·Basic Research·

Effect of 5-Aza-dC on FHIT gene expression in hepatocellular carcinoma cell line HepG2

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[Abstract] **Background and Objective:** FHIT gene methylation leads to down-regulation of its expression in hepatocellular carcinoma (HCC) cells. This study was to detect the expression of FHIT mRNA and protein in HCC cell line HepG2 after treatment of methylase inhibitor 5-Aza-2'-deoxycytidine (5-Aza-dC), and observe the effect of 5-Aza-dC on the proliferation of HepG2 cells. **Methods:** HepG2 cells were treated with 5-Aza-dC. Methylation of FHIT in HepG2 cells was detected by methylation-specific polymerase chain reaction (MSP). FHIT mRNA expression was detected by reverse transcription-polymerase chain reaction (RT-PCR). FHIT protein expression was detected by immunohistochemistry and Western blot. Cell proliferation was detected by MTT assay. **Results:** Before treatment of 5-Aza-dC, FHIT gene methylation was detected in HepG2 cells, while no FHIT mRNA and protein expression was detected. The hypermethylation of FHIT gene in HepG2 cells was effectively reversed after treatment of 5-Aza-dC. When HepG2 cells were treated with 1.0, 2.0, and 4.0 μmol/L of 5-Aza-dC for 48 h, the mRNA levels of FHIT were 0.80±0.32, 1.41±0.54, and 1.51±0.61, respectively; the protein levels of FHIT were 0.33±0.20, 1.00±0.26, and 1.12±0.38, respectively. Cell proliferation was significantly inhibited after being treated with 5-Aza-dC. **Conclusion:** 5-Aza-dC can reverse the abnormal methylation of FHIT gene, activate the silenced gene and induce FHIT mRNA and protein expression in HepG2 cells.

Key words: 5-Aza-2'-deoxycytidine, liver neoplasm, FHIT gene, methylation, methylation-specific PCR

DNA methylation is one of the most common epigenetic modifications.[1] Except for gene mutation and deletion, promoter CpG hypermethylation is the third mechanism for inactivation of tumor suppressor gene (TSG).[2] FHIT gene was found as the first TSG that combines fragile site with human cancer,[3] and abnormality of FHIT gene is an early event in tumorigenesis and development.[4] Zhao et al.[5] found that FHIT protein was missing or lowly expressed in 65% of hepatocellular carcinoma (HCC), it was correlated to pathologic type and tumor size. Yuan et al.[6] found FHIT gene alterations in HCC.

Demethylation treatment has became a new tumor therapy through suppressing the proliferation and inducing the

differentiation of tumor cells, which has a bright application prospect in cancer therapy. 5-Aza-2-deoxycytidine (5-Aza-dC), a demethylation reagent, supresses the function of DNA methyltransferases (DNMT).[7] In this study, we investigated the effects of 5-Aza-dC on FHIT expression and proliferation of HepG2 cells.

Materials and methods

Main reagents. 5-Aza-dC (Sigma, USA) was dissolved in 50 mL phosphate buffer at the concentration of 0.1 μg/μL, then filtrated, sterilized, and stored at -70℃. High-glucose Dulebecco-modified Eagle medium (H-DMEM) was purchased from Qingdao ALP Biotech Ltd. Co., China. Methyldetector bisulfite modification kit was purchased from Active Motif, USA. FHIT antibody was purchased from Zymed, USA. TRIquick Reagent was purchased from Beijing Solarbio Science & Technology Co., Ltd, China. MmLV first strand cDNA synthesis kit and PCR amplification kit were both purchased from Shanghai Sangon Biotech Ltd, China.

Cell line and cell culture. HCC cell line HepG2 was purchased from the cell bank of the Institute of Biochemistry and Cell Biology (IBCB) of Chinese Academy of Sciences (CAS), and cultured in H-DMEM containing 10% fetal serum (Hangzhou Sijiqing Ltd., China) at 37℃ in humidified atmosphere containing 5% CO_2.

Detecting FHIT gene methylation by methylation-specific PCR. HepG2 cells were treated with 0, 1.0, 2.0, and 4.0 μmol/L 5-Aza-dC for 48 h, respectively, then centrifuged to extract DNA using genomic DNA extration kit. The genomic DNA were modified and purifed following the instructions of DNA methylation detection kit, then amplified as follows: reacted at 94° C for 3 min, followed by 40 cycles of reactions at 94℃ for 40 s, at 65℃ for 40 s, and at 72℃ for 15 s, and finalized at 72℃ for 10 min. Using the same volume of double distilled water instead of template DNA in negtive control. P16 was provided as positive control. The sequences of FHIT primers were as follows:
5-TTGGGGCGCGGGTTTGGGTTTT-TACGC-3 for upstream methylation primer and 5-CGTAAACGACGCCGACCCCACTA-3 for downstream methylation primer, with a product length of 100 bp; 5-TTGGGGTGTGGGTTTG GGTTTTTATG-3 for upstream unmethylation primer and 5-CATAAACAACACCAACCCC ACTA-3 for downstream methylation primer, with a product length of 100 bp. The PCR products were detected by agarose gel electrophoresis and analyzed by BiospectrumAC gel imaging system. The expression level of FHIT was indicated by the absorbance ratio of FHIT and β-actin: the higher the ratio was, the higher FHIT expression was.

Detecting FHIT mRNA expression by RT-PCR. Total RNA of untreated and 5-Aza-dC-treated HepG2 cells were extracted using TRIquick reagent, then reversely transcribed to synthesize first-strand cDNA. Target genes were amplified by reverse transcription-polymerase chain reaction (RT-PCR). β-actin was used as internal reference. The sequences were as follows: 5-ATGTCGTTCAGATTTGGCCAAC-3 for upstream FHIT primer and 5-TCATAGATGCTGTCATTCCTGT-3 for downstream FHIT primer, with a product length of 340 bp; 5-TGGATCAGCAAGCAGGAGT ATGACGAGT-3 for upstream β-actin primer and 5-CGCAAGTTAGGTTTTGTCAAGAA AGGGT-3 for downstream β-actin primer, with a product length of 116 bp. The PCR products were detected by agarose gel electrophoresis and analyzed by BiospectrumAC gel imaging system.

Detecting FHIT protein expression by immunohistochemistry. HepG2 cells were seeded 6-well plates at a density of 1×10^6/well, and divided into 5-Aza-dC group and control group (without 5-Aza-dC). After cell attachment, HepG2 cells in 5-Aza-dC group were cultured with 4 μmol/L 5-Aza-dC (dissolved in H-DMEM with 10% fetal serum) for 48 h, while the cells in control group were cultured without 5-Aza-dC. Then, cells were fixed in cold acetone for 5 min, and dried at room

temperature. Imunohistochemistry staining was performed according to the instruction of SP immunohistochemical staining kit. PBS was used instead of FHIT antibody in negative control group; positive breast carcinoma sample was used as positive control. The results were determined as priviously discribed:[8] positive cells in 5 high power fields on each slide were counted to calculate the proportion, a slide with a proportion of positive rate of <5% was scored 0, 5%-25% scored 1, 26%-50% scored 2, 51%-75% scored 3 and >75% scored 4; a slide with no staining was scored 0, light yellow scored 1, yellow or dark yellow scored 2, brown ro dark brown scored 3. Multiplying the two scores, a slide with a final score of >4 was considered positive.

Detecting FHIT protein expression by Western blot. HepG2 cells treated with or without 5-Aza-dC were harvested, and centrifuged to extract total protein using protein extraction kit. The protein was quantified with Coomassie brilliant blue. Concentration of the protein was caculated by formula as follows: protein concentration (g/L) = (absorbance of test tube/absorbance of standard tube) × protein concentration in standard tube. The protein was separated on a 12% SDS-PAGE, and transferred onto membrane by semi-dry method. The membrane was blocked with 5% defatted milk powder (disolved in 0.05% Tween-20 phosphate buffer), and visualized by ECL chemiluminescence reagent. The films were scaned and preserved using BiospectrumAC gel imaging system.

Detecting proliferation of HepG2 cells by MTT assay. HepG2 cells at exponential growth phase were harvested and seeded in 96-well plate at a density of 5×10^3/well (100 μL/well). After the cells were cultured at 37℃ in 5% CO_2 atmosphere for 4 h for cell attachment, the culture solution containing 10% fetal bovine serum (FBS) were removed and replaced by the solution containing 2% FBS for 24-hour incubation. The cells were cultured with 1.0, 2.0, 4.0 μmol/L of 5-Aza-dC in a final volume of 200 μL, or DMSO solution of the same concentrations as experimental control, or culture medium of the same volume as blank control; four wells for each group. Then cells were incubated for 24, 48, 72 h, respectively, added with 5 g/L MTT (20μL/well) and incubated for 4 h. After that, the culture solution was removed, 200 μL DMSO was added into each well, and the plate was shaked for 10 min on the shaking table to fully dissolve the crystallization. The absorbance value at 490 nm (A_{490}) was determined by a microplate reader using the value of blank control for zero adjustment. All the experiments were carried out in triplicates. The inhibiton rate of cell proliferation was caculated by the following formula: cell proliferation inhibition rate (%) = (A_{490} of control group - A_{490} of experiment group) / A_{490} of control group × 100%.

Statistical analysis. All analyses were performed using the SPSS12.0 software package. The data of FHIT mRNA and protein expression and cell proliferation inhibition rate were presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA and SNK-q test after homogeneity of variance test. A P value of < 0.05 was considered significant.

Results

FHIT gene methylation detected by MSP. FHIT gene methylation was detected in control HepG2 cells, while no methylation was detected in 5-Aza-dC-treated HepG2 cells (Fig. 1).

FHIT mRNA expression detected by RT-PCR. No FHIT gene expression was detected in control HepG2 cells, while the mRNA levels of FHIT gene were 0.80 ± 0.32, 1.41± 0.54, and 1.51± 0.61, respectively, in HepG2 cells treated with 1.0, 2.0, 4.0 μmol/L 5-Aza-dC for 48 h, with significant differences between each other ($P<0.05$) (Fig. 2).

FHIT protein expression detected by immunohistochemistry. FHIT protein was expressed in brown in cytoplasm (Fig. 3). The expression of FHIT protein in control HepG2 cells were scored 1. When treated with 1.0, 2.0, 4.0 μmol/L 5-Aza-dC, the staining in HepG2 cells was darkened gradually and the scores were

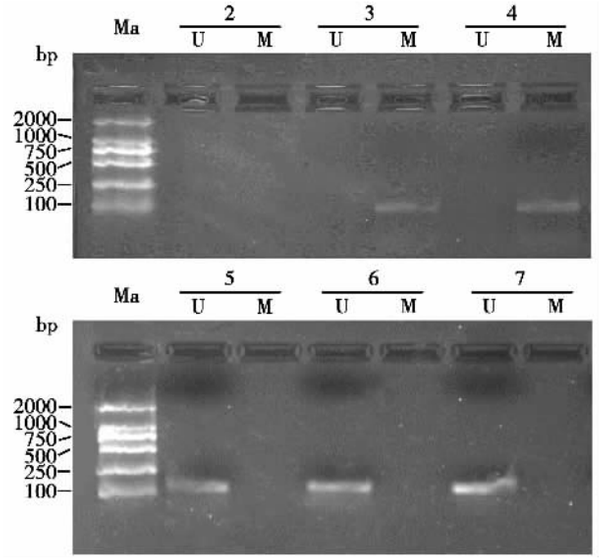

Figure 1 Methylation of FHIT gene in HepG2 cells detected by methylation-specific polymerase chain reaction (MSP)

U: unmethylation; M: methylation; lane Ma: marker D2000; lane 1: negative control; lane 2: positive control; lane 3: blank control; lanes 4–6: HepG2 cells treated with 1.0, 2.0, and 4.0 μmol/L 5-Aza-dC, respectively.

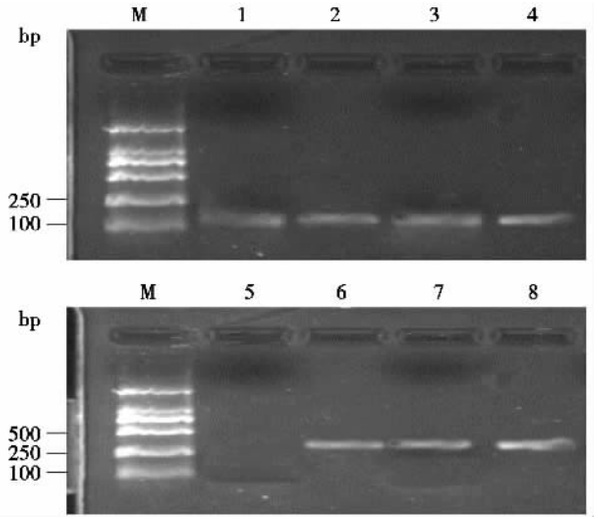

Figure 2 Expression of FHIT mRNA in HepG2 cells detected by RT-PCR

Lane M: marker; lanes 1–4: β-actin; lane 5: blank control; lane 6–8: HepG2 cells treated with 1.0, 2.0, and 4.0 μmol/L 5-Aza-dC, respectively.

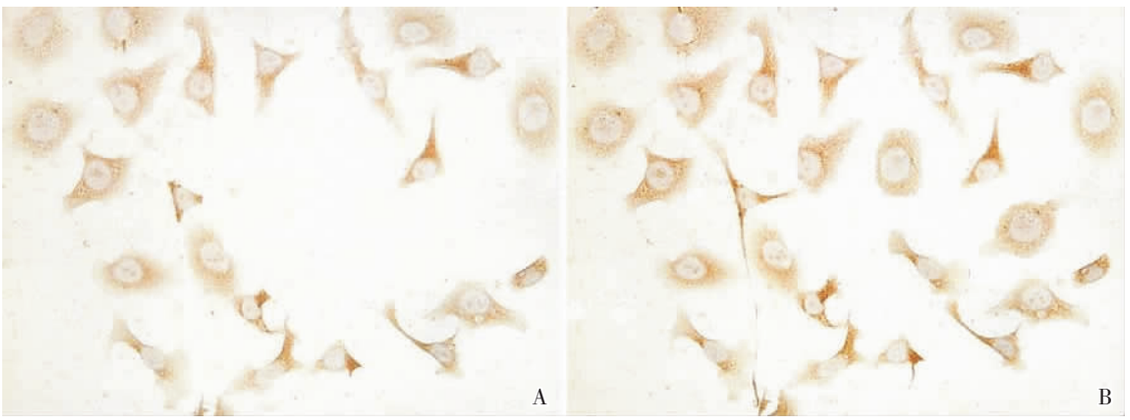

Figure 3 Expression of FHIT protein in HepG2 cells (IHC ×100)

A: Before treatment of 5-Aza-dC, FHIT protein is weakly expressed (in brown) in the cytoplasm of some cells.

B: After treatment of 5-Aza-dC, FHIT protein is intensely expressed in cytoplasm and the number of positive cells are obviously increased.

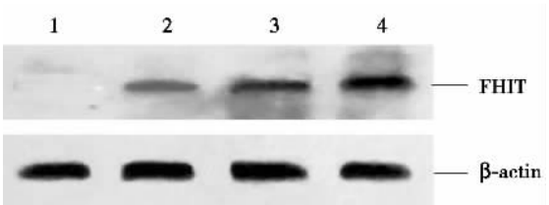

Figure 4 Expression of FHIT protein in HepG2 cells detected by Western blot

Lane 1: blank control; lanes 2–4: HepG2 cells treated with 1.0, 2.0, and 4.0 μmol/L 5-Aza-dC, respectively.

Table 1 Effect of 5-Aza-dC on proliferation of HepG2 cells

Group		Proliferation inhibition rate (%)		
		24 h	48 h	72 h
Control		0	0	0
5-Aza-dC	1.0 μmol/L	13.5±0.6	17.0±1.1	27.0±1.6
	2.0 μmol/L	28.9±1.5	37.0±1.9	43.8±2.4
	4.0 μmol/L	34.8±1.7	38.8±2.6	67.3±3.0

All $P<0.05$, vs. control; all $P<0.05$ when comparing at the same time point among groups with different concentrations of 5-Aza-dC; all $P<0.05$ when comparing the same group at different time points.

2-3, 3-4, 5-6, respectively.

FHIT protein expression detected by Western blot. No FHIT protein expression was detected in control HepG2 cells; when treated with 1.0, 2.0, 4.0 μ mol/L 5-Aza-dC for 48 h, the protein levels of FHIT were 0.33 ± 0.20, 1.00± 0.26, 1.12± 0.38, respectively, in HepG2 cells, with significant differences between each two groups ($P<0.05$) (Fig. 4).

Proliferation of HepG2 cells detected by MTT assay. 5-Aza-dC (1.0, 2.0, 4.0μ mol/L) significantly inhibited the proliferation of HepG2 cells. With the increase of 5-Aza-dC concentration, the inhibitory effect was getting significant (Table 1).

Discussion

DNA methylation is one of the main manners of epigenetic modification. It plays important roles in cell development, gene expression and stabilization, while does not change the primary structure of DNA. Low methylation of whole genome and hypermethylation of CpG island which is normally unmethylated are commonly exist in human cancer. Except for gene mutation and deletion, promoter hypermethylation is the third mechanism for inactivation of TSG. FHIT gene is the first TSG that combines fragile sites with human tumor. FHIT gene abnormality is an early event in tumorigenesis and development. It is located at the most common fragile sites in human genome, and its abnormality is associated to many tumor cell lines and human primary tumors.[9] In our study, the mRNA and protein expression of FHIT gene was decreased in HepG2 cells, indicating that FHIT gene may be down-regulated before transcription in HepG2 cells.

CpG island methylation influences gene expression mainly through binding with methyl-CpG-binding proteins (MeCPs), while its density and distribution directly influences the recognition of MeCPs.[10] Nowadays, demethylation treatment has become a new tumor therapy which suppresses tumor cell proliferation and induces cell differentiation, and has a bright application prospect. Using methylase inhibitors to treat acute myeloid leukemia, myelodysplastic syndrome, and chronic myelogenous leukemia had been widely reported, which showed good therapetic effects and had been approved to be used in the treatment of myelodysplastic syndrome by Food and Drug Administration of the USA.[11] However, the mechanisms of methylase inhibitors, such as 5-Aza-dC, are still unkown.

In this study, we treated HepG2 cells with 5-Aza-dC to investigate whether methylase inhibitor can restore the expression of FHIT gene. Our results showed FHIT gene hypermethylation in HepG2 cells, but no FHIT mRNA and protein expression. However, the situation of hypermethylation was reversed after treatment of 5-Aza-dC, FHIT mRNA and protein expression was restored, indicating that hypermethylation of FHIT promoter may be the cause of abnormal expression of FHIT in HepG2 cells. Exploring the relationship between abnormal FHIT expression and HCC as well as the moleculor mechanisms that affect FHIT expression is important for further investigation on the function of FHIT in the genesis and development of HCC, and on antitumor therapy by regulating the expression of FHIT gene.

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