Investigation of the Effect of 5-Aza-2’-Deoxycytidine on p15INK4, p16INK4, p18INK4, and p19INK4 Genes Expression, Cell Growth Inhibition, and Apoptosis Induction in Hepatocellular Carcinoma PLC/PRF/5 Cell Line

Abstract

Background: Cyclin-dependent kinases (CDKs) are the key regulators of cell-cycle transitions and characterized by needing a separate subunit, a cyclin, which provides domains essential for enzymatic activity. The activities of cyclin-CDK complexes are controlled by a group of molecules that inhibit CDK activity and CDK inhibitors (CKIs). Cancer often exhibits an aberrant CpG methylation of promoter regions of tumor suppressor genes such as CKIs. Treatment with the DNA demethylating agents, such as 5-aza-2’-deoxycytidine (5-Aza-CdR), can restore and upregulate CKIs. Previously, we reported the effect of 5-Aza-CdR and genistein on DNA methyltransferase (DNMTs) in hepatocellular carcinoma (HCC). The aim of the present study was to evaluate the effect of 5-Aza-CdR on p15INK4, p16INK4, p18INK4, and p19INK4 genes expression, cell growth inhibition, and apoptosis induction in HCC PLC/PRF/5 cell line. Materials and Methods: The effect of 5-Aza-CdR on the cell growth of PLC/PRF/5 cells, genes expression, and apoptosis induction were assessed by 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl‑2H‑tetrazolium bromide assay, real-time quantitative reverse transcription‑polymerase chain reaction analysis, and flow cytometry, respectively. Results: 5-Aza-CdR (0, 1, 5, 10, 25, and 50 μM) inhibited PLC/PRF/5 cell growth at different periods significantly. This compound induced apoptosis and reactivated p15INK4, p16INK4, p18INK4, and p19INK4 genes expression at a concentration of 5 μM significantly. Conclusion: 5-Aza-CdR can inhibit cell viability and induce apoptosis by epigenetic reactivation of p15INK4, p16INK4, p18INK4, and p19INK4 genes in HCC PLC/PRF/5.

Keywords: 5-aza-2’-deoxycytidine, cyclin-dependent kinase inhibitor, hepatocellular carcinoma

Introduction

The event of cell division is a regulated process by which the cell is divided and produced into two daughter cells. This process, in turn, is mainly attributed to the presence of two classes of molecules, including cyclin-dependent kinases (CDKs) and their partners and cyclins. CDKs are the key regulators of cell-cycle transitions and characterized by needing a separate subunit, a cyclin, which provides domains essential for enzymatic activity. Mammalian CDKs are classified into three cell cycle-related subfamilies (Cdk1, Cdk4, and Cdk5) and five transcriptional subfamilies (Cdk7, Cdk8, Cdk9, Cdk11, and Cdk20). In human, the cyclin family includes 29 protein clustered in three major groups and 16 subfamilies, the group I (cyclin B group: A, B, D, E, F, G, J, I, and O); group II (cyclin Y group: a partner of the Cdk5 subfamily); and group III (cyclin C group: C, H, K, L, and T). The activities of cyclin-CDK complexes are controlled by a group of molecules that inhibit CDK activity and CDK inhibitors (CKIs). In mammals, seven CKIs have been identified which classified into two families, including the Cip/Kip and Ink4 families. The INK4 CDKI proteins sequester CDKs and inhibit the formation of CDK-cyclin complexes. This family contains p15INK4, p16INK4, p18INK4, and p19INK4. The Cip/Kip CDKIs bind to cyclin-CDK complexes, this family includes p21Waf1/Cip1, p27Kip1 and p57Kip2. Cancer often exhibits an aberrant CpG methylation of promoter regions of tumor suppressor genes (TSGs).
that is associated with loss of gene function without DNA structural changes. Nevertheless, methylation of different regulatory genes also indicates characteristic differences between different tumor and cancer types. Frequent promoter hypermethylation of TSGs, p14, p16, and p15 and p18INK4C has been reported in hepatocellular carcinoma (HCC). Similarly, it has been revealed that reduced levels of p15INK4b and p16INK4a are a prominent cause of pancreatic cancer.[27] Several studies have been demonstrated methylated p15 INK4b and p16 INK4a genes in colon cancer[8] and p15INK4B, p16INK4A, and p57KIP2 in breast cancer.[9] DNA methyltransferases (DNMTs) are a family of the enzyme that catalyzes DNA hypermethylation. This family includes DNMT1, DNMT3a, and DNMT3b.[10] Treatment with the DNA demethylating agents, such as 5-aza-2'-deoxycytidine (5-Aza-CdR), can restore and upregulate Ink4 family such as p16INK4A mRNA and protein correlated with demethylation of the p16INK4A promoter in HCC.[11] Previously, we reported the effect of DNA demethylating agent 5-Aza-CdR and genistein (GE) on DNMTs in HCC.[12,13] The aim of the present study was to evaluate the effect of 5-Aza-CdR on p15INK4, p16INK4, p18INK4, and p19INK4 genes expression, cell growth inhibition, and apoptosis induction in HCC PLC/PRF/5 cell line.

**Materials and Methods**

**Materials**

Human HCC PLC/PRF/5 cell line was purchased from the National Cell Bank of Iran – Pasteur Institute and cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum 10% (v/v), streptomycin 100 μg/mL, and penicillin 100 IU/mL in a humidified atmosphere of 5% CO₂ in air at 37°C.

5-Aza-CdR was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma) at a final concentration of 100 μM as a stock solution. All other experimental solutions were provided by diluting this solution. Trypsin-ethylenediaminetetraacetic acid (EDTA), DMSO, antibiotics, 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT), DMEM, Annexin-V-(FITC), propidium iodide (PI), and phosphate-buffered saline (PBS) were purchased from Sigma. Total RNA extraction kit (TRIZOL reagent) and real-time polymerase chain reaction (PCR) kits (quantitative PCR MasterMix Plus for SYBR Green I dNTP) were obtained from Applied Biosystems Inc. (Foster, CA, USA).

**Cell growth and viability**

The effect of 5-Aza-CdR on the cell growth of PLC/PRF/5 was determined by MTT assay. Briefly, the cells were seeded at the density of 5 × 10⁴ cells per well onto a 96-well plate overnight and then treated with 5-Aza-CdR at a concentration of 0, 1, 2.5, 5, 10, 25, and 50 μM for 24 h, 48 h, and 72 h. Following treatment, 20 μl of 0.5% MTT in PBS was added to each well, and the incubation was continued for 4 h at 37°C. Subsequently, the culture medium was replaced with 200 μl of DMSO, and the optical density was detected by a microplate reader at a wavelength of 570 nM.

**Flow cytometry analysis**

The percentage of PLC/PRF/5 apoptotic cells was evaluated by staining the cells with annexin-V-FITC and PI according to the manufacturer’s protocol. The cells were plated at a density of 5 × 10⁴ per well in 24-well plate and treated with 5-Aza-CdR (5 μM) for 24, 48, and 72 h, and except for the control groups, which incubated with DMSO only. Subsequently, all the cells were collected by trypsinization and washed with cold PBS and resuspended in binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaOH, and 2.5 mM CaCl₂) for 10 min at room temperature in the dark. Finally, the cells were incubated with annexin-V-FITC and PI according to the manufacturer’s protocol and analyzed by flow cytometry to determine the percentage of cells by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). Three independent experiments were performed for each concentration.

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

**(Quantitative reverse transcription-polymerase chain reaction) analysis**

A total RNA was extracted from 5-Aza-CdR (5 μM) treated PLC/PRF/5 cell line using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated by RNase-free DNase (Qiagen). Subsequently, cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The expression of p15INK4, p16INK4, p18INK4, and p19INK4 genes were quantified using the quantitative SYBR Green PCR kit (TaKaRa Bio) according to the manufacturer’s protocol and measured by quantitative real-time PCR using StepOnePlus (Applied Biosystems, USA) instrument. Primer sequences of the genes are indicated in Table 1.

| Primer       | Primer sequences (5’ to 3’) | Reference |
|--------------|----------------------------|-----------|
| p15INK4b     | GGGAAAGAAGGGAAGGATGCCTTG  | [14]      |
| Forward      | GCGACCTTGTAAGGATTCTCCTC   | [15]      |
| Reverse      | GCATGCCCTTGTTCTCCCTC      |           |
| p16INK4a     | CCGCTTTTCGTAGTTCCAT       | [16]      |
| Forward      | TTATTTGAGCTTGGTTCTG        |           |
| Reverse      | GGGGACCTAGAGCAACTTACT      |           |
| p18INK4      | GGCCTGCCCCGGCGATCAGAAG    | [17]      |
| Forward      | GGGGACCTAGAGCAACTTACT      |           |
| Reverse      | GGCAATCCGGCGATCAGGCTT     |           |
| p19INK4      | GTGCATGCCTGCTGAGGCTA      |           |
| Forward      | GGACCATCAGGCGCTGCTGAGGCTT |           |
| Reverse      | TGGCACCTTGCTGAGGCTT       |           |
in Table 1. GAPDH was used as an endogenous control. Data were analyzed using the comparative Ct (ΔΔct) method. The thermocycling condition was as described previously. [13]

Statistical analysis
The database was setup with the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA) for analysis. The data were acquired from three tests and are shown as means ± standard deviations. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA) and Turkey test. P < 0.05 was considered a statistically significant difference.

Results
Result of cell viability assay
The MTT assay measures the activity of cellular enzymes that reduce the tetrazolium dye to its insoluble form, formazan, which can reflect the number of viable cells. The viability of HCC PLC/PRF/5 treated with 5-Aza-CdR (0, 1, 5, 10, 25, and 50 μM) at different time periods (24, 48, and 72 h) was determined by MTT assay. As shown in Figure 1, the inhibitory effect of this compound on the PLC/PRF/5 cell line was dependent on the dose and incubation time. The agent indicated a significant inhibitory effect with all used doses (P < 0.001). IC50 values were obtained with approximately 5 μM of 5-Aza-CdR.

Result of apoptosis assay
Cell apoptosis was quantified by the combined staining of Annexin V and PI. To determine the effect of 5-Aza-CdR (5 μM) on cell apoptosis, the PLC/PRF/5 was stained using annexin-V-(FITC) to detect the cells undergoing apoptosis in an early stage and PI to distinguish annexin V-single positive cells from those subjected to necrotic processes.

Table 2: The percentage of apoptotic cells treated with 5-Aza-CdR at different time periods

| Drug     | Dose (μM) | Duration (h) | Apoptosis (%) | P     |
|----------|-----------|--------------|---------------|-------|
| 5-Aza-CdR| 5         | 24           | 12.2          | 0.006 |
| 5-Aza-CdR| 5         | 48           | 18.16         | 0.001 |
| 5-Aza-CdR| 5         | 72           | 88.3          | 0.001 |

Discussion
Cell division consists of two processes, including DNA replication and segregation of replicated chromosomes into two daughter cells. This process is divided into two stages: mitosis (M) and interphase, which interlude between two M phases and contains G1, S, and G2 phases. The transition from one phase to another is regulated by the key regulatory proteins CDKs. Activated

![Figure 1: The effect of 5-Aza-CdR on PLC/PRF5 cell viability. The cells were treated with different concentrations of 5-Aza-CdR (1, 2.5, 5, 10, 25, and 50 μM) for 24, 48, and 72 h, and the cell viability was assessed by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay. From left to right, the first column of each group belongs to the control group and other columns belong to experimental groups treated with various concentrations of 5-Aza-CdR (1, 2.5, 5, 10, 25, and 50 μM) for 24, 48. Each experiment was conducted in triplicate. Asterisks (*) indicate significant differences between treated and untreated cells.](image1)

![Figure 2: The apoptosis-inducing effect of 5-Aza-CdR on PLC/PRF5 cells investigated by flow cytometric analysis. As shown above, 5-Aza-CdR demonstrated a time-dependent apoptotic effect. Asterisks (*) indicate significant differences between treated and untreated cells. (a) Control group, (b) Experimental group treated with 5-Aza-CdR (5 μM) for 24h, (c) Experimental group treated with 5-Aza-CdR (5 μM) for 48h, (d) Experimental group treated with 5-Aza-CdR (5 μM) for 72h.](image2)
CDKs induce downstream processes by phosphorylating selected proteins. Two families of CDK inhibitors have been recognized, the INK4 and Cip/Kip families. Hypermethylation of the promoter region of these families leads to tumorigenesis. DNMT activity is one of the mechanisms by which CDK inhibitors are hypermethylated and inactivated. DNMT inhibitors can reactivate silenced CDK inhibitors. Previously, we reported the effect of DNMT inhibitors 5-Aza-CdR and GE in HCC.[12,13] In the present study, we report that 5-Aza-CdR can inhibit PLC/PRF cell and induce apoptosis by epigenetic reactivation of p15INK4, p16INK4, p18INK4, and p19INK4 genes. Similarly, a regulatory link has been reported between DNMT downregulation and cell cycle regulator p21WAF1 protein upregulation.[19,20] Other studies have been indicated that treatment with the demethylating agent 5-Aza-CdR induces the prolonged expression of p16INK4 protein in 33 tumor cell lines.[21] Furthermore, 5-Aza-CdR induces p21WAF1/Cip1 expression through two p53 binding sites in the p21 promoter.[22] Meanwhile, 5-aza-dC treatment reactive p14ARF and p16INK4a gene expression in colon cancer Colo320, HCT116, HT29, RKO, and SW480 cell lines.[23] At the molecular level, the INK4 family (p16INK4A, p15INK4B, p18INK4C, and p19INK4D) interact specifically with CDK4 and CDK6 resulting in CDK inhibition. The D-type cyclins and their partner and CDK4/6 have proto-oncogenic properties, and their activity is regulated at different levels, including negative control by two families of CDK inhibitors.[24] The cyclin D-Cdk4-6/INK4/Rb/E2F pathway has a key role on cell growth by integrating multiple mitogenic and antimitogenic stimuli. The members of the INK4 family block the progression of the cell cycle by binding to either Cdk4 or Cdk6 and inhibiting the action of cyclin D.[25] The p15INK4b and p19INK4d transcription is associated with FOXO (Forkhead box O)-mediated G1 cell cycle arrest. FOXO transcription factors are involved in apoptosis induction and cell cycle arrest by p27KIP1 transcription or apoptosis-related genes.[26] The tumor suppressor protein, p16INK4a plays as an inhibitor of CDK4 and CDK6 and the D-type CDKs that initiate the phosphorylation of the retinoblastoma, RB, and tumor suppressor protein. Thus, p16INK4a can arrest the cells in the G1-phase of the cell cycle.[27] Finally, The INK4 family can inhibit specifically Cdk4 or Cdk6 and cause G1 arrest when overexpressed.[28]  

**Conclusion**

5-Aza-CdR can inhibit cell viability and induce apoptosis by epigenetic reactivation of p15INK4, p16INK4, p18INK4, and p19INK4 genes in HCC PLC/PRF/5.

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**Conflicts of interest**

There are no conflicts of interest.
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