

Promoter hypermethylation of PTPL1, PTPN6, DAPK, p16 and 5-azacitidine inhibits growth in DLBCL

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Abstract. Aberrant hypermethylation of CpG islands of tumor suppressor is one of the mechanisms for epigenetic loss of gene function. In the present study, the methylation status of the promoter regions of protein tyrosine phosphatase (PTPN) 6, DAPK, and p16 were studied using methylation-specific polymerase chain reaction (MSP) in 26 diffuse large B cell lymphoma (DLBCL) lymphomas. In OCI-LY1 cell line, gene methylation status, expression of PTPL1 and its reactivation by DNA demethylation was determined by PCR and on the protein level by western blotting. ELISA-like reaction was used to detect global DNA methylation measurement. Induction of apoptosis by 5-azacitidine was analyzed by Annexin V/PI staining and flow cytometry. Our results show that hypermethylation of the PTPN6 gene promoter region was found in 15.4% (4/26), the DAPK gene promoter region in 30.8% (8/26), the p16 gene promoter region in 7.7% (2/26). Notably, we identified that PTPL1 was hypermethylated and transcriptionally silenced in OCI-LY1 cell line. The expression of PTPL1 was re-inducible by 5-azacytidine. 5-azacytidine also inhibits the proliferation and decreases the global methylation level of the OCI-LY1 cell line. We can conclude from our study that a higher prevalence of methylation of PTPL1, PTPN6, DAPK and p16 occur in DLBCL. Our data also highlights 5-azacytidine as a potential therapeutic candidate for DLBCL. Further studies are required to substantiate the role of methylation of PTPL1, PTPN6, DAPK and p16 as a marker in diffuse large B cell lymphoma.

Introduction

Lymphomas are solid tumors of the immune system. Diffuse large B cell lymphoma (DLBCL) is the most common lymphoid neoplasm (1). The most common symptom of

DLBCL is lymphadenopathy. General symptoms may include loss of weight, decrease in performance status, fever and night sweats (2). Although the majority of patients with DLBCL can be cured with standard rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), patients who fail R-CHOP have dismal outcomes (3). DLBCLs inherit the cytosine methylation patterns instability that exist in the germinal center B cells from which DLBCLs arise and display variable degrees of epigenetic heterogeneity. Greater epigenetic heterogeneity is linked with poor clinical outcome (4). Many novel classes of drugs are in development that may improve the treatment of DLBCL, either as single agent or in combination (5). It is clear, however, that epigenetic alterations provide an additional series of targets that can be pharmacologically modified and offer great potential to improve patient outcomes (6).

In recent years, it has become apparent that aberrant DNA hypermethylation of tumor suppressor genes can result in their inappropriate transcriptional silencing, which contributes to loss of checkpoints and related functions in cancer (7). 5-aza-2-deoxycytidine and 5-azacitidine are applied to the treatment of MDS that incorporate into DNA and inactivate DNMT by forming a covalent bond between the 5-azacytosine ring and the enzyme (8). DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B, can methylate DNA specifically at cytosines were found to be overexpressed in 48, 13 and 45% of *de novo* DLBCLs, respectively, and correlate with advanced clinical stage (9).

Protein tyrosine phosphatase (PTPL1), is also known as PTPN13, FAP-1, PTP-BAS, PTP1E, is a large non-transmembrane PTP at 270 kDa (10). Hypermethylation of the PTPL1 gene promoter is involved in a variety of cancers, such as non-small cell lung cancer, esophageal cancer, gastric and hepatocellular tumors (11,12). Hypermethylation of PTPN6, DAPK, p16 has been reported in many human tumors (13-15).

In the present study we applied MSP to determine the methylation status of the PTPN6, DAPK, p16 gene in 26 DLBCL. To evaluate the effects of 5-azacytidine, we studied cell proliferation, and apoptosis, as well as DNA methylation status in association with mRNA and protein expression levels of PTPL1 in the DLBCL cell line OCI-LY1. The results obtained from this study contributes to a better understanding of the role of methylation of tumor suppressor

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Table I. Sequences of primers used for PCR analysis.

Gene	Forward	Reverse	Temperature (°C)
MS-PCR			
PTPL1-M	CGAGTAGTTTTAGCGGTTAC	AAAACCTTCTAACGCGAACGA	58
PTPN6-M	TGTAGTTTTTCGGAGTTAGTGTCGCGC	CCTACGATCGAAAACGACGCGAACG	59
DAPK-M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	60
p16-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	60
RT-PCR			
PTPL1	GCGCTCCAGTAGCAGGAC	TCATCTGTAAATGACACACTAC	55
BCL2	CCTCTGTTTGATTCTCCTGGCTGT	TGGAAAGCGAATCTATGTTTACAGG	56
BAX	AACATGGAGCTGCAGAGGATG	CTGGTCTTGATCCAGCCAGCCAACAG	56
hCNT1	CCTCACCTGTGTGGTCCTCA	AGACCCCTCTTAAACCAGAGC	57
hENT1	TCTCCAACCTCTCAGCCACCAA	CCTGCGATGCTGGACTTGACCT	58
hENT2	ACCATGCCCTCCACCTACAG	GGGCCTGGGATGATTTTATTG	55
β-actin	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT	60

gene in DLBCL and epigenetic pathways represent relevant and promising therapeutic targets in DLBCL, highlighting that 5-azacytidine may offer a potential new therapeutic approach to improve the poor outcomes associated with DLBCL.

Materials and methods

Cell line. The OCI-LY1 cell line, derived from DLBCL, was maintained in IMDM medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), and 1% antibiotics (Gibco-Invitrogen). Cells were incubated at 37°C in a humid atmosphere at 5% CO₂ and split every 2-3 days depending on cell density.

Patients. The formalin-fixed paraffin-embedded (FFPE) tissues of 26 patients with *de novo* DLBCL with available tumor specimen at diagnosis were included in this study. The archived FFPE tissues were obtained from the Department of Pathology, Peking University Third Hospital. The histological diagnoses were established according to World Health Organization classifications. All participants were well informed, and provided a written consent form.

Bisulfite modification. For the methylation analysis, genomic DNA was extracted from cell lines and clinical samples using the E.Z.N.A.[®] Tissue DNA kit (Omega Bio-Tek, Lilburn, GA, USA). DNA (200 ng) was processed for bisulfite modification using a BisulFlash DNA Modification kit (Epigentek, New York, NY, USA) according to the manufacturer's instructions. The bisulfite-modified DNA was stored at -20°C or used immediately.

Methylation-specific PCR. DNA methylation patterns were determined by methylation-specific PCR (MSP) after bisulfite treatment of DNA. Bisulfite-modified DNA was amplified using the previously described primers PTPL1-M, PTPN6-M, DAPK-M, p16-M (Table I). Each MSP reaction was repeated to confirm the results.

RT-PCR. Briefly, total RNA was isolated from cultured cells with TRIzol reagent (Invitrogen) and concentrations assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, 1 µg of total RNA was reverse-transcribed with the Goscript[™] Reverse Transcription system (Thermo Fisher Scientific). The primer sequences used are shown in Table I.

Global DNA methylation measurement. Genome-wide methylation pattern was measured by the MethylFlash kit (Epigentek, Farmingdale, NY, USA) following the manufacturer's instructions. Cells were seeded in a 6-well plate at the density of 2.5x10⁵/ml, and then treated with 1, 5, 10, 20 µM 5-azacytidine for 3 days for an additional 24 h. DNA (total 100 ng) is detected using capture and detection antibodies and then quantified through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at 450 nm. All investigations were performed in triplicates using 2 independent replicates.

Western blotting. OCI-LY1 cells were seeded at the density of 2.5x10⁵/ml and treated with 1, 5, 10, 20 µM 5-azacytidine at 0, 24 and 48 h, a total of three times. Total protein was isolated from cultured cells and quantified using a BCA protein assay kit. Samples (60 µg protein/lane) were separated by 8 and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a nitrocellulose membrane, proteins were incubated with the following primary antibodies overnight at 4°C: anti-PTPL1 (1:200); anti-caspase-3 (1:500), and anti-β-actin (1:1,000) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Fluorescently labeled secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) were used to detect the binding of the primary antibodies. The bound proteins were visualized by scanning the membranes in an Odyssey Infrared Imaging system (LI-COR Biosciences) (16).

Cell viability assay. Cellular viability was assessed with CCK-8 assay as described previously. In brief, OCI-LY1 cells

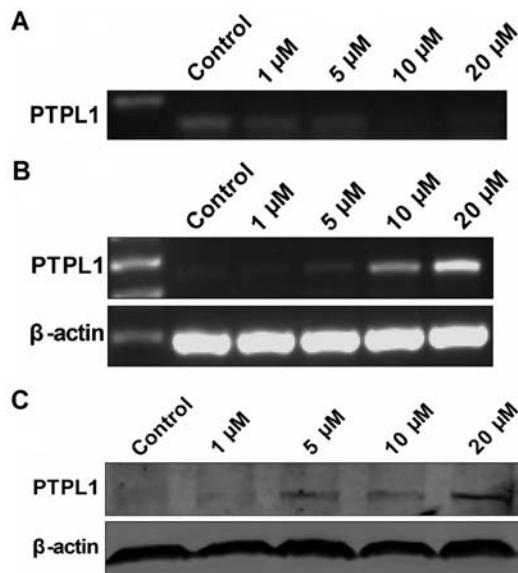


Figure 1. PTPL1 gene expression and methylation in OCI-LY1 cell line. (A) PTPL1 is methylated in OCI-LY1 cell line and can be demethylated by 5-azacytidine. (B and C) RT-PCR and western blotting showing the restored PTPL1 expression in OCI-LY1 cell lines.

were plated in 96-well plates at ~25,000 cells/well with 100 μ l culture medium. The cells were randomly divided into the control group and the test groups medially with three duplicates per group. They were then treated with 5-azacytidine at 0.1, 0.5, 1, 2, 5, 10, 20 and 50 μ M for 12, 24, 48, and 72 h, respectively. Ten microliters of CCK-8 (Dojindo, Kumamoto, Japan) accompanied every sample in each well. Non-treated cells were considered as control and incubated in similar conditions for the same period of time. After 2 h incubation at 37°C in the dark, the absorption value A of each well was detected at the wavelength of 450 nm in a Quant spectrophotometer. All procedure was repeated three times and average values were calculated. Cell inhibition rate (I%) was calculated using the following equation: $I\% = [A(\text{control}) - A(\text{treated})] / [A(\text{control}) - A(\text{blank})] \times 100\%$.

Mitochondrial membrane potential. OCI-LY1 cells were treated with 5-azacytidine at 0.1, 0.5, 1, 2, 5, 10, 20 and 50 μ M for 24, 48, and 72 h, respectively. The collapse of mitochondrial transmembrane potential ($\Delta\psi$ m) was assessed with the probe JC-1, a sensitive fluorescent dye. Cells were analyzed with FACSCanto (BD Biosciences, San Jose, CA, USA). In healthy cells, where mitochondrial potentials remain depolarized, JC-1 forms complexes of J-aggregates showing punctate red fluorescence at 590 nm emission wavelength, in apoptotic cells, JC-1 remains in the monomeric form showing diffused green fluorescence at 530 nm emission wavelength.

Flow cytometric analysis for apoptosis. To further quantify the percentage of cells undergoing apoptosis, we performed double staining with Annexin V-FITC and propidium iodide (PI) staining. Briefly, 2.5×10^5 cells/ml were plated in 6-well plates as described above treated with 1, 5, 10, and 20 μ M 5-azacytidine for 3 days for an additional 24 h. After treatment, the cells were stained

Table II. Results of DNA methylation.

Gene	Methylation	Demethylation
PTPN6	15.4% (4/26)	84.6% (22/26)
DAPK	30.8% (8/26)	69.2% (18/26)
p16	7.7% (2/26)	92.3% (24/26)

with Annexin V-FITC/PI according to the manufacturer's instructions. The apoptotic cells were analyzed by flow cytometry (BD Biosciences).

Immunohistochemistry. For immunohistochemical analysis, paraffin sections were deparaffinized in xylene for 15 min three times and rehydrated through decreasing concentrations of ethanol (100, 95, and 80% 5 min, 2 times each). Endogenous peroxidase activity was blocked by incubation of the slides in 3% H_2O_2 /phosphate-buffered saline, and non-specific binding sites were blocked with goat serum. Antigens were retrieval by microwave irradiation for 3 min in pH 6.0 citric buffer and thawed for 15 min. Primary antibody was anti-PTPL1 (1:50; Santa Cruz Biotechnology, Inc.) and the detection of bound antibodies was performed using HRP polymer kit, employing diaminobenzidine (DAB) as a chromogen (17).

Statistical analysis. The data are expressed as mean \pm SEM and analyzed using SPSS 21.0 for Windows (SPSS, Inc., Chicago, IL, USA). For comparisons of multiple groups, one-way ANOVA tests followed by LSD multiple comparison tests was performed. Statistical significance was set at $P < 0.05$.

Results

PTPL1 gene promoter methylation pattern and 5-azacytidine treatment for demethylation and re-expression of PTPL1 in OCI-LY1 cell line. Methylation of the PTPL1 gene was found in OCI-LY1 cell line (Fig. 1A). To experimentally assess whether PTPL1 promoter hypermethylation affects PTPL1 expression, we first examined the PTPL1 levels in OCI-LY1 cell line (Fig. 1B and C). To test whether PTPL1 expression is epigenetically regulated via promoter methylation in lymphoma, we treated cell lines with 5-azacytidine, leading to DNA demethylation. OCI-LY1 cell line with hypermethylated promoters failed to express OCI-LY1, except when subjected to prior treatment with 5-azacytidine.

Treatment with 5-azacytidine was able to restore PTPL1 expression in OCI-LY1 cell line at both mRNA and protein levels (Fig. 1B and C). Dosage of 5-azacytidine (10 and 20 μ M) invariably produced a relatively clear response.

To further clarify the relationship between PTPL1 promoter methylation and expression, the methylation status of PTPL1 after 5-azacytidine treatment was observed. Treated with 5-azacytidine was able to reduce PTPL1 promoter methylation in the OCI-LY1 cell line (Fig. 1A). The results confirmed that hypermethylation of PTPL1 was responsible for gene silencing since DNA demethylation resulted in reactivation of PTPL1 transcription in OCI-LY1 cell lines.

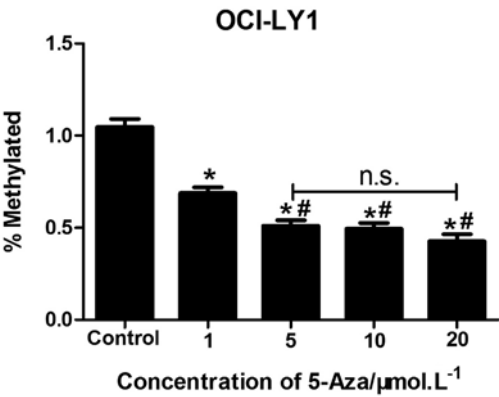


Figure 2. Genome-wide methylation pattern changes after 5-azacytidine treatment. Compared with the control cells, treatment of OCI-LY1 cell line with 1, 5, 10, and 20 μM 5-azacytidine led to a significantly decreased methylation level for both concentrations. * $P<0.05$ vs. control group; # $P<0.05$ vs. 1 μM group.

PTPN6, DAPK and p16 gene promoter hypermethylation by MS-PCR analysis. We examined the DNA from 26 formalin fixed paraffin-embedded (FFPE) tissues of patients with DLBCL (Table II). Hypermethylation of the PTPN6 gene promoter region was found in 15.4% (4/26), the DAPK gene promoter region in 30.8% (8/26), the p16 gene promoter region in 7.7% (2/26).

Genome-wide methylation pattern changes after 5-azacytidine treatment. A low global methylation status was observed

in all groups. Compared with the control cells, treatment of OCI-LY1 cell line with 1, 5, 10, and 20 μM 5-azacytidine led to a significantly decreased methylation level for both concentrations (Fig. 2). $P<0.05$ vs. control group; $P<0.05$ vs. 1 μM group.

5-azacytidine inhibits OCI-LY1 cell line proliferation. To determine whether 5-azacytidine affects the growth of OCI-LY1 cells, we measured the proliferation of OCI-LY1 cells treated with 5-azacytidine. To confirm the effects of 5-azacytidine on OCI-LY1 cell proliferation, the CCK-8 assay was used to assess cell proliferation and quantify cell viability. Fig. 3 shows that 5-azacytidine markedly inhibited the growth of OCI-LY1 cells in a dose- and time-dependent manner.

5-azacytidine decreases mitochondrial membrane potential, translocates Bax and Bcl2, and increases apoptosis in OCI-LY1 cell line. Loss of mitochondrial membrane potential is a sign of the apoptotic process in cells. The mitochondria-mediated apoptosis pathway plays a central role in apoptosis induced by the caspase-dependent pathway. 5-azacytidine treatment of OCI-LY1 resulted in a dosage- and time-dependent increase in the percentage of green fluorescent positive cells (Fig. 4A).

Members of the Bcl2 family play a critical role through their pro-apoptotic (Bax) or anti-apoptotic (Bcl2) activities. Results shows that the expression of Bax mRNA and Bcl2 mRNA was decreased concentration-dependently at 24, 48, and 72 h, but on the whole, Bax/Bcl2 ratio in favor of apoptosis was increased (Fig. 4B).

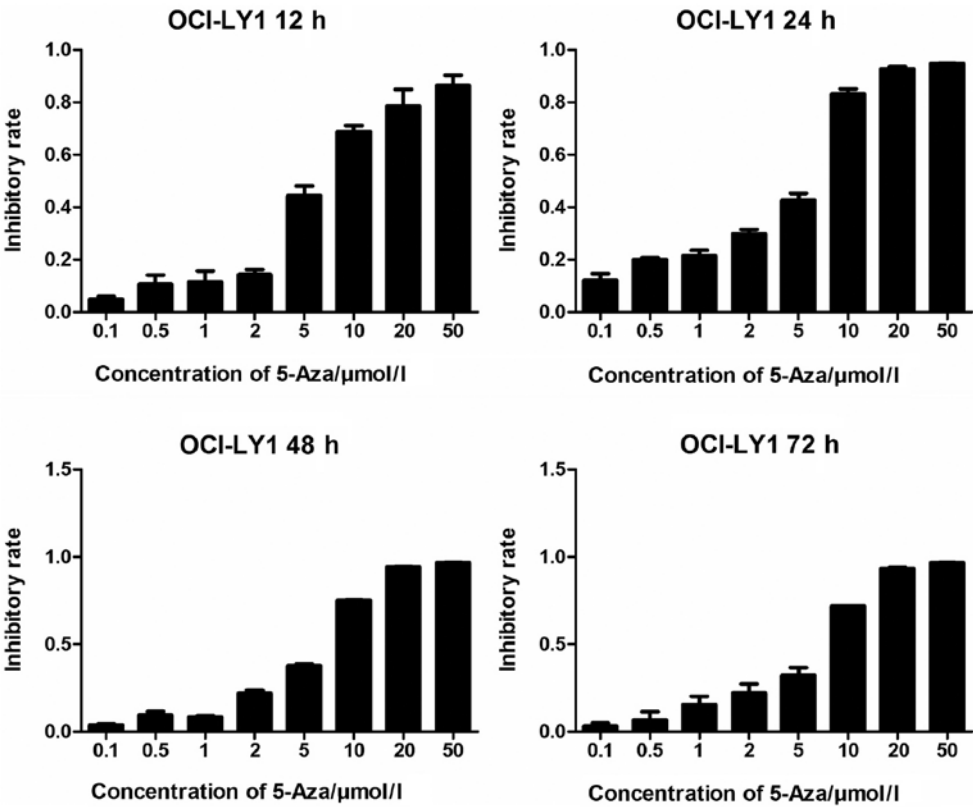


Figure 3. 5-azacytidine inhibits growth of the OCI-LY1 cell line. Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8). OCI-LY1 cells were seeded at a density of $2.5 \times 10^5/\text{ml}$ and incubated in triplicate in the presence of various concentrations of 5-azacytidine for 12, 24, 48, and 72 h, respectively.

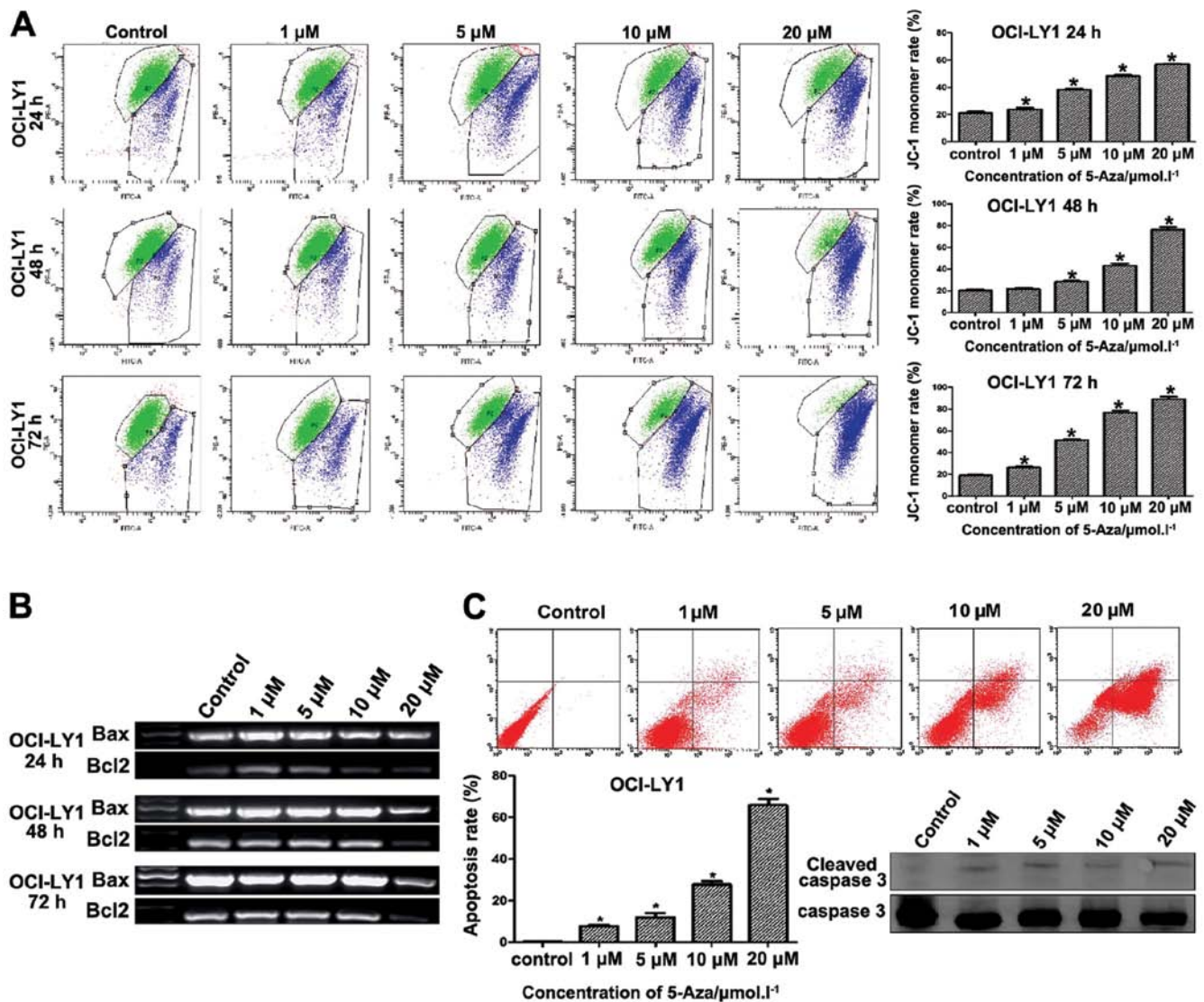


Figure 4. 5-azacytidine suppresses growth and promotes apoptosis of U266 and H929 cell lines. (A) 5-azacytidine reduces mitochondrial membrane potential. (B) 5-azacytidine upregulates the Bax/Bcl2 ratio. (C) 5-azacytidine-mediated apoptosis. * $P < 0.05$.

We next determined whether 5-azacytidine-mediated decrease of OCI-LY1 cell viability was the result of apoptosis. With the increasing dose of 5-azacytidine, for OCI-LY1 the apoptosis rate considerable increase of early (Annexin V⁺/PI⁺) apoptotic cells at the concentration of 1 μ M, 5 μ M, 10 μ M, 20 μ M of 5-azacytidine, respectively. Caspases are important regulators of apoptosis. It is well known that the activation of caspase-3 during apoptosis causes the cleavage of PARP, a major indicator enzyme of apoptosis. We also measured the expression of caspase-3 protein. After 72 h for an additional 24 h of treatment with 5-azacytidine (1 μ M, 5 μ M, 10 μ M, 20 μ M), the levels of cleavage caspase-3 in OCI-LY1 cells were increased (Fig. 4C).

Immunohistochemical study. The expression pattern of PTPL1 in DLBCL tissue was analyzed by immunohistochemistry using a specific antibody reacting against PTPL1 protein. Three DLBCLs in our series were PTPL1-negative. The remaining 7 cases were PTPL1-positive (Fig. 5).

Expression of hCNT1, hENT1 and hENT2 after 5-azacytidine treatment. RT-PCR was used to observed the expression level of hNT, to clarify their role in antitumor efficacy of 5-azacytidine (Fig. 6). Compared with control, for hCNT1, the expression was decreased after treatment with 5-azacytidine in OCI-LY1 cell lines; for hENT1 and hENT2, the changes of expression were complicated, there was a slight increase before the decrease.

Discussion

Advances in molecular genetics have vastly improved our understanding of the molecular pathways that may drive oncogenesis. Both genetic and epigenetic phenomena interact in tumorigenesis (18). DNA methyltransferase and histone methyltransferase inhibitors are emerging as viable therapeutic approaches to erase aberrant epigenetic programming, suppressing DLBCL growth, and overcome chemotherapy resistance (19,20). 5-azacytidine shows antitumor effect

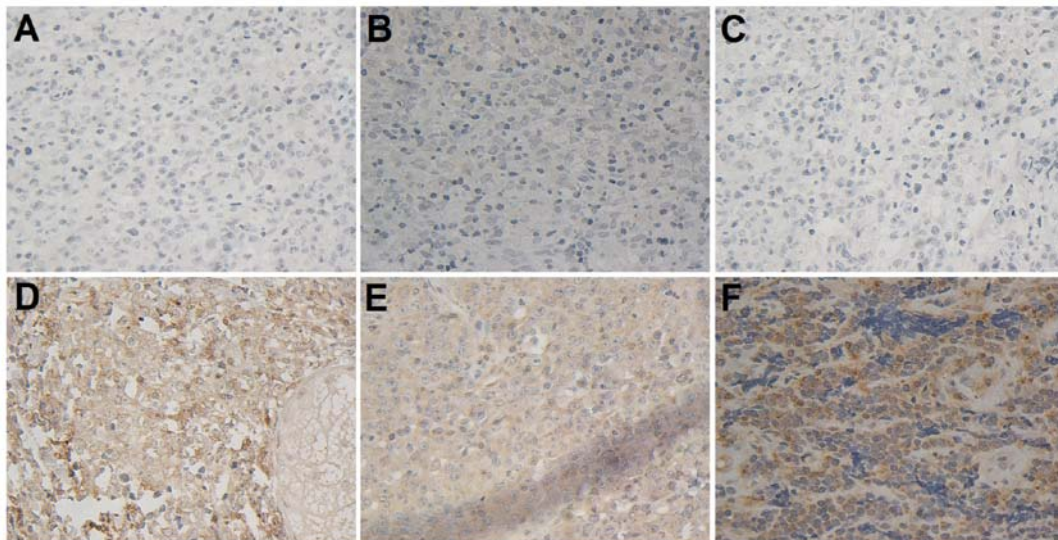


Figure 5. Immunohistochemical staining of DLBCL. Three DLBCLs (A-C) in our series were PTPL1-negative. The remaining 7 cases (D-F as representative) were PTPL1-positive.

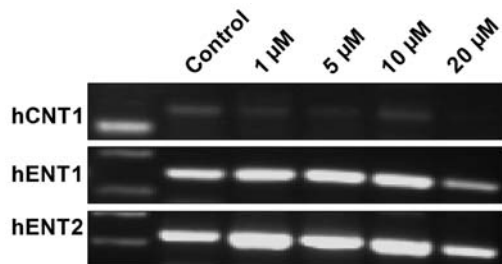


Figure 6. The expression level of hCNT1, hENT1 and hENT2 after 5-azacytidine treatment. RNA was isolated from OCI-LY1 cell line treated with 1, 5, 10, and 20 μ M 5-azacytidine for 3 days for an additional 24 h. Expression of hCNT1, hENT1 and hENT2 was evaluated by RT-PCR.

through two major mechanisms, suppressing methylation and developing cytotoxicity by inhibiting protein synthesis. The proportion of each mechanism mainly depends on concentration, its cytotoxic activity increases with concentration; whereas, DNMT inhibition peaks at low concentrations and abates at higher concentrations. Although there have been numerous studies on the antitumor effects of 5-azacytidine, its action on DLBCL is still obscure. Our experiments explored the effects of 5-azacytidine on cell proliferation, apoptosis, the alterations of gene-specific methylation status and global methylation status, to clarify the relationship between 5-azacytidine and DNA methylation, and suggested new potential clinical uses as an epigenetic therapy in DLBCL.

The loss of tumor suppressor gene (TSG) function can lead to a set of functional capabilities for malignant growth, usually including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (21). TSG may become epigenetically silenced by hypermethylation of CpG islands located in their promoter regions and epigenetic silencing of TSG is potentially reversible (22). In our study, the frequency of methylation of the PTPN6, DAPK, p16 gene in DLBCL was

demonstrated. DAPK is involved in the regulation of apoptosis, p16 is a well-known tumor suppressor gene that plays a key role in cell cycle control, PTPN6 usually functions as a negative regulator in signal transduction (23). In a study of 148 DLBCL patients, DAPK methylation was observed in 89% and the methylation of p16 is linked with poor clinical outcome (24). In addition, DAPK methylation is associated with poor overall- and disease-specific survival in a large cohort of DLBCL patients uniformly treated with rituximab. One previous study showed an incidence of promoter hypermethylation of 23% each for p16 and p15 in oral squamous cell carcinoma. The methylation of the PTPN6 gene promoter was detected in many B-cell leukemia/lymphoma cell lines as well as in patient specimens, including diffuse large B-cell lymphoma (methylation frequency, 93%), MALT lymphoma (82%), mantle cell lymphoma (75%), plasmacytoma (100%) and follicular lymphoma (96%), and also found in high-grade glioma, childhood acute lymphoblastic leukemia, and multiple myeloma (25). In our previous study, the promoter of gene PTPL1 was methylated in 59.6% (28/47). We show here that hypermethylation of the PTPN6 gene promoter region was found in 15.4% (4/26), the DAPK gene promoter region was found in 30.8% (8/26), the p16 gene promoter region was found in 7.7% (2/26). On the whole, our results are consistent with the previous results, but the methylation rate is lower than in the previous study. We assume different type of specimens is the reason. Our specimens were taken from formalin-fixed paraffin-embedded (FFPE) tissues and some of them are kept for many years.

Thus, the global DNA methylation level was decreased after treatment with 5-azacytidine for three days. For 5-azacytidine, DNMT inhibition decreases with concentration and abates at higher concentrations. Compared with control group, all groups were decreased significantly, compared with 1 μ M group, 5, 10, and 20 μ M groups were decreased significantly, but there is no statistical difference among the 5, 10, and 20 μ M groups. The demethylation of genomic DNA can promote the differentiation of DLBCL cells, but in our study, this was not

detected. Further experiments are necessary to show whether the changes in differentiation status of DLBCL cells were due to 5-azacytidine concentration. Our experiments indicated that 5-azacytidine inhibited the proliferation of OCI-LY1 cells for 12, 24, 48, and 72 h, its biological effects on cell growth varied depending on concentration and time of exposure.

In our present study, PTPL1 was methylated in OCI-LY1 cells and was silenced in these two cell lines at mRNA and protein levels. After treatment with 5-azacytidine for three days, all cell lines show PTPL1 mRNA and protein re-expression and reduced methylation level of the PTPL1 promoter. In a previous study, using a total of 82 tumor cell lines, showed that the expression of PTPL1 was frequently downregulated or silenced in non-Hodgkin's lymphoma (94%), Hodgkin's lymphoma (50%), breast (30%), gastric (60%) and hepatocellular (67%) carcinoma cell lines. In summary, PTPL1 may be a putative tumor suppressive gene in DLBCL. In our study, PTPL1 expression was downregulated in the 30% DLBCL cases. The result supports that the downregulation of PTPL1 may have a relation with the occurrence and development of DLBCL.

The present study aimed to explore the molecular mechanisms underlying the induction of cytotoxic effects by 5-azacytidine in OCI-LY1 cells. Following 5-azacytidine treatment, dose-dependent increases in the apoptotic rate were observed. The BCL2 family members play a central role in regulating programmed cell death and arbitrating the cellular fate through an accurate balance between pro-apoptotic (BAX, BAK, and BH3-only proteins) and pro-survival (BCL2 and its closest homologues, BCLXL, BCLW and MCL-1) factors. The ratio of Bax/Bcl2 could ultimately tip the balance toward cell survival or cell death (26). Reverse transcription-polymerase chain reaction analysis demonstrated that 5-azacytidine treatment upregulated the ratio of Bax/Bcl-2 and flow cytometry showed loss of mitochondrial membrane potential. The decreased mitochondrial membrane potential involved the release of cytochrome *c* and Smac/DIABLO, leading to activation of the caspase cascade, and ultimately apoptosis. Caspase-3 is a member of the caspase family of enzymes, which are the major inducers of apoptosis and is often measured in the context of research into antitumor drugs that target apoptosis (27,28). Further studies were performed to examine the effect of 5-azacytidine on cell apoptosis and the activity of caspase-3. The induction of cell death by 5-azacytidine mainly occurs via programmed cell death, as indicated by Annexin V-fluorescein isothiocyanate and PI staining. Cells treated with 1, 5, 10, and 20 μ M 5-azacytidine for 3 days detected by FACS demonstrated 5-azacytidine induced apoptosis in a dose-dependent manner. In addition our results showed that the activity of caspase-3 after treated with 5-azacytidine for 3 days increased significantly. This increase of caspase-3 was concomitant with the apoptosis of cell lines. Accordingly, the decrease in Bcl-2, increase in Bax and, finally, the activation of caspase-3 in OCI-LY1 cells indicated that the apoptotic process induced by 5-azacytidine was irreversible.

Nucleoside transporters play important physiological roles by regulating intra- and extra-cellular concentrations of purine and pyrimidine (deoxy) nucleosides, including inflammation, cardiovascular function and nutrient transport across the blood-brain barrier (29). In addition, nucleoside transport

plays its role in pathological conditions such as cardiovascular disease and cancer. Focusing on nucleoside analogs as anti-neoplastic agents, it may be a potential therapeutic strategy to manipulate the activity of nucleoside transporters. In our study, for hENT1 and hENT2, the change of expression was complicated, there was a slight increase before the decrease; for hCNT1, the expression was decreased after treatment with 5-azacytidine in OCI-LY1 cell line. Moreover, hENT1 expression in leukemia cell lines appears to be a key determinant of 5-azacytidine triggered cytotoxicity. Nucleoside transporters are responsible for the cellular uptake of currently used anticancer nucleoside-derived drugs, thus these membrane proteins might play a significant role in nucleoside-based chemotherapy.

Drug resistance is a major problem for patients treated with small molecular reagents such as 5-azacytidine and decitabine, but the mechanisms are poorly understood (30). The final decrease of hCNT1, hENT1 and hENT2 after treatment with 5-azacytidine may suggest that decreasing levels of these transporters is a possible mechanism for acquiring resistance to DNMT inhibitors. We believe our study will give some clues for the development of novel chemical entities to measure nucleoside transport activity at the cellular and organismal level.

Taken together, our observations demonstrated that 5-azacytidine has potent effects on growth inhibition, induction of caspase-dependent apoptosis in the OCI-LY1 cell line. Epigenetic dysregulation is involved in PTPN6, DAPK, p16. The loss of PTPL1 mRNA is the consequence of PTPL1 methylation and can be reversed by 5-azacytidine. The study highlights the value of 5-azacytidine as epigenetic potential therapeutic candidate for DLBCL.

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