

# Inhibitory effect of methylation inhibitor 5-aza-2-deoxycytidine on bile duct cancer cell line in vivo and in vitro

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**BACKGROUND:** Since the resection rate is low for bile duct cancer and the drugs used for chemotherapy are less effective, we studied the inhibitory effects of 5-aza-2-deoxycytidine (ZdCyd) on bile duct cancer cell line QBC-939 in vivo and in vitro and its possibility in clinical treatment.

**METHODS:** The survival and apoptosis rates of QBC939 after treatment with different dose of ZdCyd were detected by methyl thiazoy tetrazolium (MTT) and flow cytometry. The cooperative effect of ZdCyd with other chemotherapeutic drugs was also studied with MTT. The cancer cells were transplanted into nude mice, which were pre-treated with ZdCyd after tumor occurrence.

**RESULTS:** ZdCyd decreased the cell survival rate, blocked the cell cycle at G1 phase, and increased the apoptosis rate. These effects were dose- and time-dependent. ZdCyd also increased the anti-tumor effects of other chemotherapeutic drugs when used in combination. The tumor occurrence rate was lower in the ZdCyd pre-treated cells than in the untreated cells in nude mice, and ZdCyd was found to inhibit tumor growth.

**CONCLUSION:** ZdCyd can inhibit the growth of QBC939 in vivo and in vitro through induction of cell apoptosis and has the cooperative effect on bile duct cancer cell when it is used with other chemotherapeutic drugs.

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**KEY WORDS:** bile duct cancer; cell cycle; apoptosis; chemotherapy

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## Introduction

In addition to the classical genetic mechanisms of deletion or inactivating point mutations, growth regulatory gene can be functionally inactivated by promoter CpG island methylation without alterations of the primary sequence.<sup>[1-7]</sup> CpG island comprises stretches of DNA approximately 1 kb long that are rich in dinucleotides. The CpG site in these gene-associated regions is rarely methylated in normal cells with the exception of CpG island of genes on the inactivated X chromosome and CpG associated with imprinted genes.<sup>[8-11]</sup> It is now accepted that abnormal methylation is not restricted to cultured cells but also occurs during aging and tumor development.<sup>[12]</sup> The mechanism by which hypermethylation of selected CpG island occurs in cells remains to be elucidated. However, it has been shown that unmethylated CpG islands associated with a variety of genes become partially or fully methylated in tumors, leading to the abnormal expression of mRNA and protein.<sup>[13-18]</sup> This methylation status can be reactivated by 5-aza-2-deoxycytidine (ZdCyd).<sup>[19-23]</sup> ZdCyd, a methylation inhibitor, can eliminate the methylation status of tumor suppressor gene promoter, resulting in the re-expression of these genes and tumor inhibition.<sup>[24-26]</sup> Although the tumor suppressor gene is methylated, the DNA sequence is unchanged and this kind of change is reversible. This makes it possible that a methylated tumor suppressor gene can be demethylated and reactivated by ZdCyd for the purpose of cancer therapy.

ZdCyd has been used in the chemotherapy for a variety of cancers such as leukaemia.<sup>[27-29]</sup> Some studies discovered recently that many tumor suppressor genes were methylated and inactivated, suggesting that the promoter methylation of tumor suppressor gene may play an important role in the occurrence and progress of bile duct cancer. Since the surgical removal rate is low for bile duct cancer and the drugs used for chemotherapy are less effective,<sup>[30,31]</sup> new chemotherapeutic drugs are in pressing need. It is a possible way for bile duct cancer therapy via elimination of the tumor suppressor gene methylation status with ZdCyd. By now, there is still no systemic study of ZdCyd in the treatment of bile duct

cancer. We studied the inhibitory effect of ZdCyd on bile duct cell line in vivo and in vitro and its possibility in chemotherapy of bile duct cancer.

## Methods

### Cell line and reagents

Bile duct cell line QBC939 was kindly provided by Professor Shu-Guang Wang from the Third Military Medical University, Chongqing, China, and was kept in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in a 5% CO<sub>2</sub> incubator. ZdCyd was bought from Sigma, USA, and dissolved with dimethylsulfoxide (DMSO).

### Inhibitory effect of ZdCyd on QBC939

QBC939 cells were placed in RPMI1640 supplemented with 10% FCS at 37 °C in a 5% CO<sub>2</sub> incubator, and digested with 0.25% trypsin in the log phase to make a single cell suspension with a concentration of  $2 \times 10^5$ /ml counted under a microscope. The cells were planted into a 96-well plate with 200 µl per well. The blank well was only added with RPMI1640 medium without cells. The cells were incubated for 18 hours under the above conditions, then the cell culture medium was renewed, and added with ZdCyd with the final concentrations of 0.1 µmol/L, 0.5 µmol/L, 1.0 µmol/L, 5.0 µmol/L, 25 µmol/L, and 125 µmol/L. The cells of each concentration were added into 5 wells, and the control group was only given renewed culture medium without ZdCyd.

The plate was incubated in an incubator for additional 4, 12, 24, 36, 48 hours separately and then added with 20 µl methyl thiazoyl tetrazolium (MTT) per well with the concentration of 5 mg/ml. The medium was discarded after incubation for additional 4 hours, and added with 150 µl DMSO per well. The optical density (OD) level under 490 nm was measured and the cell survival rate was calculated with the following formula: cell survival rate (%) = (OD level of experimental group - OD level of blank group) / (OD level of control group - OD level of blank group) × 100%.

### Cooperative effect of ZdCyd and other chemotherapeutic drugs

QBC939 cells was cultured and single cell suspension was made similarly. The cells were divided into two groups A and B. In group A, cells were treated with different concentrations of 5-FU, adriamycin (ADM), cisplatin; and in group B, 1.0 µmol/L ZdCyd was added into each subgroup. MTT was used, and the cell survival rate was calculated with the same formula.

### Evaluation of cell apoptosis rate and cell cycle change by flow cytometry after use of ZdCyd

QBC939 cell suspension was made according to the process of MTT, and then planted into a 6-well plate. ZdCyd was added after the cells were incubated for 18 hours. The groups of different ZdCyd dosage and different time were set up as those of the process of MTT. Cells were harvested using trypsin, washed twice with PBS, and then added with 70% cold ethanol which was pre-cooled at -20 °C drop by drop. The cells were subsequently stained with propidium iodide and the cell cycle and apoptosis rate were detected by flow cytometry.

### Inhibitory effect of ZdCyd on QBC939 in nude mice

Twenty-five healthy nude mice of 4 weeks old were chosen and divided randomly into 3 groups A, B and C, and the number of mice of each group was 10, 10, 5 respectively. In group A, QBC939 was treated with 5 µmol/L ZdCyd for 24 hours, and single cell suspension was made with a concentration of  $1 \times 10^7$ /ml; 0.2 ml of the suspension was injected subcutaneously into the flanks of the nude mice. In group B, all were the same except that the cells were not treated with ZdCyd before injection into the nude mice. In group C, 0.2 ml PBS solution was injected instead of the cells. After 6 weeks, when the tumor occurred, group B was subdivided into 2 groups equally and randomly. One group was injected with ZdCyd 2 µg/g (weight) × day, and the other was injected with saline instead. The mice were kept in sterile conditions and the size of tumors was measured every week with the following formula: tumor volume = transverse diameter<sup>2</sup> × largest diameter/2.

### Statistical analysis

Statistical software SPSS was used, and a *P* value less than 0.05 was considered significant.

## Results

### Effect of ZdCyd on QBC939 proliferation

The cells were observed under an inverted microscope after different dose of ZdCyd was added. The cells treated by ZdCyd were found shrank, dead and had a lower density (Figs. 1-3). The effect of ZdCyd on cell proliferation was dose- and time-dependent. When the dose was 0.5 µmol/L, the inhibitory effect appeared, and the IC<sub>50</sub> for 24 hours was 5.0 µmol/L. The effect was markedly increased with increased dose and prolonged time.

### Cooperative effect of ZdCyd and other chemotherapeutic drugs

The IC<sub>50</sub> of 5-FU, ADM was 0.25 mg/ml and 12.5 µg/ml respectively when 1.0 µmol/L ZdCyd was added. The cell survival rate decreased to 31% and 20% respectively for 5-FU (0.25 mg/ml) and ADM (12.5

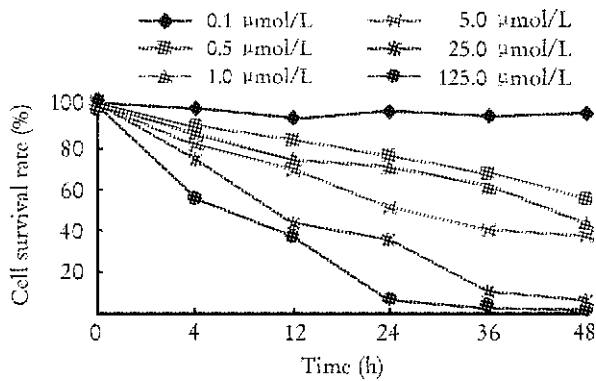


Fig. 1. Effect of ZdCyd on QBC939 proliferation.

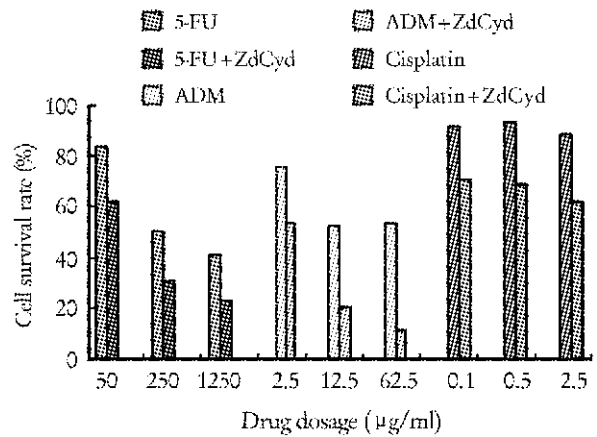


Fig. 4. The cooperative effect of ZdCyd with other chemotherapeutic drugs.

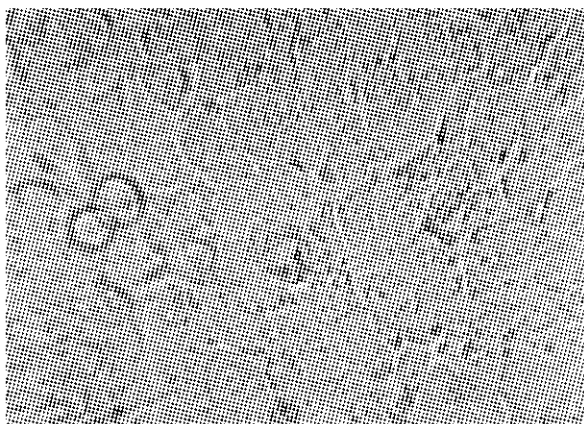


Fig. 2. The cells not treated with ZdCyd under an inverted microscope (original magnification  $\times 200$ ).

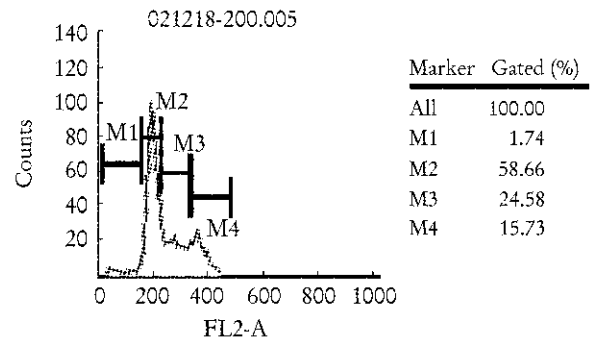


Fig. 5. Flow cytometry results of the cells untreated with ZdCyd.

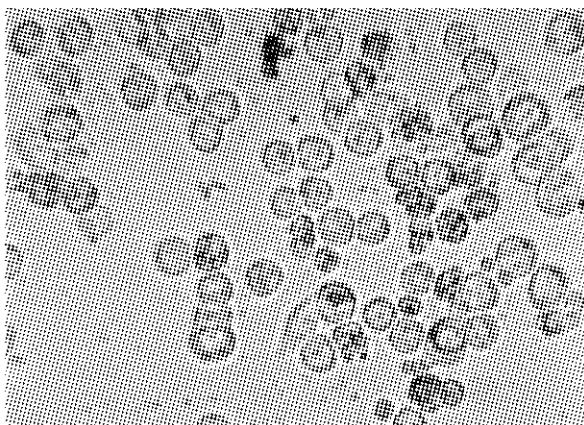


Fig. 3. Treated with 5  $\mu\text{mol/L}$  ZdCyd for 24 hours, the cells were shrunken, dead and decreased in density (original magnification  $\times 200$ ).

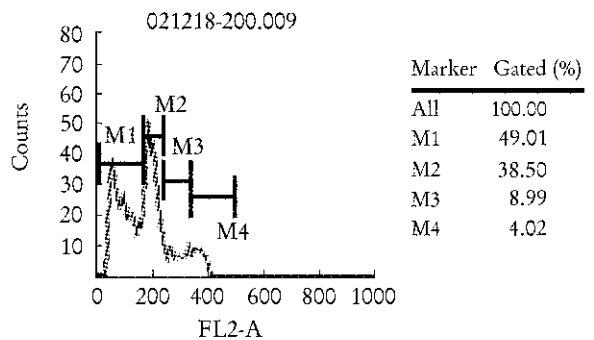


Fig. 6. Flow cytometry results of the cells treated with 5  $\mu\text{mol/L}$  ZdCyd for 24 hours.

$\mu\text{g/ml}$ ). As to cisplatin, it had no effect on cell proliferation (Fig. 4).

**Effect of ZdCyd on QBC939 cell cycle and apoptosis rate**

ZdCyd blocked the cell cycle at G1 phase and increased the apoptosis rate. The effect characterized by dose dependence is most significant at 5  $\mu\text{mol/L}$ . However, when the concentration reached 125  $\mu\text{mol/L}$ , most of the cells showed necrosis instead of apoptosis (Figs. 5 and 6, Table).

**Effect of ZdCyd on QBC939 in vitro**

**Table.** Effect of ZdCyd on QBC939 cell cycle and apoptosis rate

	M1	M2	M3	M4
0 $\mu\text{mol/L}$	1.85	58.39	25.10	14.74
0.1 $\mu\text{mol/L}$	2.07	60.14	23.26	15.82
0.5 $\mu\text{mol/L}$	18.38	58.96	14.14	9.63
1.0 $\mu\text{mol/L}$	37.54	45.38	10.27	7.05
5.0 $\mu\text{mol/L}$	48.17	39.56	8.03	4.58
25.0 $\mu\text{mol/L}$	40.36	49.53	7.04	3.95

M1, M2, M3, M4 stand for sub-G1, G1, S, G2 separately, and sub-G1 indicates the apoptosis of cells. When the dose reached 125  $\mu\text{mol/L}$ , flow cytometry could not be performed because of the number of cells survived was not enough.

The tumor occurrence rate was 20% (2/10), 100% (10/10), and 0% (0/5) for groups A, B, C respectively in the flank of the nude mice in 6 weeks after QBC939 cell injection, and the tumor size varied from 5  $\text{mm}^3$  to 2700  $\text{mm}^3$ . In group B, tumor growth of the subgroup treated with ZdCyd was slow down, and some of the tumors became smaller, even disappeared (one tumor). In the subgroup treated with saline, tumor growth was not stopped and 2 mice died of cachexia in additional 6 weeks.

## Discussion

A recent examination of more than 600 primary tumor samples of 15 tumor types showed that CpG island promoters of three or more genes were hypermethylated in 5%–10% of the samples.<sup>[32]</sup> At least one CpG island was methylated in 80% or more of samples for each tumor type. Using methods that allow genome wide screening of CpG islands has estimated that, on average, 1% of CpG islands in DNA from tumor tissues are abnormally methylated.

Caca et al<sup>[33]</sup> studied 9 bile duct cell lines and 21 cases of primary extrahepatic bile duct cancer and found that P16 expression inactivated in all the cell lines and in 11 of the 21 cases of primary extrahepatic bile duct cancer. In the 9 cell lines, 8 were inactivated by loss of heterozygosity (LOH) in chromosome 9P21, and the other was inactivated by promoter region hypermethylation. In 11 cases of bile duct cancer, however, 9 cases were inactivated by promoter methylation and they were P16 inactivated. The reported rates of P14, P16 promoter methylation in 51 cases of cholangiocarcinoma were 25%, 76% respectively, shown by the method of methylation specific PCR (MSP-PCR).<sup>[34]</sup> Moreover, mRNA expression was lost in all the cases in which methylation occurred.

In our study, ZdCyd inhibited the proliferation of bile duct cancer cell line QBC939 at the concentration of 0.5  $\mu\text{mol/L}$ , and this effect increased with increased drug doses and prolonged time in vitro. When the do-

sage reached 125  $\mu\text{mol/L}$ , almost all the cells died in 24 hours, suggesting that ZdCyd could inhibit the proliferation of bile duct cancer cells. We also found that ZdCyd could have the cell cycle blocked at G1 phase and the number of apoptotic cells increased. The apoptosis rate was 49.01% when the drug dosage was 25  $\mu\text{mol/L}$ . The survival rate decreased as long as the dosage increased, and the apoptosis rate decreased too. Broken cells and lots of cell pieces were found microscopically as the characteristics of cell necrosis. These findings were similar to cell apoptosis in low dosage and necrosis in high dosage of other drugs.

The curative effect of traditional drugs for the treatment of bile duct cancer is low when they were used singly.<sup>[35]</sup> We tried to combine ZdCyd with traditional drugs in dealing with bile duct cell proliferation and found that the cell proliferation inhibitory effect of the combination therapy is more powerful than that of single drug, suggesting that there is a cooperative killing effect on bile duct cancer cells. Possibly, ZdCyd may reactivate the inactivated tumor suppressor gene, while eliminating the methylation status of the gene promoter region and changing the cell cycle. ZdCyd could also inhibit the proliferation of bile duct cancer cell line QBC939 in vitro. In this study, the tumor occurrence rate of the pre-treated cells in nude mice was much lower than that of the untreated cells (20% vs 100%), although the number of cells injected was identical ( $2 \times 10^6$  cells/per mouse). ZdCyd also prevented tumor growth in nude mice, and reduced tumor size after treatment with 2  $\mu\text{g/g}$  per day in contrast to the control mice.

We conclude that ZdCyd can inhibit the proliferation of bile duct cancer cell line QBC939, block cell cycle at G1 phase, and induce cell apoptosis in vivo. Also it can prevent tumor recurrence and growth in nude mice, and its clinical implications await study.

## Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

## References

- Zochbauer MS, Fong KM, Virmani AK, Gerads J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res* 2001;61:249-255.
- Roncalli M, Bianchi P, Bruni B, Laghi L, Destro A, Di Gioia S, et al. Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. *Hepatology* 2002;36:427-432.
- Yu XJ, Long J, Fu DL, Zhang QH, Ni QX. Analysis of gene expression profiles in pancreatic carcinoma by using cDNA microarray. *Hepatobiliary Pancreat Dis Int* 2003;2:467-470.

- 4 Coombes MM, Briggs KL, Bone JR, Clayman GL, El-Naggar AK, Dent SY. Resetting the histone code at CDKN2A in HNSCC by inhibition of DNA methylation. *Oncogene* 2003;22:8902-8911.
- 5 Hennessy BT, Garcia-Manero G, Kantarjian HM, Giles FJ. DNA methylation in haematological malignancies: the role of decitabine. *Expert Opin Investig Drugs* 2003;12:1985-1993.
- 6 Yan PS, Shi H, Rahmatpanah F, Hsiao TH, Hsiao AH, Leu YW, et al. Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. *Cancer Res* 2003;63:6178-6186.
- 7 Sasaki M, Dharia A, Oh BR, Tanaka Y, Fujimoto S, Dahiya R. Progesterone receptor B gene inactivation and CpG hypermethylation in human uterine endometrial cancer. *Cancer Res* 2001;61:97-100.
- 8 McGregor F, Muntoni A, Fleming J, Brown J, Felix DH, MacDonald DG, et al. Molecular changes associated with oral dysplasia progression and acquisition of immortality: potential for its reversal by 5-azacytidine. *Cancer Res* 2002;62:4757-4766.
- 9 Oue N, Oshimo Y, Nakayama H, Ito R, Yoshida K, Matsusaki K, et al. DNA methylation of multiple genes in gastric carcinoma: association with histological type and CpG island methylator phenotype. *Cancer Sci* 2003;94:901-905.
- 10 Domann FE, Rice JC, Hendrix MJ, Futscher BW. Epigenetic silencing of maspin gene expression in human breast cancers. *Int J Cancer* 2000;85:805-810.
- 11 Chan AS, Tsui WY, Chen X, Chu KM, Chan TL, Chan AS, et al. Downregulation of ID4 by promoter hypermethylation in gastric adenocarcinoma. *Oncogene* 2003;22:6946-6953.
- 12 Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, et al. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. *Cancer Res* 2002;62:6456-6461.
- 13 Karpf AR, Jones DA. Reactivating the expression of methylation silenced genes in human cancer. *Oncogene* 2002;21:5496-5503.
- 14 Daskalakis M, Nguyen TT, Nguyen C, Guldberg P, Kohler G, Wijermans P, et al. Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-aza-2'-deoxycytidine (decitabine) treatment. *Blood* 2002;100:2957-2964.
- 15 Bae SI, Lee HS, Kim SH, Kim WH. Inactivation of O6-methylguanine-DNA methyltransferase by promoter CpG island hypermethylation in gastric cancers. *Br J Cancer* 2002;86:1888-1892.
- 16 Wu GS, Wang JH, Liu ZR, Zou SQ. Expression of cyclooxygenase-1 and -2 in extrahepatic cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2002;1:429-433.
- 17 Claus R, Lubbert M. Epigenetic targets in hematopoietic malignancies. *Oncogene* 2003;22:6489-6496.
- 18 Zochbauer-Muller S, Lam S, Toyooka S, Virmani AK, Toyooka KO, Seidl S, et al. Aberrant methylation of multiple genes in the upper aerodigestive tract epithelium of heavy smokers. *Int J Cancer* 2003;107:612-616.
- 19 Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM. Predicting aberrant CpG island methylation. *Proc Natl Acad Sci USA* 2003;100:12253-12258.
- 20 Chen JT, Cheng YW, Chou MC, Sen-Lin T, Lai WW, Ho WL, et al. The correlation between aberrant connexin 43 mRNA expression induced by promoter methylation and nodal micrometastasis in non-small cell lung cancer. *Clin Cancer Res* 2003;9:4200-4204.
- 21 Christman JK. 5-azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 2002;21:5483-5495.
- 22 Liang G, Gonzales FA, Jones PA, Orntoft TF, Thykjaer T. Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2'-deoxycytidine. *Cancer Res* 2002;62:961-966.
- 23 Nicoll G, Crichton DN, McDowell HE, Kernohan N, Hupp TR, Thompson AM. Expression of the hypermethylated in cancer gene (HIC-1) is associated with good outcome in human breast cancer. *Br J Cancer* 2001;85:1878-1882.
- 24 Venkatasubbarao K, Ammanamanchi S, Brattain MG, Mimari D, Freeman JW. Reversion of transcriptional repression of Sp1 by 5-aza-2'-deoxycytidine restores TGF-beta type II receptor expression in the pancreatic cancer cell line MIA PaCa-2. *Cancer Res* 2001;61:6239-6247.
- 25 Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res* 2000;60:6039-6044.
- 26 Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, et al. Low-dose 5-aza-2'-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. *J Clin Oncol* 2000;18:956-962.
- 27 Garcia-Manero G, Daniel J, Smith TL, Kornblau SM, Lee MS, Kantarjian HM, et al. DNA methylation of multiple promoter-associated CpG islands in adult acute lymphocytic leukemia. *Clin Cancer Res* 2002;8:2217-2224.
- 28 Nagy E, Beck Z, Kiss A, Csoma E, Telek B, Konya J, et al. Frequent methylation of p16INK4A and p14ARF genes implicated in the evolution of chronic myeloid leukaemia from its chronic to accelerated phase. *Eur J Cancer* 2003;39:2298-2305.
- 29 Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, et al. 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. *Blood* 2000;96:2379-2384.
- 30 Shi JS, Wang JS, Liu G, Yu YY, Lu Y, Jiao XY, et al. Early diagnosis of primary gallbladder carcinoma. *Hepatobiliary Pancreat Dis Int* 2002;1:273-275.
- 31 Liu XF, Zou SQ, Qiu FZ. Pathogenesis of cholangiocarcinoma in the porta hepatitis and infection of hepatitis virus. *Hepatobiliary Pancreat Dis Int* 2003;2:285-289.
- 32 Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61:3225-3229.
- 33 Caca K, Feisthammel J, Klee K. Inactivation of the INK4a/ARF locus and p53 in sporadic extrahepatic bile duct cancers and bile tract cancer cell lines. *Int J Cancer* 2002;97:481-488.
- 34 Tannapfel A, Sommerer F, Benicke M, Weinans L, Katalinic A, Geissler F, et al. Genetic and epigenetic alterations of the INK4a-ARF pathway in cholangiocarcinoma. *J Pathol* 2002;197:624-631.
- 35 He ZP, Fan LJ. Diagnosis and treatment of portal biliopathy. *Hepatobiliary Pancreat Dis Int* 2002;1:581-586.

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