Methylated *BNIP3* gene in colorectal cancer prognosis

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Abstract. The DNA methylation of apoptosis-related genes in various cancers contributes to the disruption of the apoptotic pathway and results in resistance to chemotherapeutic agents. Irinotecan (CPT-11) is one of the key chemotherapy drugs used to treat metastatic colorectal cancer (CRC). However, a number of metastatic CRC patients do not benefit from this drug. Thus, the identification of molecular genetic parameters associated with the response to CPT-11 is of interest. To identify apoptosis-related genes that may contribute to CPT-11 resistance, microarray analysis was conducted using colon cancer cells in which 5-aza-2'deoxycytidine (DAC) enhanced sensitivity to CPT-11. Microarray analysis identified 10 apoptosis-related genes that were up-regulated following treatment with DAC. Among the genes, Bcl-2/adenovirus E1B 19 kDa protein interacting protein 3 (*BNIP3*), a Bcl-2 family pro-apoptotic protein, was identified as being involved in CPT-11 resistance following methylation of its promoter. An analysis of 112 primary CRC cases revealed that approximately 58% of cases showed *BNIP3* methylation, and that patients with methylation exhibited a poorer outcome compared to those without methylation. In addition, in 30 patients who received first-line CPT-11 chemotherapy, patients with methylation exhibited resistance to chemotherapy compared to patients with no methylation. The results suggest that methylation of *BNIP3* is a predictive factor in the prognosis and response to CPT-11 treatment in CRC patients.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, and the second most common cause of cancer-related death (1). Approximately 20-40% of CRC patients that undergo curative surgery develop local recurrence or distant metastasis, and exhibit a poor outcome that is generally less than 2 years (2). Chemotherapy is an important strategy in the treatment of metastatic CRC, and irinotecan (CPT-11) is one of the major chemotherapy drugs used in metastatic CRC treatment. Treatment with a combination of CPT-11, 5-fluorouracil (5-FU) and leucovorin is generally approved as the standard chemotherapy for metastatic disease and somewhat increases survival (3). However, the majority of patients eventually succumb to the disease. Various predictive factors of chemosensitivity were previously investigated (4). Regarding 5-FU chemotherapy, the target enzyme thymidylate synthase and the metabolic enzyme dihydropyrimidine dehydrogenase were suggested as predictive factors (4). The predictive factors underlying CPT-11 chemosensitivity have yet to be sufficiently investigated. Thus, identification of the molecular genetic parameters associated with response to CPT-11 is of clinical interest.

Apoptosis or programmed cell death occurs in various physiological and pathological situations (5). It occurs not only in normal tissues but also in malignant tumors, and plays an important role in both the pathogenesis of tumors and their biological behavior (6). Defects in the apoptotic pathway contribute to cell accumulation in the colon, promoting malignancy and subsequent metastasis, and allow tumor cells to survive in a suspended state, thereby permitting their hematogenous or lymphatic dissemination (7). The apoptotic pathway may also be a final common step in the cytotoxicity exerted by a number of anticancer drugs with various underlying mechanisms of action (8,9). The altered expression of genes that encode apoptotic proteins provide cells with inherent resistance to anticancer drugs. Thus, the expression levels of apoptotic proteins serves as a predictor of chemotherapeutic agent response.

In addition to genetic aberrations such as mutation and deletion, increasing evidence suggests that epigenetic changes play an important role in CRC pathogenesis (10,11). The aberrant methylation of gene promoter regions leading to gene silencing is currently the most widely studied epigenetic abnormality in human malignancies, affecting multiple cellular functions including cell growth and differentiation, cell cycle control and DNA repair (11,12). Given that DNA methylation affects the transcription of crucial genes involved in the regulation of apoptosis (13,14), it may also contribute to the evolution of resistance to chemotherapeutic agents.

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In a previous study, it was shown that the inactivation of apoptosis-related genes due to DNA hypermethylation may predict the response to the 5-FU-based chemotherapy treatment of gastric cancer (15). To identify the apoptosis-related genes involved in reduced sensitivity to CPT-11, the genome was screened for genes responding to the demethylating agent 5-aza-2'-deoxycytidine (DAC) using microarray analysis. Colon cancer cells were used in the event that DAC was able to enhance sensitivity to CPT-11. Based on our microarray results, an apoptosis-related gene, Bcl-2/adenovirus E1B 19 kDa protein interacting protein 3 (*BNIP3*)was selected. The methylation status of the gene was analyzed in primary CRCs. Moreover, the methylation status was compared with various clinicopathological variables and *BNIP3* methylation was investigated as a predictor of prognosis and response to CPT-11 treatment in CRC patients.

Materials and methods

Reagents. SN-38, an active metabolite of CPT-11, was provided by Yakult (Tokyo, Japan). SN-38 was dissolved in dimethyl sulfoxide at a concentration of 1 μM and stored at -20˚C, before further dilution in phosphate-buffered saline (PBS) and filter sterilization immediately prior to use. DAC was obtained from Sigma (St. Louis, MO, USA), dissolved in PBS and filter-sterilized.

Cell lines. The human colon adenocarcinoma cell lines HCT-15 and HT29 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan) and the American Type Culture Collection (Manassas, VA, USA). HCT-15 cells were maintained in RPMI-1640 (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (Gibco-BRL, Gaithersburg, MD, USA) and 1.0 mM sodium pyruvate (Gibco). HT29 cells were maintained in Mac Coy's medium containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cell lines were cultured at 37˚C in 5% CO_2. Cells ($5x10^3$ per well) were plated in 24-well plates on day 1 of the culture. On day 3, the medium was removed and new medium containing DAC was added. The cells were treated with 0.5 μM DAC (or PBS as a control) for 72 h. On day 6, the cells were rinsed twice with FBS-free medium, and fresh medium containing 0.0015 μM SN-38 (or PBS) was added. The dose of each drug was administered on the basis of its pharmacological dose as previously reported (14), and our preliminary experiments (data not shown). The cells were harvested following incubation with trypsin-EDTA on day 9, stained with trypan blue and counted. Total mRNA or genomic DNA was extracted on day 6 and utilized in the analysis of mRNA expression or methylation status following DAC treatment.

Microarray analysis. Total RNA was extracted from cultured cells using the RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA was assessed using the Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA), and the samples were confirmed to have an RNA Integrity Number >5.0 prior to gene expression analysis. Contaminant DNA was removed via digestion with RNase-free DNase (Qiagen). Using 2 μg total RNA, cRNA was prepared using the one-cycle target labeling and control reagents kit (Affymetrix, Santa Clara, CA, USA). Hybridization and signal detection of HG-U133 plus 2.0 arrays (Affymetrix) were performed following the manufacturer's instructions. The 4 microarray datasets obtained were normalized using the robust multi-array average (RMA) method (16) and R 2.4.1 statistical software (17) together with a BioConductor package. Normalized gene expression levels were log2-transformed, and 62 control probe sets were removed for further analysis. For each of the 54,613 probe sets, a fold change analysis was performed using each pair of treated and untreated HCT15 and HT29 cells. Probe sets that showed >1.5-fold increase or decrease were identified as differentially expressed genes.

Real-time polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from the treated cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and incubated with DNase as previously reported (14). cDNA was then synthesized from 10 ng extracted RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). PCR was performed as previously described (14), using the conditions: initial denaturation for 96˚C for 2 min, followed by 26 cycles of denaturation for 1 min at 96˚C, annealing for 1 min at 57-60˚C and extension for 1 min at 72˚C. As an internal control for RT-PCR analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were amplified from the same cDNA samples. Primer sequences were: GAPDH, 5'-CAACAGCCTCAAGATCATCAGC-3' (forward) and 5'-TCCTAGACGGCAGGTCAGGTC-3' (reverse); *BNIP3*, 5'-GGATGCAGGAGGAGAGCCT-3' (forward) and 5'-CGAG GTGGGCTGTCACAGT-3' (reverse); and SOCS3, 5'-GACCA GCGCCACTTCTT-CAC-3' (forward) and 5'-ACTGGATGCG CAGGTTCTTG-3' (reverse). Following amplification, PCR products were separated on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination.

DNA extraction. Tumor or normal mucosal tissue was cut into 10-μm sections from tissue blocks, with a change of blade and cleaning of the microtome between specimens. The specimens were deparaffinized and washed, and tumor tissue was manually dissected using a razor blade, using a hematoxylin and eosin (H&E)-stained slide as a guide. Dissected tissues or cultured cell lines were then incubated overnight with proteinase-K in digestion buffer and the genomic DNA was extracted using a standard phenol chloroform method.

Methylight analysis. Sodium bisulfite conversion and DNA recovery were performed using EpiTect Bisulfite (Qiagen). Following sodium bisulfite conversion, the genomic DNA was analyzed using the Methylight technique, a fluorescence-based RT-PCR (Q-PCR) assay (18) and the ABI Prism 7300 R-T PCR System (Taq Man; Applied Biosystems, Foster City, CA, USA). Two sets of primers and probes, designed specifically for bisulfite-converted DNA were used. One set detected methylation in the gene of interest and the second set served as a reference set for β-actin (ACTB) to normalize for input DNA. The reference primers and probes were designed in a region of the ACTB gene that lacks CpG dinucleotides, allowing for equal amplification regardless of the methylation levels. Primer

and probe sequences were: ACTB, 5'-AGGTTGGGGAAGTTT GTTTTTG-3'(forward),5'-CCACCACCCAACACACAATA-3' (reverse) and 5'-TGGGGTGGTGATGGAGGAGGTT-3' (probe); BNIP3, 5'-TTCGGTCGGAGGAATTTATAGG-3' (forward), 5'-CCCCAATCGCGACCAA-3' (reverse) and 5'-ACGACGCGACCGCAAAT-3' (probe); as well as SOCS3 5'-TCGCGTTTTTTT-TTTCGTAGTTT-3' (forward), 5'-CGC GACCTCCGCACA-3' (reverse) and 5'-CGACCGCTACCG CATCCCGA-3' (probe). *Sss*I-treated HCT-15 DNA was used as a fully methylated positive control (100% methylation ratio). Parallel Taq Man PCR reactions were performed with specific primers for the bisulfite-converted methylated sequence for a particular locus and with the ACTB reference primers. In each case, triplicate threshold cycle (Ct) values were obtained and averaged, and expression levels were then evaluated by the 2-ΔΔCt method (19). As an internal standard, each individual sample was normalized to its ACTB content and compared to the gene expression level of *Sss*I-treated HCT-15 DNA as positive controls (calibration sample) as follows: $2^{\Delta\Delta Ct}$, where ΔΔCt = (Ct-target-Ct-reference)-treated sample – (Ct-target-Ct-reference) calibrator sample. The percentage of fully-methylated reference (PMR) was defined as $2^{\Delta\Delta Ct}$ x 100%.

Patients and tissue samples. Patients entered into this study had undergone surgical resection for primary sporadic colorectal cancer at the Department of Surgical Oncology, Tokyo Medical and Dental University (Tokyo, Japan). This study was pre-approved by the institutional review board of Tokyo Medical and Dental University, and written informed consent was obtained from all 112 participants prior to the commencement of the study. Following surgery for both colon and rectal cancer, patients exhibiting stage III cancer received oral or intravenous 5-FU-based adjuvant chemotherapy, and those with stage IV tumor or following recurrence received 5-FU-based systemic chemotherapy. The resected specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. For all cases, archival H&E slides of the primary tumors were retrieved and reviewed to confirm pathological features. In addition to the International Union Against Cancer Tumor-Node-Metastasis (TNM) classification (20), clinicopathological factors such as age, degree of histological differentiation, pathological tumor class and lymph node metastasis at the time of diagnosis in stage I-III patients during the follow-up period were studied. The response rate to chemotherapy including CPT-11 treatment was assessed by Response Evaluation Criteria In Solid Tumors (21).

Statistical analysis. Statistical analysis was carried out using StatView Software (version 5.0). To estimate the differences between groups, the Chi-square test, Welch's t-test and log-rank test were used where appropriate. The Kaplan-Meier method was used to estimate survival. Survival was calculated from the date of surgery. Prognostic factors were examined by univariate and multivariate analysis using the Cox proportional hazards model. $P<0.05$ was considered to be statistically significant.

Results

DAC increased sensitivity to SN-38 in colorectal cancer cells. Pre-incubation with the demethylating agent DAC was examined to investigate whether it influenced the sensitivity of the colon cancer cell lines HCT-15 and HT29 to SN-38. On day 9, the total number of HCT-15 cells that had been pre-incubated with DAC prior to exposure to SN-38 was found to be significantly decreased in comparison to cells treated with SN-38 alone (p=0.0049, Fig. 1A). A similar reduction was observed in the HT29 cells (p=0.0038, Fig. 1A). Treatment with DAC or SN-38 alone resulted in no significant effect on the rate of cell proliferation in the two cell lines.

Identification of pro-apoptotic genes induced by DAC in colon cancer cells. Since inactivation of the apoptotic pathway is associated with chemoresistance (8), identification of the pro-apoptotic genes that may be reactivated by DAC was attempted. Using oligonucleotide microarrays, the global changes in gene expression following the treatment of HCT-15 and HT29 with DAC were analyzed, and the expression changes in treated and untreated cells were compared. Expression levels of 943 genes were found to be increased by >1.5-fold following DAC treatment in HCT-15 cells, and 2,720 genes were increased in the HT29 cells. A total of 234 of these genes were found to be similarly altered in the two cell lines. Among the 234 genes, 10 were identified as having known pro-apoptotic activities (Table I). As the expression levels of *BNIP3* and *SOCS3* are frequently reduced due to promoter methylation in gastrointestinal cancers (22,23), up-regulation of these genes following DAC treatment was analyzed to determine whether it was caused by DNA demethylation. The *BNIP3* mRNA expression was up-regulated and its DNA promoter was demethylated in both the HCT-15 and HT29 cell lines following treatment with DAC (Fig. 1B and C). In contrast, although *SOCS3* mRNA expression was enhanced after DAC treatment, primers and probes specific for methylated *SOCS3* failed to amplify any product in the two cell lines by Methylight analysis (data not shown). The results indicated that *SOCS3* expression may be induced by DAC treatment independently of the methylation status of the DNA promoter.

Relationship between clinicopathological factors and BNIP3 *methylation in CRC patients*. The median PMR value for *BNIP3* methylation in 16 randomly selected patients with normal colon epithelial tissue was 0. The positive methylation was defined as PMR>0. The *BNIP3* methylation status was then investigated in 112 patients who had undergone surgical resection for primary CRC between March 2000 and April 2003. Patients were prospectively followed over a median post-operative duration of 42 months and methylation of *BNIP3* was detected in 58% of the 112 patients. No significant correlations were found between *BNIP3* methylation and clinicopathological factors such as age, gender, tumor depth, vessel invasion, lymphatic invasion, lymph node metastasis and stage (Table II). Patients with *BNIP3* methylation also exhibited a significantly shorter overall survival time (OS) compared to those without methylation (p=0.012, Fig. 2A). In stage II/III tumors, patients with *BNIP3* methylation also exhibited a significantly shorter OS than those without methylation (p=0.039, Fig 2B). No difference in relapse-free survival (RFS) was observed between patients with or without methylation (p=0.16, Fig. 2C). Regarding stage II/III patients, investigation of the univariate analysis of survival using the

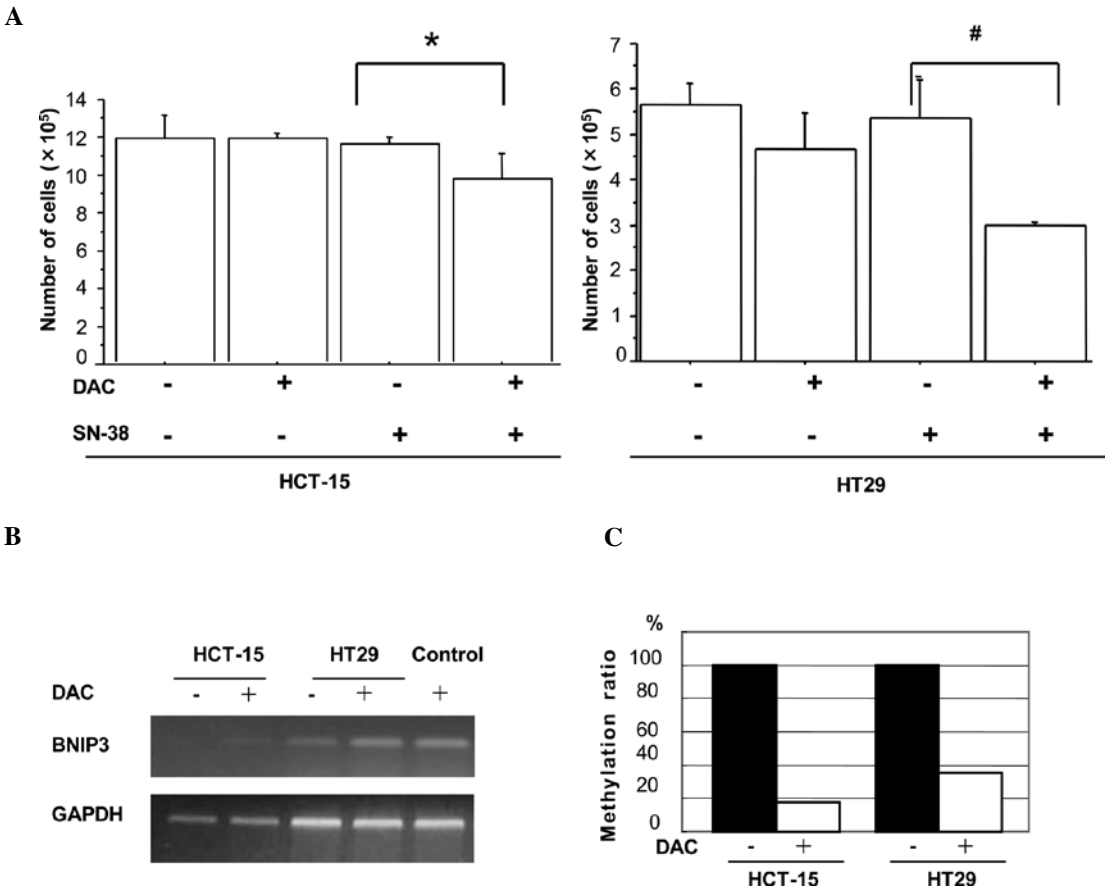

Figure 1. (A) *In vitro* growth inhibition following treatment with the demethylating agent DAC and/or SN-38. HCT-15 and HT29 cells were treated with 4 protocols (untreated, DAC alone, SN-38 alone or DAC followed by SN-38), and the total number of cells were counted on day 9. (B) Changes in mRNA expression and (C) the methylation status of *BNIP3* as examined by RT-PCR and Methylight analysis after DAC treatment, respectively. Error bars are the mean ± SD. *p=0.0049, #p=0.0038.

Table I. Apoptosis-related genes up-regulated by DAC treatment in colon cancer cell lines.

		Fold change	
Symbol	Name	HCT-15	HT29
UNC5B	Unc-5 homolog B (*C. elegans*)	5.21	11.8
NUPR1	Nuclear protein 1	2.26	5.68
ATF5	Activating transcription factor 5	4.76	3.00
IFI6	Interferon, α-inducible protein 6	2.92	3.28
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	2.07	3.34
SCIN	Scinderin	6.55	2.34
STAT1	Signal transducer and activator of transcription 1, 91 kDa	2.03	1.80
SOCS3	Suppressor of cytokine signaling 3	3.48	1.61
BNIP3	BCL2/adenovirus E 1B 19 kDa interacting protein 3	22.1	1.54
EEF1A2	Eukaryotic translation elongation factor α2	1.51	1.67

Cox proportional hazards model showed that *BNIP3* methylation (p=0.043) and lymph node metastasis (p=0.0086) were significantly associated with OS. All of the variables with a significance level at p<0.35 in the univariate analyses were subsequently used for multivariate analyses. Multivariate analysis of the variables failed to show *BNIP3* methylation to be an independent prognostic factor (Table III).

Prognostic value of BNIP3 methylation in CRC patients who received treatment with chemotherapeutic agents containing

Table II. Relationship between clinicopathological factors and *BNIP3* methylation.

	BNIP3		
	Met (n=65)	Unm (n=47)	p-value
Age			0.88
<64	30	21	
≥64	35	26	
Gender			0.48
Male	43	28	
Female	22	19	
Location			0.88
Right	24	18	
Left	41	29	
Lymphatic invasion			0.25
Negative	12	13	
Positive	53	34	
Vessel invasion			0.88
Negative	5	4	
Positive	60	43	
Depth of invasion			0.23
T1, T2	14	6	
T3, T4	51	41	
Lymph node metastasis			0.24
Negative	30	27	
Positive	35	20	
Stage			0.19
I	9	4	
II	17	21	
III	25	16	
IV	14	6	

Met, methylation group; Unm, unmethylation group.

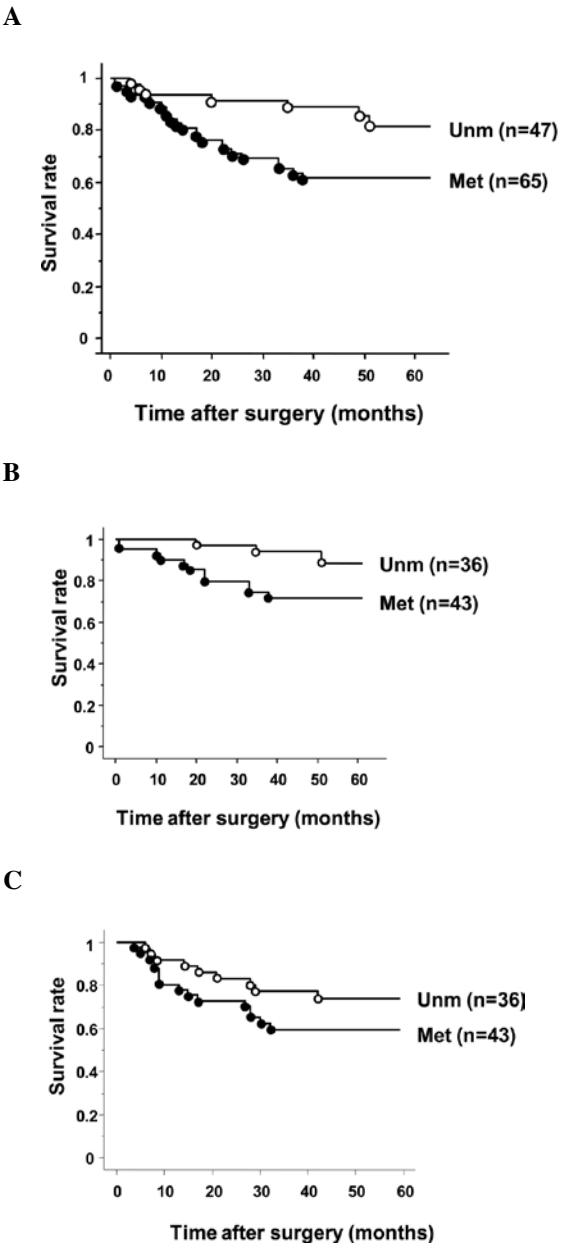

Figure 2. Survival analysis according to the presence of *BNIP3* methylation. (A) OS of the 112 patients. Patients with *BNIP3* methylation exhibited a significantly shorter OS compared to those without methylation (p=0.012). (B) OS of stage II/III patients. OS of patients with methylation was significantly shorter compared with that of patients without methylation (p=0.039). (C) RFS of stage II/III patients. RFS of patients with methylation was shorter than that of patients without methylation, but this difference did not reach statistical significance (p=0.16). ●, methylation group (Met); ○, unmethylation group (Unm).

CPT-11. BNIP3 methylation was examined to determine whether it was a prognostic marker in primary CRC patients treated with CPT-11. Of the patients who had undergone surgical resection for primary CRC between May 1998 and September 2007, 30 patients (including 5 from 112 patients between 2000 and 2003) who demonstrated recurrence after radical resection or incomplete resection due to distant metastasis, received first-line chemotherapy with CPT-11. Patients were subsequently observed over a median duration of 15 months following the initial administration of CPT-11. One patient had complete response (CR), 10 partial response (PR), 7 stable disease (SD) for >6 months, and 12 progression of disease (PD). When CR and PR patients were defined as responders, the observed response rate was lower in patients with *BNIP3* methylation than those without (33.3 vs. 40%), but this difference was not statistically significant (p=0.70, Fig. 3A). Time to progression (TTP) in patients with *BNIP3* methylation was slightly shorter than that in patients without methylation, but this difference was not statistically significant (median 196.5 vs. 237 days) (p=0.10, Fig. 3B).

Discussion

In this study, 2 colon cancer cell lines were utilized in which DAC treatment enhanced sensitivity to SN-38, and identified the apoptosis-related genes that were up-regulated following treatment with DAC by microarray analysis. Among these genes, we confirmed methylation of the *BNIP3* promoter

Table III. Analysis of overall survival in stage II/III patients using the Cox proportional hazard model.

	Stage II/III patients (n=79)		
Prognosis factor	Hazard ratio	95% CI	p-value
Univariate analysis			
BNIP3 methylation	3.744	1.044-13.432	0.043
Age	1.647	0.579-4.819	0.34
Gender	2.457	0.684-8.850	0.17
Tumor differentiation	0.555	0.155-1.993	0.37
Tumor location	0.614	0.215-1.752	0.36
Depth of invasion	1.170	0.153-8.929	0.88
Lymph node metastasis	4.739	1.484-15.152	0.0086
Lymphatic invasion	2.762	0.361-21.277	0.33
Multivariate analysis			
BNIP3 methylation	3.260	0.887-11.984	0.075
Age	1.366	0.468-3.985	0.57
Gender	2.105	0.574-7.752	0.26
Lymph node metastasis	3.040	0.670-13.889	0.15
Lymphatic invasion	1.481	0.130-16.928	0.75

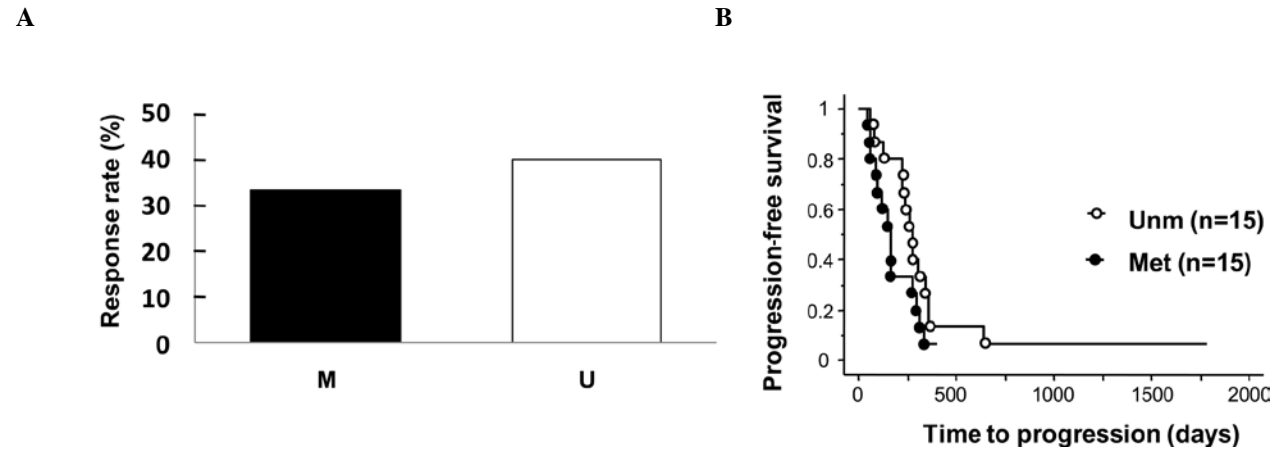

Figure 3. Relationship between the response to CPT-11 and *BNIP3* methylation in 30 patients that received first-line CPT-11 chemotherapy. (A) Response rate to CPT-11. Response rate of patients with *BNIP3* methylation (M) was lower than that of patients without (U) (33.3 vs. 40%), although differences did not reach statistical significance (p=0.70). (B) Time to progression (TTP) among patients who received CPT-11. TTP of patients with methylation was shorter than that of patients without methylation (196.5 vs. 237 days), but this difference was not statistically significant (p=0.10). ●, methylation group (Met); ○, unmethylation group (Unm).

in colon cancer cell lines, and subsequently detected its methylation in more than half of the cases of primary CRC. Our results showed that *BNIP3* methylation is associated with poor clinical outcome and chemoresistance.

Hypoxia is a common phenomenon observed in solid tumors and is known to stabilize hypoxia-inducible factor-1 (HIF-1), which is involved in the pathways underlying angiogenesis, glucose metabolism and cell proliferation (24-26). HIF-1 transactivates a large number of genes that may either promote or inhibit the growth and survival of individual tumor cells (27). Thus, selection of an individual tumor cell may maintain the expression of beneficial HIF-1 transcriptional targets, while silencing pro-death hypoxia-induced signals may result in lethal malignancy (28). *BNIP3* is a pro-apoptotic member of the Bcl-2 family whose role is mediated by HIF-1 (29-31). *BNIP3* is also known to play an important role in the regulation of apoptosis (32-34). An increased *BNIP3* expression induces cell death through mitochondrial dysfunction, membrane depolarization, mitochondrial permeability transition pore opening and increased production of reactive oxygen species (35). A reduced *BNIP3* expression has been identified in a wide range of cancer cells and primary malignancies (23,36,37). A number of recent studies showed that methylation of the *BNIP3* promoter may play an important role in silencing expression in a range of tumor types. These studies also showed that *BNIP3* methylation is detected in various primary cancers (23,36,37). In this study, *BNIP3* methylation was present in almost 60% of the primary CRC cases. Taken together, silencing *BNIP3* by

methylation may be important in tumorigenesis processes in the colon and rectum. Given the potential ability of HIF-1 to evoke apoptosis through its target gene, the down-regulation of *BNIP3* by methylation may disrupt the HIF-1-BINP3 apoptotic pathway and permit cancer cells with high malignant potential to survive, since CRC patients with *BNIP3* methylation had a poorer outcome than those with without. It was reported that *BNIP3* silencing induces metastatic growth of breast cancer in the liver, lung and bone (28). In this study, 70% (14/20) of patients with distant metastasis exhibited *BNIP3* methylation in the 112 CRC cases examined (Table II), suggesting that *BHIP3* silencing may contribute to the acquisition of metastatic potential in cancer cells.

CPT-11 is a topoisomerase I (topo-I) inhibitor that forms stable topo-I DNA-cleavable complexes and inhibits the progression of the replication fork. However, the relationship between the expression levels of topo-I mRNA and chemosensitivity of CPT-11 is unclear (38,39). In a previous study, we showed that DAC increases the growth inhibitory effects of CPT-11 on the colon cancer cell line HCT-15, despite showing no effect on topo-I expression levels (14). Furthermore, our microarray experiment did not identify changes in topo-I expression following treatment with DAC. Thus, topo-I may not be involved in the enhanced sensitivity to CPT-11 following DAC treatment.

Apoptosis is a significant mechanism through which chemotherapeutic agents exert their cytotoxic effects on cancer cells. However, cancer cells acquire resistance to apoptosis due to an altered expression or mutation of apoptosis-related genes during the tumorigenesis processes (40). Numerous studies have shown a relationship between disruption of the apoptosis pathway and chemoresistance in various tumors (8,9). In addition, the susceptibility of cancer cells to cytotoxic drugs appears to be at least partially due to a dependence on the balance between pro- and anti-apoptotic members of the Bcl-2 family (41). Given that *BNIP3* is a member of the Bcl-2 family of pro-apoptotic proteins, and that it appears to antagonize the activity of pro-survival proteins, including Bcl-2 and Bcl-xL (35), it may also contribute to chemosensitivity. Previously, *BNIP3* expression was found to be down-regulated in clones with acquired resistance to 5-FU compared to their parental CRC cell lines (33). Additionally, *BNIP3* expression was found to be associated with paclitaxel response in an ovarian cancer model (42). *BNIP3* down-regulation results from the addition of small interfering RNA enhanced chemoresistance in pancreatic cancer cells (43,44). By contrast, the overexpression of *BNIP3* in rat fibroblast cells increased sensitivity to apoptosis induced by topo-I and -II inhibitors (45). Our previous study showed that the inhibitory effects of SN-38 on tumor tissue are increased in a CRC xenograft model when *BNIP3* expression is restored via promoter demethylation following treatment with DAC (14). In this study, *BNIP3* was up-regulated following treatment with DAC in SN-38-resistant CRC cells when DAC increased sensitivity to SN-38. Moreover, CRC patients with *BNIP3* methylation exhibited a shorter TTP for CPT-11 treatment. Thus, *BNIP3* may play an important role in the reduced response to CPT-11 treatment in CRC patients.

In conclusion, the relatively high frequency of *BNIP3* methylation in primary CRC suggests that this gene is involved in carcinogenesis of the colon and rectum. Moreover, since *BNIP3* methylation is associated with poor OS and decreased sensitivity to CPT-11, the methylation status of this gene may be a predictive factor for prognosis and responsiveness to CPT-11 in CRC patients. Since methylation appears to be reversed by a chemical agent, *BNIP3* reactivation via a demethylating agent may be a novel target for the treatment of CRC.

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