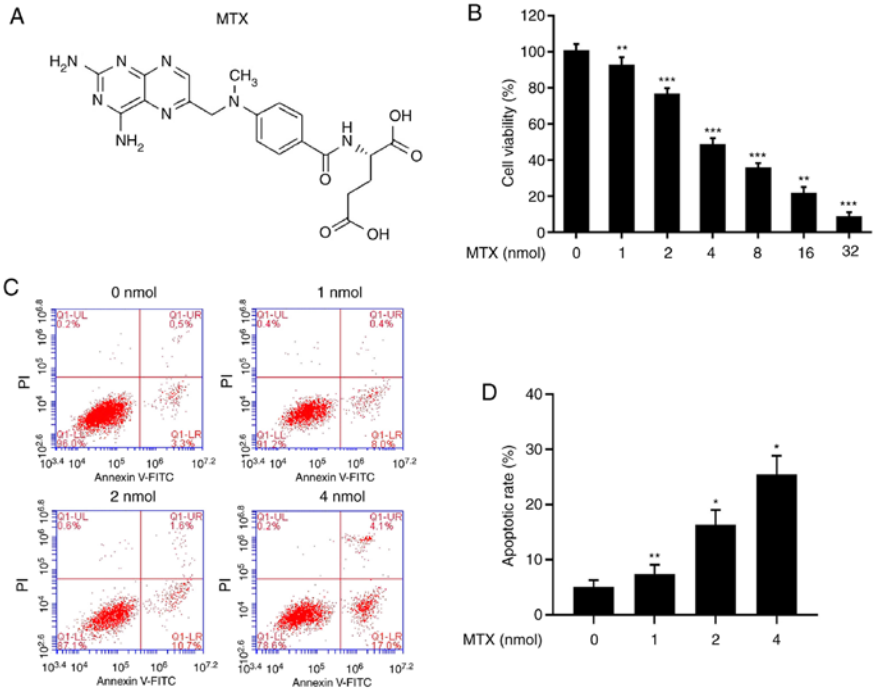

Figure 4. Effect of MTX on the biological function of HUT-78 cells. (A) Chemical formula of MTX. (B) Effect of different concentrations of MTX on cell survival. (C) Flow cytometric analysis of (D) the effects of different concentrations of MTX on cells effects of apoptosis. Data are presented as the mean ± SD, n=3, and were analysed using a one-way ANOVA with Tukey's test. *P<0.05, **P<0.01, ***P<0.005 vs. NC. MTX, methotrexate; NC, negative control.

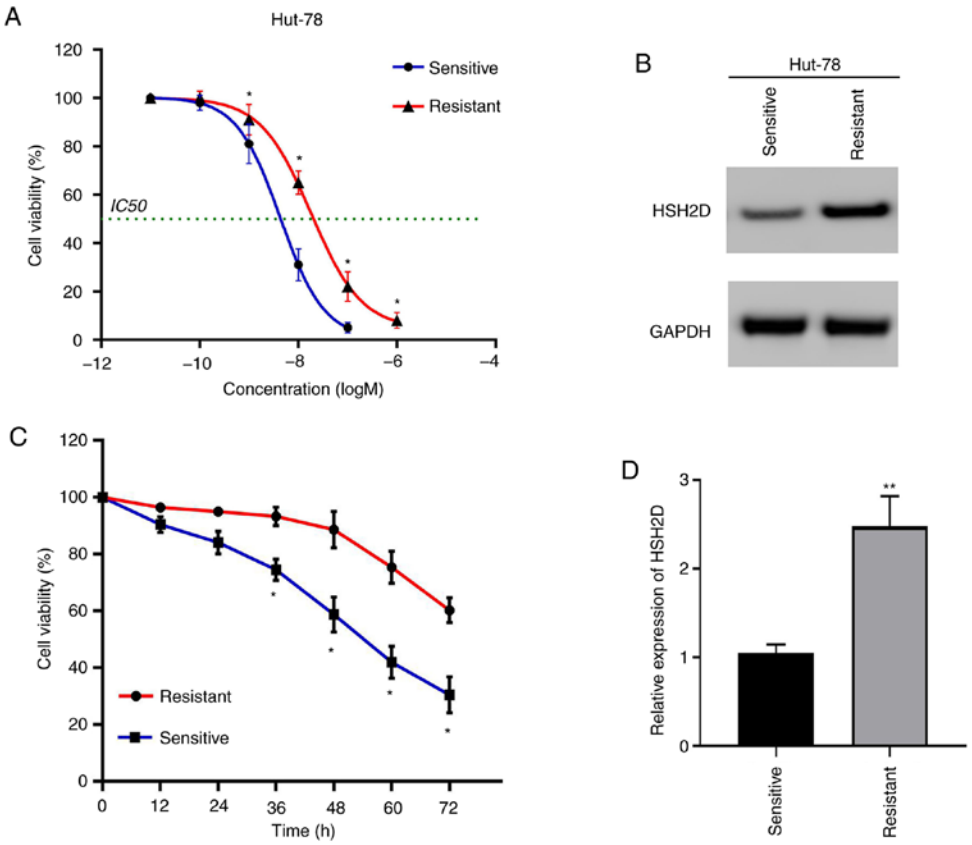

Figure 5. Relationship between HSH2D and methotrexate resistance. (A) Culture of MTX-resistant strains of HuT-78 cells. (C) Proliferation of MTX-resistant strains of HuT-78 cells. HSH2D expression in MTX-resistant strains of HuT-78 cells was detected using (B) western blotting and (D) reverse transcription-quantitative PCR. Data are presented as the mean ± SD, n=3, and were analysed using a two-tailed Student's t-test. *P<0.05, **P<0.01 vs. sensitive group. MTX, methotrexate; NC, negative control; HSH2D, haematopoietic SH2 domain containing.

that siHSH2D-1 significantly silenced HSH2D in HuT-78 cells compared with the NC, and to a greater extent than si-HSH2D-2 (Fig. 6A). Silencing HSH2D promoted MTX treatment (20 nM)-induced cytotoxicity compared with the control group (Fig. 6B). Furthermore, knockdown of HSH2D resulted in a decrease in the IC_{50} value and in the inhibition of cell proliferation after MTX treatment (Fig. 6C and D). In addition, flow cytometry analysis demonstrated that after MTX treatment and HSH2D silencing in drug-resistant cell lines, the G_1 phase of the cells was shortened and the S phase was increased (Fig. 6E and F). Collectively, these results indicated that HSH2D serves a crucial role in MTX resistance.

Discussion

The treatment strategies for leukaemia include systemic chemotherapy, local radiotherapy and surgical treatment, among which chemotherapy is the most important clinical method (23,24). Single drug low-dose chemotherapy has been improved to multi-drug high-dose treatment, which greatly increases the therapeutic effect in paediatric patients with ALL (25). At present, the chemotherapy drugs for T-ALL include MTX and etoposide. Although the majority of paediatric patients with T-ALL go into remission after treatment due to the presence of drug-resistant leukaemia cells, 20% of paediatrics do not go into remission, which is a burden on the patients and their families (26,27). Therefore, an in-depth study of the mechanism of T-ALL drug resistance has important social and medical value. In the early stage, the present results demonstrated that the expression of HSH2D was significantly increased in lymphoblastic leukaemia cells and decreased in T-ALL, which suggested that HSH2D may be associated with the malignancy and drug resistance of T-ALL tumours.

Immunotherapy has occupied a certain position in the treatment of leukaemia. IL-2, as a therapeutic agent to improve the immune function of cells, has been widely used in clinical tumour treatment (28). Previous studies have reported that, in ALL, autogenous bone metastasis often leads to leukaemia recurrence (29). To produce anti-leukemic immunity, researchers have used recombinant human IL-2, which is given immediately after transplantation (30). The clinical toxicity, haematopoiesis recovery and immune activation of patients treated with IL-2 have been compared with a group of patients without IL-2 (31). Compared with IL-2 controls, patients receiving IL-2 tended to recover neutrophils, platelets and red blood cells earlier and were discharge earlier (28). Moreover, the expression of IL-2Rα (CD25) on the cell surface is considered a powerful predictor of poor prognosis in patients with acute myeloid leukaemia (AML) (32,33). After overexpression and knockdown of HSH2D in HuT-78 cells, the present study identified that HSH2D affected the expression of IL-2 and may inhibit the CD28-mediated activation of the RE/AP element of IL-2. Du *et al* (34) revealed that serum concentrations of IL-2 in paediatric patients with ALL

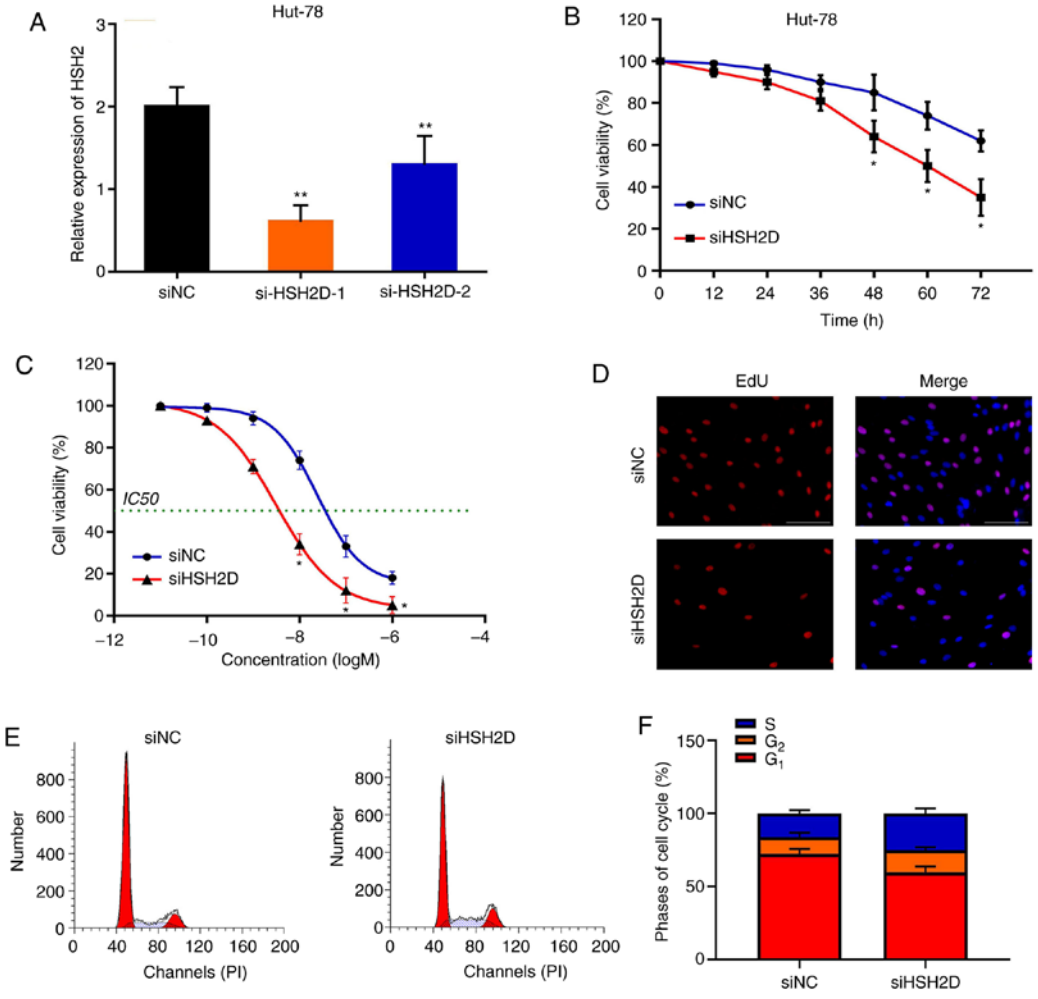

Figure 6. HSH2D is essential for MTX resistance of HuT-78 cells. (A) Silencing efficacy was evaluated using reverse transcription-quantitative PCR after transfection of two siRNAs and a NC of HSH2D. (B) Knockdown of HSH2D promoted apoptosis induced by MTX treatment in both MTX-resistant cell lines. (C) IC_{50} value of MTX was detected for both sensitive and resistant cells using a Cell Counting Kit-8 assay. (D) EdU results of the cell proliferation of drug resistant strains with HSH2D knockdown. (E) Flow cytometry was used to detected (F) the cell cycle in drug resistant strains with HSH2D knockdown. Data are presented as the mean ± SD, n=3, and were analysed using a two-tailed Student's t-test or one-way ANOVA. *P<0.05 and **P<0.01 vs. siNC, siRNA negative control; MTX, methotrexate; HSH2D, haematopoietic SH2 domain containing.

decreased significantly, and along with the analysis of the current findings, it was suggested that the change of HSH2D expression in patients with ALL results in the decrease of IL-2. IL-2 receptors, CD25 and CD28, are important genes for regulatory T-cells (35). The changes in IL-2 caused by HSH2D may be an important factor in the changes in the content of regulatory T-cells in ALL, and previous studies are using the effects of anti-CD3/CD28 coated beads and IL-2 on expanded T-cells for immunotherapy (36). Thus, HSH2D may be a potential therapeutic target for ALL.

MTX is an important component in the treatment of ALL. Currently, 80% of patients diagnosed with early pre-B-ALL are treated with combined chemotherapy, but this treatment is ineffective for 20% of patients (37). The failure of the treatment is partly due to the resistance of the cells to the therapeutic drugs (38), and drug resistance is more common in the T-lineage phenotype. Intensive treatment including high-dose MTX can improve the survival rate of patients with T-ALL to that of patients with B-ALL (39). Thus, cell resistance to drugs can be partially overcome by increased doses. The study of MTX resistance is ongoing (40). It has been revealed that the increase of dihydrofolate reductase and the damage of MTX transport are key factors in MTX resistance in childhood ALL (41). Previous studies have measured the difference in MTX resistance-related mRNA expression levels in childhood leukaemia by standardized RT-qPCR based on a competitive template, and observed that in T-ALL, compared with ordinary/pre-B-ALL, the difference in MTX resistance, dihydrofolate reductase (DHFR), thymidylate synthetase (TS) and folylpolyglutamate synthase mRNA expression levels are increased (22,42,43). The present results suggested that HSH2D is one of the important factors affecting MTX resistance, and identified via gene manipulation that HSH2D expression is necessary for HuT-78 cells resistance to MTX. The intracellular accumulation of MTX polyglutamic acid in leukaemia cells is an important determinant of the anti-leukaemia activity of MTX in paediatric patients with ALL (44). The traditional explanation for MTX resistance is due to inactivating mutations or downregulation affecting the replication factor C gene, as well as increased levels of DHFR and TS enzymes and mutations with reduced affinity for antifolates (45). Wojtuszkiewicz *et al* (22) reported that

the low cellular level of long-chain polyglutamates of MTX is an important predictor of MTX resistance and is associated with dismal therapeutic outcome. This study demonstrated the mechanism of MTX resistance to HuT-78 cells from the perspective of signal regulation. It also suggested that the regulation of HSH2D on ALL may participate in the expression of RFC and TS (22). However, this requires further in-depth investigation.

In conclusion, drug resistance is an obstacle in the use of chemotherapy for the treatment of lymphoblastic leukaemia. The present results demonstrated that in HuT-78 cells, HSH2D inhibited CD28-mediated activation of IL-2, and via the cultivation of the MTX-resistant cell line, it was identified that HSH2D expression was necessary for HuT-78 cells to be resistant to MTC. Therefore, the current results provide a potential target for the reversal of MTX resistance.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

YX conceived and designed the experiments. JW performed the experiments and analyzed the data. JW and YX wrote the manuscript. Both 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. Singh N, Frey NV, Grupp SA and Maude SL: CAR T cell therapy in acute lymphoblastic leukemia and potential for chronic lymphocytic leukemia. Curr Treat Options Oncol 17: 28, 2016.
2. Swaminathan S, Klemm L, Park E, Papaemmanuil E, Ford A, Kweon SM, Trageser D, Hasselfeld B, Henke N, Mooster J, *et al*: Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia. Nat Immunol 16: 766-774, 2015.
3. Belver L and Ferrando A: The genetics and mechanisms of T cell acute lymphoblastic leukaemia. Nat Rev Cancer 16: 494-507, 2016.
4. Roberts KG and Mullighan CG: Genomics in acute lymphoblastic leukaemia: Insights and treatment implications. Nat Rev Clin Oncol 12: 344-357, 2015.
5. Conter V, Valsecchi MG, Buldini B, Parasole R, Locatelli F, Colombini A, Rizzari C, Putti MC, Barisone E, Lo Nigro L, *et al*: Early T-cell precursor acute lymphoblastic leukaemia in children treated in AIEOP centres with AIEOP-BFM protocols: A retrospective analysis. Lancet Haematol 3: e80-e86, 2016.
6. Cooper SL and Brown PA: Treatment of pediatric acute lymphoblastic leukemia. Pediatr Clin North Am 62: 61-73, 2015.
7. Kantarjian H, Stein A, Gökbuget N, Fielding AK, Schuh AC, Ribera JM, Wei A, Dombret H, Foà R, Bassan R, *et al*: Blinatumomab versus chemotherapy for advanced acute lymphoblastic leukemia. N Engl J Med 376: 836-847, 2017.
8. Pui CH, Yang JJ, Hunger SP, Pieters R, Schrappe M, Biondi A, Vora A, Baruchel A, Silverman LB, Schmiegelow K, *et al*: Childhood acute lymphoblastic leukemia: Progress through collaboration. J Clin Oncol 33: 2938-2948, 2015.
9. Grohmann T, Penke M, Petzold-Quinque S, Schuster S, Richter S, Kiess W and Garten A: Inhibition of NAMPT sensitizes MOLT4 leukemia cells for etoposide treatment through the SIRT2-p53 pathway. Leuk Res 69: 39-46, 2018.
10. Bartram I, Erben U, Ortiz-Tanchez J, Blunert K, Schlee C, Neumann M, Heesch S and Baldus CD: Inhibition of IGF1-R overcomes IGFBP7-induced chemotherapy resistance in T-ALL. BMC Cancer 15: 663, 2015.
11. Porter DL, Hwang WT, Frey NV, Lacey SF, Shaw PA, Loren AW, Bagg A, Marcucci KT, Shen A, Gonzalez V, *et al*: Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci Transl Med 7: 303ra139, 2015.
12. Jain N, Lamb AV, O'Brien S, Ravandi F, Konopleva M, Jabbour E, Zuo Z, Jorgensen J, Lin P, Pierce S, *et al*: Early T-cell precursor acute lymphoblastic leukemia/lymphoma (ETP-ALL/LBL) in adolescents and adults: A high-risk subtype. Blood 127: 1863-1869, 2016.
13. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, Easton J, Chen X, Wang J, Rusch M, *et al*: The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 481: 157-163, 2012.
14. Neumann M, Heesch S, Schlee C, Schwartz S, Gökbuget N, Hoelzer D, Konstandin NP, Ksienzyk B, Vosberg S, Graf A, *et al*: Whole-exome sequencing in adult ETP-ALL reveals a high rate of DNMT3A mutations. Blood 121: 4749-4752, 2013.
15. Oda T, Muramatsu MA, Isogai T, Masuho Y, Asano S and Yamashita T: HSH2: A novel SH2 domain-containing adapter protein involved in tyrosine kinase signaling in hematopoietic cells. Biochem Biophys Res Commun 288: 1078-1086, 2001.
16. Lapinski PE, Oliver JA, Bodie JN, Marti F and King PD: The T-cell-specific adapter protein family: TSAd, ALX, and SH2D4A/SH2D4B. Immunol Rev 232: 240-254, 2009.
17. Pegram HJ, Purdon TJ, van Leeuwen DG, Curran KJ, Giralt SA, Barker JN and Brentjens RJ: IL-12-secreting CD19-targeted cord blood-derived T cells for the immunotherapy of B-cell acute lymphoblastic leukemia. Leukemia 29: 415-422, 2015.
18. Shapiro MJ, Powell P, Ndubuizu A, Nzerem C and Shapiro VS: The ALX Src homology 2 domain is both necessary and sufficient to inhibit T cell receptor/CD28-mediated up-regulation of RE/AP. J Biol Chem 279: 40647-40652, 2004.
19. Haferlach T, Kohlmann A, Wieczorek L, Basso G, Kronnie GT, Béné MC, De Vos J, Hernández JM, Hofmann WK, Mills KI, *et al*: Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: Report from the International microarray innovations in leukemia study group. J Clin Oncol 28: 2529-2537, 2010.
20. Gutierrez A, Kentsis A, Sanda T, Holmfeldt L, Chen SC, Zhang J, Protopopov A, Chin L, Dahlberg SE, Neuberg DS, *et al*: The BCL11B tumor suppressor is mutated across the major molecular subtypes of T-cell acute lymphoblastic leukemia. Blood 118: 4169-4173, 2011.
21. 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.
22. Wojtuszkiewicz A, Peters GJ, Van Woerden NL, Dubbelman B, Escherich G, Schmiegelow K, Sonneveld E, Pieters R, van de Ven PM, Jansen G, *et al*: Methotrexate resistance in relation to treatment outcome in childhood acute lymphoblastic leukemia. J Hematol Oncol 8: 61, 2015.
23. Ishida T, Jo T, Takemoto S, Suzushima H, Uozumi K, Yamamoto K, Uike N, Saburi Y, Nosaka K, Utsunomiya A, *et al*: Dose-intensified chemotherapy alone or in combination with mogamulizumab in newly diagnosed aggressive adult T-cell leukaemia-lymphoma: A randomized phase II study. Br J Haematol 169: 672-682, 2015.

24. Iyer NS, Balsamo LM, Bracken MB and Kadan-Lottick NS: Chemotherapy-only treatment effects on long-term neurocognitive functioning in childhood ALL survivors: A review and meta-analysis. Blood 126: 346-353, 2015.
25. Eiser C, Stride CB, Vora A, Goulden N, Mitchell C, Buck G, Adams M and Jenney MEM; National Cancer Research Institute Childhood Leukaemia Sub-Group and UK Childhood Leukaemia Clinicians Network: Prospective evaluation of quality of life in children treated in UKALL 2003 for acute lymphoblastic leukaemia: A cohort study. Pediatr Blood Cancer 64, 2017.
26. Rodríguez-Lirio A, Pérez-Yarza G, Fernández-Suárez MR, Alonso-Tejerina E, Boyano MD and Asumendi A: Metformin Induces cell cycle arrest and apoptosis in drug-resistant leukemia cells. Leuk Res Treatment 2015: 516460, 2015.
27. Frismantas V, Dobay MP, Rinaldi A, Tchinda J, Dunn SH, Kunz J, Richter-Pechanska P, Marovca B, Pail O, Jenni S, *et al*: Ex vivo drug response profiling detects recurrent sensitivity patterns in drug-resistant acute lymphoblastic leukemia. Blood 129: e26-e37, 2017.
28. Lee DA: Cellular therapy: Adoptive immunotherapy with expanded natural killer cells. Immunol Rev 290: 85-99, 2019.
29. Idris SZ, Hassan N, Lee LJ, Md Noor S, Osman R, Abdul-Jalil M, Nordin AJ and Abdullah M: Increased regulatory T cells in acute lymphoblastic leukaemia patients. Hematology 21: 206-212, 2016.
30. Locatelli F, Moretta F, Brescia L and Merli P: Natural killer cells in the treatment of high-risk acute leukaemia. Semin Immunol 26: 173-179, 2014.
31. Atkins MB: Interleukin-2: Clinical applications. Semin Oncol 29 (3 Suppl 7): S12-S17, 2002.
32. Terwijn M, Feller N, van Rhenen A, Kelder A, Westra G, Zweegman S, Ossenkoppele G and Schuurhuis GJ: Interleukin-2 receptor alpha-chain (CD25) expression on leukaemic blasts is predictive for outcome and level of residual disease in AML. Eur J Cancer 45: 1692-1699, 2009.
33. Cerny J, Yu H, Ramanathan M, Raffel GD, Walsh WV, Fortier N, Shanahan L, O'Rourke E, Bednarik J, Barton B, *et al*: Expression of CD25 independently predicts early treatment failure of acute myeloid leukaemia (AML). Br J Haematol 160: 262-266, 2013.
34. Du S, Jia Y, Tang H, Sun Y, Wu W, Sun L, Du J, Geng B, Tang C and Jin H: Immune regulation of hydrogen sulfide in children with acute lymphoblastic leukemia. Chin Med J (Engl) 127: 3695-3699, 2014.
35. Shen XH, Xu P, Yu X, Song HF, Chen H, Zhang XG, Wu MY and Wang XF: Discrepant clinical significance of $CD28^{+}CD8^{-}$ and $CD4+CD25^{high}$ regulatory T cells during the progression of hepatitis B virus infection. Viral Immunol 31: 548-558, 2018.
36. Martkamchan S, Onlamoon N, Wang S, Pattanapanyasat K and Ammaranond P: The effects of anti-CD3/CD28 coated beads and IL-2 on expanded T cell for immunotherapy. Adv Clin Exp Med 25: 821-828, 2016.
37. Faganel Kotnik B, Grabnar I, Bohanec Grabar P, Dolzan V and Jazbec J: Association of genetic polymorphism in the folate metabolic pathway with methotrexate pharmacokinetics and toxicity in childhood acute lymphoblastic leukaemia and malignant lymphoma. Eur J Clin Pharmacol 67: 993-1006, 2011.
38. Kodidela S, Suresh Chandra P and Dubashi B: Pharmacogenetics of methotrexate in acute lymphoblastic leukaemia: Why still at the bench level? Eur J Clin Pharmacol 70: 253-260, 2014.
39. Park E, Gang EJ, Hsieh YT, Schaefer P, Chae S, Klemm L, Huantes S, Loh M, Conway EM, Kang ES, *et al*: Targeting survivin overcomes drug resistance in acute lymphoblastic leukemia. Blood 118: 2191-2199, 2011.
40. Lopez-Lopez E, Gutierrez-Camino A, Bilbao-Aldaiturriaga N, Pombar-Gomez M, Martin-Guerrero I and Garcia-Orad A: Pharmacogenetics of childhood acute lymphoblastic leukemia. Pharmacogenomics 15: 1383-1398, 2014.
41. Ansari M, Sauty G, Labuda M, Gagné V, Laverdière C, Moghrabi A, Sinnett D and Krajinovic M: Polymorphisms in multidrug resistance-associated protein gene 4 is associated with outcome in childhood acute lymphoblastic leukemia. Blood 114: 1383-1386, 2009.
42. Xin N, Fen Z, Li C, Yan X and Runming J: Intracranial hemorrhage following oral low-dose methotrexate after multiple toxicities caused by high-dose methotrexate in childhood acute lymphoblastic leukemia. Front Pharmacol 10: 1072, 2019.
43. Gervasini G and Mota-Zamorano S: Clinical implications of methotrexate pharmacogenetics in childhood acute lymphoblastic leukaemia. Curr Drug Metab 20: 313-330, 2019.
44. Gonen N and Assaraf YG: Antifolates in cancer therapy: Structure, activity and mechanisms of drug resistance. Drug Resist Updat 15: 183-210, 2012.
45. Kager L, Cheok M, Yang W, Zaza G, Cheng Q, Panetta JC, Pui CH, Downing JR, Relling MV and Evans WE: Folate pathway gene expression differs in subtypes of acute lymphoblastic leukemia and influences methotrexate pharmacodynamics. J Clin Invest 115: 110-117, 2005.