Silencing of Bidirectional Promoters by DNA Methylation in Tumorigenesis

Jingmin Shu, Jaroslav Jelinek, Hao Chang, Lanlan Shen, Taichun Qin, Woonbok Chung, Yasuhiro Oki, and Jean-Pierre J. Issa

Department of Leukemia, M.D. Anderson Cancer Center, Houston, Texas

Abstract

CpG island methylation within promoters is known to silence individual genes in cancer. The involvement of this process in silencing gene pairs controlled by bidirectional promoters is unclear. In a screen for hypermethylated CpG islands in cancer, bidirectional promoters constituted 25.2% of all identified promoters, which matches with the genomic representation of bidirectional promoters. From the screen, we selected three bidirectional gene pairs for detailed analysis, WNT9A/CD558500, CTDSPL/BC040563, and CKNK15/BF195580. Levels of mRNA of all three pairs of genes were inversely correlated with the degree of promoter methylation in multiple cancer cell lines. Hypermethylation of these promoters induced by 5-aza-2-deoxycytidine treatment reactivated or enhanced gene expression bidirectionally. The bidirectional nature of the WNT9A/CD558500 promoter was confirmed by luciferase assays, and hypermethylation downregulated expression of both genes in the pair. Methylation of WNT9A/CD558500 and CTDSPL/BC040563 promoters occurs frequently in primary colon cancers and acute lymphoid leukemias (ALL), respectively, and methylation was correlated with decreased gene expression in ALL patient samples. Our study shows that hypermethylation of bidirectional promoter-associated CpG island silences two genes simultaneously, a property that should be taken into account when studying the functional consequences of hypermethylation in cancer.

Introduction

Bidirectional gene organization is defined as two genes arranged head to head on opposite strands with <1,000 bp between their transcription start sites (1). Generally, about 20% of genes are organized in this way (1, 2). It was previously suggested that bidirectional organization may provide stronger resistance to invasion by transposable elements (2), which is possibly one of the reason why many important genes (e.g., 30% of housekeeping genes) are arranged in this way (1). Remarkably, bidirectional promoters are particularly common in DNA repair genes with a frequency of 40% (1). It remains to be determined if this is also true for tumor suppressor genes. The GC content of bidirectional promoters was reported to be higher than that of regular promoters (3), and it was proposed that these promoters could be more resistant to methylation for protection of essential genes.

Like other promoters, bidirectional promoters are also frequently associated with CpG islands (2). A CpG island is a short region of DNA in which the frequency of the CG sequence is less suppressed (4, 5). Hypermethylation of CpG islands in promoter regions usually results in gene silencing, and several tumor suppressor genes are hypermethylated in their promoter regions in cancers, which is thought to contribute to tumorigenesis (3, 6, 7). Most bidirectional promoters coordinately regulate transcription of the gene pair (3), but it remains to be determined whether hypermethylation of CpG islands in such promoters is also able to silence genes in both directions. If this were true, silencing of bidirectional promoters by hypermethylation would possibly be an important mechanism for oncogenesis because a single “hit” within these promoters could potentially disable two tumor suppressor genes simultaneously, which could accelerate tumor development, according to the multiple hit theory of tumorigenesis (8).

Here, we sought to determine the effect of hypermethylation of bidirectional promoters on the expression of either gene in the gene pair and study the likelihood of this event in cancer. We report on three bidirectional promoters hypermethylated in cancer, with silencing of both genes in each case. Our data expand the effects of CpG island methylation in tumor development.

Materials and Methods

Cell lines and tissues. Cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA). All of the patient samples, both cancerous and normal, were obtained from an established tissue bank at the University of Texas M.D. Anderson Cancer Center (Houston, TX). Patients gave informed consent for the collection of residual tissue as per institutional guidelines. The studies were approved by the institutional review board of the M.D. Anderson Cancer Center.

5′ Rapid amplification of cDNA ends PCR. Human fetal brain marathon-ready cDNA (Clontech, Mountain View, CA) was used as template for PCR according to the manufacturer’s instruction with primers listed in Supplementary Table S1. The largest-size PCR products were gel purified by the QIAprep Gel Purification kit (Invitrogen, Carlsbad, CA) and cloned into a pcDNA 4.0-TOPO vector (Invitrogen). Individual clones were sequenced at the DNA sequencing core facility at the University of Texas M.D. Anderson Cancer Center.

DNA bisulfite treatment. After DNA extraction, DNA was treated with bisulfite as reported previously (9). Two micrograms of genomic DNA were denatured by 0.2 mol/L NaOH at 37°C for 10 minutes followed by incubation with freshly prepared 30 μL of 10 mmol/L hydroquinone and 520 μL of 3 mol/L sodium bisulfite (pH 5) at 50°C for 16 hours. DNA was purified with a Wizard miniprep Column (Promega Co., San Diego, CA), desulfonated with 0.3 mol/L NaOH at 25°C for 5 minutes, precipitated with ammonium acetate and ethanol, and resuspended in 30 μL distilled water.
Combined bisulfite restriction analysis. We used bisulfite-PCR followed by combined bisulfite restriction analysis (COBRA; ref. 10) to analyze the methylation status of cell lines. PCR reactions were carried in 50 μL reactions, including 1 μL bisulfite-treated DNA, 2 mmol/L MgCl₂, 0.25 mmol/L deoxynucleotide triphosphate, 1 unit Taq polymerase, 16 mmol/L (NH₄)₂SO₄, 67 mmol/L Tris-HCl (pH 8.8), 1 mmol/L 2-mercaptoethanol, and 100 mmol/L primers. PCR products of WNT9A/CD558500 and KCNK15/BF195580 promoters were digested with HpyCH4IV and HınII, respectively. The digestion products were separated in nondenaturing polyacrylamide gels followed by densitometric analysis to obtain quantitative methylation levels. Densitometric analysis was done using a Bio-Rad Geldoc 2000 digital analyzer equipped with the Quantity One version 4.0.3 software (Bio-Rad, Hercules, CA). Primer sequences are described in Supplementary Table S1.

Bisulfite pyrosequencing. To extend our study to more cell lines and patient samples, we used the pyrosequencing method (11). Two-step PCRs were done. In the first step, we used 100 nmol/L forward primer and reverse primer, 1 μL bisulfite treated DNA, and the PCR buffer mentioned above. DNA was amplified at proper PCR conditions for 25 cycles. For the second step, we used 100 nmol/L forward (or reverse) primer, 10 nmol/L of 20-bp universal sequence connected to reverse (or forward) primer, 100 nmol/L biotinylated universal primer, 0.5 μL 1st step PCR product, and PCR buffer and ran at proper conditions for 40 cycles. Primer sequences are shown in Supplementary Table S1. The final biotin-labeled PCR product was captured by Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden). PCR product bound on the bead was purified, precipitated, and ligated into the pCR 4.0-TOPO vector (Invitrogen), and individual clones were sequenced at the DNA sequencing core facility at the University of Texas M.D. Anderson Cancer Center. About 10 clones were sequenced for each gene pair in each cell line, respectively. Methylation was calculated as the average value of the 10 clones.

RNA purification and reverse transcription-PCR. Gene expression was analyzed by reverse transcription-PCR (RT-PCR). Total RNA was isolated from different cell lines and normal and patient blood samples with TRIZOL reagent (Molecular Research Center, Inc., Cincinnati, OH). Genomic DNA was removed with the DNA-Free kit (Ambion, Austin, TX). Reverse transcription was done with High Capacity cDNA kit (Applied Biosystems, Foster City, CA). cDNA for the three gene pairs and β-actin (internal control) were amplified by PCR. Taqman real-time PCR was used to confirm the results of conventional RT-PCR with Taqman ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer’s instruction. Transcription levels were determined by the average of independent triplicate results. The sequence of all primers, Taqman probes, and their positions are shown in Supplementary Table S1.

5-Aza-2′-deoxycytidine treatment. Cell lines were treated with 1 μmol/L of the hypomethylating reagent 5-aza-2′-deoxycytidine (DAC; Sigma, St. Louis, MO). Fresh DAC was added daily for 5 days, before DNA and RNA were extracted for analysis.

Construction of luciferase reporter constructs. The 254-bp common promoter region between WNT9A and CD558500 translational start sites was synthesized and cloned into the pGL3-basic luciferase reporter vector (Promega) in both orientations (A–B-pGL3 and B–A-pGL3 constructs). For in vitro methylation, the promoters in either orientation were released with KpnI and NcoI digestion and treated with SSSI methylase (New England BioLabs, Beverly, MA) according to the manufacturer’s instruction. The methylation status of the two promoters was confirmed by BstU digestion. Methylation promoters were purified, precipitated, and ligated back into the pGL3-basic vector. For negative control, the same amount of DNA fragments without SSSI treatment was subjected to the same procedure.

**Figure 1.** Schematic illustration of three bidirectional promoters: (A) WNT9A/CD558500 promoter, (B) CTDSPL/BC040563 promoter, (C) KCNK15/BF195580 promoter. Arrows, direction of gene transcription. Vertical bars, CpG sites. Open boxes, first exons of each gene. Gray areas, range tested by pyrosequencing or COBRA for DNA methylation. Thick black lines, range tested by bisulfite sequencing. R, 5′-end of RACE PCR products.
Transfection and luciferase assays. HCT116 cells were plated and transiently transfected with the pGL3-basic vector and methylated and unmethylated A→B-pGL3 and B→A-pGL3 constructs using the Lipofectamine 2000 kit (Invitrogen). Cells were harvested after 24 hours. Luciferase activity was determined using the Luciferase assay system (Promega Co., Madison, WI). Luciferase activities were normalized to total protein concentration of the harvested cells determined by the Bio-Rad protein assay (Bio-Rad).

Results

Three newly found hypermethylated bidirectional promoters in human cancer. Methylated CpG island amplification (MCA) coupled with representational difference analysis (RDA) is an unbiased method to identify DNA fragments hypermethylated in cancer (12). Using MCA/RDA in multiple tumor types, we identified >150 genes hypermethylated in cancer (12).

From these, three bidirectional promoters (WNT9A/CD558500, CTDSPL/BC040563, and KCNK15/BF195580) were randomly selected (out of 34, as discussed later) for detailed analysis. All three promoters reside in CpG islands. The WNT9A island is at chromosome 1q42.13 and spans the transcription start sites of WNT9A and the CD558500 spliced expressed sequence tag (EST; Fig. 1A). The distance between the transcription start sites is about 240 bp. WNT9A is a member of the WNT family, members of which have been reported to be oncogenes or tumor suppressor genes (13). The CTDSPL island maps at chromosome 3p33.3 and spans the transcription start sites of CTDSPL and the spliced EST BC040563 (Fig. 1B). The two transcription start sites are within 130 bp of each other. CTDSPL is a small phosphatase-like protein that may be involved in the regulation of cell growth and differentiation and is a reported tumor suppressor gene (14). The KCNK15 island is located at chromosome 20q13.12 and is shared by KCNK15 and the spliced EST BF195580 (Fig. 1C) with an overlapping exon 1. KCNK15, or potassium channel subfamily K member 15, encodes a potassium channel subunit. Other ion channels have previously been reported to be silenced in cancer and to have potential tumor suppressor gene properties (15, 16). To make sure the transcription start sites of the three ESTs are accurate, we did 5′-rapid amplification of cDNA ends (RACE) PCR with 5′-RACE Ready cDNA from fetal brain. We found that the transcription start sites of BF195580 and BC040563 are the same as those in Invitrogen’s full-length cDNA clone collection and those in the Genbank database. For CD558500, our results showed that the transcription start site is 312 bp downstream of that in the Genbank database (depicted in Fig. 1), suggesting the possibility of alternative transcription start sites, a common finding in TATA less CpG island promoters. However, this promoter still qualifies as bidirectional because both transcription start sites of CD558500 lie within 1 kb of that of WNT9A.

We then tested methylation levels of these gene pairs in 33 cell lines from different tissues by COBRA, pyrosequencing, and bisulfite sequencing in selected cases. Sites analyzed are shown in Fig. 1, and the data are shown in Fig. 2 and summarized in Table 1. For WNT9A, both COBRA and pyrosequencing were done. The results from these two methods were consistent with each other (Table 1). Methylation densities were >50% in 8 of 25 cell lines, most of which were colon cancer and B-cell leukemia cell lines. To confirm our COBRA and pyrosequencing results, we did bisulfite sequencing in three selected cell lines: SW48 (colon cancer), RKO (colon cancer), and RAJI (B-cell leukemia). A region 538-bp long covering 66 CpG sites was tested for its methylation status. Bisulfite-treated genomic DNA extracted from blood samples of healthy donors was used as normal control (Fig. 2A). No methylation was detected in normal controls, whereas in all three cancer cell lines, the methylation levels were >90% (RAJI shown in Fig. 2A), consistent with the COBRA and pyrosequencing results. Methylation spanned the transcription start sites of both genes in the pair, with no preference for one over the other, within the region analyzed. The CTDSPL promoter was hypermethylated in several leukemia cell lines (RAJI, BJAB, and HL-60). Bisulfite sequencing was also done in these three cell lines with consistent
results (HL-60 shown in Fig. 2B). Again, methylation spanned both genes. For KCNK15, hypermethylation was found in 10 cell lines, including colon cancer, leukemia, and bladder cancer. Four of five colon cancer cell lines were hypermethylated, and methylation status of all four B-cell leukemia cell lines was >70%. Bisulfite sequencing results of the normal control and SW48 are shown in Fig. 2C and were consistent with the COBRA and pyrosequencing data. For this pair of genes as well, methylation spanned both transcription start sites. We calculated the correlation between methylation measured by the three different methods with a nonparametric correlation (Spearman) statistical method. For all three CpG islands, the correlation between COBRA and pyrosequencing was 0.90, between pyrosequencing and bisulfite sequencing was 0.71, and between COBRA and bisulfite sequencing was 0.78. These correlations are highly significant, showing that these three different methods are consistent with each other.

Methylation of bidirectional promoters is inversely correlated with expression of both genes in a pair. We examined expression levels of these three gene pairs in cell lines with various methylation densities but with the same tissue origin. Pyrosequencing results were used to compare the methylation status of the different cell lines, and RT-PCR was done to test gene expression. Based on Table 1 results, we compared the expression levels of WNT9A and CD558500 in HT29, RKO, SW48, and HCT116 cell lines, all of which were isolated from colon carcinoma with methylation densities of 89%, 86%, 77%, and 2%, respectively (Fig. 3A). HCT116, which had the lowest level of methylation, had the strongest expression of both WNT9A and CD558500 among these four cell lines, whereas the expression level of these two genes was undetectable in RKO and HT29, the two most heavily methylated cell lines. The WNT9A promoter transcription activity was also studied in B-cell and myeloid leukemia cell lines, and a similar inverse correlation between expression and methylation was observed (Fig. 3B).

B-cell and myeloid leukemia cell lines were used to study the gene pair CTDSPL and BC040563. Both CTDSPL and BC040563 were silenced in RAJI and BJAB, in which methylation levels were >70%, but were strongly expressed in two cell lines with low methylation levels [i.e., RS4;11 (3%) and ALL-1 (3%); Fig. 3B]. The KCNK15 promoter was hypermethylated in colon cancer, B-cell leukemia, and myeloid leukemia cell lines. We, thus, selected four colon cancer cell lines and two myeloid leukemia cell lines for

| Table 1. Methylation level of three bidirectional promoters in human cancer cell lines |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Tissue          | Cell lines      | Wnt9A (%)       | CTDSPL (%)      | KCNK15 (%)      |
|                 | COBRA | Pyrosequencing | Bisulfite       | Pyrosequencing | Bisulfite       | COBRA | Pyrosequencing | Bisulfite       |
| Colon cancer    | HT29  | 50             | 89              | 5              | 0              | 40               |
|                 | SW48  | 64             | 77              | 91             | 5              | 86               | 97               | 97               |
|                 | RKO   | 48             | 86              | 98             | 4              | 40               | 81               | 70               |
|                 | HCT116| 0              | 2               | 5              | 5              | 88               | 96               |
|                 | CaCO2 | 0              | 4               | 5              | 0              | 0                | 0                | 0                |
| B-cell leukemias| Raji  | 72             | 93              | 95             | 98             | 74               | 90               | 97               | 85               |
|                 | BJAB  | 74             | 91              | 82             | 3              | 72               | 72               | 51               | 85               |
|                 | RS4;11| 2             | 82              | 3              | 2              | 0                | 0                | 0                | 0                |
|                 | ALL-1 | 0              | 2               | 3              | 3              | 0                | 0                | 0                | 0                |
| Myeloid leukemias| HL-60| 83             | 90              | 95             | 98             | 0                | 0                | 0                | 0                |
|                 | K562  | 0              | 2               | 4              | 0              | 0                | 0                | 0                | 0                |
|                 | MV4;11| 0             | 0               | 0              | 0              | 0                | 0                | 0                | 0                |
|                 | BV173 | 5              | 5               | 5              | 5              | 5                | 5                | 5                | 5                |
| T-cell leukemias| CEM   | 0              | 23              | 5              | 0              | 20               | 0                | 20               | 20               |
|                 | Jurkat| 0              | 2               | 7              | 0              | 12               | 0                | 12               | 12               |
|                 | TALL  | 84             | 62              | 12             | 6              | 60               | 0                | 60               | 60               |
| Liver cancer    | HepG2 | 0              | 5               | 5              | 5              | 5                | 5                | 5                | 5                |
|                 | HeP3B | 0              | 5               | 5              | 5              | 5                | 5                | 5                | 5                |
| Bladder cancer  | UC2   | 3              | 2               | 96             | 6              | 0                | 0                | 0                | 0                |
| Breast cancer   | Cama-1| 0              | 5               | 0              | 0              | 0                | 0                | 0                | 0                |
|                 | MB-435| 27             | 5               | 47             | 4              | 0                | 0                | 0                | 0                |
|                 | SKBr3 | 44             | 6               | 0              | 0              | 0                | 0                | 0                | 0                |
|                 | 231   | 0              | 4               | 0              | 0              | 0                | 0                | 0                | 0                |
|                 | BT474 | 0              | 4               | 0              | 0              | 0                | 0                | 0                | 0                |
|                 | HTB126| 0              | 4               | 0              | 0              | 0                | 0                | 0                | 0                |
|                 | MCF-12A| 5             | 5               | 5              | 5              | 5                | 5                | 5                | 5                |
| Prostate cancer | LNCaP | 0              | 6               | 35             | 6              | 35               | 35               | 35               | 35               |
|                 | PC3   | 0              | 10              | 0              | 0              | 0                | 0                | 0                | 0                |
| Gliomas         | U373  | 0              | 4               | 0              | 0              | 0                | 0                | 0                | 0                |
| Lung cancer     | H69   | 7              | 7               | 11             | 11             | 11               | 11               | 11               | 11               |
| Normal fetal lung| IMR90| 0              | 12              | 0              | 0              | 0                | 0                | 0                | 0                |
gene expression studies (Fig. 3C). Once again, we found the expression of both KCNK15 and BF195580 was inversely correlated with methylation density of the promoter. In each case, the results obtained by conventional RT-PCR were independently confirmed by real-time PCR (Fig. 3). Our study strongly suggests that hypermethylation of bidirectional promoters in cancer results in silencing of both genes in a pair, in either direction.

Bidirectional restoration of gene expression by induction of hypomethylation. We then determined whether manipulation of methylation status in bidirectional promoters by the DNA methyltransferase inhibitor DAC is able to up-regulate the expression of the silenced genes in several cell lines. HL-60, RKO, and RS4;11 were selected because of high methylation levels (>80%) in WNT9A/CD558500 and low expression levels of both WNT9A and CD558500. After DAC treatment, the methylation levels in these cell lines were tested by pyrosequencing (Fig. 3D) and were found to decrease to varying degrees. At the same time, RT-PCR showed that the expression levels of both WNT9A and CD558500 increased after DAC treatment, and the expression levels were again inversely correlated with the density of methylation (Fig. 3D). The same pattern was also detected in the KCNK15 and BF195580 gene pairs (Fig. 3E). In CTDSP/BC040563, we saw restoration of the expression of CTDSP but not BC040563 in hypermethylated cell lines (HL-60 and RAJI) after DAC treatment (Fig. 3E; data not shown). The reason could be the low transcriptional activity of BC040563 in myeloid leukemia cell lines because the expression level of BC040563 is very low even in K562 cells whose BC040563 promoter was unmethylated, as shown in Fig. 3B. For RAJI, DAC treatment only lowered the methylation density of this promoter from 97% to 90%, and the expression might still be difficult to detect for BC040563 at this level. We have also done real-time PCR whose results again confirmed that of conventional RT-PCR (Fig. 3), except in CTDSP and BC040563 pair because of the low expression levels in HL-60 and RAJI even after DAC treatment.

WNT9A/CD558500 promoter functions bidirectionally. To confirm the bidirectional nature of the WNT9A/CD558500 promoter, we cloned the 254-bp promoter region between the translation start sites of WNT9A and CD558500 promoter, we cloned the 254-bp promoter region between the translation start sites of WNT9A and CD558500 in either orientation into a luciferase reporter (Fig. 4A). The promoter displayed bidirectional transcriptional activity in luciferase assays (Fig. 4B). Interestingly, WNT9A expression was reproducibly twice that of CD558500, which could be related to posttranscriptional mechanisms. When the promoter was methylated in vitro by SSSI

Figure 3. Expression levels of three bidirectional gene pairs. A to C, mRNA expression in colon cancer, B-cell lymphoid leukemia, and myeloid leukemia cells was measured by conventional RT-PCR and by real-time quantitative PCR. Methylation level (%) for each cell line studied (top). The quantitative PCR data is shown normalized to the most unmethylated cell line in each tissue type (left-most line), which was arbitrarily assigned a value of 100. A, WNT9A and CD558500. B, CTDSP/ and BC040563. C, KCNK15 and BF195580. D to F, mRNA expression level before and after DAC treatment. Results of conventional PCR. Methylation level (%) of these genes before (−) and after (+) DAC for each cell line studied (top). When available, confirmatory results by quantitative PCR are shown, expressed as fold increase compared to pretreatment. H*, quantitative PCR confirmation of activation, but inability to derive a fold increase because expression at baseline was undetectable. ND, not done. D, WNT9A and CD558500. E, CTDSP/ and BC040563. F, KCNK15 and BF195580.
methylase, its transcriptional activity in either direction was greatly reduced compared with the unmethylated control (Fig. 4C), suggesting that promoter methylation can lead to bidirectional silencing of gene transcription.

**DNA methylation of the three gene pairs in primary cancers.** The promoter CpG islands of the three selected gene pairs were frequently hypermethylated in colon cancer and leukemia cell lines. To determine their relevance to tumorigenesis, we next examined methylation levels of these three promoters in normal colon, colon cancer tissues, normal blood, and blood samples from acute lymphoid leukemia (ALL) patients using bisulfite pyrosequencing (Table 2). Hypermethylation in patient samples was defined as methylation density >10% (which is twice higher than the typical average tumor methylation level). We found that hypermethylation of each gene pair occurs in primary colon cancer and ALL. The WNT9A/CD558500 promoter was found hypermethylated in 31% (5 of 15) of colon cancer samples, including two samples with methylation densities >40%. CTDSPL/BC040563 hypermethylation was observed in 24% (8 of 34) of ALL patients. KCNK15/BF195580 hypermethylation was found in 22% (7 of 32) of all samples. We then tested gene expression in ALL patient blood samples by real-time PCR. We found that the expression levels of WNT9A/CD558500 and CTDSPL/BC040563 gene pairs were significantly lower in tumor samples with hypermethylation (Fig. 5). KCNK15 and BF195580 were not tested because their expression levels were too low to detect in blood samples.

**Relative frequency of methylation in bidirectional promoter CpG islands.** A previous study reported that there was no evidence that CpG islands have particular preference to bidirectional promoters (2). However, it has been suggested that the divergent organization of promoters could help resist invasion by transposable elements, which may trigger DNA methylation (17). Thus, it is possible that bidirectional promoters may be more resistant to attack by de novo methylation complexes. We evaluated the frequency of bidirectional promoters in methylated CpG islands in cancers by comparing them to adjacent genes. We examined all 135 CpG islands methylated in eight types of tumors that overlapped with the promoter region of genes from the MCA/RDA database. For each gene, we selected two controls, consisting of the immediately adjacent 5′ and 3′ promoter-associated CpG islands. Bidirectional promoters were found in 25.2% (34 of 135) of the methylated CpG islands and 25.9% (70 of 270) in the neighboring CpG islands (Supplementary Table S2). Thus, there is no indication that bidirectional promoters are particularly predisposed to or protected against de novo methylation in cancer.

**Discussion**

Bidirectional promoters have been studied for years as an interesting pattern of genome organization. Housekeeping genes are relatively enriched around these promoters, especially DNA repair genes (1) and possibly also other tumor suppressor genes. Therefore, hypermethylation of bidirectional promoters is of special interest in tumor development because of the chances of simultaneous silencing of two tumor suppressor genes in a single hypermethylation event. In this study, we provide evidence that aberrant methylation of bidirectional promoters is inversely correlated with expression of both genes in the pair in cancer cells and in vitro luciferase reporter assays. Methylation showed no preference to any particular transcription start site in CpG sites within the sequenced area. DAC treatment was able to bidirectionally restore gene expression silenced by hypermethylated divergent promoters. The frequency of bidirectional promoters in a database of hypermethylated CpG islands was comparable with that of bidirectional promoters among controls (adjacent CpG islands). Thus, hypermethylation of bidirectional promoters is a potentially efficient gene silencing mechanism in tumorigenesis, which may be physiologically relevant given the higher ratio of DNA repair genes in bidirectional gene pairs.

Several genes important to cancer, such as the DNA repair genes ATM (18), MSH3 (19), and BRCA1 (20), are controlled by
bidirectional promoters, and some of these genes are indeed hypermethylated in cancers. For example, ATМ has been reported to show hypermethylation in breast cancer (21). Its opposite gene NPAT was found to play an important role in regulation of cell cycle (22). The effect of ATМ promoter hypermethylation might, therefore, be more profound than it seems, which indicates the necessity of evaluating the combined effect of silencing of the divergent gene pairs by promoter hypermethylation on carcinogenesis.

Our study identifies hypermethylation of the promoters of six genes in cancer. WNT9A is a member of the WNT gene family, which are ligands for frizzled receptor family (23). WNT9A mRNA is expressed in various types of human cancer, such as gastric cancer, pancreatic cancer, and breast cancer (24). WNT9A members have been proposed to act as oncogenes [e.g., in lung cancer (25) and intestinal cancers (26)]. Previous studies have suggested that WNT5A, another WNT family member, may also function as a tumor suppressor gene (27). WNT9A hypermethylation in cancer points to its possible role as a tumor suppressor gene. However, oncogenes can also show hypermethylation in cancer (28), and further studies are needed to address the role of WNT9A in carcinogenesis. CTDSPL functions as a phosphatase involved in the regulation of cell growth and differentiation (14). It was reported that CTDSPL was homozgyously deleted in about 15% of major epithelial cancers. Significant down-regulation of CTDSPL expression was detected in many cancer cell lines and tumor samples. CTDSPL was implicated as a tumor suppressor gene, and its suppressor function was found to be dependent on the blocking of cell cycle at G1-S boundary through dephosphorylating RB (14). Previous researchers were able to find hypermethylation of the CTDSPL gene but not in the promoter region (14). In our study, we clearly showed dense methylation of CTDSPL promoter in several leukemia cell lines and 24% of ALL patient samples. It seems likely that methylation of CTDSPL may affect leukemogenesis. KCNK15 is a potassium channel, and a potential role for this gene in oncogenesis is unknown. Other ion channels, such as the Ca2+ channel CACNA1G (15) and the Na+ channel SLC5A8 (16), have been reported to be hypermethylated in cancer, with some evidence of tumor suppression function. The other three genes (CD558500, BC040563, and BTF195580) are all functionally characterized at present.

In summary, we found an inverse correlation between methylation of CpG islands containing bidirectional promoters and transcription of the gene pair, suggesting that silencing of bidirectional gene pairs are co-coordinate regulated. About 25% of hypermethylated promoter associated CpG islands in cancer are bidirectional, implicating the possibility that hypermethylation of bidirectional promoters might be an efficient way for silencing of tumor suppressor genes in tumor development. We also report for the first time the potential involvement of hypermethylation of the promoters of WNT9A, CTDSPL, and KCNK15 in tumorigenesis, and there is plausible evidence implicating CTDSPL as a bona fide tumor suppressor gene in ALL.

Acknowledgments

Received 7/31/2005; revised 2/2/2006; accepted 3/10/2006.

Grant support: NIH grants P50CA10632, RO1CA098006, and R33CA89837. DNA sequencing in the Core Sequencing facility at the M.D. Anderson Cancer Center is supported by the NIH core grant CA16672.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

16. Li H, Myeroff L, Smiraglia D, et al. SLC5A8, a sodium transporter, is a tumor suppressor gene silenced by
Silencing of Bidirectional Promoters by DNA Methylation in Tumorigenesis

Jingmin Shu, Jaroslav Jelinek, Hao Chang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/10/5077

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2006/05/19/66.10.5077.DC1

Cited articles
This article cites 28 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/10/5077.full#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/10/5077.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.