Identification of HRK as a Target of Epigenetic Inactivation in Colorectal and Gastric Cancer

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ABSTRACT

Purpose: Aberrant methylation of CpG islands can be a good molecular marker for identifying genes inactivated in cancer. We found the proapoptotic gene HRK to be a target for hypermethylation in human cancers and examined the role of such methylation in silencing the gene’s expression.

Experimental Design: Methylation of HRK was evaluated by bisulfite-PCR and bisulfite sequencing in a group of colorectal and gastric cancer cell lines and primary cancers. Gene expression and histone acetylation were examined by reverse transcription-PCR and chromatin immunoprecipitation analyses, respectively. Apoptosis of cancer cells after treatment with a DNA methyltransferase inhibitor and/or histone deacetylase inhibitor was examined with fluorescence-activated cell-sorting analysis.

Results: The region around the HRK transcription start site was methylated in 36% of colorectal and 32% of gastric cancer cell lines and was closely associated with loss of expression in those cell types. HRK expression was restored by treatment with a methyltransferase inhibitor, 5-aza-deoxycytidine, and enhanced further by addition of histone deacetylase inhibitor trichostatin A or depsipeptide. Such restoration of HRK expression was well correlated with induction of apoptosis and enhancement of Adriamycin-induced apoptosis. Expression of other proapoptotic genes, including BAX, BAD, BID, and PUMA, was unaffected by treatment with 5-aza-deoxycytidine. Aberrant methylation of HRK was also frequently detected in primary colorectal cancers that showed methylation of multiple genes, including p16INK4A and hMLH1, and was associated with wild-type p53.

Conclusion: HRK methylation can be a useful molecular target for cancer therapy in a subset of colorectal and gastric cancers.

INTRODUCTION

Dysregulation of proliferation, together with defective control of apoptosis, are hallmarks of human tumors (1). Among the genes involved in the regulation of apoptosis, p53 is a major player whose downstream target genes participate in several apoptosis pathways and are disrupted in cancer (2). BAX, one of the target genes of p53, has been shown to be mutated in a subset of colorectal and gastric cancers (3). In addition to genetic changes, much evidence now suggests that by silencing transcription of certain genes, aberrant methylation of CpG islands (CGIs) also plays a key role in tumorigenesis (4, 5). Indeed, an increasing number of genes related to cell cycle control, DNA damage repair, the invasiveness of tumors, and growth factor responses are now known to be inactivated by DNA methylation in gastrointestinal malignancies. Much less is known about epigenetic inactivation of proapoptotic genes, however (6–8).

Aberrantly methylated CGIs can be good molecular markers with which to identify genes inactivated in cancer. Several techniques, including restriction landmark genome scanning and methylation-sensitive arbitrary primer PCR, have been used to screen for aberrantly methylated DNA fragments as molecular markers of genes inactivated by DNA methylation (9–11). We have developed a novel methylation screening technique, methylated CGI amplification, which we used to identify DNA fragments hypermethylated in cancer (12). Moreover, coupling methylated CGI amplification to representational difference analysis allowed us to identify genes inactivated by hypermethylation (13, 14). One of the DNA fragments identified with methylated CGI amplification, MINT17 (12, 15), was mapped to a part of the CGI of harakiri (HRK), a proapoptotic gene belonging to the BH3-only subfamily of bcl-2. HRK was originally isolated as a bcl-2- and bcl-2-interacting protein (16) whose expression was induced by withdrawal of growth factors and which was involved in apoptosis of hematopoietic and neuronal cells (17, 18). Little is known about the function of HRK in human tumors. The aim of the present study, therefore, was to determine the extent to which DNA methylation affects expression of HRK and induction of apoptosis in a group of human cancer cell lines and primary colorectal and gastric cancers.

MATERIALS AND METHODS

Cell Lines and Specimens. We used 11 colorectal cancer cell lines (Caco2, RKO, SW48, HCT116, DLD-1, LoVo, HT29, Colo205, Colo201, SW480, and Colo32Dv1), 19 gastric cancer cell lines (MKN7, MKN28, MKN45, MKN74, JRST, KatoIII, AZ521, NUGC3, NUGC4, SNU1, SNU638, SH101, HSC39,
and 23 stomach mucosa specimens have been described previously
specimens, as well as the 38 normal colorectal mucosa specimens
search Bioresources (Tokyo, Japan). The 58 primary colorectal
lection (Manassas, VA) or from the Japanese Collection of Re-
lines were obtained from either the American Type Culture Col-
Center, Research Institute, Tokyo, Japan) (19, 20), all of the cell
lines (HSC39, HSC40, HSC41, HSC42, HSC43, HSC44, HSC45,
ATL2) in this study. With the exception of eight gastric cancer cell
BALL1, Jurkat, Hs-sultan, RPMI1788, SCC3, Raji, IM9, and
cell lines (HO3238, KMS12PE, RPMI8226, K562, KR12, Daudi,
CHC20, CHC32, HLE, and HuH7), and 14 hematopoietic cancer
atic cancer cell lines (Panc-1, BxPC3, MIAPACA2, KP-1NL, KP3,
HSC40, HSC41, HSC42, HSC43, HSC44, and HSC45), 7 pancre-
ativa (number of cycles).
F, forward; R, reverse; RT-PCR, reverse-transcription-PCR.

HSC40, HSC41, HSC42, HSC43, HSC44, and HSC45), 7 pancre-
atic cancer cell lines (Panc-1, BxPC3, MIAPACA2, KP-1NL, KP3,
CF-PAC, and Capan2), 5 hepatocellular cancer cell lines (CHC4,
CHC20, CHC32, HLE, and HuH7), and 14 hematopoietic cancer
cell lines (HO3238, KMS12PE, RPMI8226, K562, KR12, Daudi,
BALL1, Jurkat, Hs-sultan, RPMI1788, SCC3, Raji, IM9, and
ATL2) in this study. With the exception of eight gastric cancer cell
lines (HSC39, HSC40, HSC41, HSC42, HSC43, HSC44, HSC45,
and SH-101) kindly provided by Dr. Yanagihara (National Cancer
Center, Research Institute, Tokyo, Japan) (19, 20), all of the cell
lines were obtained from either the American Type Culture Col-
lection (Manassas, VA) or from the Japanese Collection of Re-
search Bioresources (Tokyo, Japan). The 58 primary colorectal
cancers, 50 colorectal adenomas, and 23 primary gastric cancer
specimens, as well as the 38 normal colorectal mucosa specimens
and 23 stomach mucosa specimens have been described previously
(21, 22).

**Bisulfite-PCR and Sequencing.** Bisulfite modification of
data was carried out essentially as described previously (23), after
which the DNA was purified by a DNA purification system (Pro-
mega), precipitated with ethanol, resuspended in water, and stored
at −20°C until use. Bisulfite-PCR was carried out with primers that
amplify both methylated and unmethylated alleles. After amplifi-
cation, the PCR products were digested with restriction enzymes
that cleaved the regions containing the CpG sites retained after
bisulfite treatment as a result of cytosine methylation. The PCR
primers and cycling parameters used to examine methylation of
various regions of HRK and five other proapoptotic genes are
shown in Table 1. To sequence the bisulfite-PCR products, frag-
ments amplified with primers HRKGM1F/HRKGM1R and
demonstrated in Table 1. To sequence the bisulfite-PCR products, frag-
ments amplified with primers HRKGM1F/HRKGM1R and
HRKGM2F/HRKGM2R were cloned into pCR4 vector by use of
a TOPO-cloning kit (Invitrogen). Cycle sequencing was then car-
ried out with a BigDye terminator kit (Applied Biosystems), after
which the DNA was sequenced on an ABI 3100 automated se-
quencer (Applied Biosystems).

**Reverse transcription-PCR (RT-PCR).** Total RNA was
prepared from normal stomach and colon tissues and from lym-
phocytes and cancer cell lines, after which 5-μg samples were
reverse-transcribed with Superscript II (Life Technologies) to pre-
pare first-strand cDNA. The primer sequences and PCR parameters
used are shown in Table 1. Controls consisted of RNA treated
identically but without the addition of reverse transcriptase and
are labeled as negative (−). The integrity of the cDNA was con-
ferred by amplifying glyceraldehyde-3-phosphate dehydrogenase

### Table 1. Sequences for primers used in this study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequences</th>
<th>PCR conditions</th>
<th>Size (bp)</th>
<th>Enzyme</th>
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<tr>
<td>HRK-GM1</td>
<td>F: 5'-AAAAGTATAATATAAAGGAGAATTTG-3'</td>
<td>60 (3), 58 (4), 56 (5), 54 (26)</td>
<td>161</td>
<td>TaqI</td>
</tr>
<tr>
<td></td>
<td>R: 5'-RATACAAAAAAGACACATACA-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>170</td>
<td>HhaI</td>
</tr>
<tr>
<td>HRK-GM2</td>
<td>F: 5'-GGYGGATTTAGGTTATGTT-3'</td>
<td>64 (3), 62 (4), 60 (5), 58 (26)</td>
<td>181</td>
<td>BspT104I</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CRCCRCACATATACTACAATAC-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>184</td>
<td>NruI</td>
</tr>
<tr>
<td>MINT17</td>
<td>F: 5'-TTTTTTTTTTTTTTGGAAGGTT-3'</td>
<td>62 (3), 60 (4), 58 (5), 56 (26)</td>
<td>186</td>
<td>TaqI/Tail</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CITCRACCACACACGTTTAC-3'</td>
<td>62 (3), 60 (4), 58 (5), 56 (26)</td>
<td>179</td>
<td>TaiI</td>
</tr>
<tr>
<td>HRK-GM7</td>
<td>F: 5'-TGYGGATGGAAGGGGGA-3'</td>
<td>60 (35)</td>
<td>237</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'-CACAACGCTTCGCTCGCT-3'</td>
<td>60 (35)</td>
<td>129</td>
<td></td>
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<tr>
<td>BAD-GM1</td>
<td>F: 5'-GGTGATGGGGTAGTTGAGGTGGTGAT-3'</td>
<td>60 (3), 58 (4), 56 (5), 54 (26)</td>
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<td>TaqI</td>
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<td></td>
<td>R: 5'-ACTTAAAAATTCTCAATCTTCCCTC-3'</td>
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<td>172</td>
<td>HinI</td>
</tr>
<tr>
<td>BAX-GM1</td>
<td>F: 5'-TTATTTTGTTATATTATGTTGAT-3'</td>
<td>60 (3), 56 (4), 54 (5), 52 (26)</td>
<td>176</td>
<td>TaqI</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AATACRATAAAAAACCRRCTAA-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>189</td>
<td>TaqI</td>
</tr>
<tr>
<td>BID-GM1</td>
<td>F: 5'-AAAGGTTGGAAGTTGTTGGTTATG-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>176</td>
<td>TaqI</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TCRACCTACCCTCCTCTGTAT-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>176</td>
<td>TaqI</td>
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<td>BIM-GM1</td>
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<td>TaqI</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAAAAACAAACCTTTACCAAC-3'</td>
<td>58 (3), 56 (4), 54 (5), 52 (26)</td>
<td>185</td>
<td>TaqI</td>
</tr>
<tr>
<td>PUMA-GM1</td>
<td>F: 5'-GGGGGGYGGGGTGTGTGTGTGAT-3'</td>
<td>60 (3), 58 (4), 56 (5), 54 (26)</td>
<td>185</td>
<td>TaqI</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ACRACACCAACRATCCCAACAA-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
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<tr>
<td>PS4-RT</td>
<td>F: 5'-GAAGCCACAGACCCGACACCACT-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGTTTCCAACAACGAGCAG-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>BID-RT</td>
<td>F: 5'-CCTAGAGACATGGAGAAGGAGAAGAC-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGAATAAAGAGCACTGGTTGTAGAT-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>BAX-RT</td>
<td>F: 5'-CTGCCCGCGGGAAGACCTTGAG-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGCAGCTCCCGCCACAAGATG-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>NOXA-RT</td>
<td>F: 5'-CCAGTTGGAGGCTTGAGGTTC-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGTTTCCAAGGCGGCCCCCATG-3'</td>
<td>60 (35)</td>
<td>320</td>
<td></td>
</tr>
</tbody>
</table>

a Temperature in °C (number of cycles).

b F, forward; R, reverse; RT-PCR, reverse-transcription-PCR.
GAPDH as described previously (21). Samples (10 μl) of amplified product were then subjected to 2.5% agarose gel electrophoresis and stained with ethidium bromide.

**Chromatin Immunoprecipitation (ChIP) Analysis.**

ChIP analysis was carried out as described previously (24). Briefly, 1 × 10⁶ cells were incubated with 1.0% formaldehyde for 10 min at 37°C to cross-link the DNA. The cells were then washed with ice-cold PBS containing protease inhibitors and resuspended in lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), protease inhibitor]. Nucleoprotein complexes were sonicated to reduce the size of the DNA fragments to 200-1000 bp, after which immunoprecipitation with antiacetylated histone H3 or antiacetylated histone H4 antibody (Upstate Biotechnologies, Lake Placid, NY) was carried out for 16 h at 4°C with rotation, and the resultant immune complexes were collected on protein A-agarose beads. The DNA was then purified by phenol–chloroform extraction, precipitated with ethanol, and resuspended in water, after which they were subjected to PCR amplification with primers HRK-ChIPF and HRK-ChIPR (Table 1). The amplified products were subjected to agarose gel electrophoresis, and the intensities of resultant bands were determined by a Lane and Spot Analyzer (Atto, Japan).

**Detection of Apoptotic cells by Fluorescence Microscopy.**

Cells (5 × 10⁶) grown in 60-mm plates at 37°C under an atmosphere of 5% CO₂-95% air were treated first for 72 h with 1 μM 5-aza-deoxycytidine (5-aza-dC) and/or a histone deacetylase (HDAC) inhibitor and Adriamycin (Sigma). They were then trypsinized, fixed with methanol, rehydrated with PBS, treated with 2 mg/ml RNase at 37°C for 30 min, and stained with PI (50 μg/ml of solution). Fluorescence-activated cell-sorting analysis was then carried out with a Becton Dickinson FACScan flow cytometer (Branntree, MA).

**RESULTS**

**Identification of HRK as a Target for Aberrant Methylation in Cancer.** We previously used the genome screening technique, methylated CGI amplification, to identify DNA frag-

![Fig. 1 Schematic diagram of the sequence around MINT17. A, genomic structure of HRK: CpG sites are indicated by vertical bars; the transcription start site is indicated by an arrow; and the positions of the PCR products analyzed by bisulfite-PCR are indicated by horizontal bars. B, bisulfite-PCR analysis of the 5’ region of HRK in the indicated (top) cancer cell lines. The primer sets used are shown on the left. M, methylated alleles.](image-url)
ments aberrantly methylated in colorectal cancer (12). One of those fragments, termed MINT17, matched a genomic DNA sequence on chromosome 12 previously identified by the Human Genome Project. Subsequent Blast analysis revealed that MINT17 is situated within the 2-kb CGI in the 5’ region of a proapoptotic gene, HRK (Fig. 1A). By comparing genomic sequences obtained from the Human Genome Project (AC025687) and cDNA sequences obtained with an expressed sequence tag (BE247231) from a 5’-end-enriched cDNA library constructed by the oligo-capping method (25), the HRK transcription start site was determined to be 120 bp upstream of the translation start site (Fig. 1A, arrow). Using the promoter prediction program TESS (BCM launcher), we identified three putative SP1 binding sites, an AP1 binding site, and a transcription factor ID (TFIID) binding site, indicating a role for the CGI in the transcriptional regulation of the gene’s expression.

The HRK CGI is relatively large, and it is not clear whether it is coordinately regulated with respect to protection from methylation. To address that issue, we designed six primer sets that together spanned the entire CGI and assessed the methylation status in a panel of colorectal and gastric cancer cell lines (Fig. 1). Methylation of the 5’ edge of the CGI (Fig. 1, GM7) was detected in all 30 cell lines tested, as was methylation of the 3’ edge (Fig. 1, GM6). By contrast, methylation of the region around transcription start site (Fig. 1, GM1 and GM2) was observed in only 4 of 11 (36%) colorectal cancer cell lines and 6 of 19 (32%) gastric cancer cell lines. Such methylation around the transcription start site was specific to gastrointestinal cancer and was not detected in any of the 7 pancreatic, 14 hematopoietic, or 5 hepatocellular cancer cell lines tested (Table 2; data not shown).

Bisulfite sequencing was then carried out in five cell lines to assess the methylation of HRK in more detail (Fig. 2). HSC44 and KatoIII cells, which bisulfite-PCR showed to be densely methylated, were methylated at almost all CpG sites in the region analyzed. The majority of CpG sites were unmethylated in HCT116 cells, as would be predicted from the bisulfite-PCR. With respect to RKO and HT29 cells, which bisulfite-PCR showed to be partially methylated, the former exhibited heterogeneous methylation, whereas the latter showed methylation in only a limited region.

**Table 2.** Frequency of HRK methylation in various human tumors

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Frequency, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>4/11 (36)</td>
</tr>
<tr>
<td>Gastric</td>
<td>6/19 (32)</td>
</tr>
<tr>
<td>Liver</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>Primary tumors</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>14/58 (24)</td>
</tr>
<tr>
<td>Colorectal adenoma</td>
<td>1/50 (2)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>4/23 (17)</td>
</tr>
<tr>
<td>Hepatocellular cancer</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>Normal tissue</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>0/38 (0)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0/23 (0)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0/6 (0)</td>
</tr>
</tbody>
</table>

**Fig. 2.** Bisulfite sequencing of the 5’ region of HRK. PCR products were cloned into pCR4-TOPO vectors by use of a TOPO DNA cloning kit, and at least seven clones were sequenced. The CpG sites analyzed are indicated by vertical bars and shown at the top. Methylated and unmethylated CpG sites are indi- cated by ● and ○, respectively. The CpG sites examined by bisulfite-PCR are indicated at the top. The positions of TATA box and restriction sites used for bisulfite-PCR are shown at the top. The translation start site was shown by a horizontal arrow at the top.

Absence of Methylation in Other Bcl-2 Family Proapoptotic Genes in Gastric and Colorectal Cancers. We next examined the methylation status of various other proapop- totic bcl-2 family genes to determine the extent to which they are also affected by epigenetic inactivation in colorectal and gastric cancers. Of six BH3-only family genes tested, four (BAD, BID, PUMA, and BIM) contained CpGs in their 5’ regions; BIK and BLK did not have CGIs. BAX, another bcl-2 family proapoptotic gene, was also found to contain a CGI. When bisulfite-PCR was carried out with primers that covered the regions around transcription start sites, no methylation was detected in any of the five CGI-containing genes in 11 colorectal and 19 gastric cancer cell lines (Fig. 3). Apparently, epigenetic inactivation of bcl-2 family genes other than HRK is not common in gastrointestinal cancers.

DNA Methylation and Histone Deacetylation Are Associated with Loss of HRK Expression. Although initial reports suggested that HRK is expressed mainly in hematopoietic cells (16), our RT-PCR analysis revealed that HRK mRNA is expressed in a wide variety of tissues, including stomach and...
intestine (Fig. 4A). To determine whether aberrant methylation of HRK correlates with the silencing of its expression, we carried out RT-PCR with cDNA from 8 colorectal and 14 gastric cancer cell lines (Fig. 4, B and C). We found that expression of HRK was readily detectable in the 6 colorectal and 10 gastric cancer cell lines that did not show methylation of HRK. Expression of HRK was not detected in the two colorectal (SW48 and HT29) and four gastric cancer cell lines (MKN28, KatoIII, HSC44, and HSC45) that showed dense methylation of HRK, however, which indicates that methylation of the 5’/H11032 region of HRK is associated with loss of expression.

The levels of methylation in each region of the CGI and expression of HRK are summarized in Fig. 5. The cell lines tested could generally be divided into two groups based on the methylation densities determined by bisulfite-PCR. Although methylation of the 5’ and 3’ edges of the CGI occurred frequently, it did not affect HRK expression, and all cell lines belonging to group 1 expressed HRK mRNA at readily detectable levels. On the other hand, cell lines belonging to group 2, which were more densely methylated around the transcription start site, did not express the gene, indicating that methylation of that region plays a critical role in silencing HRK expression. Notably, RKO cells did not fall into either group 1 or 2. They were methylated on ~50% of the alleles throughout the entire region analyzed, but nonetheless expressed the gene. This may mean that RKO cells are a heterogeneous population in which some cells are unmethylated or that expression is derived from unmethylated alleles in all cells.

It was recently shown that histone modification is also involved in the gene silencing caused by DNA methylation (26, 27). We therefore treated various cell lines with the HDAC inhibitor TSA and found that, by itself, TSA did not restore HRK expression (Fig. 6A). It did, however, synergistically enhance the response to 5-aza-dC, indicating a role for histone deacetylation in gene silencing. Furthermore, ChIP analysis using antiacetylated histone H3 and H4 antibodies showed that acetylation of histones H3 and H4 correlated directly with gene expression and inversely with DNA methylation (Fig. 6B).

Re-expression of HRK Is Correlated with Induction of Apoptosis. To determine whether the silencing of proapoptotic genes correlated with the escape of cancer cells from apoptosis, we treated three gastric cancer cell lines in which HRK expression was silent because of methylation (HSC44, HSC45, and KatoIII) with 5-aza-dC and then examined expression of HRK, BAX, BAD, BID, and NOXA. We found that 5-aza-dC induced expression of HRK in all three cell lines (Fig. 7). Expression of the other genes was unaffected by 5-aza-dC, which is consistent with the finding that DNA methylation in the 5’ region of these genes was absent in cancers (Fig. 3).
incidence of apoptosis roughly paralleled the expression of HRK, reaching a peak after 72 h of treatment with 5-aza-dC (Fig. 8). Moreover, the effect was synergistically enhanced by addition of TSA or depsipeptide, another HDAC. By contrast, cell lines in which HRK was unmethylated were significantly less susceptible to 5-aza-dC-induced cell death (Fig. 8B; Fig. 9A).

We next examined the extent to which pretreating cells with 5-aza-dC increases the susceptibility of cancer cells to apoptosis stimulated by a chemotherapeutic drug (Fig. 9B). Cell lines were first treated with 1 μM 5-aza-dC or mock-treated for 72 h and then with Adriamycin for 24 h, after which the percentage of apoptotic cells was determined by flow cytometry. In the cell lines showing HRK methylation, the numbers of apoptotic cells were significantly higher in cells pretreated by 5-aza-dC than in mock-treated cells. On the other hand, no significant effect of 5-aza-dC was seen in the cell lines in which HRK was unmethylated.

**Aberrant Methylation of HRK in Primary Tumors.**

To examine the methylation status of HRK in primary tumors, we performed bisulfite-PCR using samples of DNA from 58 colorectal and 23 gastric cancer cases and corresponding samples of normal gastric mucosa (Fig. 10). Methylation of the HRK CGI was detected in 14 (24%) colorectal and 4 (17%) gastric cancer cases but in only 2% (1 of 50) of colorectal adenomas and in none of the 38 samples of colorectal mucosa and 23 samples of gastric mucosa from areas adjacent to the tumors.

Fig. 5 Correlation between methylation of the HRK CGIs and gene expression. The percentages of methylated alleles were determined by bisulfite-PCR as shown in Fig. 1; their averages are indicated by the columns. All cell lines belonging to group 1 (blue columns; Caco2, HCT116, DLD-1, LoVo, SW480, MKN7, MKN45, MKN74, JRST, AZ521, NUGC3, SNU1, HSC42, and HSC43) expressed the gene. Cell lines belonging to group 2 (red columns; SW48, HT29, MKN28, KatoIII, SNU638, HSC44, and HSC45) generally did not express the gene, although a small amount of the mRNA was detected in SNU638 cells.

Fig. 4 Expression of HRK in normal tissues (A) and in colorectal (B) and gastric (C) cancer cell lines. Expression of HRK by the indicated (top) cell lines was examined by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also amplified to confirm the integrity of the cDNA. Corresponding negative controls (amplification without reverse transcription) are shown as RT-negative (−).

Fig. 6 Involvement of histone deacetylation in DNA methylation-induced silencing of HRK. A, representative RT-PCR analysis of the HSC45 gastric cancer cell line treated with 0.2 μM 5-aza-dC, 300 nM TSA, or both. B, acetylation status of the histone around the HRK CGI. Shown are the results of a representative ChIP assay carried out with antiacetylated histone H3 antibody followed by PCR to amplify the 5' region of HRK. The amount of DNA used was monitored by a PCR that amplified DNA prepared from the input solution.
Thus, aberrant methylation of HRK appears to be a cancer-specific phenomenon.

Finally, we evaluated the correlation between HRK methylation and the clinicopathological features of colorectal cancers. There was no correlation between HRK methylation and age (69.4 years versus 64.8 years; \( P = 0.175 \)), gender, or location of the tumor. On the other hand, HRK methylation was detected exclusively in tumors that showed simultaneous methylation of multiple CGIs, i.e., tumors that exhibited the CGI methylator phenotype (22). Among the cases studied, 14 of 34 (41%) CGI methylator phenotype-positive cases showed methylation of HRK, whereas none of the 24 CGI methylator phenotype-negative cases did so (\( P < 0.001 \)). In addition, there was a significant correlation between methylation of HRK and the absence of p53 mutations in colorectal cancers [1 of 21 (5%) in cases with mutant p53 versus 13 of 37 (35%) in cases with wild-type p53; \( P < 0.05 \)].

**DISCUSSION**

HRK, which was originally identified as a proapoptotic gene mediating apoptosis induced by diminished levels of cytokine in hematopoietic cells (16), belongs to the BH3-only subfamily of Bcl-2 genes, whose products act at points upstream in the apoptotic signal cascade (28). Alteration of these proteins may be a critical feature of cancer cells, enabling them to escape apoptosis resulting, for example, from the DNA damage caused by chemotherapeutic drugs (29, 30). In this report we have shown that expression of a proapoptotic gene, HRK, is silenced in colorectal and gastric cancer by DNA methylation and histone deacetylation. Interestingly, HRK is located in chromosome 12q13, where loss of heterozygosity has frequently been detected in human tumors (31–33), which means that HRK may be inactivated not only by DNA methylation but also by gene deletion. However, it was aberrant methylation that we found to be a specific feature of HRK in the various tumor cells studied and that was not seen in any of the other BH3-only family genes studied (BAD, BID, and PUMA). The reason for this specificity remains unclear but may be related to the fact that expression of HRK, like that of NOXA, is regulated at the transcriptional level, whereas the others are regulated by post-transcriptional mechanisms. For example, whereas NOXA is induced by p53 and HRK by cellular stress caused by absence of growth factors (18,
34), the proapoptotic activity of BAD is controlled by the level of its phosphorylation (35). Thus, inactivation of BAD in cancer, if it occurs, is likely caused by altered protein modification (e.g., phosphorylation) rather than by transcriptional silencing.

The role of HRK in apoptosis has mainly been described in hematopoietic and neuronal cells: loss of the growth signal in these cells induces transcription of HRK, leading to cell death (18, 36). Our findings, however, suggest that HRK is much more widely expressed, and significant increases in the numbers of apoptotic cells were observed when HSC44 and HSC45 gastric cancer cells were treated with 5-aza-dC plus TSA or depsipeptide. Indeed, the fact that significant induction of apoptosis was observed when cancer cells re-expressing HRK were treated with 5-aza-dC suggests that the gene may be a good molecular target for chemotherapy. In that regard, recent studies have shown that the combined use of inhibitors of DNA methyltransferase and HDAC synergistically enhances expression of otherwise methylated genes (27, 37). Notably, cell lines in which HRK was unmethylated tended to be less sensitive to 5-aza-dC (Fig. 8B; Fig. 9). Further study will be necessary to determine whether cell lines that express HRK have defects in chemotherapeutic drug-activated pathways leading to apoptosis.

Ablation of HRK was frequently detected in colorectal cancers that showed methylation of multiple CGIs, as well as in colorectal cancers that did not have p53 mutations. Epigenetic inactivation of HRK may thus lead to loss of apoptosis, even in cancer cells expressing wild-type p53. It was previously demonstrated that cancers expressing wild-type p53 tend to be resistant to gene therapy using Ad-p53 (38). Epigenetic therapy using 5-aza-dC may thus represent a useful alternative approach for treating such cases. It is also noteworthy that a majority of genes methylated in tumors tend to also be methylated in premalignant regions, such as colorectal adenomas (13, 22, 39). This was not the case with HRK, however, suggesting that inactivation of HRK may involve a critical step in the progression from adenomas to cancers.

In summary, we have shown that a proapoptotic gene, HRK, is inactivated by aberrant methylation of its CGI, which sheds new light on the mechanism by which cancers expressing wild-type p53 escape apoptosis. Furthermore, the fact that proapoptotic genes other than HRK appear to be unaffected by 5-aza-dC suggests that HRK might be selectively targetable for antimethylation and histone deacetylation therapy aimed at inducing apoptosis in cancer cells.

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