***DNMT3A* Low-Expression is Correlated to Poor Prognosis in Childhood B Cell Precursor Acute Lymphoblastic Leukemia and Confers Resistance to Daunorubicin on Leukemic Cells**

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Abstract

Background

Little is known about *DNMT3A* expression and its prognostic significance in childhood B cell precursor acute lymphoblastic leukemia (BCP-ALL).

Methods

We determined *DNMT3A* mRNA expression in 102 children with BCP-ALL. Correlations with relapse-free survival (RFS) and common clinical characteristics were analyzed. *DNMT3A* was stably knocked out by CRISPR/Cas9 gene editing technology in 697 cell line. Cell proliferation activity after treated with daunorubicin was determined by CCK8 assay in *DNMT3A* KO 697 cell line.

Results

DNMT3A expression in BCP-ALL patients who were in CCR was higher than in those who got relapse ($P=0.0111$). Receiver operating characteristic curve showed prognostic significance of *DNMT3A* expression ($P=0.003$). Low expression of *DNMT3A* (<0.197) was significantly correlated with poor RFS ($P<0.001$) in children with BCP-ALL. Knock-out of *DNMT3A* in 697 cell line significantly increased IC50 of daunorubicin ($P=0.0057$), indicating elevated resistance to daunorubicin.

Conclusions

Low expression of *DNMT3A* associates with poor prognosis in children with BCP-ALL. Knock-out of *DNMT3A* confers resistance to daunorubicin on leukemic cells.

Introduction

B cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common childhood malignancy. Although cure rate of childhood BCP-ALL has been greatly improved with risk-adjusted therapy [1, 2], relapsed leukemia is still a leading cause of death for children mainly due to therapy resistance [2–4]. Thus, it is of importance to clarifying the mechanisms of therapy resistance and relapse of BCP-ALL.

DNA methyltransferase 3A (DNMT3A) catalyzes de novo DNA methylation and plays important roles in the pathogenesis of malignancies including leukemia. Furthermore, *DNMT3A* mutations in acute myeloid leukemia (AML) and T cell ALL are associated with poor prognosis of the patients [5–11]. Our previous studies have shown that *DNMT3A* mutations can be found in a few of children with BCP-ALL, and are correlated with poor prognosis [12]. However, the expression level of *DNMT3A* and its prognostic significance in BCP-ALL remains unclear.

In this study, we assessed the relationship between expression level of *DNMT3A* and prognosis in Chinese childhood BCP-ALL. Moreover, CRISPR/Cas9 has been used to knock out *DNMT3A* gene in

leukemic 697 cell line in order to explore the role of *DNMT3A* expression playing in resistance to chemotherapeutic drugs. We showed that low expression of *DNMT3A* was correlated with poor treatment outcome, knock-out of this gene resulted in obvious resistance to daunorubicin (DNR), a common chemotherapeutic drug in treatment of ALL.

Materials And Methods

Patients

A total of 102 BCP-ALL patients with available diagnostic bone marrow (BM) samples were enrolled in this study. These patients were diagnosed and treated in accordance with the Chinese Children's Leukemia Group ALL 2008 Protocol (CCLG-ALL 2008) from July 2010 to May 2014 at Beijing Children's Hospital [4]. There were 64 boys and 38 girls, aged from 1 to 13 years with a median age of 4. Thirty-four patients carried 4 types of fusion genes including *TEL-AML1*, *E2A-PBX1*, *BCR-ABL*, and *TLS-ERG*. The details of stratification and treatment according to CCLG-ALL 2008 were described previously [4, 13]. Ninety-four patients were in continuous complete remission (CR), 8 patients relapsed 2 to 62 months after diagnosis. The follow-up time ranged from 1 to 92 months (median, 59 months). Additionally, BM samples from 11 patients at continuous CR were collected and used as control.

MRD at d33 (the end of induction of remission) and d78 (before consolidation therapy) were detected using RQ-PCR targeted at Ig/TCR (*immunoglobulin* and *T cell receptor* gene rearrangements) according to European MRD laboratory guidelines [14–17].

Informed consents were obtained from all the children's parents or legal guardians.

Cell lines

Human BCP-ALL cell line 697, as a kind gift from Dr. Suning Chen at the first affiliated Hospital of Soochow University (Suzhou, China), was cultured in RPMI 1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, AusGeneX, Brisbane) and 1% penicillin/streptomycin. HEK293T cell, kindly provided by Dr. Fen Chang at Peking University Health Science Center (Beijing, China), was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Nucleic acid extraction

Mononucleated cells were separated from 1 ml of patients' BM aspirate by centrifugation with Ficoll 400 (MD Pacific Technology CO., Ltd.) and stored at -70°C until use. Total RNA of samples was extracted and reverse transcribed using Trizol Reagent (Invitrogen, USA) and MMLV reverse transcriptase (Promega, USA) according to the manufacturers' instructions respectively. Genomic DNA of 697 cell line was extracted using a Blood & Cell Culture DNA Midi Kit (TIANGEN, China) according to the manufacturer's protocol.

Quantitative analysis of DNMT3A expression

Real-time quantitative polymerase chain reaction (RQ-PCR) was performed using Power SYBR™ Green PCR Master Mix (Applied Biosystems 4367659) by an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). *GUS* (β-Glucuronidase) expression was used as an internal control. The cycling condition included pre-denaturation at 95°C for 30 seconds, followed by 40 cycles of 5 s at 95°C, 30s at 55°C and 30s at 72°C. Primers are shown in Table 1. We used the cDNA samples obtained from 697 cell line as a calibrator. The relative expression of *DNMT3A* was calculated by the method of $2^{-\Delta\Delta Ct}$. The levels of *DNMT3A* and *GUS* were tested in triplicates.

Table 1
Oligo sequences

Oligo name	Sequence	Description
DNMT3A ex7 sg F	CACCGGGGGCCCCGGGGAGTCTCAGA	sgRNA primer
DNMT3A ex7 sg R	AAACTCTGAGACTCCCCGGGCCCCC	
DNMT3A ex7 F	TTTCACGGCAAGGCAGCTGGTTG	PCR primer (445bp) for T7e1 assay
DNMT3A ex7 R	AGAGGAGAGCAGGACGGGAGGAG	
DNMT3A ex23 F	GCCACCTCTTCGCTCCGCTG	RQ-PCR primer(239bp)for clinical samples
DNMT3A ex23 R	GATGATGTCCAACCCTTTTCGCAA	
GUS F	GAAAATATGTGGTTGGAGAGCTCATT	RQ-PCR primer(101bp)as internal control for clinical samples
GUS R	CCGAGTGAAGATCCCCTTTTTA	
The capital letters underlined indicate Bsmbl sticky end.		

Lenti DNMT3A-sgRNA-Cas9 constructs

The cDNA sequence encoding sgRNA which targets a conserved sequence in exon 7 of human *DNMT3A* gene was synthesized and subcloned into LentiCRISPR-v2 plasmid (Addgene 52961, kindly provided by Dr. Jian Huang at Temple University, Philadelphia, PA) to make the lentiDNMT3A-sgRNA-Cas9 constructs. Briefly, the forward and reverse primers including 20 bp target *DNMT3A* sequence and Bsmbl sticky end were annealed and inserted into the lentiCRISPR-v2 plasmid digested with FastDigest Esp3I (Thermo Fisher Scientific, #FD0454) (Fig. 1). sgRNA primer sequences have been reported by Gundry MC et al. previously and are shown in Table 1 [18].

Lentivirus production and infection

To produce lentivirus, 6.0µg of transfer plasmid lentiDNMT3A-sgRNA-Cas9 or control plasmid lenti-CRISPR-v2 were co-transfected into HEK 293T cells with 4.5µg of packaging plasmids psPAX2 (AddGene 12260) and 3.0µg of VSV-G (AddGene 8454) using FuGENE® 6 Transfection Reagent (Promega E2692) according to the manufacturer's instructions. After incubation for 48h or 72h, the culture supernatants containing lentivirus were harvested and filtered with 0.45µm filter and stored at – 80°C. The 697 cell line (5×10^5) was infected with the lentivirus at an M.O.I. of 40, using spin-transduction (centrifuging the plate coated with 8µg/ml polybrene (SANTA CRUZ) at 1200g for 2 hours at 25°C), then were cultured for 24 hours in the incubator. On the next day, the medium was changed with fresh RPMI 1640 complete medium and the cells were cultured for another 24 hours.

T7EN1 assays for quantifying frequencies of indel mutations

Lentivirus-infected cells were selected by 1µg/ml puromycin for 2 days. Genomic DNA was extracted and used to amplify the genomic region flanking the *DNMT3A* sgRNA target site with KAPA2G Robust HotStart ReadyMix (KAPA BIOSYSTEMS KK5702) and PCR primers listed in Table 1. Then T7EN1 assay was performed using T7 Endonuclease I (NEB #M0302L) according to the Instruction Manual. The digested DNA was analyzed on electrophoresis system using a 2% agarose gel.

Western blotting

A fraction of lentivirus-infected cells was lysed in NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA). The lysates were denatured in 5× SDS loading buffer by boiling at 95°C for 10minutes and were subjected on a NuPAGE™ 4–12% Bis-Tris Protein Gels (Invitrogen). After transferred to Biotrace NT nitrocellulose Transfer Membrane (PALL, 66485), the expression of proteins was detected using following antibodies: DNMT3A (D23G1) Rabbit mAb (CST 3598), DNMT3A (D2H4B) Rabbit mAb (CST 32578), Lamin B1 Mouse mAb (proteintech 66095-1-Ig), Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680 (Invitrogen A21058),Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 800 (Invitrogen A32735). The bands were scanned by LICOR Odyssey CLX.

Cell viability

The lentivirus-infected 697 cell line was plated into 96-well plate, 10^4 /well. After treatment with 100µl DNR (0.005, 0.01, 0.05, 0.1, 0.5, 1µg/ml), the cells were cultured for 48h at 37°C in a humidified atmosphere containing 5% CO₂. Twenty microliters of Cell Counting Kit-8 (CCK-8, Yeasen 40203ES60*, Shanghai, China) solution were added to each well and mixed gently. After incubation for 1 h, optical density (OD) at 450 nm was determined using a Spectra MAX 190 microplate reader. After calibrated with non-cellular background, cell viability was calculated using a non-treatment control regarded as 100% of cell viability.

Statistical analysis

Receiver Operating Characteristic (ROC) curve was used to decide the cut-off value of low- and high-expression of *DNMT3A* (*DNMT3A*^{low} and *DNMT3A*^{high}) in leukemic cells of children with BCP-ALL. Fisher's exact test was used to test the differences in clinical characteristics and relapse rates between *DNMT3A*^{low} and *DNMT3A*^{high} patients. Relapse free survival (RFS) was defined as the date of leukemia diagnosis to the date of recurrence. Survival estimates were calculated using the Kaplan-Meier method, and the groups were compared using the log-rank test. The independent prognostic significance of *DNMT3A* expression and the common clinical features was analyzed by Cox proportional hazards model (Method: Enter). All data were analyzed with the SPSS 16.0 software package and a *P* value < 0.05 was considered statistically significant. The fitting curves of inhibitory effects of DNR on cell proliferation were plotted by GraphPad Prism 8, and half maximal inhibitory concentration (IC50) was also calculated by the software.

Results

DNMT3A expression in childhood BCP-ALL

Firstly, we determined *DNMT3A* expression in 102 newly diagnosed (ND) BCP-ALL patients and 11 patients with non-malignant hematological disease (control) by relative quantitative PCR. As a result, *DNMT3A* expression in ND patients with BCP-ALL, ranged from 0.0006594 to 1.713 with a median of 0.4363, was significantly higher than that in control patients (range: 0.08055 to 0.1865, median: 0.1147; *P* = 0.0004, Fig. 2a). Interestingly, *DNMT3A* expression in ND BCP-ALL patients who got relapse was significantly decreased compared with that in patients who were in continuous complete remission (CCR) at the last follow-up (*P* = 0.0111, Fig. 2b).

Low expression of DNMT3A indicated poor prognosis in ND BCP-ALL patients

ROC curve was used to examine the prognostic value of *DNMT3A* expression in BCP-ALL patients. It was found that the area under curve (AUC) was 0.819 (*P* = 0.003, Fig. 3a), which indicated that *DNMT3A* expression could be a potential prognostic biomarker for ND BCP-ALL patients.

A cut-off of 0.197 was chosen as both the sensitivity (75.0%) and the specificity (87.2%) were acceptable. We then divided 102 patients into two groups, 22 cases with low *DNMT3A* expression (≤ 0.197 , *DNMT3A*^{low}) and 80 cases with high *DNMT3A* expression (> 0.197 , *DNMT3A*^{high}). There was a significantly higher relapse rate in *DNMT3A*^{low} group (6 out of 22 vs 2 out of 88, Fisher's exact test, *P* = 0.001). Moreover, poor RFS was observed in the patients of *DNMT3A*^{low} group (*P* < 0.001) (Fig. 3b).

Furthermore, Cox proportional hazards regression analysis indicated the independent prognostic significance of *DNMT3A* expression with common prognostic factors as multivariates (HR = 19.195, 95% CI: 3.159 ~ 116.651, *P* = 0.001, Table 2). These findings indicated that low expression of *DNMT3A* in leukemic cells at diagnosis could be a useful indicator for disease relapse in childhood BCP-ALL.

Table 2
The independent prognostic significance of *DNMT3A* expression and other common clinical features

Features	Hazard ratio (HR)	<i>P</i>	95% CI for HR	
			Lower	Upper
<i>DNMT3A</i> expression	19.195	0.001	3.159	116.651
Age	0.000	0.988	0.000	
WBC	1.353	0.738	0.231	7.929
<i>TEL-AML1</i>	0.252	0.093	0.51	1.258
<i>E2A-PBX1</i>	1.025	1.000	0.000	
<i>BCR-ABL1</i>	1.446	1.000	0.000	
MRD at day33	3.517	0.212	0.487	25.380
MRD at day78	0.000	0.996	0.000	

Comparison of clinical features between *DNMT3A*^{low} and *DNMT3A*^{high} patients

In the next step, we analyzed the correlation of *DNMT3A* expression with common clinical characteristics such as age, gender, white blood cell (WBC) count at diagnosis and fusion genes. However, no correlation was found between *DNMT3A* expression and above clinical characteristics (Table 3).

Table 3
Correlation of *DNMT3A* expression with clinical characteristics in BCP-ALL

	<i>DNMT3A</i> ^{low} , n (%)	<i>DNMT3A</i> ^{high} , n (%)	<i>P</i>
Age			
< 1 or ≥ 10	1(4.5)	6 (7.5)	1.000
1 ~ 10	21(95.5)	74 (92.5)	
Gender			
Male	16 (72.7)	48 (60)	0.327
Female	6 (27.3)	32 (40)	
WBC(*10 ⁹ /L)			
< 50	15 (68.2)	68 (85)	0.118
≥ 50	7 (31.8)	12 (15)	
Fusion gene			
Negative	19 (86.4)	49 (61.25)	0.264
<i>TEL-AML1</i>	3 (13.6)	25 (31.25)	
<i>E2A-PBX1</i>	0 (0)	3 (3.75)	
<i>BCR-ABL1</i>	0 (0)	2 (2.5)	
<i>TLS-ERG</i>	0 (0)	1 (1.25)	
MRD at d33			
< 10 ⁻³	18(85.7)	67 (89.3)	0.701
≥ 10 ⁻³	3(14.3)	8(10.7)	
MRD at d78			
< 10 ⁻⁴	21 (100)	72 (96)	1.000
≥ 10 ⁻⁴	0(0)	3(4)	

We further analyzed the association of *DNMT3A* expression with MRD at d33 and MRD at d78 respectively, but no significant correlation between them was found (Fisher's exact test, *P* > 0.05, Table 3).

Knock-out of *DNMT3A* enhanced resistance of 697 cell line to DNR

To confirm the correlation of low expression of *DNMT3A* with poor prognosis of children with BCP-ALL, firstly, we disrupted *DNMT3A* in 697 cell line. T7 endonuclease I (T7EN1) assay showed high efficiency of the sgRNA to direct Cas9-mediated ablation of *DNMT3A* (Fig. 4a). Furthermore, as expected, Western blotting indicated that *DNMT3A* expression was remarkably reduced after infection with *DNMT3A*-sgRNA lentivirus (Fig. 4b).

DNR is one of the main chemotherapeutic drugs in induction therapy of BCP-ALL. We next tested whether knock-out of *DNMT3A* gene could cause 697 cell line to be tolerant to DNR by CCK8 assay. These cells were treated by different concentrations of DNR for 24h. It was shown that IC50 was significantly increased in the *DNMT3A*-knockout cells, indicating decreased cell viability (Fig. 5, Control vs. *DNMT3A* KO, 0.06449 vs. 0.1052 $\mu\text{g/ml}$, $P = 0.0057$). These results demonstrated that sgRNA mediated Cas9 knock-out of *DNMT3A* can causes 697 cell line to be resistant to DNR, implying that *DNMT3A* expression plays an important role in the sensitivity of leukemic cells to chemotherapeutic drugs such as DNR.

Discussion

In recent two decades, *DNMT3A* mutations have been found in approximately 20% of adult AML patients, 9% of adult T-ALL and 0 ~ 1.4% of childhood AML, and the hotspots of mutations are mainly located in exon 23 which encodes the catalytic methyltransferase domain [5–11]. *DNMT3A* mutations are associated with poor prognosis and used for risk stratification in AML [5–10], and is associated with increased age and adverse outcome in adult T-ALL [11]. However, few studies focused on the role of *DNMT3A* in BCP-ALL. Our previous study has shown that *DNMT3A* mutations can be found in exon 23 and its adjacent intron regions in a few of children with BCP-ALL (5/182, 2.7%), and may have adverse impact on prognosis [12].

As there are only a few BCP-ALL patients with *DNMT3A* mutations, we sought to determine the prognostic significance of *DNMT3A* expression in BCP-ALL. As expected, low expression leading to decreased methyltransferase activity was associated with relapse in 102 patients with BCP-ALL. Furthermore, knock-out of *DNMT3A* increased IC50 of DNR in 697 cell line, indicating the relationship of low expression of *DNMT3A* and chemoresistance.

It was reported that *Dnmt3a* loss in HSCs leads to hypomethylation of genes with a causal role in cancer, such as *Runx1* and *Gata3*. *Runx1* promotes murine erythroid progenitor proliferation and inhibits differentiation by preventing Pu.1 downregulation [19]. *Gata3* targets *Runx1* in the embryonic hematopoietic stem cell niche [20]. Thus, previous studies and ours suggest that deletion or low-expression of *Dnmt3a* result in differentiation inhibition of HSCs and allow HSCs to be propagated indefinitely in vivo [21–24], which may play an important role in leukemogenesis and resistance to chemotherapy. This may provide us with an alternative target of therapy for childhood BCP-ALL.

It has been reported that *DNMT3A* expression is directly transactivated by transcription factor WT1 in Wilms' tumor cells [25] and is negatively regulated by p53 at the transcriptional level in lung cancer[26]. In fact, overexpression of the WT1 transcript was demonstrated in children with B-ALL at diagnosis [27],

which may contribute to increased expression of *DNMT3A* in leukemic cells. However, increased expression of *TP53* by 2 to 20-fold higher in pediatric primary B-ALL than in healthy controls [28] would inhibit *DNMT3A* expression. Thus, the regulation mechanism of *DNMT3A* expression is quite complicated in different types of cancer cells and needs to be clarified especially in childhood BCP-ALL.

In summary, we associated low expression of *DNMT3A* with poor prognosis in Chinese pediatric patients with B-ALL and resistance to DNR. Furthermore, successful disruption of *DNMT3A* in 697 cell line may facilitate the studies on mechanism of relapse and chemotherapeutic resistance for childhood BCP-ALL.

Declarations

Ethics approval and consent to participate: Yes

Consent for publication: Yes

Availability of data and materials: Emails to corresponding author Zhigang Li

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Authors' contributions

Zhigang Li designed this research and revised the manuscript. Weijing Li performed experiments, interpreted/analyzed data, prepared figures and wrote the manuscript. Chanjuan Wang also performed part of the experiments and Lei Cui helped in analyzing data and preparing figures. Wei Liu and Ruidong Zhang also contributed to study design and organized informed consent. Shuguang Liu and Xiaoxi Zhao contributed patient material and provided clinical data.

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Figures

target

A

B

Figure 1

Schematic diagram of sgRNA targeting DNMT3A. a. The structure of DNMT3A gene and the three common transcripts. Black vertical lines: exons. Horizontal lines: introns. Arrow: the location of sgRNA targeting exon 7. b. The structure of lentiCRISPR v2 plasmid. The arrows indicate the sgRNA sequence.

Figure 2

DNMT3A expression in ND BCP-ALL patients and controls. a. DNMT3A expression was significantly increased in ND BCP-ALL patients compared with that of controls. b. DNMT3A expression was significantly decreased in ND BCP-ALL patients who relapsed compared with that in ND patients in CCR.

Figure 3

Prognostic significance of DNMT3A expression in 102 children with BCP-ALL. a. ROC curve analysis of DNMT3A expression with relapse as an event. B. The patients in DNMT3A_{low} group had poorer RFS than those in DNMT3A_{high} group (P<0.001).

Figure 4

LentiCRISPR/Cas9 mediated editing of DNMT3A gene in 697 cell line. a. T7e1 assay analysis of specific sgRNA-mediated indels at DNMT3A locus in 697 cell line. The lower migrating bands marking by a white arrow represent the disrupted gene alleles. b. Expression of three DNMT3A protein variants was significantly reduced in 697 cell line infected by DNMT3A-sgRNA lentivirus.

Figure 5

Knock-out of DNMT3A gene increased resistance of 697 cell line to DNR. IC50 of DNR significantly increased in DNMT3A-knockout cells (Independent-samples T test, P = 0.0057). The standard errors of the means are shown (n = 3 experiments for each drug concentration).