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ORIGINAL ARTICLE

Hydralazine inhibits human cervical cancer cell growth in vitro in association with APC demethylation and re-expression

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Abstract
Purpose The tumor suppressor adenomatous polyposis coli (APC) is frequently silenced by promoter hypermethylation in human cervical cancer. Clinically, it has been approved that DNA methylation inhibitors, such as 5-aza-2′-deoxycytidine (5-Aza-dC), can reverse APC promoter methylation, but widespread clinical use of these inhibitors is limited by their toxicity and instability in aqueous solution. Hydralazine is a stable DNA methylation inhibitor that has minimal toxicity in vitro and in vivo. The purpose of this study was to evaluate the effects of hydralazine on APC reactivation and the inhibition of human cervical cancer cells in vitro.
Methods Expression of APC gene, and methylation status were analyzed by RT-PCR, quantitative real time RT-PCR, and methylation-specific PCR methods. β-Catenin protein that correlates closely with APC was detected by immunohistochemistry method after treatment with hydralazine. MTT and FCM assays were used to observe the changes of proliferation activity, cell cycle, and apoptosis of the cells.
Results Methylated APC was not expressed in HeLa cell, hemimethylated APC was expressed in CaSki cells, and unmethylated APC was expressed normally in SiHa cells. Hydralazine induces APC expression and promotes demethylation in HeLa and CaSki cells. After treatment with 40 μmol/L hydralazine for 72 h, growth inhibitive rates (%) of HeLa, CaSki, and SiHa cell lines were 52.12 ± 3.78, 44.31 ± 2.59, and 47.73 ± 4.73, respectively. On the contrary, the normal cell ECV304 growth inhibitory rate was only 27.18 ± 0.79. The expression of APC mRNA in HeLa, CaSki, and SiHa cell lines increased 10.35-, 11.40-, and 0.73-fold, respectively. HeLa and CaSki cells were arrested in S phase of the cell cycle by hydralazine, and the percentage of apoptotic cells in the two cell lines treated with hydralazine was increased significantly compared to the untreated cells ($P < 0.01$). The expression of β-catenin protein in the cell membrane was observed after the treatment with hydralazine.
Conclusions Hydralazine, an effective inhibitor of APC methylation and promoter of APC re-expression, can inhibit cell growth in human cervical cancer in vitro and be potentially used for the clinical treatment of human cervical cancer.

Keywords Hydralazine · Cervical cancer · APC · DNA methylation, β-catenin

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Abbreviations

5-Aza-C	5-Azacytidine
5-Aza-dC	5-Aza-2′-deoxycytidine
APC	Adenomatous polyposis coli
FAP	Familial adenomatous polyposis
FCM	Flow cytometry
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IHC	Immunohistochemistry
MSP	Methylation-specific PCR

Introduction

Tumor suppressor gene APC (adenomatous polyposis coli) has been identified as the responsible gene mutated in familial adenomatous polyposis (FAP), an inherited disease characterized by the development of thousands of polyps in the colon, eventually leading to colon cancer [1]. APC, a ubiquitously expressed multifunctional protein, interacts with various cellular proteins in which the most thoroughly studied function is its ability to induce degradation of β-catenin, a subunit of the cadherin protein complex and a key Wnt signaling effector. When bound to β-catenin, APC promotes the phosphorylation of highly conserved serine and threonine residues of β-catenin targeting this protein for degradation via the proteosome system [2, 3]. The defective APC protein is not able to bind or degrade β-catenin. The impairment of β-catenin degradation results in its accumulation and formation of a stable, constitutively active complex with T cell factor. The increased β-catenin activates growth-promoting genes such as c-myc via the interaction of β-catenin/Tcf-4 complexes, ultimately causing the activation of Wnt signaling target genes leading to loss of cellular growth control [4–6]. Thus, APC plays an important role in the Wnt signaling pathway.

Recently, it has become recognized that inactivation of APC protein may occur via multiple mechanisms, including allelic loss, gene mutation, or methylation of CpG sites in promoter regions, which leads to abnormal and uncontrolled growth of cancer cells [7, 8]. Hypermethylation of APC gene has been described in a variety of human cancers including colorectal, breast, lung, endometrial, esophageal, bladder and gastric tumors, as well as T-cell leukemia/lymphoma [9–16]. However, hypermethylation of the APC gene has not been analyzed systematically in human cervical cancer cell. Clinically approved DNA methylation inhibitors, such as 5-aza-2′-deoxycytidine (5-Aza-dC), have demonstrated reversing APC promoter methylation, but widespread clinical use of these inhibitors is limited due to their toxicity and instability in aqueous solution. These nucleosides have shown no efficacy in solid tumors used alone, and in combination with chemotherapy add substantial myelosuppression rendering them not promising as demethylating agents in solid tumors. Hydralazine is a stable DNA methylation inhibitor that has minimal toxicity in vitro and in vivo [17–20].

In the current study, we evaluated hydralazine's effects on APC reactivation and cell growth in vitro, and investigated the potential of hydralazine in treating human cervical cancer. In addition, we detected the expression of β-catenin protein by immunohistochemisty (IHC), after the treatment with hydralazine. This study provided a clinically relevant strategy using hydralazine for the treatment of human cervical cancer.

Materials and methods

Human cervical cancer cell lines and cell culture

Human cervical cancer cell lines HeLa (adenocarcinoma of the cervix), CaSki and SiHa (squamous carcinoma of the cervix) and human umbilical vein endothelial cell (ECV304) were used in the current study. SiHa and ECV304 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines HeLa and CaSki were from the immunological laboratory of Three Gorges University, China. These cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and then sub-cultured using 0.25% trypsin with 1 mmol/l EDTA. The medium was changed every other day. Exponentially growing cells were used for the experiments.

DNA preparation

Genomic DNA from the human cervical cancer cell lines and ECV304 cell line was extracted by Tissue/Cell Genomic DNA Isolation Kit (Watson Biotechnologies Inc., China). The cell cultures were trypsin-EDTA digested before collection, and cell line DNA was then prepared with the method described in the manufacturer instructions. DNA was dissolved in low TE buffer and stored at −20°C.

RNA isolation and semiquantitative reverse transcription (RT)-PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen, Co., USA). Agarose gel electrophoresis at 1.5% and spectrophotometric analysis ($A_{260\ nm}$:$A_{280\ nm}$ ratio) were used to assess RNA quality. RNA was stored at −70°C before use. First-strand cDNA was synthesized using oligo-(dT) primer (ToYoBo Co., Ltd, Japan). Five microgram of total RNA was used for each reaction. After first strand synthesis, the reaction mixture was diluted to 100 μl with water. Subsequently, 5 μl of the diluted cDNA mixture was used for PCR amplification in a final 25 μl reaction volume. PCR amplification for APC was carried out with primer sets derived from the published [16, 20]: APC-sense, 5′-GAGACAGAATGGAGGTGCTGC-3′; APC-antisense, 5′-GTAAGATGATTGGAATTATCTTCT-3′, which amplified a 170 bp product. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to ensure cDNA quality and quantity for each RT-PCR. GAPDH gene primer sets were: GAPDH-sense, 5′-AACGGATTTGGTCGTATTGGG-3′; GAPDH-antisense, 5′-TTGATTTTGGAGGGATCTCGC-3′, which

amplified a 233 bp product. The program used for each of the RT-PCR experiments was 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 56°C for 60 s, and 72°C for 60 s, then extended at 72°C for 7 min. Amplified products were analyzed on 2% agarose gels.

Quantitative real time RT-PCR assay for APC expression

Total cellular RNA was isolated and the reverse transcription step was performed as described above. Quantitative real time PCR was carried out with primers described above also. Each reaction was set up in a final 25 μl reaction volume containing 10 pmol of each primer, 5 μl of the diluted cDNA mixture, 3.5 mmol/L $MgCl_2$ and 0.56× SYBR Green I (Generay Biotech Co., Ltd, Shanghai, China). The PCR conditions used were as follows: 5 min denaturation at 95°C, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Amplification of the target gene was monitored as a function of increased SYBR Green I fluorescence. An analysis threshold was set, and the cycle threshold (Ct) was computed for each sample. The comparative difference of APC gene expression was determined by $2^{-\Delta\Delta Ct}$, and ΔΔCt was determined by the following formula:

$$\Delta\Delta Ct = (Ct_{APC-treated} - Ct_{GAPDH-treated}) - (Ct_{APC-untreated} - Ct_{GAPDH-untreated}).$$

Methylation-specific PCR

Genomic DNA from cervical cancer cell lines was bisulfite modified with EZ DNA Methylation Kit (Zymo Co., USA), following manufacturer's instructions. Bisulfite treatment converts unmethylated cytosines to uracils while leaving the methylated cytosines unaffected. Modified DNA was stored at −70°C until used. PCR was performed essentially as described: APC promoter 1A (GenBank accession No. U02509) was amplified with the following primers [12]: unmethylated primers APC-1A-U-sense, 5′-TGTGAGGG TATATTTTTGAGGGGTAT-3′ and APC-1A-U-antisense, 5′-CTTCTCTCTCCACTTCCCAACCCA-3′, which amplified a 109 bp product (nucleotides 629-737); and methylated primers APC-1A-M- sense, 5′-GGTATATTTTCGA GGGGTACG-3′ and APC-1A-M-antisense, 5′-TTCCCGA CCCGCACTCCGC-3′, which amplified a 90-bp product (nucleotides 635–724). Each methylation-specific PCR reaction incorporated 100 ng of bisulfite-treated DNA as template, 0.5 μmol/L of each primer, 0.2 mmol/L deoxynucleoside triphosphate, 10× PCR buffer, and 0.75 unit of Taq Hot Start Polymerase (TaKaRa, Japan) in a final reaction volume of 25 μl. The program used for each of the PCR experiments was 95°C for 5 min, followed by 40 cycles at 95°C for 45 s, 56°C for 45 s, and 72°C for 45 s, then extended at 72°C for 7 min. The methylation control (in vitro methylated DNA served as the positive methylation controls) were kindly provided by professor Han Yu (Molecular Biology Institute of Three Gorges University), and normal human peripheral lymphocytes was included as an unmethylated control (negative control). Water blank was used as a blank control in each set of PCR reactions. Amplified products were analyzed on 2.5% agarose gels.

MTT assay

Cells were seeded into 96-well plates (Costar, USA) at 2×10^3 cells/well in 0.1 ml complete medium. After incubation for 24 h, the cells were treated with various concentrations of hydralazine [0, 5, 10, 20, 40, 80, 160, and 320 μmol/L hydralazine (Sigma, St. Lois, MO, USA)] for 72 h with fresh media being added every 24 h. Subsequently, the cell viability was measured by MTT dye reduction assay at 24, 48, and 72 h, respectively. Briefly, 50 μl of 5 mg/mL MTT (Amresco, USA) reagent in 1× PBS were added to each well. Viable cells with active mitochondria reduce the MTT to an insoluble purple formazan precipitate that is solubilized by the subsequent addition of 150 μl of DMSO. The formazan dye was measured spectrophotometrically using an ELISA reader (TECAN GENios, Austria). All assays were performed in triplicate. Inhibition was used to indicate the suppressive effect of hydralazine on human cervical cancer cell lines and ECV304 cell line. Inhibition (%) is defined as $[(A_{570\ nm\text{-}control} - A_{570\ nm\text{-}experiment})/A_{570\ nm\text{-}control}] \times 100\%$.

Hydralazine treatment

The human cervical cancer cell lines HeLa, CaSki, and SiHa were seeded at the density of 2×10^5 cells/culture flask in the volume of 50 ml, respectively. After 24 h, the cells were treated with different doses of hydralazine in RPMI 1640 for 72 h and RPMI 1640 medium was used as a control. The medium was changed every 24 h. On day 4, the cells were harvested for analyses of gene expression and DNA methylation.

IHC staining

To detect the expression of β-catenin protein which correlates closely with APC, HeLa and CaSki cell lines were seeded into 6-well plates placed beforehand with sterile coverslips at the density of 1×10^5 cells/well, respectively. After 24 h, the cells were treated with RPMI 1640 containing either 0 or 40 μmol/L hydralazine for 72 h, respectively, exchanging fresh medium every 24 h. The cells on each coverslip were analyzed by

standard IHC method with UltraSensitive™ SP kit (Maixin.bio Co., China) and anti-human β-catenin mAb (Maixin.bio Co).

Cell cycle analysis and apoptosis measurement

Flow cytometry was used to analyze the cell cycle and apoptosis. CaSki cell lines, ECV304 cell line and 1×10^6 cells of HeLa were treated with various concentrations of hydralazine (0, 10, 20 and 40 μmol/L) for 72 h. Then, cells were harvested with 0.25% trypsin and precipitated with the help of centrifugation at 1,000 rpm for 5 min at room temperature. After the supernatant was removed, the sediments were fixed in 70% ethanol at 4°C for 24 h. After centrifugation at 1,000 rpm for 5 min at 4°C, the cell pellets were permeabilized by suspension in 0.5 ml of 0.1% Triton X-100 and 0.1% trisodium citrate for 30 min, washed, and then mixed with 1 ml of 50 μg/ml propidium iodide (PI) plus 50 μg/ml RNase in the dark. After 30 min at 37°C, samples were filtered through nylon mesh into glass tubes, and the DNA contents were analyzed using a Coulter Flow Cytometer (Beckman Coulter, USA). The distributions of three phases were estimated according to standard procedures [21]. The percentages of cells in different cell cycle phases (G0/G1, S, or G2/M) were calculated by means of Coulter Epics XL-4 Multicycle for Windows Software (Beckman Coulter). The sub-G1 peak was considered as apoptosis peak.

Statistical analysis

All numerical data were expressed as average of values obtained ± standard deviation (SD) of experiments made by triplicate. Comparisons were evaluated by unpaired *t* test, and a value of $P < 0.05$ was considered significant (SPSS software 10.0).

Results

Hydralazine induces APC expression and promotes demethylation in HeLa and CaSki cells

Using RT-PCR, we initially analyzed the expression of APC gene among three established human cervical cancer cell lines (Fig. 1a). APC gene was not expressed in HeLa cell line and was expressed less in CaSki cell line, whereas APC gene was expressed normally in SiHa cell. In the cell lines wherein the APC gene was not expressed or expressed less, treatment with the demethylating agent hydralazine at 40 μmol/L for 72 h resulted in a robust restoration of the expression of APC gene (Fig. 1b).

To determine a more detailed map of the expression of APC gene, quantitative real-time RT-PCR assays were performed. The expression of APC gene in HeLa, CaSki and SiHa cell lines, treated by 40 μmol/L hydralazine for 72 h, increased to 10.35 ± 0.51, 11.40 ± 1.43 and 0.73 ± 0.15-fold, respectively (Fig. 1c).

Methylation-specific PCR was then performed to determine if the silencing of APC gene was related to promoter region methylation, as suggested by the demethylating experiments (Fig. 2a). APC gene methylation was not present in SiHa cell line, complete methylation of APC gene was detected in HeLa cell line, and CaSki cell line were hemimethylated. APC gene in both HeLa and CaSki cell lines was demethylated after the treatment with 40 μmol/L hydralazine (Fig. 2b). These results show that hydralazine induces demethylation and APC expression in HeLa and CaSki cells.

Hydralazine induces the change of β-catenin protein expression

To determine the expression of β-catenin protein after treatment with hydralazine, we performed IHC staining of HeLa, CaSki and SiHa cells with either 0 or 40 μmol/L

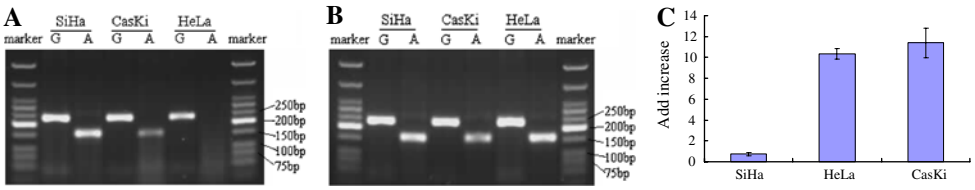

Fig. 1 Hydralazine induces APC expression in HeLa and CaSki cells. **a** APC gene expression in cervical cancer cell lines before treatment of hydralazine with RT-PCR; strong APC expression was present in SiHa cell line, whereas in HeLa cell line APC expression was absent and APC was expressed less in CaSki cell line. **b** APC gene expression in cervical cancer cell lines treated with 40 μmol/L hydralazine for 72 h with RT-PCR; strong APC expression was present in the three cervical cancer cell lines. **c** APC gene expression in cervical cancer cell lines following treatment with 40 μmol/L hydralazine for 72 h with quantitative real time RT-PCR The expression of APC gene in HeLa, CaSki, and SiHa cell lines increased to 10.35 ± 0.51, 11.40 ± 1.43 and 0.73 ± 0.15-fold (mean ± SD), respectively, as compared with the untreated control (*G* GAPDH; *A* APC; GAPDH expression is an internal control, a low molecular weight DNA marker indicated an appropriate size for the amplified products)

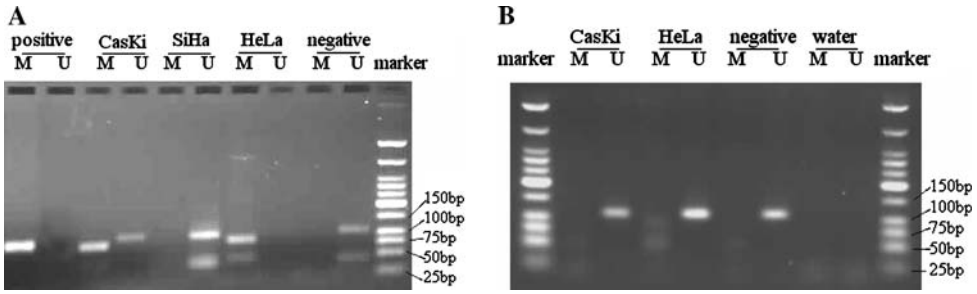

Fig. 2 Hydralazine induces APC promoter demethylation in HeLa and CaSki cells. **a** APC gene methylation-specific PCR in cervical cancer cell lines before treatment of hydralazine. The presence of methylation of APC corresponded directly to the loss of its expression in each of the cell lines shown in Fig. 1a. **b** APC gene methylation-specific PCR in cervical cancer cell lines with 40 μmol/L hydralazine for 72 h. A low molecular weight DNA marker indicated an appropriate size for the amplified product. (*U* amplification of unmethylated alleles, *M* methylated alleles, *positive* in vitro methylated DNA; and *negative* normal human peripheral lymphocytes)

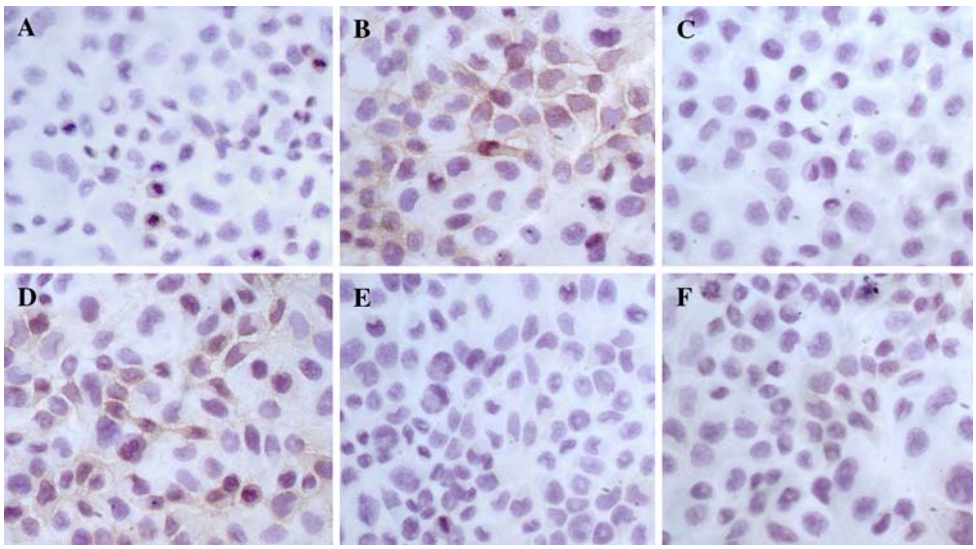

Fig. 3 Expression of β-catenin protein in cervical cancer cell lines (×400). **a** Expression of β-catenin protein in HeLa cell line without treatment. **b** Expression of β-catenin protein in HeLa cell line treated with hydralazine. **c** Expression of β-catenin protein in CaSki cell line without treatment. **d** Expression of β-catenin protein in CaSki cell line treated with hydralazine. **e** Expression of β-catenin protein in SiHa cell line without treatment. **f** Expression of β-catenin protein in SiHa cell line treated with hydralazine

hydralazine for 72 h, respectively (Fig. 3). β-Catenin protein was not expressed or was just slightly expressed on the cell membrane in HeLa, CaSki and SiHa cells without treatment. After treatment with 40 μmol/L hydralazine, HeLa and CaSki cells highly expressed β-catenin protein on cell membrane, while on the contrary, SiHa cell still did not express β-catenin protein on cell membrane.

Hydralazine inhibits human cervical cancer cell line proliferation

We analyzed the effect of hydralazine on cell proliferation in cervical cancer cell lines and ECV304 cell line using MTT assay. The time-dependent and dose-dependent inhibitory fashions were found in cervical cancer cell lines (Fig. 4a–c). We observed that growth inhibitive rates (%) of the three cervical cancer cell lines, HeLa, CaSki and SiHa, treated with 40 μmol/L hydralazine for 72 h were 52.12 ± 3.78 versus control ($P = 0.008$), 44.31 ± 2.59 versus control ($P = 0.001$), and 47.73 ± 4.73 versus control ($P = 0.0001$), respectively, whereas ECV304 was insensitive to hydralazine, 27.18 ± 0.79 (Fig. 4d). The result indicates that hydralazine is not specific to HeLa and CaSki cell lines, in which APC promotes hypermethylation or hemimethylation. At the same time, the MTT results show that hydralazine at demethylating doses only inhibits human cervical cancer cell line proliferation remarkably, but has no obvious inhibitive effect on the growth of normal cell line ECV304.

Hydralazine induces apoptosis and S phase arrest

We tested whether hydralazine alters cell cycle populations in cervical cancer cell lines, including HeLa, CaSki and ECV304, using PI staining. Hydralazine increased the percentage of apoptotic cells (sub-G1) and cells arrested in S phase of the cell cycle (Fig. 5a, b). When compared with the untreated HeLa cells, the HeLa cells treated with 10, 20 and 40 μmol/L hydralazine for 72 h caused a 10.29, 20.69, and 23.99% increase in the sub-G1

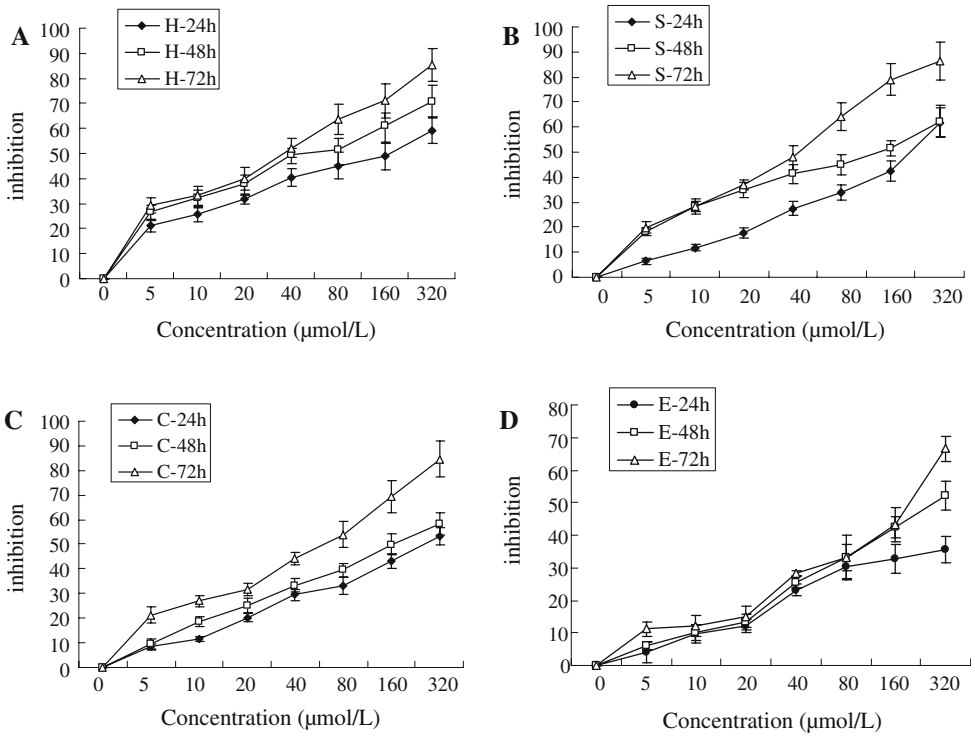

Fig. 4 Effect of hydralazine on the growth of human cervical cancer cells and ECV304. All cell lines were treated with hydralazine at concentrations of 0, 5, 10, 20, 40, 80, 160, and 320 μmol/L for 24, 48 and 72 h, respectively. The absorbance "A" in the groups was measured at 570 nm. **a** MTT assay of HeLa cells treated with hydralazine. **b** MTT assay of SiHa cells treated with hydralazine. **c** MTT assay of CaSki cells treated with hydralazine. **d** MTT assay of ECV304 cells treated with hydralazine

population, respectively, and a 24.9, 6.7, and 30.8% increase in S phase cells, respectively. When compared with the untreated CaSki cells, the CaSki cells treated with 10, 20 and 40 μmol/L hydralazine for 72 h caused a 2.29, 14.22 and 15.52% increase in the sub-G1 population, respectively, and a 0.5, 7.8, and 7.9% increase in S phase cells, respectively. Consistent with the increases in apoptotic and S phase cells, we also observed concurrent decreases in the percentage of cells in G0/G1 phase following hydralazine treatments. When compared with the untreated ECV304 cells, the ECV304 cells treated with 10, 20 and 40 μmol/L hydralazine for 72 h almost caused no changes in the sub-G1 population, respectively, and little change in S and G2/M populations ($P > 0.05$, Fig. 5c). These results indicate that hydralazine, when used at demethylating doses, induces apoptosis and S phase arrest in cervical cancer cells but has little effect on the normal cell line ECV304.

Discussion

Aberrant methylation in promoter regions is recognized as an alternate mechanism for transcriptional silencing in many tumor suppressor genes. Hypermethylation of APC gene has been described in a variety of human cancers [9–16]. Our study demonstrated for the first time that the promoter of the APC gene was hypermethylated, hemimethylated, and unmethylated in HeLa, CaSki, and SiHa cells, respectively. One study demonstrated that APC promoter was hypermethylated in 60% of cervical adenocarcinoma samples and 13% of squamous carcinoma of the cervix (SCC) [22], but another study detected that APC promoter was hypermethylated in 94% of SCC [20]. The rates of APC promoter methylation in SCC samples are different in the two studies probably because the samples of two studies were obtained from different countries and numbers of samples were different. Our study showed that APC promoter was not methylated completely in the three cervical cancer cell lines, which was coincident with the reports described above. Our study indicates that the methylation of APC promoter is a frequent event which silences APC gene, and then APC gene loses its function as tumor suppressor gene, which is one of the mechanisms in cervical carcinogenesis.

At present, 5-Aza-C (5-azacytidine) and cytidine analogs are commonly used as DNA methylation inhibitors. 5-Aza-C and 5-Aza-dC have shown significant clinical activity against myeloid malignancies [23, 24]. However, clinical use of these agents is complicated because of their toxicity and instability in aqueous solutions [25, 26].

Hydralazine, a widely available peripheral vasodilator agent, has been extensively used for high blood pressure, heart failure, and pregnancy-associated hypertensive disorders [27–29]. Hence, its evaluation as a demethylating agent in a clinical trial involving cancer patients could proceed with no major concerns regarding unexpected toxicity and long-term side effects aside of its known

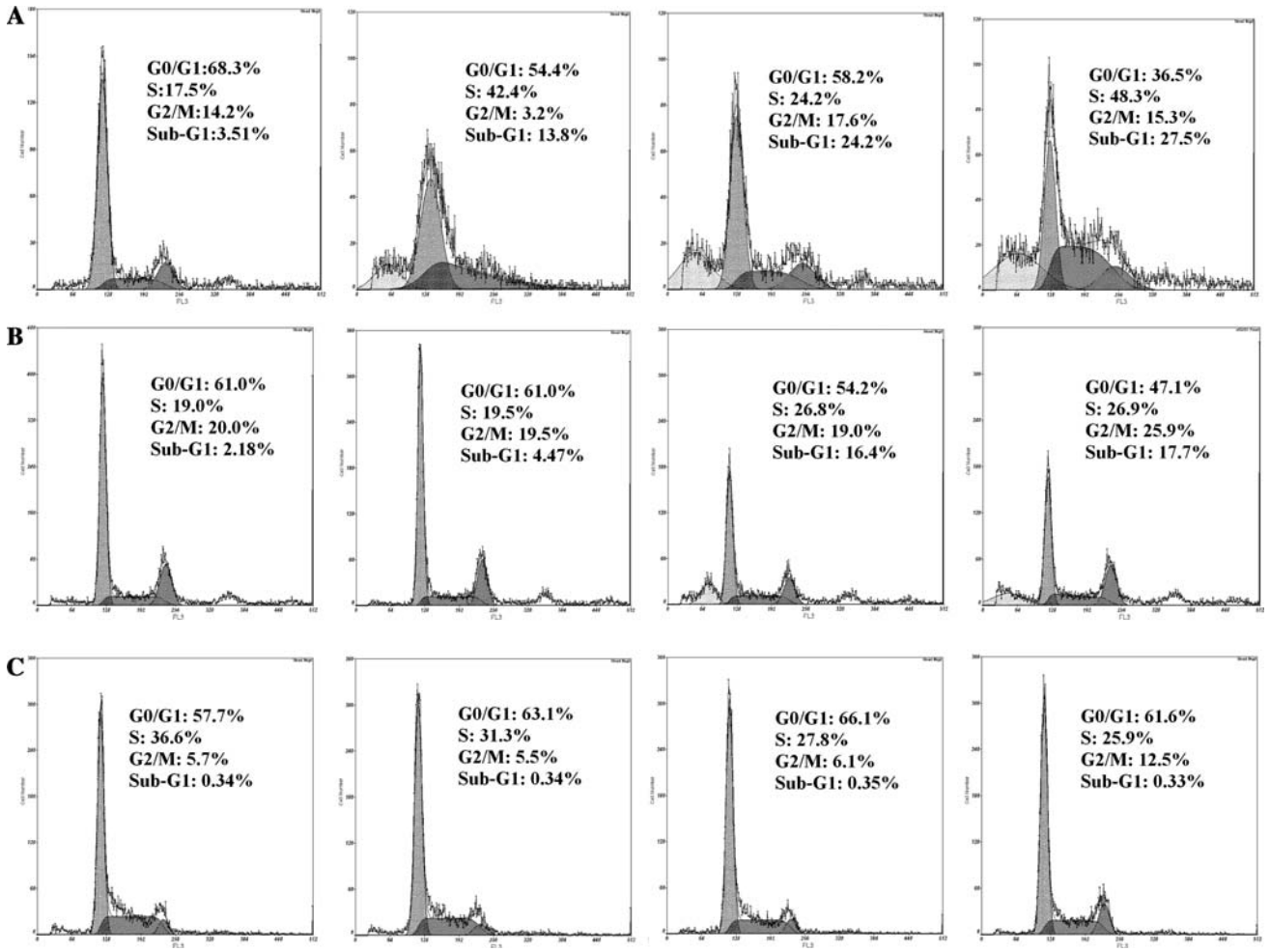

Fig. 5 Effects of hydralazine on the cell cycle in cell lines. Shown are representative histograms of PI-stained DNA content following treatment with hydralazine. **a** 0, 10, 20, and 40 μmol/L hydralazine treatment of HeLa for 72 h. **b** 0, 10, 20 and 40 μmol/L hydralazine treatment of CaSki for 72 h. **c** 0, 10, 20, and 40 μmol/L hydralazine treatment of ECV304 for 72 h

capacity to induce a lupus-like condition [30]. Segura-Pacheco et al. [17] previously reported that hydralazine induced demethylation and transcriptional reactivation at the mRNA and protein levels of the ER, RARβ, and p16 genes in MDA-231, MCF-7, and T24 cell lines treated with10 μmol/L for 5 days. Chavez-Blanco et al. [31] demonstrated that 10 μmol/L hydralazine had no significant effect on the proliferation inhibition in HeLa cell line treated for 5 days, whereas the proliferation of HeLa cell line treated with 10 μmol/L hydralazine and 1 mmol/L valproic acid was inhibited significantly. Our study indicated that 10 μmol/L hydralazine had no obvious inhibition effect on cervical cancer cell lines including HeLa, CaSki and SiHa, which is coincident with the study of Chavez-Blanco et al. When three cervical cancer cell lines and ECV304 cell line were treated with 40 μmol/L hydralazine for 72 h, we detected that the proliferation of the three cervical cancer cell lines was inhibited significantly, whereas ECV304 was insensitive to hydralazine. Therefore, a higher concentration of hydralazine (40 μmol/L) is required to treat cervical cancer cell lines. APC gene in Hela cell line, in which APC promoter is hypermethylated was demethylated and re-expressed at the mRNA level after having been treated with 40 μmol/L hydralazine for 72 h, and APC gene in CaSki cell line in which APC promoter is hemimethylated was also demethylated and had a stronger expression at the mRNA level after the same treatment described above. These results suggest that 40 μmol/L hydralazine can induce demethylation and transcriptional reactivation at the mRNA level of APC gene in cervical cancer cell lines. Furthermore, the normal cell line ECV304 is insensitive to hydralazine, indicating that hydralazine has little toxicity and is quite promising for cervical cancer therapy. Furthermore, hydralazine should be given in continuous plasma concentrations that are reached in patients.

MTT assay demonstrates that the three cervical cancer cells lines without hydralazine treatment proliferate fast, but proliferate slowly at different levels after hydralazine

treatment. At the same time, the results of MSP (methylation-specific PCR) and RT-PCR assays show that methylation or hemimethylation of HeLa and CaSki cell lines are demethylated and the APC gene re-express after hydralazine treatment. Therefore, we can conclude that APC promotes demethylation, and transcriptional reactivation of the gene is one mechanism in the inhibition of growth of cervical cancer cells lines. However, hydralazine does not only inhibit significantly the proliferation of HeLa and CaSki cells lines, but also has a strong inhibitive effect on the proliferation of SiHa cell line in which the APC promoter is unmethylated. Thus, we suggest that APC promoter demethylation and transcriptional reactivation of the gene is not the only mechanism in the inhibition of growth of cervical cancer cells lines; there are probably other mechanisms that remain unknown. In this study, we detected that hydralazine inhibited the growth of cervical cancer cell lines in a dose-dependent and time-dependent manner. Our data show that part of the anti-carcinogenic effects of hydralazine may derive from S phase arrest and/or apoptosis induction. A previous study found that hydralazine arrested human bladder cancer cell line T24 at G1 phase of the cell cycle [17]. This means that multiple mechanisms might be responsible for the anti-carcinogenic effects of hydralazine. In the study, we discovered that β-catenin protein was re-expressed on the cell membrane of HeLa and CaSki cell lines after treatment with 40 μmol/L hydralazine for 72 h, which indicated that Wnt signal transduction was inhibited and the target genes were not transcripted.

In conclusion, hydralazine, a demethylating agent, induces APC promoter demethylation and inhibits HeLa and CaSki cell growth. Our study provides a clinical potential strategy using hydralazine for human cervical cancer therapy.

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