Aberrant Methylation and Silencing of the BNIP3 Gene in Colorectal and Gastric Cancer

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ABSTRACT

BNIP3 protein is a proapoptotic member of the Bel-2 family that is expressed in hypoxic regions of tumors. To examine its role in the progression of gastrointestinal cancer, we examined the expression and DNA methylation status of BNIP3 gene in a panel of colorectal and gastric cancer cell lines. BNIP3 was not expressed in 14 of the 24 cell lines tested, and its absence was not caused by gene mutation or by altered expression of hypoxia inducible factor-1, a key transcription factor that regulates BNIP3 expression. On the other hand, methylation of the 5’ CpG island of BNIP3 was closely correlated with silencing the gene. Moreover, treating methylated cells with the methyltransferase inhibitor 5-aza-deoxycytidine restored hypoxia-induced expression of BNIP3 mRNA and protein, which in turn led to cell death. Aberrant methylation of BNIP3 was also detected in 66% of primary colorectal and 49% of primary gastric cancers, but progression of some gastrointestinal cancers and that it inactivation of BNIP3 may play a key role in the progression of some gastrointestinal cancers and that it may be a useful molecular target for therapy.

INTRODUCTION

Hypoxia is an important cellular stress involved in a variety of human diseases, including cancer (1, 2). Solid tumors often contain hypoxic regions, which are associated with a poor prognosis because hypoxic tumor cells often develop resistance to chemotherapy and radiation (1). This is in part because within hypoxic regions hypoxia inducible factor-1 (HIF-1), a transcription factor complex composed of HIF-1α and HIF-1β/ARNT, up-regulates various genes involved in glycolysis, angiogenesis, and cell survival (3, 4). Notably, however, HIF-1 also induces expression of a number of proapoptotic genes (5), and it remains unclear how cancer cells adapt to hypoxia despite such apoptotic signaling.

The hypoxia-inducible proapoptotic gene BNIP3 was originally identified as a protein that interacts with adenovirus E1B 19-kDa protein (6). It is capable of homodimerization as well as heterodimerization with the antiapoptotic protein bcl-2 and contains a COOH-terminal transmembrane domain that is required for mitochondrial localization and proapoptotic activity (7–9). Expression of BNIP3 is induced by hypoxia, such as that which occurs during cardiac ischemia and in the hypoxic regions of tumors (10–13). Although it was recently shown that BNIP3 expression is controlled by both positive and negative regulators, including HIF-1α (5), epidermal growth factor (14), and PLAGL2 (15), the transcriptional regulation of BNIP3 in tumor cells is still not fully understood.

Many tumor suppressor genes are known to be inactivated by epigenetic alterations that include DNA methylation of their 5’ regions and histone deacetylation (16, 17). To date, moreover, epigenetic alteration of several proapoptotic genes, including DAP-kinase, TMS1/ASC, and HRK, has been shown (18–20), although little is known about the epigenetic alteration of the genes involved in hypoxia-induced apoptosis in gastrointestinal cancers. In the present study, therefore, we examined the relationship between the methylation of BNIP3 and its expression in a panel of colorectal and gastric cancer cells.

MATERIALS AND METHODS

Cell Lines and Specimens. Eight colorectal and 10 gastric cancer cell lines were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) or the American Type Culture Collection (Manassas, VA). Eight of the gastric cancer cell lines (HSC39, HSC40, HSC41, HSC42, HSC43, HSC44, HSC45 and SH-101; refs. 21, 22) as well as the 61 primary colorectal cancers and 73 primary gastric cancers were described previously (20, 23–25). In addition, 12 gastric cancer xenografts established in our laboratory were used to examine BNIP3 expression. Briefly, gastric cancer specimens were resected and transplanted into the dorsum of severe combined immunodeficient mice without in vitro culture. When the xenografts reached about 1 cm in diameter, they were harvested and stored at −80°C until use. DNA was extracted from both
cultured and primary cells using the phenol-chloroform method. In some cases, cell lines were incubated for 96 hours with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC, Sigma, St. Louis, MO) before their harvest for further analysis. When subjected to hypoxia, cells were placed in a chamber (Wakemakaku, Tokyo, Japan) containing an atmosphere of 1% O2, 5% CO2, and 94% N2.

**Reverse Transcription–PCR.** Total RNA was extracted using Isogen (NIPPON GENE, Tokyo, Japan) after which cDNA was prepared using SuperScript III (Invitrogen, San Diego, CA). After PCR, the amplified products were electrophoresed in 2.5% Nuseive gels. Real-time PCR was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems), Foster City, CA) in a solution containing 1 × SYBR PCR Mater Mix (Applied Biosystems), 1 μL of cDNA, and 0.5 μmol/L each primer. Levels of BNIP3 expression were normalized to the signal from glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR parameters and primer sequences are shown in Table 1.

**Western Blot Analysis.** Samples (20 μg) of the cell lysate were subjected to 10% SDS-PAGE, after which the resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were then blocked with 5% nonfat milk and 0.1% Tween 20 in TBS and probed with mouse monoclonal antibodies (Abcam, Cambridge, United Kingdom), after which the blots were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**Bisulfite-PCR and Sequencing.** Bisulfite treatment and combined bisulfite restriction analysis (COBRA), a semiquantitative methylation analysis, was carried out as described previously (26, 27). PCR was carried out using primers designed from the nucleotide sequences obtained from Genbank (AL162274) to amplify both methylated and unmethylated alleles. Samples (20 μL) of the amplified product were then digested with restriction enzymes that cleave CpG sites retained because of methylation. After ethanol precipitation, the DNA was subjected to 3% agarose gel electrophoresis and stained with ethidium bromide. The PCR conditions, primer sequences, and restriction enzymes used are listed in Table 1. To sequence the bisulfite-PCR products obtained from cell lines, fragments of amplified product were cloned into pCR4-TOPO vector using a TOPO-TA cloning kit (Invitrogen), after which 11 clones from each cell line were sequenced. In addition, bisulfite sequencing of primary tumors was carried out by direct sequencing using bisulfite-PCR products as templates. Briefly, 10 μL of bisulfite-PCR product were electrophoresed in 1% SeaPlaque agarose gel, excised, purified using a PCR purification system (Promega, Madison, WI) and eluted in 50 μL of water. Samples (1 μL) were then used as templates for the sequencing reaction carried out as described above.

**Confocal Immunofluorescence Microscopy.** A total of $2 \times 10^4$ cells on slides were fixed for 5 minutes in 4% paraformaldehyde solution. After blocking with 3% bovine serum albumin in PBS, the cells were incubated for 16 hours with anti-BNIP3 antibody (Abcam), washed with PBS, and stained with FITC-conjugated secondary antibody (Molecular Probes, Eugene, OR). In addition, the nucleus was stained with Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc, Burlingame, CA), and the mitochondria were stained with Mitotracker (Molecular Probes). The stained cells were examined using an Olympus IX70 inverted confocal laser scanning microscope driven with Fluview 2.0 software.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation assays were carried out as described previously (28).

### Table 1 Primer sequences for BNIP3 analysis

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Annealing, °C (cycles)</th>
<th>Size (bp), enzymes</th>
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<td><strong>Bisulfite-PCR</strong></td>
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| BNIP3 region 1                    | F: 5′-TGYGGYGGAGGATTTATAGGGTAG-3′<sup>Y</sup>  
R: 5′-CCCTCRCCACCRCAAAAC-3′        | 58(3), 56(4), 54(5), 52(26)  
Dde1                                  | 156                   |
| BNIP3 region 2                    | F: 5′-GATAGGCTTGAAGGTGTAATG-3′<sup>R</sup>  
R: 5′-CCCRCCTTACCTATAAATTC-3′        | 58(3), 56(4), 54(5), 52(26)  
Mbo1                                  | 170                   |
| NIX                               | F: 5′-TGGTAGYGGTAGGTAGG-3′<sup>C</sup>  
R: 5′-ACTRACAAATATAAAAACRACATTATC-3′<sup>R</sup> | 58(3), 56(4), 54(5), 52(26)  
Taq1                                  | 152                   |
| Caspase 3                         | F: 5′-TGGATGGYGGTGTATG-3′<sup>Mbo</sup>  
R: 5′-CTAAACCCCAATCTAAACTAATTAC-3′<sup>F</sup> | 58(3), 56(4), 54(5), 52(26)  
BstUI                                 | 187                   |
| Caspase 9                         | F: 5′-TGTTTTAGYGGAGTTAAAGA-3′<sup>Taq</sup>  
R: 5′-ACTCTCAAAACRAAAACRACCT-3′<sup>Taq</sup> | 58(3), 56(4), 54(5), 52(26)  
HinF1                                 | 164                   |
| APAF-1                            | F: 5′-TTATAGGTTTTTATTGGYGTAGTGT-3′<sup>Mbo</sup>  
R: 5′-CCRCRTTCCTCACCAATACC-3′<sup>Mbo</sup> | 58(3), 56(4), 54(5), 52(26)  
HinF1                                 | 164                   |
| **Reverse transcription–PCR**     |                        |                    |
| BNIP3                             | F: 5′-CCACCTCGCTCGAGACACCAAC-3′<sup>R</sup>  
R: 5′-GAGACGAGACGAGATGGAAGGAAAC-3′<sup>Taq</sup> | 66(3), 64(4), 62(5), 60(23)  
BstUI                                 | 317                   |
| **Real-time PCR**                 |                        |                    |
| BNIP3                             | F: 5′-CCGGGTATGAGGAGGAGGAG-3′<sup>R</sup>  
R: 5′-TTATAATAGAAACCGAGGTGAAC-3′<sup>Taq</sup> | 60(40)                 |
| NIX                               | F: 5′-GGACAGAGATGTCTCCAGGGACTTC-3′<sup>Taq</sup> | 60(40)                 |
| **Chromatin immunoprecipitation** |                        |                    |
| BNIP3                             | F: 5′-CCGGCCGGCGCTTCCGGCTCAC-3′<sup>R</sup>  
R: 5′-GCTCCGGACCTCCGTTTCCCCACCGCC-3′<sup>R</sup> | 70(35)                 |

Note. Y, C or T; R, A or G.
using a chromatin immunoprecipitation assay kit (Upstate Biotechnologies, Lake Placid, NY). PCR was carried out in a solution containing 1× PCR buffer (TaKaRa, Tokyo, Japan), 1 μmol/L primers, 0.25 mmol/L deoxynucleotide triphosphate mixture, and 1.0 unit of Hot Start Taq polymerase (TaKaRa). The primer sequences for the PCR reaction are shown in Table 1. The amplified products were subjected to agarose gel electrophoresis.

**Flow Cytometry Analysis.** A total of 2 × 10⁵ cells were plated and incubated for 96 hours with either mock or 2.0 μmol/L 5-aza-dCyd, after which they were exposed to either hypoxia (1% O₂, 5% CO₂, 94% N₂) or normoxia (20% O₂, 5% CO₂, 75% N₂) for 48 or 96 hours, harvested, fixed in 90% ethanol, incubated for 30 minutes at 37°C with 2 mg/mL of RNase (Sigma), and stained with 50 μg/mL propidium iodide. About 5 × 10⁴ cells were then analyzed using a Becton Dickinson FACScalibur (BD Biosciences, Bedford, MA).

**RESULTS**

**Absence of BNIP3 Expression in Colorectal and Gastric Cancer Cell Lines.** We initially used reverse transcription–PCR to examine expression of BNIP3 in a panel of colorectal and gastric cancer cell lines (Fig. 1A). Although we detected expression of BNIP3 in 10 cell lines, the gene was apparently not expressed in 14 lines. Mutational analysis carried out by direct sequencing using six primer sets that amplify the 6 exons covering the entire coding region of BNIP3 failed to identify any mutations, except one polymorphism that does not affect amino acid sequence (data not shown). Thus, the absence of BNIP3 expression seems to be caused by mechanisms other than genetic alteration. In addition, by carrying out real-time PCR using cDNA prepared from cells incubated under hypoxic conditions, we observed that hypoxia enhanced BNIP3 expression in cells previously shown to express BNIP3, but had no effect on cells previously shown not to express BNIP3 (Fig. 1B). As a control, we also evaluated the expression of the hypoxia-inducible gene NIX and found it to be expressed in all of the cell lines tested (Fig. 1B).

To better understand the molecular mechanism underlying the absence of BNIP3 expression, we first used Western blot analysis to examine expression of HIF-1α, a major component of the hypoxia-induced transcription factor HIF-1 (Fig. 1C). After subjecting the cells hypoxia, expression of HIF-1α was readily detectable, regardless of whether or not BNIP3 was also expressed. Thus, the absence of BNIP3 expression seems to be caused by mechanisms other than impaired HIF-1 expression.

**Aberrant Methylation of BNIP3 Is Associated with Its Silencing.** The 5' region of BNIP3 fulfills the criteria for a CpG island (CpG:GpC = 0.65; GC% = 55%; Fig. 2A; ref. 29). We therefore used COBRA to determine whether DNA methylation plays a role in silencing BNIP3 expression in a panel of colorectal and gastric cancer cell lines (Fig. 2B). We detected significant methylation (>80%) of the BNIP3 5' CpG island in 5 (63%) of 8 colorectal and 9 (50%) of 18 gastric cancer cell lines, and methylation was closely associated with silencing the gene. Moreover, LoVo and NUGC2 cells, which showed partial methylation (LoVo, 21% in region 1 and 37% in region 2; NUGC2, 0% in region 1 and 11% in region 2), expressed correspondingly lower levels of BNIP3.
We then used bisulfite sequencing to examine the methylation status of BNIP3 in more detail (Fig. 2C). In KatoIII and AZ-521 cells, which COBRA showed to be methylated (KatoIII, 91% in region 1 and 100% in region 2; AZ-521, 65% in region 1 and 100% in region 2, respectively), BNIP3 was found to be methylated on almost all of the CpG sites examined, which confirms that detection of methylation by COBRA is a reliable means of evaluating regional DNA methylation. In addition, when we determined the methylation status of four other genes known to mediate hypoxia-induced apoptosis (NIX, Caspase-3, Caspase-9, and APAF-1) using bisulfite-PCR with primers that covered the region around the respective transcription start sites, we detected no methylation in any of these genes (Fig. 2D). Apparently, the observed methylation is specific for BNIP3.

**Treatment with 5-Aza-dCyd Restores Expression of BNIP3 in Colorectal and Gastric Cancer Cells.** Further confirming the role of DNA methylation in gene silencing was our finding that treating cell lines not expressing BNIP3 with the DNA methyltransferase inhibitor 5-aza-dC induced BNIP3 transcription (Fig. 3A). Moreover, 5-aza-dC also restored capacity of hypoxia to enhance expression of BNIP3 mRNA (Fig. 3B). Likewise, Western blot analysis showed that 5-aza-dC restored expression of BNIP3 protein and that the effect was enhanced in cells subjected to hypoxia (Fig. 3C).

BNIP3 is reportedly localized in the mitochondria (30) of hypoxic cells. When we examined the expression and cellular localization of BNIP3 in methylated and unmethylated cell lines, we found that only low levels of BNIP3 are expressed under normoxic conditions, even in unmethylated cell lines. Under hypoxic conditions, however, BNIP3 was detected in both the cytoplasm and mitochondria of unmethylated cells (Fig. 3D). No BNIP3 expression was detected in the methylated RKO cell line, but expression was restored by treating the cells with 5-aza-dC before subjecting them to hypoxia, and the protein was localized in the mitochondria.

We then determined the extent to which restoration of BNIP3 expression would restore hypoxia-induced apoptosis. We found that although untreated DLD-1 cells were resistant to hypoxia-induced apoptosis, significant numbers of apoptotic cells were detected when the cells were treated with 5-aza-dC before subjecting them to hypoxia. Thus, restoration of BNIP3 seems to make DLD-1 cells susceptible to hypoxia-induced cell death (Fig. 3E).

**Role of Histone Deacetylation in Silencing BNIP3.** It was recently shown that DNA methylation affects gene expression by modifying the structure of the chromatin (16, 17). We therefore examined expression of BNIP3 after treating cells with a low dose of 5-aza-dC (0.2 μmol/L) and/or trichostatin A (300 nmol/L), a histone deacetylase inhibitor (Fig. 4A). Expression of BNIP3 was not restored when cells were treated with trichostatin A alone, and only relatively low levels of expression were observed when cells were treated with the 5-aza-dC alone. By contrast, higher levels of BNIP3 expression were detected when the cells were treated with both 5-aza-dCyd and trichostatin A, suggesting a role for histone deacetylation in DNA methylation–mediated silencing of BNIP3 expression. This idea was substantiated by...
chromatin immunoprecipitation assays in which antiacetylated histone H3 and H4 antibodies were used to examine the acetylation status of histones H3 and H4 in the 5' region of BNIP3 (Fig. 4B). Whereas Colo320 and MKN45 cells, which express BNIP3, showed high levels of histone acetylation, that acetylation was absent or negligible in methylated cell lines not expressing BNIP3 (RKO, DLD-1, HT29, MKN28, and MKN74 cells).

**Aberrant Methylation of BNIP3 in Primary Colorectal and Gastric Cancers.** Finally, to verify that BNIP3 methylation also occurs in primary tumors and is not a phenomenon limited to cultured cells, we evaluated a panel of primary colorectal and gastric cancers (Fig. 5A). We found that BNIP3 was methylated in 40 (66%) of 61 primary colorectal and 36 (49%) of 73 primary gastric cancers examined. No methylation was detected in samples of normal colorectal and gastric mucosal tissue collected from areas adjacent to the tumors, indicating that BNIP3 methylation is cancer specific.

To determine the extent of BNIP3 methylation in more detail, the bisulfite-PCR products from four primary colorectal cancers, four primary gastric cancers, and eight normal tissue samples were directly sequenced. The representative results in Fig. 5B show that samples determined by COBRA to be methylated showed dense methylation of all CpG sites in a region that includes the hypoxia-responsive element, the HIF-1 binding site. By contrast, methylation was barely detectable in two unmethylated tumors and in the eight normal tissue samples collected from regions adjacent to the tumors.

We then confirmed that BNIP3 methylation correlates with down-regulation of its expression in primary tumors by carrying out real-time PCR using cDNA prepared from xenografts established from primary gastric cancers (Fig. 5C). Note that levels of BNIP3 expression were significantly lower in tumors with BNIP3 methylation than in those without such methylation.

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**Fig. 3** Restoration of BNIP3 expression in colorectal and gastric cancer cell lines. **A**, expression of BNIP3 following treatment with 5-aza-dC; GAPDH was amplified to confirm the integrity of cDNA. **B**, induction of BNIP3. Real-time PCR was carried out using cDNA prepared from the indicated (top) cell lines treated with mock or 5-aza-dC and then incubated for 24 hours under normoxic or hypoxic conditions. BNIP3 signals were normalized to that of GAPDH. **C**, Western blot analysis of BNIP3 carried out after treating the indicated (top) cell lines for 96 hours with 5-aza-dC and then subjecting them to normoxic (Hypoxia−) or hypoxic (Hypoxia+) conditions for 24 hours. DAPI, immunohistochromosomal analysis of the intracellular distribution of BNIP3. HSC45 and RKO cells were plated on coverslip glass, incubated for 24 hours under either normoxic or hypoxic conditions, and stained with anti-BNIP3 antibody (green). Mitochondria were visualized using MitoTracker (red) and nuclei using 4',6-diamidino-2-phenylindole (blue). **E**, restoration of hypoxia-induced cell death mediated by BNIP3. RKO and DLD-1 cells (2 × 10^5 cells) were treated for 96 hours with mock or 2 μmol/L 5-aza-dC and then incubated under normoxic (top) or hypoxic (bottom) conditions for 48 or 96 hours. The cells were then harvested, fixed, and stained with propidium iodide. Apoptotic cells were identified using a flow cytometer.
DISCUSSION

Although solid tumors often contain hypoxic regions, the cells in those regions survive and continue to grow. One way in which this is accomplished is through induction of angiogenesis mediated in part by HIF-1 (3). Because HIF-1 activates prosurvival as well as proapoptosis signaling, it has been hypothesized that during progression, cancer cells acquire the ability to escape apoptosis (5, 11, 12, 14). In that regard, BNIP3 expression is regulated largely by HIF-1 and is thus highly responsive hypoxia (5, 10). Furthermore, our present findings show that BNIP3 is silenced by methylation of its 5' CpG island and deacetylation of histone in that region. That BNIP3 methylation occurred frequently among both gastric and colorectal tumors, and was cancer specific, suggests it likely contributes to tumorigenesis in gastrointestinal malignancies. In addition, epigenetic alteration of other proapoptotic genes involved in hypoxia-induced cell death was not observed, and induction of BNIP3 by 5-aza-dC caused cell death. Additional studies should enable determination of whether there is a correlation between BNIP3 methylation and such clinicopathologic parameters as prognosis and resistance to chemotherapy or radiation.

Hypoxia activates apoptotic signaling pathways involving the activation of p53 target genes and APAF-1/caspase, as well as HIF-1-mediated expression of BNIP3 (31). Increased BNIP3 expression induces cell death through mitochondrial dysfunction, but is independent of APAF-1/caspase activation and cytochrome c release (30). To date, studies evaluating cancer therapies making use of hypoxia-induced cell death have focused largely on inhibiting HIF-1 and angiogenesis (32, 33). In the
present study, we show that induction of BNIP3 using a methyltransferase inhibitor does indeed sensitize cancer cells to hypoxia. Thus, activation of hypoxia-induced apoptotic signaling may be a useful approach to treating some cancers. What’s more, Kothari et al. recently reported that growth factors such as epidermal growth factor protect cancer cells from BNIP3-mediated apoptosis and that inhibiting epidermal growth factor signaling sensitized cancer cells to apoptosis (14). This suggests that a combination therapy making use of anti-epidermal growth factor molecules and a methyltransferase inhibitor may be more efficacious than either alone.

The molecular mechanism by which DNA methylation silences gene expression is not fully understood. It may be that methylation directly interferes with the binding of transcription factors, or it may act indirectly via the action of methylated DNA binding proteins or chromatin modification (16, 17). Consistent with the last, we found that histone deacetylation does play a role in the silencing of BNIP3. In cell lines lacking BNIP3 expression, acetylation levels of histones H3 and H4 were very low, which may alter the interaction of HIF-1α with the hypoxia-responsive element of BNIP3, but this remains to be determined.

In summary, we have shown that BNIP3 is silenced by DNA methylation and histone deacetylation. Restoration of BNIP3 expression using a methyltransferase inhibitor sensitized cancer cells to hypoxia, suggesting that BNIP3 may be a good molecular target for a cancer therapy aimed at increasing the susceptibility of cancer cells to hypoxia-induced apoptosis.

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REFERENCES

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