



Regulation of DNA methylation on the expression of the FHIT gene contributes to cervical carcinoma cell tumorigenesis

YING WU*, LI MENG*, HUI WANG, QIAN XU, SHIXUAN WANG, SUFANG WU, LING XI, YUN ZHAO, JIANFENG ZHOU, GANG XU, YUPING LU and DING MA

Cancer Biology Research Center, Tongji Hospital, Tongji Medical School, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

Received February 24, 2006; Accepted May 4, 2006

Abstract. The purpose of this study was to determine the effect of the FHIT gene on tumorigenesis of cervical cancer. RT-PCR and MTT were used to detect the expression of FHIT and cell proliferation respectively. Flow cytometry was used to test cell cycle and cell apoptosis. The expression of FHIT was not induced at all four cervical cancer cells treated with demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR). However, a constant level of FHIT expression was detected in the human umbilical vein endothelial cell before and after 5-aza-dC treatment. The proliferation of all four cervical cancer cells was inhibited evidently when treated with 5-aza-dC and the inhibiting rate of cell growth along with the increasing concentration of 5-aza-dC. There was no obvious inhibiting effect on the growth of the human umbilical vein endothelial cell treated with 5-aza-dC. An increasing G1 phase and high apoptosis rate were detected in all four cervical cancer cells. However, a negligible change was seen in the human umbilical vein endothelial cell. These results suggest that aberrant methylation of the FHIT gene might be a key mechanism for silenced FHIT gene, which could be reactivated and whose tumor suppressing function could be restored by demethylating agent treatment.

Introduction

Cervical cancer is the third most common cancer in women, with approximately 371,200 new cases diagnosed each year

worldwide (1). Overall, the ratio of mortality to incidence is 51%. Hence, cervical cancer remains a significant public health concern (1). Whereas the exact etiology of cervical cancer remains unknown, it appears that an early event is infection by human papilloma virus (HPV), which has been found in 99.7% of squamous cell cervical carcinomas (2). The role of HPV infection in the development of cervical dysplasia involves the inactivation of cellular proteins p53 and Rb by the HPV proteins E6 and E7 (3). However, not all patients with HPV infection develop cervical dysplasia or invasive cervical carcinoma. Most infected women do not develop invasive cancer (4,5). Hence, other genetic events, such as oncogene activation or tumor suppressor gene inactivation, are likely to be required in addition to infection with HPVs for the development of cervical cancer.

The candidate tumor suppressor gene, FHIT, has been cloned and mapped to the chromosome region 3p14.2, one of the regions most frequently deleted in cervical carcinoma (6-10). The FHIT gene encompasses a region of over 1 Mb, which includes the common frequent break-point in 3p14.2 fragile site (FRA3B), and encodes a 16.8-kDa protein that has been shown to function as a 5', 5'''-P₁, P₃-triphosphate (Ap₃A) hydrolase *in vitro* (11). Studies have provided evidence supporting a role for FHIT in the regulation of apoptosis and the cell cycle (12,13). In addition, recent studies have reported aberrant FHIT transcripts and/or loss of Fhit protein expression in 43-61% of invasive cervical cancers and a third of high-grade cervical intraepithelial lesions but only rarely in low-grade cervical intraepithelial lesions. Integration of HPV into the FRA3B/FHIT locus has also been observed in a primary cervical carcinoma. Collectively, the findings suggest that FHIT gene alterations may play an important role in cervical tumorigenesis.

DNA methylation is a frequent epigenetic event in many human cancers (14,15). A growing number of cancer-related genes are being recognized that harbor dense methylation of cytosine in normally unmethylated CpG-rich sequences, called CpG islands, located within the 5' gene promoter regions (16). Several studies have indicated that methylation was essential for normal development (17), X chromosome inactivation (18) and imprinting (19,20). Aberrant promoter methylation is an important mechanism for loss of gene function in tumors and may be more frequent than mutations in coding regions (21-24). Furthermore, genes silenced by hypermethylation can be

Correspondence to: Dr Ding Ma, Cancer Biology Research Center, Tongji Hospital, Tongji Medical School, Huazhong University of Science and Technology, 1095 Jiefang Ave., Wuhan, Hubei 430030, P.R. China
E-mail: dma@tjh.tjmu.edu.cn; dingma424@yahoo.com

*Contributed equally

Abbreviations: HPV, human papilloma virus; FHIT, fragile histidine triad; 5-Aza-CdR, 5-aza-2'-deoxycytidine

Key words: HPV, FHIT, cervical cancer, DNA methylation, 5-Aza-CdR

reactivated by 5-Aza-2'-deoxycytidine (5-Aza-CdR) (25), which inhibit DNA methylation and are also effective anti-leukemic agents (26,27).

In this study, we sought to further define the role of FHIT in cervical cancer by restoring FHIT expression in four human cervical cancer cell lines (CaSki, C-33A, HeLa, SiHa) and one human umbilical vein endothelial cell line (ECV-304), following treatment with demethylating agent 5-Aza-CdR. The purpose of this study was designed to evaluate whether aberrant methylation of the FHIT gene is an important mechanism for loss of gene function, or if FHIT gene alterations play an important role in cervical tumorigenesis.

Materials and methods

Cell lines. CaSki, C-33A, HeLa and SiHa (cervical carcinoma-derived cell lines) and ECV-304 (human umbilical vein endothelial cell line) were obtained from the American Type Culture Collection. C-33A, HeLa and SiHa were cultured in DMEM supplemented with 10% FCS and 5% penicillin/streptomycin. CaSki and ECV-304 were cultured in RPMI-1640 supplemented with 10% FCS and 5% penicillin/streptomycin.

5-Aza-CdR treatment. Cells were plated (2×10^5 cells/60-mm dish) and treated 24 h later with 5×10^{-7} M, 10^{-6} M, 2×10^{-6} M, 5×10^{-6} M or 10^{-5} M 5-Aza-CdR (Sigma). The medium was changed 24 h after drug treatment and every subsequent 2 days.

RNA extraction and cDNA synthesis. RNA was extracted using TRIzol (Promega) according to the manufacturer's protocol. RNA was solubilized in RNase free water and stored at -80°C . First-strand cDNA was synthesized from 2 μg of DNase I-treated total-RNA. RNA was reverse transcribed in a 25- μl volume of 5X first-strand buffer, 10 mM dNTPs, 0.5 $\mu\text{g}/\mu\text{l}$ random hexamers, 25 units of RNasin (Promega), and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) by incubation at 42°C for 60 min. Each cDNA synthesis reaction was paired with a control reaction without the addition of reverse transcriptase.

RT-PCR and cDNA sequencing. Amplification of FHIT cDNA encompassing the entire open reading frame was performed using non-nested (28) RT-PCR strategies as described previously. Primers for non-nested PCR (MUR5 and RP2) yield expected PCR products of 747 bp, extending from the terminal portion of exon 2 into exon 10. The PCR reactions were performed in 25- μl reaction volumes using 175 ng of each FHIT primer, 300 μM of dNTPs, 2.5 units of Taq polymerase (Promega), and 2 μl of cDNA. Initial denaturation for 90 sec at 95°C was followed by 35 cycles of 95°C for 30 sec, 62°C for 60 sec and 70°C for 60 sec, and a final extension at 70°C for 5 min. To exclude RNA degradation as an explanation for the absence of FHIT expression, RT-PCR using GAPDH-specific primers (product of 508 bp) was performed on all RNA samples. The GAPDH amplifications were performed in 25- μl reaction volumes using 0.4 μM GAPDH-specific primers (sense, 5'-GAGAAGTATGACAACAGCCTC-3'; antisense, 5'-AGTGGTCGTTGAGGGCAATG-3'), 200 μM dNTPs, and 1.5 units of Taq polymerase. Reactions were initially denatured at 95°C for 2 min and then amplified with

30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 2 min, and a final extension at 72°C for 5 min.

Determination of cell growth rate. Inhibition of cell growth by the drug treatment was assayed by MTT staining of viable cells. In brief, when cells were in the logarithmic growth phase, they were inoculated to the 96-pore plate, with 200 μl cell suspension in each pore. After 24-h incubation, 5-Aza-CdR of different concentrations (5×10^{-7} M, 10^{-6} M, 2×10^{-6} M, 5×10^{-6} M and 10^{-5} M) was added to the cells and cells were cultured for another 24 or 72 h, then 50 μl of MTT (2 mg/ml in PBS) was added to each well, and each plate was incubated for an additional 4 h at 37°C until a purple precipitate was visible. The medium was then carefully removed, and precipitates were dissolved in 100 μl of DMSO. The growth rate was plotted as the percentage of viable cells in PBS-treated controls (a value arbitrarily set at 100%). Each experiment was repeated three times. Data were presented as an average of the results from individual experiments.

Apoptosis and cell cycle analysis. Cells (10^5 /60-mm dish) were plated and treated 24 h later with 5×10^{-7} M, 10^{-6} M, 2×10^{-6} M, 5×10^{-6} M or 10^{-5} M 5-Aza-CdR. Cells were washed and fixed after 24 or 72 h with 75% ethanol for 15 min at 4°C , then treated with RNasin (20 $\mu\text{g}/\text{ml}$), and stained with propidium iodide (50 $\mu\text{g}/\text{ml}$; Sigma). DNA content at each cell cycle stage was determined via flow cytometry.

Statistical analysis. Standard error for all measured biological parameters is displayed in the appropriate figures. Student's two-sided t-test was used to compare the values of the test and control samples. A value of $P < 0.05$ was taken as significant.

Results

Methylation of specific CpG sites in the promoter region results in transcriptional silencing of the FHIT gene in cervical-carcinoma cell lines. Human umbilical vein endothelial cell line ECV-304 and cervical carcinoma-derived cell lines CaSki, C-33A, HeLa and SiHa were treated with increasing concentrations of 5-Aza-CdR by 24 or 72 h, and RT-PCR was performed to determine whether FHIT was induced by drug treatment. A PCR product of 747 bp was detected in ECV-304 cells, but was not observed in all cervical carcinoma-derived cell lines (Fig. 1). As shown in Fig. 1B, treatment with the demethylating agent, 5-Aza-CdR, restored FHIT expression in CaSki, SiHa, C-33A and HeLa (data of the last three cell lines is not shown). The FHIT expression in cervical cancer cell lines strongly increased with treatment of 10^{-6} M and 2×10^{-6} M 5-Aza-CdR at 24 h of treatment and 2×10^{-6} M 5-Aza-CdR at 72 h of treatment, which might be suitable concentrations and time-points to reactivate FHIT function. In contrast, there was a negligible change in ECV-304 cells. These results strongly suggested that methylation-mediated inactivation of FHIT in cervical cancer cells could be reversed by demethylating with 5-Aza-CdR treatment.

The tumor cell growth inhibition of FHIT after treatment with 5-Aza-CdR. To determine whether 5-Aza-CdR, an inhibitor of

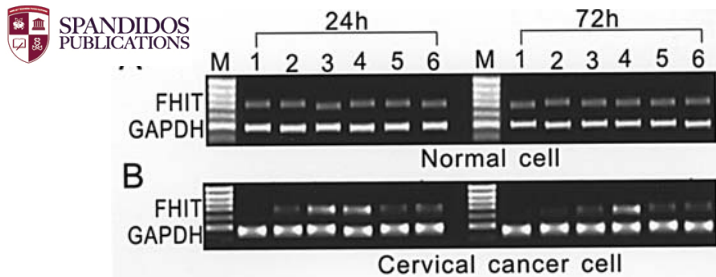


Figure 1. 5-Aza-CdR induced FHIT expression. FHIT expression was measured via RT-PCR. A PCR product of 747 bp corresponding to the expected size of the FHIT RT-PCR product was detected. Lane M, 100 bp DNA ladder; lane 1, non-treatment; lane 2, treatment with 5×10^{-7} M 5-Aza-CdR; lane 3, treatment with 10^{-6} M 5-Aza-CdR; lane 4, treatment with 2×10^{-6} M 5-Aza-CdR; lane 5, treatment with 5×10^{-6} M 5-Aza-CdR; lane 6, treatment with 10^{-5} M 5-Aza-CdR. (A) FHIT expression in normal cells. Total-RNA was isolated from ECV-304 cells. GAPDH expression was measured to be a control for cDNA input and integrity (product of 508 bp). (B) 5-Aza-CdR induced FHIT expression in Caski cervical cancer cells. Total-RNA was isolated from these cells lines after 5-Aza-CdR treatment (as described in Materials and methods). Untreated cells were analyzed under similar conditions as a control. GAPDH expression was measured as a control for cDNA input and integrity.

DNA methylation, could restore the growth inhibition effect of the FHIT gene on cervical cancer cell proliferation, the growth curves of tumor proliferation were observed after treatment with increasing 5-Aza-CdR concentrations. Normal umbilical vein endothelial cells ECV-304 were given the same treatment as control (Fig. 2). During the five days of treatment with 5-Aza-CdR, Caski cell proliferation was strongly inhibited at concentrations of 10^{-6} M and 2×10^{-6} M 5-Aza-CdR and showed significant difference when compared with non-treated Caski cells. C-33A, HeLa and SiHa cells showed a similar trend (data not shown). However, ECV-304 cell proliferation was not inhibited in the same conditions. The reason may be that abnormal methylation patterns do not exist in these normal cells; thus tumor cells, but not normal cells, were responsive to the growth-suppressive effects of this DNA methylation inhibitor.

Effects of FHIT on cell cycle kinetics after treatment with 5-Aza-CdR. To further investigate the effects of 5-Aza-CdR on cell cycle, Caski, C-33A, HeLa and SiHa cells were treated with increasing concentrations of 5-Aza-CdR (5×10^{-7} M, 10^{-6} M, 2×10^{-6} M, 5×10^{-6} M and 10^{-5} M). Flow cytometry was used to analyze the changes of the cell cycle profiles in Caski after 24 h treatment with 5-Aza-CdR (Fig. 3A, Table I). Most cells in the cell cycle were intercepted before S-phase when treated with 5-Aza-CdR by any concentration below 10^{-5} M, resulting in a decreased number of cells in S-phase compared with the non-treated group. Similar changes were detected in the other three cervical cancer cell lines (data not shown). The data in this study demonstrated that FHIT expression induced by 5-Aza-CdR results in most tumor cells being arrested in G1-phase and blocks cell immortalization.

Tumor cell apoptosis induction by FHIT gene reactivation by 5-Aza-CdR treatment. Next, we observed the effects of FHIT reactivation on tumor cell apoptosis at increasing concentrations of 5-Aza-CdR. The induction of apoptosis of four

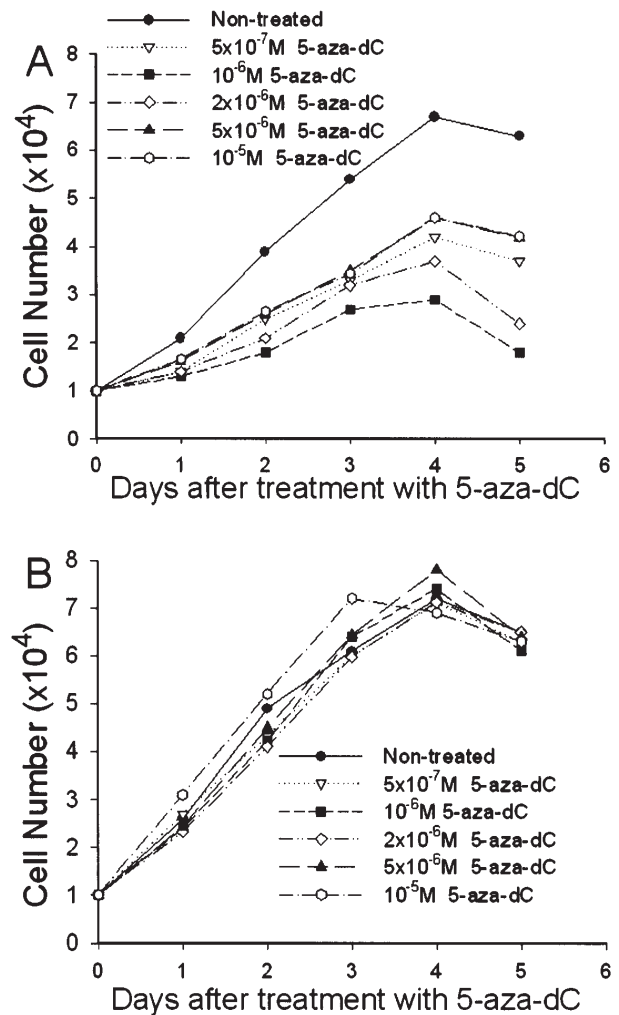


Figure 2. Effects of 5-aza-dC on growth suppression. (A), 5-aza-dC induced growth suppression in Caski cervical carcinoma-derived cell lines. Cells were plated and treated with 5×10^{-7} M, 10^{-6} M, 2×10^{-6} M, 5×10^{-6} M or 10^{-5} M 5-aza-dC. Non-treated cells were analyzed under similar conditions as a control. (B), The cell proliferation of human umbilical vein endothelial cell line ECV-304 with the same drug treatment.

Table I. Effects of 5-aza-dC on cell cycle profiles in cervical cancer cells.

Concentration of 5-aza-dC	G1 (%)	G2/M (%)	S (%)
Non-treatment	71.43	14.53	14.13
5×10^{-7} M	74.23	20.11	5.66 ^a
10^{-6} M	80.35	17.15	2.50 ^a
2×10^{-6} M	78.43	21.57	0.00 ^a
5×10^{-6} M	78.49	16.63	4.88 ^a
10^{-5} M	70.55	13.41	16.04

^aCompare with non-treatment group ($P < 0.05$).

cervical cancer cells was determined by microscopic examination of Propidium Iodide (PI)-stained cells, and the DNA content (apoptotic nuclei) was measured by using FACScan

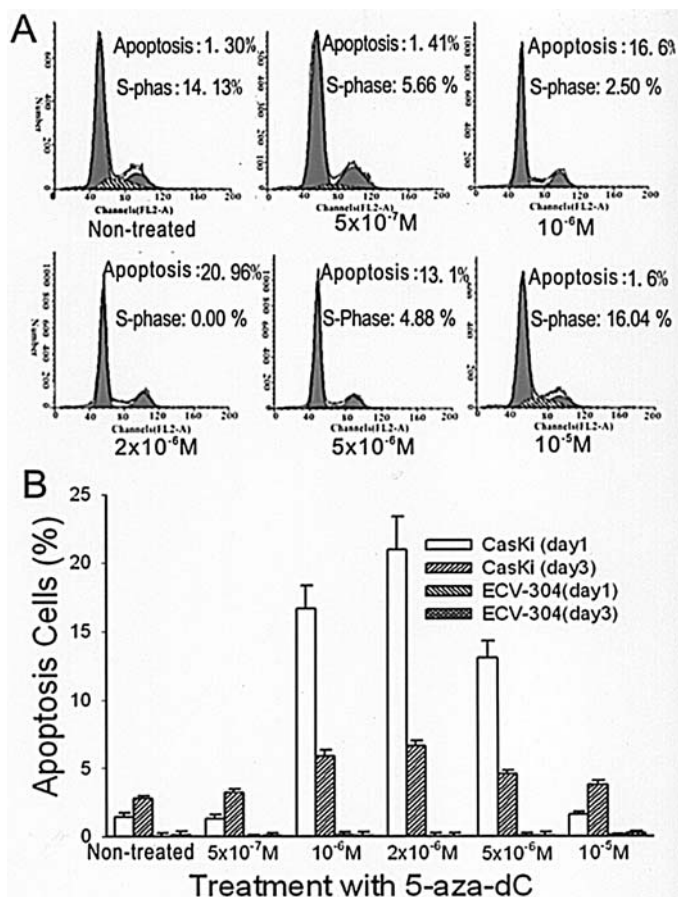


Figure 3. 5-aza-dC induced apoptosis and effect on cell cycle kinetics in cervical cancer cell. (A) Cells were treated with 5-aza-dC by 5×10^{-7} M, 10^{-6} M, 2×10^{-6} M, 5×10^{-6} M or 10^{-5} M. Representative results from flow cytometry of propidium iodine-stained cervical cancer cell. The percentages of apoptotic cells are indicated as the proportion of cells that contained sub-G₁ DNA. The distribution of tumor cells in cell cycle was counted. (B) After 24 or 72 h treatment with 5-aza-dC, apoptosis of cells was analyzed by flow cytometry. The rate of apoptosis is expressed as the percentage of PI-stained cells in the total cell population. Human umbilical vein endothelial cell line ECV-304 was tested as control. The means and errors results come from triple experiments.

analyses (Fig. 3). We found that exposure of CasKi cells to increasing concentrations of 5-Aza-CdR resulted in morphologic alterations characteristic of apoptosis, including nuclear condensation and fragmentation. The number of apoptotic cells increased with the concentration of 5-Aza-CdR. The inducing apoptotic peak of tumor cells was reached at the concentration of 2×10^{-6} M 5-Aza-CdR. A similar apoptosis tendency was detected in the other three cervical cancer cell lines when treated with 5-Aza-CdR (data not shown). In contrast, ECV-304 cells had a poor apoptotic response to the treatment of 5-Aza-CdR. These observations provide evidence that reactivation of FHIT can induce apoptosis in cervical cancer.

Discussion

Cervical carcinomas, as other tumors, likely result from an accumulation of genetic alterations such as activating oncogenes, inactivating tumor suppressor genes, and genes involved in DNA damage recognition and repair. Although infection with HPVs has been established as an important initiating

event in the development of pre-invasive lesions of the cervix, relatively few will progress to invasive carcinoma if left untreated (28). So, HPV infection is insufficient for tumor development. Efforts to characterize the genetic alterations that occur in cervical carcinogenesis have revealed several chromosomal regions that have a high frequency of allelic loss in cervical cancers (29,30). Those alterations that have been identified include amplification of c-myc, HER2-neu, and an as-yet-unidentified gene in the distal region of chromosome 3q (31-33). The allelic losses of chromosome 3p have also been seen in a number of analyses of cervical cancer (30). Despite considerable efforts, the tumor suppressor gene targeted by 3p allelic losses in cervical carcinoma has not been identified conclusively. One of the regions of 3p commonly deleted in cervical carcinomas includes the fragile histidine triad (FHIT) gene.

The FHIT gene, located at 3p14.2, has demonstrated an altered function in numerous cancers. Abnormal FHIT transcripts have been identified previously in a variety of tumor cell lines and primary carcinomas, integration of human papillomavirus DNA has been especially identified at a fragile site (FRA3B) within the FHIT locus in cervical cancer. Reexpression of this gene in tumor cells has yielded conflicting results. It has been reported that the FHIT gene is involved in the regulation of the cell cycle and its tumor suppressor activity derived from proapoptotic activity (12). However, other study did not discover any regulation of the cell cycle or function with respect to induction of apoptosis (30,34). These conflicting data suggest that the mechanisms involved with the function of the FHIT gene may be different from other classic tumor suppressor genes such as p53 and Rb1, and FHIT functions may exert tumor suppressive activity in a tissue-specific fashion or at a particular point in the multistage process of carcinogenesis.

To clarify the molecular mechanism of the FHIT gene in the cytogenesis of cervical cancer and determine whether the hypermethylation of the FHIT gene played an important role in cervical tumorigenesis, we analyzed the effects of 5-Aza-CdR reintroduction of FHIT expression in cervical carcinoma-derived cells. Our results showed that demethylation through 5-Aza-CdR treatment results in cell proliferation inhibition and apoptosis reduction in cervical carcinoma-derived cell lines restored the depressed expression of FHIT; whereas, no substantial changes were detected in human umbilical vein endothelial cell line ECV-304 treated with 5-Aza-CdR. 5-Aza-CdR is well known for its ability to induce the expression of genes silenced by demethylation, which was further demonstrated by the data of increasing expression of the FHIT gene after treatment with the demethylating agent 5-Aza-CdR in the present study. A possible inference from our data is that FHIT methylation leads to suppressed tumor gene inactivation or silence and promotes tumorigenesis and development in cervical cancer, in spite of the sustained infection of HPVs.

The data in this study has confirmed that methylation of FHIT is important for tumorigenesis in cervical cancer, and this situation might be inverted by demethylation through 5-Aza-CdR treatment. A major question we address pertains to the role of the active FHIT gene in immediate cell apoptosis responses induced by 5-Aza-CdR. We have shown that most cervical cancer cells were constrained in G₁-phase, and a few



cells went into S-phase when treated with 5-Aza-CdR. r cells (0%) were detected in S-phase when the concentration of 5-Aza-CdR reached 2×10^{-6} M. Our study suggested that loss of FHIT function might potentiate the transition of cells into S-phase and increase cellular proliferation, and FHIT gene silencing dissolved by 5-Aza-CdR treatment might lead to tumor cell arrest in G1-phase and block cell immortalization.

In conclusion, we demonstrated that aberrant methylation of the FHIT gene might be an important mechanism of loss of gene function. This candidate tumor suppressor gene silenced by hypermethylation could be overturned with demethylation, and the tumor suppressing functions of FHIT could also be aroused by 5-Aza-CdR treatment. This instigation will help us to better understand the interaction between two factors strongly involved in cervical carcinogenesis, FHIT gene inactivation and HPV infection, and the mechanism of FHIT-mediated apoptosis. It therefore seems that restoration of the FHIT gene by demethylation through 5-Aza-CdR treatment may become a therapeutic approach for the prevention and treatment of cervical cancer.

Acknowledgements

This study was supported by grants from the National Science Foundation of China (no. 30025017 and no. 30271358) and the '973' Program of China (no. 2002CB513100).

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