Anti-tumor effect of 5-aza-2'-deoxycytidine by inhibiting telomerase activity in hepatocellular carcinoma cells

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AIM: To investigate the effect of the demethylating reagent 5-aza-2'-deoxycytidine (DAC) on telomerase activity in hepatocellular carcinoma (HCC) cell lines, SMMC-7721 and HepG2.

METHODS: The related gene expression in cell lines was examined by real-time reverse transcription-polymerase chain reaction and Western blotting analysis. The telomerase activity was examined by telomeric repeat amplification protocol-enzyme-linked immunosorbent assay and DNA methylation was determined by methylation-specific polymerase chain reaction.

RESULTS: The telomerase activity was significantly reduced in both cell lines treated with DAC, accompanied by downregulation of telomerase reverse transcriptase (hTERT). We also observed the effect of DAC on the methylation status of hTERT promoter and the expression of regulatory genes, such as c-myc, p15, p16, p21, E2F1, and WT1. The methylation status of hTERT promoter could be reversed in SMMC-7721 by DAC, but not in HepG2 cells. However, p16 expression could be reactivated by demethylation of its promoter, and c-Myc expression was repressed in both cell lines. Moreover, DAC could enhance the sensitivity to the chemotherapeutic agents, such as cisplatin, by induction of apoptosis of HCC cells.

CONCLUSION: The DAC exerts its anti-tumor effects in HCC cells by inhibiting the telomerase activity.

Key words: 5-aza-2'-deoxycytidine; Telomerase; Hepatocellular carcinoma; DNA methylation

INTRODUCTION

The demethylating reagent 5-aza-2'-deoxycytidine (DAC) inhibits DNA methyltransferases and reverses DNA me-
talation\(^{\text{[3]}}\). It has been found that DAC can inhibit cancer cell growth, particularly the leukemia cells, and it has been applied for the treatment of myelodysplastic syndromes. A phase 1 study was finished using DAC as an antineoplastic drug for hematopoietic malignancies\(^{\text{[2,3]}}\). However, the mechanisms underlying its anticancer activity and other biological effects are not fully understood. It is believed to reactivate genes, including the tumor suppressor genes p16, E-cadherin, and hMLH1 in cancer cells. Reactivation of these genes is associated with cell cycle arrest and apoptosis, which leads to inhibition of tumor cell growth\(^{\text{[3]}}\). However, the mechanisms of telomerase activity and telomerase reverse transcriptase (hTERT) down-regulated by DAC remain unclear. Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA sequences and almost universally provides the molecular basis for tumor cell proliferative capacity\(^{\text{[6,7]}}\). Telomerase reactivation is a critical step in cellular immortality and carcinogenesis\(^{\text{[8,9]}}\). The enzyme consists of three major components: hTERT, telomerase associated protein (TEP1), and telomerase RNA (TERC)\(^{\text{[10]}}\). Transcriptional regulation of hTERT is believed to be the major mechanism of telomerase regulation in human cells. Transient transfection experiments with hTERT promoter-luciferase reporters showed that the hTERT promoter is inactive in normal and transformed premortal cells, but, like telomerase, is activated in immortal cells\(^{\text{[9]}}\). Expression of hTERT was observed at high levels in malignant tumors and cancer cell lines, but not in normal tissues or telomerase-negative cell lines, and a strong correlation has been found between hTERT expression and telomerase activity in a variety of tumors\(^{\text{[10,11]}}\). These findings suggest that expression of hTERT might be a critical event in carcinogenesis. Thus, the mechanisms of hTERT activation are essential for understanding the molecular basis of telomerase activation and carcinogenesis. The hTERT promoter contains binding sites for many transcription factors that could be involved in its regulation. The abundance of these potential transcription factor binding sites was subjected to various factors in different cellular contexts\(^{\text{[12,13]}}\). Several transcription factors are known to participate in hTERT gene expression, including positive regulators: c-Myc, Sp1, human papillomavirus 16 E6, and steroid hormones; and negative regulators: Mad1, p53, p15, p16, p21, E2F, pRB, WT1, interferon-α, tumor growth factor-β and myeloid cellspecific zinc finger protein\(^{\text{[14,15]}}\). Our previous studies revealed that methylation status of P21, P15, P16, WT1 and E2F-1 was significantly associated with cancer tissues in hepatocellular carcinoma (HCC)\(^{\text{[16]}}\). In the present study, we observed the effect of DAC on telomerase activity and hTERT expression in HCC cell lines, and found that DAC down-regulated the telomerase activity and hTERT expression by p16 promoter demethylation.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

Human hepatoma cell lines SMMC-7721 and HepG2 were maintained in Dulbecco’s modified Eagle’s medium (high glucose) (Gibco, Invitrogen, United States) and supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, United States), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator under 95% air and 5% CO\(_2\) at 37 °C. Cells from exponentially growing cultures were used in all the experiments. This study was approved by the Institutional Ethics Committee of the Second Military Medical University, China.

**DAC**

Human hepatoma cell lines (SMMC-7721 and HepG2) were seeded at a density of 5 × 10\(^4\) cells per well in 6-well tissue culture plates and were allowed to attach over a 24-h period. The demethylating reagent DAC (Merk, Calbiochem, United States) was added to a final concentration of 1 μmol/L, 2 μmol/L and 4 μmol/L and the cells grew for 1 d, 3 d and 5 d. At indicated time intervals, cells were harvested by trypsinization and washed with phosphate-buffered saline.

**Assay of telomerase activity by telomeric repeat amplification protocol-enzyme-linked immunosorbent assay**

Telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA) was performed using the telomerase kit Telo TAGGG Telomerase PCR ELISA PLUS (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Extracts from HEK293 cells were used as positive controls for the assay, and cell lysates heat-inactivated for 10 min at 85 °C were used as negative controls. Absorbance values were reported as the A\(_{450}\) nm reading.

**Real-time reverse transcription-polymerase chain reaction**

RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA, United States). cDNA was synthesized using 2 μg total RNA and a moloney murine leukemia virus-reverse transcriptase kit with random hexamer primers and an RNase inhibitor (Takara Biotechnology Co. Ltd., Dalian, China). Polymerase chain reaction (PCR) amplifications of the respective genes were carried out with 40 ng complementary DNA, 500 nmol/L forward and reverse primer, and iTaqSYBRGreen Supermix (Bio-Rad Laboratories, Hercules, CA) in a final volume of 20 μL. Primer sequences and annealing temperature are summarized in Table 1. Real-time RT-PCR was performed on a Mastercycler cycler (Eppendorf, Hamburg, Germany), and all experiments were performed twice. Amplification of the housekeeping gene β-actin was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed by the -ΔΔct method.

**Methylation-specific polymerase chain reaction**

The bisulfite modification of DNA was done as described previously\(^{\text{[17]}}\). DNA methylation was determined


by methylation-specific PCR (MSP). Forty ng of bisulfite-modified DNA was subjected to PCR amplification. The PCR reaction mixture contained 2.5 μL of 10 × PCR buffer, 100 pmol of each primer, 2 mmol/L of each dNTPs, and 1 U of Hotstart Taq DNA polymerase (Takara Biotechnology Co. Ltd, Dalian, China) at a final volume of 25 μL. The PCR was performed in a thermal cycler. Primer sequences and reaction conditions are summarized in Table 1. DNA methylated by SssI methylase (SssI DNA) was used as positive control for methylated alleles.

### Western blotting analysis

HepG2 and SMMC-7721 cells were lysed in RIPA lysis buffer (Beyotime, China) with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). An equal amount of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane. After blocking with 5% nonfat milk, the membrane was probed with anti-hTERT (Santa Cruz, United States), developed with the BeyoECL Plus substrate system (Beyotime, China), and re-probed with β-actin antibody (Santa Cruz, United States) to confirm equal protein loading.

### Results

#### Telomerase activity in HCC cells with DAC

To investigate the effects of DAC on telomerase activity, SMMC-7721 and HepG2 were cultured with 1 μmol/L, 2 μmol/L and 4 μmol/L DAC. Telomerase activity was measured by TRAP-PCR-ELISA assay after 1 d, 3 d and 5 d of exposure to DAC. Inhibition of telomerase activity was observed in both cell lines in a dose-dependent manner, by maximal repression on day 3 at 4 μmol/L or day 5 at 2 μmol/L DAC (Figure 1). There was a 52.7% reduction of telomerase activity in SMMC-7721 cells treated with 4 μmol/L DAC for 3 d, and a 45.6% reduction of telomerase activity in HepG2 cells. The results revealed that the effect of DAC on telomerase activity varied in different cell lines.

#### Effect of DAC on telomerase reverse transcriptase expression in HCC cells

Since the expression of hTERT is closely associated with telomerase activity, we examined whether hTERT expression is suppressed in SMMC-7721 and HepG2 cells by DAC. The expression of hTERT mRNA in SMMC-7721 cells was decreased to 82% on day 1, 34% on day 3, and 26% on day 5 after DAC treatment (2 μmol/L) (Figure 2). A decline in hTERT mRNA was also detected in HepG2 cells treated with DAC. hTERT mRNA expression was maximally down-regulated by 4 μmol/L DAC. The complete down-regulation of hTERT mRNA became apparent on day 3 of treatment and maximal on day 5 in both cell lines. Furthermore, we treated SMMC-7721 and HepG2 cells with 1 μmol/L, 2 μmol/L, and 4 μmol/L DAC respectively for 3 d and 2 μmol/L for 1 d, 3 d, 5 d, respectively, then detected the hTERT expression in protein level by Western blotting analysis (Figure 3). The hTERT protein in SMMC-7721 and HepG2 cells was also down-regulated by DAC in a dose- and time-dependent manner, with maximal repression at 4 μmol/L on day 5. The hTERT protein was notably suppressed in both HepG2 and SMMC-7721 cells after treated by 2 μmol/L DAC for 3 d; however, the effect was more significant in SMMC-7721 cells. These results were in accordance with hTERT mRNA expression. The results indicated that inhibition of telomerase activity in HCC cells treated with DAC may contribute to a striking decrease in hTERT expression.

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**Table 1** Primer sequences for reverse transcription-polymerase chain reaction and methylation-specific polymerase chain reaction analysis

| Gene   | Primer sequence (5'-3') | Annealing (℃) | Product size (bp) |
|--------|-------------------------|----------------|------------------|
| hTERT-F | CCGAAGAGTGTCGGAGCAGAA   | 58             | 422              |
| hTERT-R | GATGAAGGCAGCTTCGCA      | 65             | 132              |
| E2F1-F  | GAAGGTGATCCAGACTCTGACCTT | 65             | 204              |
| E2F1-R  | ACCACACGCGTGTCCAGGAA    | 57             | 179              |
| p16-R   | ACCACACGCGTGTCCAGGAA    | 60             | 70               |
| p21-F   | GGACGACGACATCAGAGCAGTTT | 62             | 120              |
| p21-R   | GGATTAGGGCTTCCTCTTGGA   | 65             | 275              |
| WT1-R   | ACCACACGCGTGTCCAGGAA    | 60             | 70               |
| WT1-F   | GGATTAGGGCTTCCTCTTGGA   | 60             | 70               |

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Figure 1  Effect of 5-aza-2’-deoxycitidine on telomerase activity in human hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B). Cells were incubated with DAC (1 μmol/L, 2 μmol/L or 4 μmol/L). Cell pellets were collected and subjected to telomeric repeat amplification protocol-enzyme-linked immunosorbent assay. $^aP < 0.05$ (by unpaired Student’s $t$ test). Neg: Negative control; Pos: Positive control; DAC: 5-aza-2’-deoxycitidine. All studies are representative of at least three independent experiments.

Figure 2  Effect of 5-aza-2’-deoxycitidine on telomerase reverse transcriptase mRNA in hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B). Cells were incubated with DAC (1 μmol/L, 2 μmol/L or 4 μmol/L). Cell pellets were collected and subjected to real-time reverse transcription-polymerase chain reaction assay. PC: Positive control; DAC: 5-aza-2’-deoxycitidine; hTERT: Human telomerase reverse transcriptase.
Figure 3 Expression of telomerase reverse transcriptase protein in hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B) during exposure to 5-aza-2'-deoxycytidine (1 μmol/L, 2 μmol/L or 4 μmol/L). Total proteins were extracted, and Western blotting analysis was performed. The same blot was reprobed for β-actin as a loading control; hTERT: Human telomerase reverse transcriptase.

Figure 4 Methylation of telomerase reverse transcriptase promoter in 5-aza-2'-deoxycytidine-treated hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B). M: Methylation; U: Unmethylation; PC: Positive control; DAC: 5-aza-2'-deoxycytidine.

mRNA and protein.

Methylation of telomerase reverse transcriptase promoter in HCC cells by DAC
Since promoter methylation may be involved in hTERT repression in HCC cells, we observed the effects of DAC on promoter methylation of hTERT gene using MSP [18]. According to MSP analysis, the hTERT promoter was found to be hypermethylated in SMMC-7721, but not in HepG2 cells (Figure 4). The demethylation of hTERT was found in SMMC-7721 cells treated with DAC in a dose- and time-dependent manner, and there was complete demethylation after treatment with 5 μmol/L DAC for 3 d or 2 μmol/L for 3 d (Figure 4A). However, DAC showed no effects on hTERT methylation in HepG2 cells (Figure 4B). These data suggested that the demethylation of hTERT promoter by DAC could not play an important role in down-regulation of hTERT expression.

Expression of critical regulatory genes of telomerase reverse transcriptase transcription by DAC
We focused on some regulatory genes of hTERT transcription, such as c-myc, p15, p16, p21, E2F-1, WT1. Using real-time PCR, we examined the mRNA expression of these genes during exposure to DAC at 2 μmol/L for 1 d, 3 d and 5 d. The data showed that c-myc had high expression while p16 had low expression (SMMC-7721) or lost expression (HepG2) in hepatoma cells, and the other genes showed different levels of expression (Figure 5). The down-regulation of c-myc and up-regulation of p16 mRNA expression were found in HCC cell lines treated with DAC. Western blotting analyses further revealed the significant levels of c-myc and p16 in both SMMC-7721 and HepG2 cells. These results suggested that c-myc and p16 could play an important role in down-regulating the hTERT expression by DAC.

Methylation of p16 promoter and reactivation of its expression by DAC
We reproduced the methylation pattern of p16 (Figure 6) using MSP. With DAC treatment, we detected increased demethylation of p16 promoter. The data suggested that promoter hypermethylation could causatively contribute to transcriptional silencing of p16, which up-regulated the hTERT transcription in HCC cells.

Human hepatoma cells sensitive to chemotherapeutic agents after DAC treatment
To determine whether DAC could effectively inhibit HCC cell growth, we treated SMMC-7721 and HepG2 cells with various doses of DAC for 72 h. We found that DAC inhibited the growth of cell lines in a dose-dependent manner (Figure 7). To determine whether the growth inhibition by DAC can be enhanced by chemotherapeutic agents, SMMC-7721 and HepG2 cells were treated with DAC in combination with cisplatin. Notably, while dramatic morphological changes were caused by combination of DAC with cisplatin, many cells revealed detachment and shrinkage. The growth inhibition was about 76% in
the cells treated with DAC in combination with cisplatin (20 μmol/L) as compared with 54% in the cells treated with cisplatin alone, or 48% treated with DAC alone. These data suggested that DAC could enhance the sensitivity of HCC cells to chemotherapeutic agents such as cisplatin. With the administration of cisplatin and DAC, more cells revealed nuclear condensation and fragmentation of apoptotic cell death. These results were confirmed by Annexin-V and propidium iodide staining and FACS analysis (Figure 8). The cell apoptosis was significantly enhanced by the combined administration of 2 μmol/L DAC and 20 μmol/L cisplatin. These results suggested that the enhanced effects of the combined treatment on cell death were attributed to the augmented induction of apoptosis.

**DISCUSSION**

The most widely used demethylating agent, DAC, was first characterized 30 years ago and it functions as a mechanism-dependent suicide inhibitor of DNA methyltransferases, with which genes silenced by hypermethylation can be reactivated\[^{19}\]. It has been found that telomerase activity in cancer cells was inhibited by differ-
Differentiation, inducing demethylating agent DAC. We demonstrated that telomerase activity could be inhibited by DAC in HCC cells (HepG2 and SMMC-7721), and the demethylation of p16 promoter could play an important role.

Telomerase reactivation is a critical step in cellular immortality and carcinogenesis and is considered as a target for cancer treatment. Our lab revealed that more than 85% HCC had much stronger telomerase activity than cirrhosis. Drug-induced cell killing of tumor cells is associated with a decline in detectable telomerase activity. In the present study, DAC inhibited telomerase activity and down-regulated hTERT expression in both human hepatoma cell lines SMMC-7721 and HepG2, and DAC suppressed the transcriptional activity of hTERT genes. Targeting telomerase activity is one of the mechanisms responsible for this reagent’s inhibition of cancer cell growth.

On one hand, promoter methylation was associated with transcriptional silencing of the hTERT gene, as treat-
Figure 8  Apoptosis induced by 5-aza-2’-deoxycitidine. SMMC-7721 and HepG2 cells were treated with 2 μmol/L DAC, 20 μmol/L cisplatin, or in combination for 72 h. A: The fluorescent microscopic pictures of apoptotic cells were captured by 4’,6-diamidino-2-phenylindole staining of the condensed and fragmented nuclei; B, C: Annexin V-fluorescein isothiocyanate (FITC) staining following facial action coding system analysis was shown for SMMC-7721 (B) or HepG2 cells (C). DAC: 5-aza-2’-deoxycitidine; Con: Control; CDDP: Cisplatin.
ment of cells with demethylating agent DAC resulted in an increase in hTERT transcription in an immortal fibroblast line SUSM-122,23. On the other hand, DNA hypermethylation was implicated in the positive regulation of the hTERT promoter because demethylation in several telomerase-positive tumor cell lines reduced hTERT expression and telomerase activity accompanied by telomere shortening22,23. Our present study showed that hTERT promoter was methylated in SMMC-7721 cells, but not in HepG2 cells, and almost complete demethylation of hTERT promoter only occurred in the former cell line after DAC treatment. The explanation that CpG methylation likely interfered with the binding of transcriptional repressors, thereby positively regulating the hTERT promoter, may be reasonable for SMMC-7721 but not for HepG2. These findings suggested that hTERT promoter methylation is not the sole regulator of hTERT gene expression in HCC cells treated with DAC.

Several transcription factors are known to be responsible for the regulation of hTERT expression, including c-myc, p21, p16, p15, E2F-1 and Wilms' Tumor 1 suppressor gene24. This evidence prompted us to examine the effect of DAC on the expression of these genes. The c-myc was over-expressed while p16 expression was low or lost in hepatoma cell lines, and DAC repressed c-myc expression while reactivating p16. c-myc plays a critical role in telomerase activation through up-regulating the hTERT transcription, and this could be one mechanism by which 5-aza-CR represses hTERT transcription. Inactivation of p16-dependent pathways possibly in conjunction with telomerase activation might be a critical step for immortalization25. Our findings indicated that up-regulation of p16 and subsequent down-regulation of c-myc could be major pathways for hTERT repression by DAC.

Several evidences indicated that p16 expression could be transcriptionally silenced by CpG island hypermethylation in HCC26. The absence of expression and promoter methylation of p16 suggested that aberrant methylation is a major mechanism of the inactivation of p16 expression in HCC. Our data showed that DAC reversed p16 promoter methylation status and reactivated its expression, suggesting that p16 plays an important role in the down-regulation of telomerase activity by DAC.

In conclusion, the demethylating reagent 5-aza-CR represses telomerase activity and down-regulates hTERT expression. p16 could play a key role in this regulation. Our findings may provide insights into one of the mechanisms through which 5-aza-CR exerts growth-inhibitory effects on HCC cells.

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Innovations and breakthroughs
The authors demonstrated that the demethylating reagent DAC represses telomerase activity and down-regulates telomerase reverse transcriptase (hTERT) expression. p16 could play a key role in this regulation.

Applications
By understanding the mechanism of DAC repressing telomerase activity, this study may represent a future strategy for the treatment of patients with hepatocellular carcinoma (HCC).

Terminology
Telomerase and hTERT is a critical step in cellular immortality and carcinogenesis and is considered as a target for cancer treatment. And several transcription factors such as c-myc and p16 were responsible for the regulation of hTERT expression. The DNA methylation could play an important role in telomerase activity.

Peer review
This is a nice paper that describes the role of an inhibitor of DNA methylation DAC on telomerase expression and activity in HCC cell lines. Work has been well designed, effects have been proved in more than one HCC cell lines and in general terms, conclusions are supported by the presented results.

COMMENTS

**Background**
The inactivation of tumor suppressor genes by aberrant DNA methylation plays an important role in the development of malignancies. An inhibitor of DNA methylation, 5-aza-2′-deoxycytidine (DAC), could inhibit telomerase activity in some cancer cell lines, but the molecular mechanism remains unclear.

**Research frontiers**
DAC can inhibit cancer cell growth, particularly leukemia cells, and it has been
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