Hypermethylation in Human Cancers of the RIZ1 Tumor Suppressor Gene, a Member of a Histone/Protein Methyltransferase Superfamily

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Abstract

The retinoblastoma protein-interacting zinc finger gene RIZ1 is a tumor suppressor gene and a member of a nuclear histone/protein methyltransferase superfamily. RIZ1 inactivation is commonly found in many types of human cancers and occurs through loss of mRNA expression, frameshift mutation, chromosomal deletion, and missense mutation. RIZ1 is also a tumor susceptibility gene in mice. We now show that loss of RIZ1 mRNA in human cancers is associated with DNA methylation of its promoter CpG island. Methylation of the RIZ1 promoter strongly correlated with lost or decreased RIZ1 mRNA expression in breast, liver, colon, and lung cancer cell lines as well as in liver cancer tissues. Treatment with the methylation inhibitor 5-aza-2'-deoxycytidine activated RIZ1 mRNA expression in cancer cells. Furthermore, methylation was found in 11 of 25 (44%) breast cancer specimens and 20 of 32 (62%) liver cancer specimens. Our results suggest that DNA methylation is a common mechanism in inactivating the RIZ1 tumor suppressor gene in human liver and breast cancers.

Introduction

Inactivation of TSGs plays an important role in cancer formation and progression. Either genetic or epigenetic mechanisms are known to inactivate TSGs. Whereas the importance of genetic mutations in cancer has long been recognized, the appreciation of epigenetic inactivation is more recent (1–3). Eight tumor suppressors are known to undergo promoter methylation in cancer, including Rb, CDKN2A (p16), CDKN2B, VHL, E-cadherin, MLH1, Brcal, and PTEN. The frequency with which promoter methylation contributes to TSG inactivation ranges from about 9% of Rb1 in retinoblastoma to 33% of VHL in Von Hippel Lindau disease to 84% of MLH1 in microsatellite-unstable colorectal tumors. Therefore, epigenetic silencing represents a significant contributor to human carcinogenesis.

The retinoblastoma protein-interacting zinc finger gene RIZ was isolated in a functional screening for Rb-binding proteins (4), also independently isolated as DNA-binding protein MTB-Zf (5), GATA3 transcription factor-binding protein G3B (6), and a coactivator of estrogen receptor (7). RIZ contains the canonical Rb-binding motif LXXE and the nuclear hormone receptor-binding motif LXXLL. In addition, RIZ contains a novel protein methyltransferase domain, called the PR domain or SET domain (8, 9), which is present in ~50 human genes. Two products of the gene exist: (a) RIZ1, which contains the PR domain; and (b) RIZ2, which lacks the domain (10). RIZ1 but not RIZ2 has tumor-suppressive properties. The gene maps to chromosome 1p36, a region commonly deleted in more than a dozen different types of human cancers (11). RIZ1 expression, but not RIZ2 expression, is commonly silenced in many types of human tumors, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma (12–14). RIZ1 missense inactivating mutations also occur in human cancer tissues and cell lines, and all target the PR/SET domain (15). Frequent frameshift mutations of the RIZ gene are common in microsatellite instability-positive tumors and truncate a PR-interacting domain (14, 16, 17).

Importantly, mouse gene knockout models show that RIZ1 inactivation can indeed cause tumor susceptibility (15). In keeping with its role as a bona fide TSG, RIZ1 has strong tumor-suppressive activities. Adenovirus-mediated RIZ1 expression causes G1-M cell cycle arrest and/or apoptosis in breast cancer, liver cancer, and microsatellite instability-positive colon cancer cells (12–14). Adenovirus RIZ1 can also inhibit growth of colon cancer xenografts (18). Finally, an independent line of investigation supports the notion of methyltransferases such as RIZ1 as TSGs. A methyltransferase inhibitor, MTA, is known to accumulate in many cancers due to the homozygous deletion of the MTAP gene, which encodes the enzyme MTA phosphorlase that degrades MTA (19).

In this study, we examined the role of DNA methylation in the loss or decrease in RIZ1 mRNA expression in human cancers. We cloned the RIZ1 promoter and found that it has characteristics of CpG island. The promoter was commonly methylated in breast and liver cancers, and methylation correlated strongly with loss of mRNA expression. Treatment with methylation inhibitors reactivated mRNA expression. Thus, DNA methylation appears to be a common mechanism of inactivating the RIZ1 TSG.

Materials and Methods

Cell Lines and Tissues. We obtained cancer cell lines from the American Type Culture Collection. Human cancer cell lines were grown in DMEM plus 10% FCS. Genomic DNAs from breast cancer and liver cancer have been described previously (20, 21).

RIZ1 Promoter Cloning. To clone full-length RIZ1 cDNA, we made cDNAs from mRNA of Y79 retinoblastoma cells using primer RP99 (5'-TAGTAAATGGCTCTGTTG-3'), derived from the RIZ gene 5' region and packaged the cDNAs into the λ phages using the cDNA library kit from Pharmacia. The library was next screened using RIZ1 cDNA probe. One clone was isolated that had an extended 5' region with high similarity to the 5' end of the rat RIZ cDNA. We next made an oligonucleotide primer from this region, RP279 (5' -CAGGGAAGCCGATCAGAGCAGG-3'), and used it to screen a chromosome 1 cosmid library from the Reference Library Data base at the Max Planck Institute in Berlin, Germany (22). From one of the positive cosmids clones, we then isolated an ~1-kb Pst1 fragment, which was next subcloned into pBSK vector and sequenced.

Promoter Reporter Assay. To test for promoter activity, we subcloned a fragment of the CpG island amplified using primer set RP280 (5'-GC-
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DNA Methylation Analysis. Sequence analysis of genomic DNA templates treated with bisulfite was performed according to the procedure described by Frommer et al. (24) and modified by Clark et al. (25). Genomic DNAs from tumor tissues and cells (50 ng) were treated with sodium bisulfite, and treated DNA was purified using the Wizard column (Promega). For DNA sequencing analysis, the DNA was used for PCR with the primer set RP294 (5'-GGTTGGTTATTGGGCGACGGC-3') and RP295 (5'-CAAAAACCGCCCTGCGCCACTCCTTACC-3'). The PCR products were subcloned into pCR script cloning vector (Invitrogen), and individual clones were subjected to DNA sequencing analysis.

Bisulfite-treated DNA was also used for MSP analysis (26). We generated PCR primers that distinguish between bisulfite-modified and unmodified cytosines in the RIZ1 CpG island. To amplify methylated DNA, we used the primer set RP217MF (5'-GGTTGGTTATTGGGCGACGGC-3') and RP217MR (5'-CAAAAACCGCCCTGCGCCACTCCTTACC-3'). The PCR reaction was 68°C. To amplify unmethylated DNA, we used the primer set RP217UF (5'-GGTTGGTTATTGGGCGACGGC-3') and RP217UR (5'-CAAAAACCGCCCTGCGCCACTCCTTACC-3'). The annealing temperature of the PCR reaction was 68°C.

Reexpression of RIZ1 by 5-Aza-dC Treatment. Cancer cells (5 × 10^6 cells) were grown for 4 days in the presence of various concentrations of 5-Aza-dC. RNA was isolated, and RT-PCR was performed as described previously (12, 13). Briefly, reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and oligonucleotide primer RP217 (5'-CTCTTGACAGCTTTAAGAGT-3'). The first-strand cDNA sample was then amplified using RP168 (5'-TGCTACAGTGTAAAATT-3') and RP217. The primers for amplification of human β-actin are 5'-GGGCGGCCGCCCAGGCAACAC-3' and 5'-CTCTCTTATGTCACCGGCAAGTTC-3'. PCR reactions were run for 30 cycles. The PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining.

Results

RIZ1 Promoter Cloning and Characterization. We have previously isolated a full-length RIZ1 cDNA (10). Using the 5' region of the cDNA, we cloned the corresponding genomic DNA fragment and tested it for promoter activities. However, no promoter activity was detected. This region, now designated exon 1b (Fig. 1A), appears to represent an alternatively spliced exon. To clone the authentic 5' region of RIZ1 cDNA, we made a cDNA library using RIZ primer-primed cDNA. From this library, we isolated a cDNA clone that contains a GC-rich region with significant similarity to the published 5' region of the rat cDNA (Fig. 1B; Ref. 4). We made an oligonucleotide primer RP279 and used the oligonucleotide to screen a cosmid library. We then isolated a 0.98-kb Pst1 genomic fragment containing this GC-rich exon from a cosmid clone. Sequence analysis of this fragment revealed a 0.64-kb CpG island (Fig. 1A; 340–980 bp). This region has a GC content of 76.9%, a CG/GC ratio of 1.09, and a CpG ratio of 1.07.

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\frac{\text{number of CpG}}{\text{number of nucleotides analyzed}} \times \frac{\text{number of G}}{\text{number of C}} \times \frac{\text{G/GC ratio}}{1}
\]

characteristic of CpG island (27, 28). The 5'-end region of the cDNA clone that was similar to rat cDNA is located within this CpG island (Fig. 1, A–C), and was designated exon 1a (Fig. 1, A and C).

Fig. 1. RIZ1 promoter cloning and mapping. A, schematics of RIZ gene genomic structure. Exon and intron distances are drawn to scale and based on public human genome (exon 3–10) and Celera human genome (exon 1–3 with gaps). B, comparison of human exon 1 sequence (nucleotide 557–591 of the Pst I fragment) with rat cDNA 5' sequence. C, nucleotide sequence of the 992-bp Pst I fragment. The transcription start site is indicated by arrows. The primer sequences used in this study are underlined, except for primer RP294 sequences, which are in italic. D, RNase protection analysis. Total RNAs isolated from RIZ1 mRNA-positive SW480 and DLD1 cells and RIZ1 mRNA-negative Huh2 cells were analyzed using the RIZ1 probe as indicated in A. Actin probe was analyzed as a quantitation control.
To map the transcription start sites, we performed the RNAse protection assay using a PCR fragment of the genomic DNA as probe. This fragment was amplified using primers RP280 and RP279 (Fig. 1C) and subcloned into pBlueScript vector. Total RNAs from hepatoma cell line Huh2 (RIZ1 negative), colon cancer cell line SW480 (RIZ1 positive), and colon cancer cell line DLD1 (RIZ1 positive) were used for hybridization with the RIZ1 antisense probe. As shown in Fig. 1D, a protected fragment of 145 nucleotides was specifically observed in RIZ1 mRNA-positive cell lines SW480 and DLD1, but not in RIZ1-negative cell line Huh2. The result mapped the transcription start site within the CpG island as shown in Fig. 1C.

To test for promoter activity, we subcloned a fragment of the CpG island amplified using primer set RP280 and RP279 into the luciferase reporter vector pGL3 basic. Plasmid pGLR1P279-280 contains a 0.315-kb fragment (330–645bp) at the middle of the Pst1 fragment (Fig.2A). When transfected into human kidney 293 cells, strong promoter activity was observed for construct pGLR1P279-280, which was comparable to that of the SV40 promoter/enhancer as represented by pGL3 control plasmid (Fig.2, B and C). Thus, the CpG island at the 5’ end of the RIZ1 gene functions as a promoter.

**Methylation of the RIZ1 Promoter in Cancer Cell Lines and Tumor Tissues.** The observation that the RIZ1 gene promoter consists of a CpG island suggests that DNA methylation may play a role in silencing RIZ1 gene expression. To study RIZ1 promoter DNA methylation, genomic DNA was isolated from cancer cell lines and treated with sodium bisulfite. Bisulfite-treated DNA was then analyzed by sequencing and MSP (24–26). The bisulfite genomic sequencing technique allows methylated cytosines to be distinguished through their resistance to bisulfite-mediated deamination; unmethylated cytosines are converted to uridine by bisulfite. The sequence under investigation is then amplified by PCR to yield a product in which all uracil and thymine residues have been amplified as thymine, and only 5-methylcytosine residues have been amplified as cytosine. The PCR products can then be cloned and sequenced. We used the primer set RP294 and RP295 to amplify the bisulfite-treated genomic DNA. The PCR products were cloned, and individual clones were randomly selected for DNA sequence determination. The positions of CpGs were represented by vertical lines and numbered. Promoter start site and MSP primers are indicated by arrows. Closed circles represent methylated CpGs, and open circles represent unmethylated CpGs. Gray circles represent the CpG at position 22 that was unusually changed to CpA by bisulfite treatments.

![Fig. 3. Sequence analysis of RIZ1 promoter DNA methylation. Genomic DNAs from liver cancer cell lines HepG2, Ha22T, and Hep3B; breast cancer cell lines MB435 and ZR75; and normal lymphocytes were treated with sodium bisulfite and amplified by primer set RP294 and RP295. The PCR products were cloned, and individual clones were randomly selected for DNA sequence determination. The positions of CpGs were represented by vertical lines and numbered. Promoter start site and MSP primers are indicated by arrows. Closed circles represent methylated CpGs, and open circles represent unmethylated CpGs. Gray circles represent the CpG at position 22 that was unusually changed to CpA by bisulfite treatments.](image-url)
RIZ1. Nevertheless, partial methylation was noted in these two cell lines, consistent with the possibility that a fraction of the RIZ1 gene was methylated and silenced in these cell lines. Also in these two cell lines, the CpG at position 22 was changed to CpA by the bisulfite treatment for unknown reasons. Finally, no methylation was observed for normal lymphocyte DNA.

We then developed a MSP assay to distinguish methylated and unmethylated DNA. To establish the specificity of our PCR primers, we used RIZ1 promoter plasmid DNA before and after treatment with SsoI DNA methylase as controls. This enzyme methylates all CpG cytosines. We confirmed that the primer pair specific for methylated sequences generated a PCR product with methylated DNA but not unmethylated DNA; whereas the primer pair specific for nonmethylated sequences generated a PCR product with only unmethylated DNA (data not shown). We also made sure that none of the primer pairs generated any PCR products using non-bisulfite-treated DNA (data not shown), indicating that DNA that has been incompletely treated with sodium bisulfite was unlikely to be amplified by primer sets specific for methylated sequences and therefore inadvertently interpreted as being methylated. As shown in Fig. 4 and summarized in Table 1, methylation of RIZ1 promoter CpG island was found in all cancer types tested and was most common in liver cancer cell lines, which correlates well with the previously known expression status of RIZ1 mRNA in these cell lines.

Previously, we have studied liver cancer tissue samples and found that RIZ1 expression is commonly lost or decreased in these tumors (13). To correlate whether loss of expression in tumor tissues is correlated with DNA methylation, we studied the DNAs of the previous tumor samples that showed decreased RIZ1 expression. We found that three of the four tumor samples showed DNA methylation (data not shown), and, as summarized in Table 1, these three tumor samples also show lost or reduced RIZ1 expression.

We then determined the frequency of RIZ1 methylation in liver and breast tumor tissues by MSP analysis (Fig. 4, C and D). A total of 32 pairs of DNAs from liver cancer tissues and matched normal tissues were analyzed, and 20 tumor tissues (62.5%) showed DNA methylation. Of the 20 matched normal tissue DNA pairs, 4 showed DNA methylation, whereas 16 did not. In 12 cases where tumor DNA did not show methylation, 3 of the matched normal tissue DNA samples showed methylation. A total of 25 breast cancer DNAs were analyzed, and 11 (44%) showed DNA methylation. The unmethylated form was detected in all samples in both tumors and nonmalignant tissues. This was expected because the tumor specimens represented macroscopically isolated samples that contained both tumor and nonmalignant tissue. We evaluated the DNA methylation changes in the tumors and the clinical data obtained from these patients. We found no correlation between methylation and any of the clinicopathological characteristics of the patients. We also analyzed the DNA methylation changes and the 1p36 or RIZ1 LOH changes in these liver and breast cancers, which were characterized previously (20, 21). We found no correlation between methylation and 1p36 or RIZ loss of heterozygosity.

Promoters silenced by methylation can be reactivated by treatment with 5-Aza-dC, which is a well-documented inhibitor of DNA methylation. We treated liver cancer cell lines HepG2 and Huh1 and colon cancer cell line SW48. By RT-PCR analysis, RIZ1 expression was detected after treatment with increased amounts of 5-Aza-dC.
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RIZ1 (Fig. 4E). The data confirm that methylation plays an important role in silencing RIZ1.

Discussion

Loss of expression of the RIZ1 TSG is commonly found in many types of human cancers and is causally linked with carcinogenesis, as suggested by the tumor susceptibility of mice deficient in RIZ1. This report shows that such lost or decreased RIZ1 expression occurs largely through gene silencing or DNA methylation of the RIZ1 gene promoter. This conclusion is based on several observations presented here. The RIZ1 promoter has characteristics of a CpG island, which is present in most of the known TSG promoters that suffer DNA methylation in cancers. The methylation of the RIZ1 promoter is strongly correlated with reduced mRNA expression. Finally, RIZ1 expression can be reactivated by 5-Aza-dC in several cancer cell lines. However, our study cannot exclude other potential mechanisms of RIZ1 silencing. One such potential mechanism is that RIZ1 silencing could be caused by a defect in a certain transcription factor that normally activates RIZ1 promoter. Another potential mechanism is mutation in the RIZ1 promoter. However, given the prevalence of methylation, these other mechanisms are not likely to be commonly involved.

The RIZ1 promoter we cloned in this study has strong promoter activity. The observation that this promoter has a CpG island is consistent with the largely ubiquitous expression of RIZ1 (4, 10). Most widely expressed housekeeping genes are known to contain CpG islands. By DNA sequencing analysis, we have determined the positions of many of the methylated CpGs. Whereas not all CpGs around the transcription start site are methylated, most of them are. We also developed a MSP assay to conveniently study RIZ1 methylation in primary tumor sample DNAs. Based on the analysis of 32 tumor cell lines and tissues, methylation as detected by our MSP assay is strongly associated with lost or reduced RIZ1 mRNA expression. In addition, the PCR primers in our MSP assay cover eight CpGs, all of which are commonly methylated in liver and breast cancers as shown by DNA sequencing analysis. Therefore, our MSP assay developed here represents an accurate measure of RIZ1 gene expression status.

Our results show that the RIZ1 promoter CpG island is commonly methylated in liver and breast cancer tissue samples, consistent with the results obtained from cancer cell lines. A majority of the liver tumor tissue cases showed methylation in the tumor tissues but not in the corresponding nonmalignant tissues, suggesting that in these cases, methylation is a tumor-specific event. However, several cases showed methylation in the nonmalignant liver tissues, but not in the corresponding tumor tissues. A possible explanation for detection of methylated alleles in the nonmalignant liver samples is that they represent premalignant changes. Methylation in TSGs in normal tissues but not tumor tissues has also been reported in lung cancer, colon cancer, and breast cancers (29–31). Alternatively, it has been described that methylation of certain genes is common in aging tissues. Thus, the aging process may also be a possible explanation for detection of methylation in nonmalignant liver tissues.

Data accumulated in the past have made it notable that TSGs tend to be preferentially inactivated by a preferred mechanism. For example, p53 is mostly inactivated by point mutation, p16/CDKN2A is generally inactivated by homozygous deletion and DNA methylation, and MLH1 is inactivated by mutation in familial colon cancer but by DNA methylation in sporadic colon cancer. The implication of these observations is that there might be a group of TSGs for which the primary mechanisms of inactivation may be DNA hypermethylation (1). Given that silencing would relieve selective pressure for mutations, these genes may rarely suffer mutations. However, in the absence of genetic mutations, it will be challenging to prove that a gene that is silenced in human cancers is in fact causally linked with carcinogenesis. Among the numerous genes that are known to be silenced in human cancers, RIZ1 is one of the few with a proven role in causing cancer as demonstrated by genetic data in both animals and humans (15). Whereas previous studies demonstrate reduced RIZ1 gene expression to be common in cancers, this study confirms that RIZ1 is commonly silenced by DNA methylation. RIZ1 methylation is found in 62% of liver cancers and 44% of breast cancers. However, RIZ1 methylation has not been detected in these cancers (20, 21). Thus, DNA methylation may represent the preferred mechanism of RIZ1 inactivation in these cancers. Furthermore, because many types of human cancer cell lines exhibit reduced RIZ1 expression, we predict that RIZ1 gene methylation will be commonly found in many types of human cancer tissues. Finally, the MSP assay we developed here can conveniently assay RIZ1 expression status in primary tissues. Our small sample-size study here was not informative with regard to a correlation between RIZ1 silencing and specific stages/characteristics of cancer. However, definitive conclusions on this issue will require future investigations of large sets of tumor samples, which would also address whether RIZ1 methylation may prove to be useful in cancer diagnosis/prognosis.

References


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