Regulation of demethylation and re-expression of RASSF1A gene in gastric cancer cell lines by combined treatment of 5-Aza-CdR and NaB

Wen-Jing Shen, Dong-Qiu Dai, Yue Teng, Hong-Bo Liu

Abstract

AIM: To investigate the changes of methylation state and expression of RASSF1A gene in human gastric cancer cell lines SGC7901 and BGC823 which were treated in vitro with demethylating agent 5-Aza-CdR in combination with histone deacetylase inhibitor NaB.

METHODS: After SGC7901 and BGC823 cells were treated with 5-Aza-CdR and/or NaB, the methylation state of RASSF1A gene was detected by methylation-specific PCR, and the changes in expression of mRNA and protein level of RASSF1A gene were observed by RT-PCR and Western-blotting before and after drug treatment.

RESULTS: Hypermethylation was detected in the promoter region of RASSF1A gene in both SGC7901 and BGC823 cells, and there was no expression of this gene at both mRNA and protein level. After treatment with 5-Aza-CdR, demethylation occurred in the promoter region of RASSF1A gene, which subsequently induced re-expression of this gene. The treatment with NaB alone showed no effect on the methylation state and expression of RASSF1A gene. The combined treatment of 5-Aza-CdR and NaB induced complete demethylation of RASSF1A gene, leading to a significantly higher re-expression of the mRNA and protein of RASSF1A than those treated with 5-Aza-CdR alone (P < 0.05).

CONCLUSION: Hypermethylation in the promoter region is related to inactivation of RASSF1A gene in human gastric cancer cell lines SGC7901 and BGC823, while demethylating agent 5-Aza-CdR can reverse the methylation state of RASSF1A gene and induce its re-expression. Histone deacetylase inhibitor NaB had a synergistic effect with 5-Aza-CdR in both demethylation and gene transcriptional regulation.

INTRODUCTION

Gastric cancer is a malignant tumor which seriously threatens the human health worldwide. It has been shown that the occurrence of gastric cancer is a multi-gene-involved, multi-stage pathological process under the action of both genetic and epigenetic factors, and that epigenetic mechanism plays an important role in the occurrence and development of gastric cancer[1-4]. Various factors can lead to the loss of function of tumor suppressor genes (TSGs). It has been confirmed that DNA methylation is the third, sometime the only mechanism for the inactivation of TSG, besides gene mutation and deletion[5]. The Ras associated domain family gene 1A (RASSF1A) is an established TSG that can inhibit cell growth, and promote apoptosis and senescence through various ways[6-9]. The methylation in the promoter region of RASSF1A gene can cause depletion of RASSF1A expression, thereby leading to occurrence of tumors, which has been confirmed in human gastric cancer[10,11]. Since the regulation of gene expression by abnormal methylation is reversible, demethylating agents could be used to induce re-expression of the inactivated genes due to methylation[12]. Therefore, changing the CpG island state in the gene promoter region is a potential method for clinical treatment of cancers[13]. The demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-CdR) is a cyto-
sine analogue formed by modified C-5 position of pyrimidine, and used as a DNMT (DNA methyltransferase) inhibitor for the clinical treatment of leukemia[14,15]. Histone acetylation may be related to the occurrence of human gastric cancer[16]. DNA methylation and histone acetylation are associated with each other in the epigenetic regulation of gene expression, and they together play an important role in the occurrence of tumors. They affect each other and likely have the synergistic effect[17]. Therefore, the combined treatment of demethylating agent and histone deacetylase inhibitor can induce the gene demethylation, reverse the transcriptional inhibition, and make the gene re-express, which might be used for the clinical treatment of tumors[18].

In this study, the gastric cancer cell lines were treated in vitro with demethylating agent 5-Aza-CdR in combination with histone deacetylase inhibitor NaB (sodium butyrate) so as to investigate the changes in methylation state and expression of RASSF1A gene before and after drug treatment, and to further explore the roles of these two drugs in regulating RASSF1A gene expression. The results will provide the experimental evidences for the clinical therapy.

MATERIALS AND METHODS

Materials

The main materials used included human gastric cancer cell lines SGC7901 and BGC823 (Department of Cell Biology of China Medical University), RPMI 1640 medium (Hyclone), 5-Aza-CdR (Sigma), NaB (Sigma), hydroquinone (Sigma), sodium bisulfitie (NaHSO3) (Sigma), Wizard DNA Clean-up (Promega), glycogen (New England Biolabs), Sssl (New England Biolabs), Taq enzyme (Takara), dNTPs (Takara), DMSO (Sigma), the RT-PCR Kit (TaKaRa), the sheep anti-human RASSF1A polyclonal antibody (Santa Cruz) and the TRIzol reagent for total RNA extraction (GIBCO BRL).

Methods

Cultivation of human gastric cancer cell lines: BGC823 and SGC7901 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37°C and 5% CO2. At the logarithmic phase of cell growth, with a percentage of viable cells over 95%, the cells were remained hungry for 24 h in the RPMI 1640 medium containing 1% fetal bovine serum, followed by the drug treatment. Cells were transferred every day into the fresh medium containing 10% fetal bovine serum at 37°C and SGC7901 cells were grown in RPMI 1640 medium containing 1% fetal bovine serum at 37°C.

Experimental groups: (1) 5-Aza-CdR treatment groups at the dosage of 1.0 or 3.0 μmol/L; (2) NaB treatment groups at the dosage of 1.0 or 3.0 mmol/L; (3) Combined treatment groups: 5-Aza-CdR at 1.0 μmol/L in combination with NaB at 1.0 mmol/L, and 5-Aza-CdR at 3.0 μmol/L in combination with NaB at 3.0 mmol/L. The gastric cancer cells were first treated with 5-Aza-CdR at 1.0 or 3.0 μmol/L for 48 h, followed by washing, and then were treated with NaB at 1.0 or 3.0 mmol/L for 72 h; the cells were then harvested for further analysis.

Detection of methylation state of RASSF1A by MSP: Different groups of BGC823 and SGC7901 cells were harvested after five days treated with different drugs, and cellular DNA was isolated from the harvested cells using phenol-chloroform-isoamyl alcohol assay. The concentration and purity of DNA were detected by the UV spectrophotometry. DNA modification and purification were conducted as described previously[19].

Methylation-specific PCR reaction: Total reaction mixture volume of 25 μL, containing 2 μL DNA, 2.5 μL 10 × PCR buffer, 0.5 μL each of the sense and antisense primers, 2 μL dNTPs, 0.2 μL Taq enzyme, and 17.3 μL double-distilled water. Reaction condition: pre-denatured at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s, and with a final extension at 72°C for 10 min. The annealing temperature was 58°C for the methylated reaction, while 57°C for the un-methylated reaction. The methyltransferase SssI-treated and untreated peripheral blood cell DNA from the healthy adults were used as the positive and negative controls respectively, while the double-distilled water as the blank control. The 1000 bp DL2000 was used as the molecular weight markers. The sequences of the methylated primers are as follows: the upstream primer 5'-GTGTTGAGTGTGTGTTAATG TG-3' and the downstream primers 5'-TACCAACCCACAA ACTAAAAACAA-3', generating a 108 bp PCR product. The PCR products were subjective to the 2% agarose gel electrophoresis at 100 V for 40 min. The gels were photographed using a laser density scanner (Pharmacia LKB Ultrascan) for analysis. Each experiment was repeated five times.

Analysis of mRNA expression of RASSF1A by semi-quantitative RT-PCR: The cellular total RNA was isolated using the Trizol reagent from the harvested cells. The cDNAs were synthesized from the templates in presence of reverse transcriptase and oligo (dT)20 primers. The upstream and downstream RASSF1A mRNA primers were 5'-GGC GTGGTGCGCAAACAGCC-3' and 5'-GGGTGTACAGTCT TGCTGGAGGG-3' respectively, generating a 330 bp PCR product at the annealing temperature of 67°C. The β-actin was used as the internal control, and its upstream and downstream primers were 5'-TCTTGTACACGCTGTACAC-3' and 5'-CTTTACACTGACCTACTG-3' respectively, generating a 498 bp PCR product. Twenty-five μL PCR reaction mixture was pre-denatured at 95°C for 2 min, and 35 cycles of 94°C for 40 s, 67°C for 40 s, and 72°C for 60s were performed with a final extension at 72°C for 5 min. The RT-PCR products were subjective to the 2% agarose gel electrophoresis. The gels were photographed using an Alpha Image 2000 automatic formatter, and the amount of each PCR product was analyzed using the Fluorchem V 2.0 Stand Alone software. The scanning
optical density of each band was taken as the indicator of the gene expression level. Taking the β-actin as the internal control, the ratio of scanning density of RASSF1A gene to that of β-actin was obtained. The ratio values used as the analyzing index were further compared between different experimental groups to investigate the changes in mRNA expression of RASSF1A. Each experiment was repeated five times.

Analysis of protein expression of RASSF1A by Western blotting: 200 μL of cell lysis buffer was mixed with 40 μL harvested cells to isolate the total cellular protein, and then quantitated by the phenol reagent method, 100 μL of the total protein was denatured for 10 min, and 25 μL was taken for the 10% polyacrylamide gel electrophoresis. The proteins separated by the electrophoresis were then transferred onto the PVDF membrane, and 10% skim milk used to block the PVDF membrane at room temperature for 1 h. The PVDF membranes were then incubated in the 1:200 dilution of the primary antibody overnight, and then in the 1:2000 diluted alkaline phosphatase-labeled secondary antibody solution at 37°C for 2 h, followed by the DAB coloration. The PVDF membranes were then photographed using a ChemiImager 5500 AlPhaInn Ch, and the subsequent data acquisition and mining were conducted with the Fluorchem V2.0 system. Taking the expression level of β-actin protein as the internal control, the ratio of scanning density of RASSF1A protein to that of β-actin was finally calculated and used as the analyzing index to further investigate the changes in protein expression of RASSF1A and compare them between different experimental groups. Each experiment was repeated five times.

Table 1 mRNA expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823

| Marker | 1    | 2    | 3    | 4    | 5    | 6    | 7 |
|--------|------|------|------|------|------|------|---|
| SGC7901 | 0.29 ± 0.03 | 0.45 ± 0.05 | 0.06 ± 0.04 | 0.75 ± 0.04 | 0.88 ± 0.09 | 0.96 ± 0.05 | 0.92 ± 0.08 |
| BGC823  | 0.36 ± 0.05 | 0.64 ± 0.07 | 0.06 ± 0.04 | 0.82 ± 0.09 | 0.89 ± 0.03 | 0.95 ± 0.06 | 0.95 ± 0.05 |

*P < 0.05, vs groups 2 and 3 (FSGC7901 = 208.83, P < 0.001, FSGC823 = 62.10, P < 0.001).

RESULTS

Methylation state of RASSF1A gene

Hypermethylation in the promoter region of RASSF1A gene was detected in gastric cancer cell lines SGC7901 and BGC823, and demethylation appeared after treatment with 5-Aza-CdR for five days, while NaB had no influence on its methylation state. The combined use of 5-Aza-CdR and NaB induced complete demethylation of RASSF1A gene in both SGC7901 and BGC823 cells (Figure 1).

mRNA transcription of RASSF1A gene

There was no mRNA transcription of RASSF1A gene in SGC7901 and BGC823 cells, and it re-expressed after treatment with 5-Aza-CdR for 5 d, while NaB had no influence on its expression. The combined use of 5-Aza-CdR and NaB induced higher re-expression of RASSF1A gene at the mRNA level than the use of 5-Aza-CdR alone (P < 0.05) (Figure 2; Table 1).

Protein expression of RASSF1A gene

There was no expression of RASSF1A protein in SGC7901 and BGC823 cells, and it re-expressed after treatment with 5-Aza-CdR for 5 d, while NaB had no influence.
influence on its expression. The combined use of 5-Aza-CdR and NaB induced higher re-expression of the RASSF1A gene at the protein level than the use of 5-Aza-CdR alone (P < 0.05) (Figure 3A and B, Table 2).

**DISCUSSION**

The gene transcriptional regulation in eukaryotes is a complex, multi-step process, and the covalent modification of chromatin participates in the regulation of transcription initiation; it can occur in both DNA and histone. DNA modification is primarily the cytosine methylation, while the covalent modifications of histone include acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation, which thereby constitute the histone code. The occurrence of tumors is a process under the mutual action of genetic and epigenetic mechanisms. The epigenetic molecular mechanisms include DNA methylation, histone modification, chromatin remodeling and RNA interference, all of which play important roles in gene transcriptional regulation.

As shown in this study, the action of 5-Aza-CdR on gastric cancer cell lines SGC7901 and BGC823 resulted in the demethylation of RASSF1A gene accompanied by the re-transcription of mRNA and re-expression of protein; this confirmed that 5-Aza-CdR regulates the transcription of RASSF1A gene. DNA methylation regulates gene expression, while the DNA hypomethylation promotes gene expression and DNA hypermethylation inhibits it. 5-Aza-CdR is a cytidine analogue; as a demethylating agent, its mechanism of action is primarily through its covalent bonding to DNMT, which will reduce the biological activity of DNMT, thus lowering the methylation level and regulating gene expression. The demethylation of TSGS by 5-Aza-CdR has been reported. It has been shown recently that the mechanism of action of 5-Aza-CdR is likely to inhibit the activity of DNMT3a and DNMT3b. After the action of 5-Aza-CdR, the transcription inhibitor MBD brake away from the demethylated DNA or the transcription repression complex that originally bonded to MBD, and then form to its activated conformation of chromatic body. Whether the gene expression is re-activated after the demethylation of this gene by 5-Aza-CdR, depended on the following two factors: (1) whether the hypermethylation of the CpG island in the gene promoter region is reversed; (2) whether there is the simultaneous existence of a transcription activator to activate the specific target promoter. For some genes, 5-Aza-CdR alone is sufficient to induce the activation and expression, which can be used to explain the reversed expression of RASSF1A gene in the gastric cancer cells, as observed in the present study. This is supported by the report by Byun et al.

With the action of NaB, there was no change in the methylation state and expression of RASSF1A gene in both SGC7901 and BGC823 cells. In the eukaryotes, histone is an important component of the elementary structure of chromatin-nucleosome, of which the “N” terminal can be covalently modified such as acetylation. The acetylation of histone is a reversible dynamic process; the acetylated chromatin was related to transcriptional activation, while the desacetylated chromatin was related to transcription inhibition. The acetylation of histone is a reversible dynamic process; the acetylated chromatin was related to transcriptional activation, while the desacetylated chromatin was related to transcription inhibition. The common mechanism of action of histone deacetylase inhibitors HDACIs is to inhibit the histone deacetylation so as to enhance the acetylation level of histone, by which the gene expression was up-regulated. Among HDACIs, the NaB is a kind of nontoxic short-chain fatty acid that has been proved to inhibit the proliferation of cancer cells in vivo and in vitro, to induce the apoptosis, to promote the cell differentiation and to block the cell cycle. It has the potential for the clinical therapy of malignant cancers such as breast cancer, colorectal cancer, endometrial cancer and so on. It has been shown that HDACIs can raise the acetylation level of histone, and change the chromatic structure, thereby inducing the transcriptional activity of some genes. Selker et al. reported that another HDACI-TSA (trichostatin A) could selectively down-regulate the methylation level of some genes in Neurospora, but the use of NaB alone has no effect on the gene expression of RASSF1A, which was supported by Lynch et al.

In this study, the up-regulation of RASSF1A gene under the combined treatment of 5-Aza-CdR and NaB was significantly higher than that under the use of 5-Aza-CdR alone, indicating that the demethylating agent 5-Aza-CdR and the deacetylase inhibitor NaB had a synergistic effect in transcriptional regulation; this was consistent

### Table 2: Protein expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823

|          | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|----------|-----|-----|-----|-----|-----|-----|-----|
| SGC7901  | 0.66±0.07 | 0.71±0.09 | 0.00 | 0.88±0.06 | 0.89±0.05 | 0.00 | 0.00 |
| BGC823   | 0.45±0.03 | 0.65±0.08 | 0.00 | 0.78±0.04 | 0.83±0.06 | 0.00 | 0.00 |

*P < 0.05, compared with groups 2 and 3 (FGC7901 = 14.41, P < 0.001, FGC823 = 46.01, P < 0.001).
with the results by Egger et al. As shown previously, both DNA methylation and histone acetylation were closely related to the regulation of gene expression. DNA methylation could induce the acetylation of the local histone, and could decrease the chromatin acetylation level. The methylated CpG-binding protein MeCP2 bonded to chromatin by a methylation-dependent manner; through its transcriptional repression domain, MeCP2 was closely related to the inhibition complex consisting of the p53 tumor suppressors Sin3A and HDAC. The methylation of promoter region and the histone deacetylation are synergistic in the inhibition of gene transcription. However, the inhibitive effect of histone deacetylation on gene transcription relies on the number of methylation sites. If the mCpG in the promoter region is at low density, the pathway of histone deacetylation plays a major role in inhibiting gene transcription. Under the condition that the number of methylation sites in the promoter region increased to the threshold value that is large enough to lead to the extensive silence of gene expression, the pathway of histone deacetylation plays a secondary role in inhibiting gene transcription. Moreover, the pathway of DNA methylation will play the leading role in the gene transcriptional regulation, and it can inhibit the gene transcription by the manner independent of the histone deacetylation. The rational explanation for the mechanism of synergistic action of 5-Aza-CdR and NaB is that NaB promotes the formation of the activated conformation of chromatic body with the action of 5-Aza-CdR. Fahrner et al. demonstrated that DNA methylation could maintain the inhibition state of the key histones in the hypermethylated promoter regions, directly or indirectly through the transcriptional inhibition. And there is inducible demethylation of hMLH1 gene in the RKO colon cancer cell line 12 h after the 5-Aza-CdR treatment, and reverse of the acetylation state of histone 48 h after the treatment. These also demonstrate the leading role of the DNA methylation in the transcriptional regulation.

The RASSF1A gene can inhibit cell growth, and promote apoptosis and senescence. This study showed that the loss of expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823 was caused by the hypermethylation in the promoter region, the use of 5-Aza-CdR could result in the demethylation and the restoration of transcriptional activity of RASSF1A gene, and that NaB and 5-Aza-CdR were synergetic in regulating the expression of RASSF1A gene. Hypermethylation of RASSF1A gene is closely related to the occurrence of gastric cancer. As RASSF1A gene promotes apoptosis and is also likely associated with the resistance to platinum chemotherapy, the effect of 5-Aza-CdR in conjunction with NaB on the regulation of RASSF1A expression is of significant value in clinical practice.

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