

Methylation of *BNIP3* and *DAPK* indicates lower response to chemotherapy and poor prognosis in gastric cancer

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Abstract. Aberrant promoter hypermethylation (methylation) is an epigenetic change that silences the expression of crucial genes, thus inactivating the apoptotic pathway in various cancers. Inactivation of the apoptotic pathway has been considered to be associated with chemoresistance. The objective of the present study was to clarify the effect of the methylation of the apoptosis-related genes, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (*BNIP3*) and death-associated protein kinase (*DAPK*), on the response to chemotherapy in metastatic or recurrent gastric cancers. Tumor samples were obtained from 80 gastric cancer patients who were treated with fluoropyrimidine-based chemotherapy for distant metastatic or recurrent disease, after surgical resection of the primary tumor. The methylation status of the apoptosis-related genes, *BNIP3* and *DAPK*, was investigated by methylation-specific PCR. Methylation in *BNIP3* was detected in 31 tumors (39%) and in *DAPK* in 33 tumors (41%). There was no correlation between the methylation status of *BNIP3* and that of *DAPK*. The response rate was significantly lower in patients with methylation of *DAPK*, than in those without (21 vs. 49% $p=0.012$). Progression-free survival time (PFS) was shorter in patients with methylation of *DAPK* than in those without ($p=0.007$). The overall survival time (OS) was shorter in patients with methylation of *BNIP3* than in those without ($p=0.031$). The response rate was significantly lower in patients with methylation of either *DAPK* or *BNIP3*, or both, than in those without methylation ($p=0.003$). PFS and OS were significantly shorter in patients with methylation of either or both of these genes than in those without ($p=0.002$,

$p=0.001$). The methylation of *BNIP3* and *DAPK* can predict lower response to chemotherapy and poor prognosis in gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer and the second most frequent cause of cancer-related death worldwide (1,2). Surgical treatment is the mainstay of therapy for gastric cancer, although chemotherapy is required for unresectable advanced disease (3). The survival time of patients with distant metastases or recurrence has been prolonged by the introduction of new regimens including oral fluoropyrimidines, oxaliplatin, taxans and irinotecan (4,5). However, the effectiveness of chemotherapy is insufficient for gastric cancer patients. It would be useful to be able to select patients whose tumor would be sensitive to chemotherapy. However, there is no reliable marker that can predict response to chemotherapy for gastric cancer.

Most chemotherapeutic agents, such as fluoropyrimidines and irinotecan, damage DNA and induce apoptosis. The inactivation of the apoptotic pathway has been considered to be associated with chemoresistance (6,7). The dysfunction of apoptosis-related genes could decrease apoptosis induced by chemotherapy. The methylation of many apoptosis-related genes, including Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (*BNIP3*) and death-associated protein kinase (*DAPK*), has been reported in various cancers. DNA methylation plays a key role in carcinogenesis, as well as in genome mutation or deletion. Methylation is associated with the transcriptional silencing of selected genes and affects cell growth and differentiation in gastric cancer (8-11).

In the present study, the methylation of the apoptosis-related genes, *BNIP3* and *DAPK*, was examined in patients who were treated with fluoropyrimidine-based chemotherapy for metastatic or recurrent gastric cancer, in order to analyze the association between the methylation of apoptosis-related genes and the response to chemotherapy.

Materials and methods

A total of 80 patients who had undergone surgical resection for primary gastric cancer at the Department of Surgical

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Oncology, Tokyo Medical and Dental University (Tokyo, Japan), were included in the present study. Forty-four patients had recurrent disease after radical resection, and 36 underwent non-curative resection due to distant metastases. All patients received oral or intravenous fluoropyrimidine-based chemotherapy. No patient received any other adjuvant therapy. Overall, 41, 18, 15 and 6 patients were treated with S-1 + irinotecan, S-1 + docetaxel, 5-fluorouracil (5-FU) + cisplatin, and 5-FU + other agents, respectively.

All resected specimens were fixed in 10% pH-neutral formalin and embedded in paraffin. In all cases, archival H&E slides of the gastric tumors were retrieved and reviewed in order to confirm the pathological features. Histological findings and tumor staging were classified according to the tumor-node-metastasis (TNM) classification advocated by the International Union against Cancer. Response to treatment was evaluated according to the Response Evaluation Criteria in Solid Tumors. The resected gastric cancer samples and clinical data were collected after obtaining appropriate institutional review board approval and written informed consent from all the patients.

DNA extraction and methylation-specific PCR. Tissue was cut into 10- μ m-thick sections from paraffin-embedded blocks. The specimens were deparaffinized and washed, and then using the H&E slide as a guide, tumor tissue was manually dissected. As much as possible, equal amounts of tissue were dissected for each case. Genomic DNA was extracted by standard proteinase K (Invitrogen, Carlsbad, CA, USA) digestion, phenol/chloroform extraction and ethanol precipitation, as previously described (12). Bisulfite treatment was performed using a CpGenome DNA modification kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's instructions. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosine (but not methylated cytosine) into uracil, which is then converted to thymidine during the subsequent PCR step, thus resulting in sequence differences between methylated and unmethylated DNA. The methylation

Table I. Clinicopathological features associated with *BNIP3* and *DAPK* methylation.

	<i>BNIP3</i> (39%)		<i>DAPK</i> (41%)	
	Met	Unm	Met	Unm
Age	NS		NS	
Range	21-81	17-78	38-81	17-78
Mean	62	61	63.3	60
Gender	NS		NS	
Male	20	40	27	33
Female	11	9	6	14
Age	NS		NS	
Differentiated	10	16	10	16
Undifferentiated	21	33	23	31
Age	NS		NS	
T1	1	1	1	1
T2	8	8	4	12
T3	19	33	24	28
T4	3	7	4	6
Age	NS		NS	
Absent	6	4	5	5
Present	25	45	28	42
Age	NS		NS	
I	4	2	2	4
II	1	2	3	0
III	12	23	14	21
IV	14	22	14	22

Met, methylation; Unm, unmethylation; NS, not significant.

status of the *BNIP3* and *DAPK* genes was determined by methylation-specific PCR (13). The following primer sequences were designed for the *BNIP3* and *DAPK* genes:

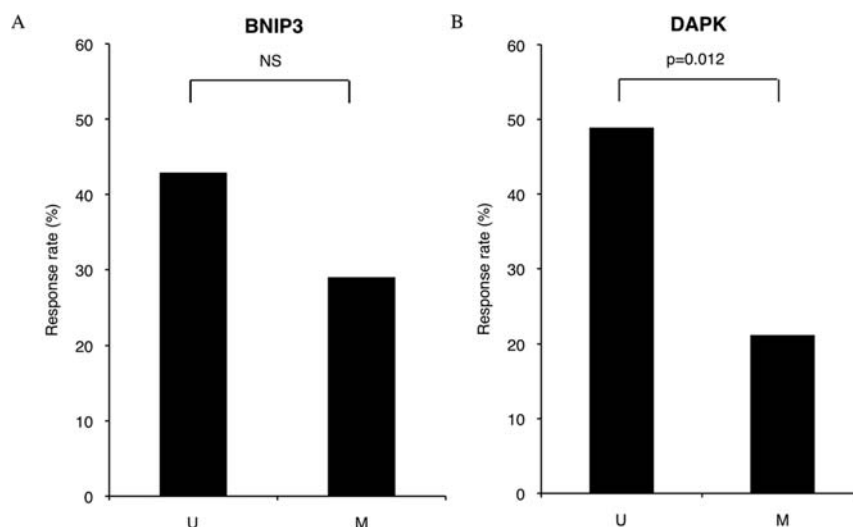


Figure 1. Response rate to chemotherapy with respect to methylation status. (A) The methylation of *BNIP3* led to a lower response rate, although the differences were not significant. (B) The methylation of *DAPK* is significantly associated with lower response rate, as opposed to unmethylation ($p=0.012$). M, methylation; U, unmethylation; NS, not significant.

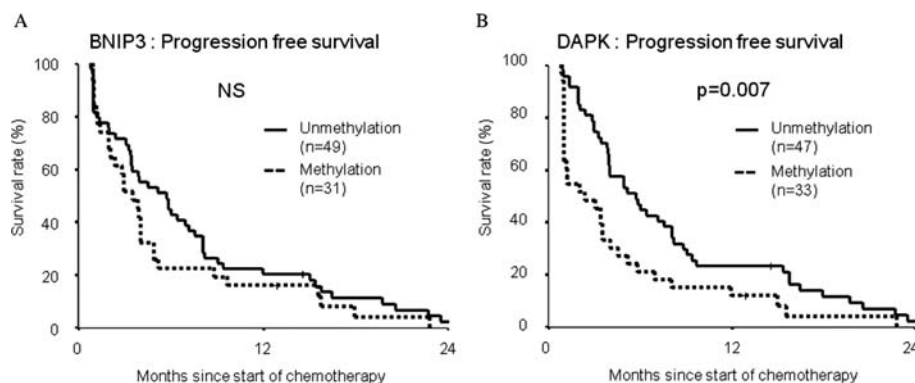


Figure 2. PFS with respect to methylation status. (A) *BNIP3* methylation was not related to PFS. (B) PFS of patients with methylation of *DAPK* was significantly shorter than that of patients without methylation ($p=0.007$). NS, not significant.

Methylated *BNIP3* forward PCR primer, 5'-ATTCGTTTC GCGTACGAGTC-3' and reverse, 5'-GCGTCGCCCAT TAACCGCGA-3'; unmethylated *BNIP3* forward PCR primer, 5'-ATTTGTTTTGTGTATGTGTTGTA-3' and reverse, 5'-ACATCACCCATTAACCACAA-3'; methylated *DAPK* forward PCR primer, 5'-GGATAGTCGGATCGAGT TAACGTC-3' and reverse, 5'-CCCTCCCAAACGCCGA-3'; unmethylated *DAPK* forward PCR primer, 5'-GGAGGATA GTTGATTGAGTTAATGTT-3' and reverse, 5'-CA AATCCCTCCCAAACACCAA-3'. PCR was performed in 25- μ l reaction mixtures as follows: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 6°C for 1 min, 72°C for 1 min, and a final 10-min extension at 72°C. The PCR products were loaded onto a 2% agarose gel, stained with 0.5 μ g/ml ethidium bromide, and visualized under ultraviolet illumination.

Statistical analysis. The correlations between *BNIP3* or *DAPK* methylation status and clinicopathological features were statistically analyzed using the t-test (for age), Chi-square test (for gender and histological type), and the Mann-Whitney U test (for tumor depth, lymph node metastases and stage). Anti-cancer drug receptivity according to methylation status was analyzed using the Chi-square test.

Overall survival time (OS) was defined as the period between the start of chemotherapy and the time of death. Progression-free survival time (PFS) was calculated from the first day of chemotherapy to the date on which progression of disease was first observed. OS and PFS were assessed using the Kaplan-Meier method, and differences between survival curves were analyzed using the log-rank test. Values of $p<0.05$ were considered to be significant. All analyses were performed using the statistical software Dr. SPSS2 (SPSS Japan Inc., an IBM company).

Results

DNA methylation status of *BNIP3* and *DAPK* in gastric cancer. Methylation was detected in *BNIP3* in 31 tumors (39%) and in *DAPK* in 33 tumors (41%). There was no correlation between the methylation status of *BNIP3* and that of *DAPK*. There was no relationship between the methylation status of the two genes and clinicopathological findings,

including age, gender, histological type, tumor depth, lymph node metastases and stage (Table I).

Correlation between response to chemotherapy and *BNIP3* and *DAPK* methylation status. Of the 80 patients who underwent fluoropyrimidine-based chemotherapy treatment, 30 patients responded (complete response and partial response) and 50 did not (stable disease and progressive disease).

The response rate was lower in patients with methylation than in those without. *DAPK* methylation was significantly associated with the response rate to chemotherapy (*BNIP3*, 29 vs. 43%, $p=0.210$; *DAPK*, 21 vs. 49%, $p=0.012$, Fig. 1A and B). PFS was significantly shorter in patients with *DAPK* methylation than in those without ($p=0.007$, Fig. 2B). *BNIP3* methylation was not related to PFS (Fig. 2A). OS was significantly shorter in patients with *BNIP3* methylation than in those without ($p=0.031$, Fig. 3A). No significant correlation was observed between OS and the *DAPK* methylation status (Fig. 3B).

The response rate was significantly lower in patients with methylation of either *BNIP3* or *DAPK*, or both, compared to that in patients with no methylation ($p=0.003$, Fig. 4). PFS and OS were significantly shorter in patients with methylation of either *BNIP3* and *DAPK*, or both, than in patients without methylation ($p=0.002$ and $p=0.001$, respectively, Fig. 5A and B). The median survival was 15.8 months with methylation of either *BNIP3* and *DAPK*, or both, and 6.9 months without methylation. PFS and OS were not associated with any other clinicopathological factors.

Discussion

In the present study, the methylation of the apoptosis-related genes, *BNIP3* and *DAPK*, was a good genetic marker of chemoresistance in gastric cancer. The results also demonstrate that apoptosis-related genes play a crucial role in response to chemotherapy in gastric cancer.

In gastric cancer, carcinogenesis and progression involve genetic alterations of tumor-related genes, such as *p53* and *PTEN*. The mutation of *p53* is one of the most prevalent genetic alterations and is mainly associated with tumor progression (14). The methylation of tumor-suppressor genes also plays a crucial role in carcinogenesis and progression.

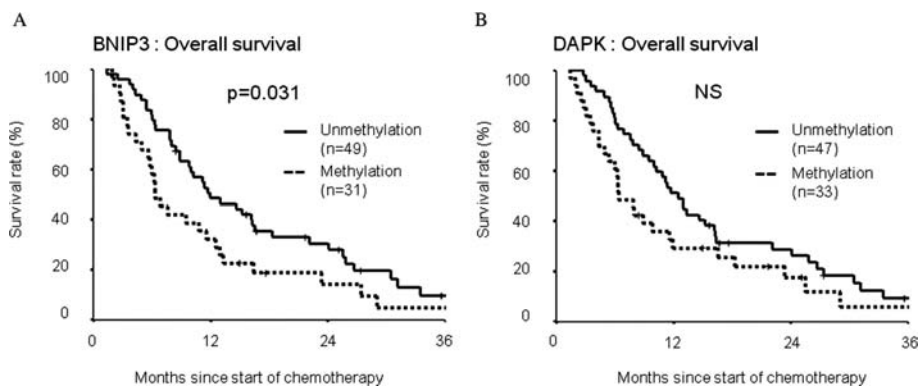


Figure 3. OS with respect to methylation status. (A) OS of patients with methylation of *BNIP3* was shorter than that of patients without methylation ($p=0.031$). (B) *DAPK* methylation was not related to OS. NS, not significant.

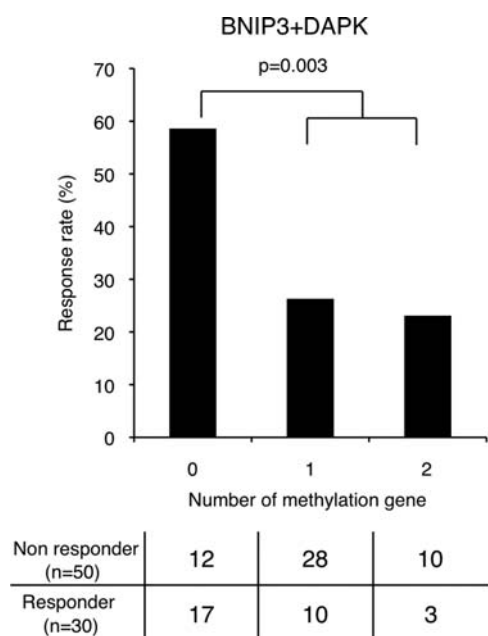


Figure 4. Analysis of two genes (*BNIP3* and *DAPK*) together with respect to response rate. The response rate with methylation of either, or both of the two genes, was significantly lower than that with no methylation ($p=0.003$).

The methylation of *E-cadherin* has been observed in >50% of early stage undifferentiated gastric cancers and has also been observed in surrounding non-cancerous epithelial cells (15,16). *E-cadherin* methylation could thus play a major role in the development of undifferentiated cancer of the stomach (17). Furthermore, methylation could provide useful information regarding prognosis. Yu *et al* reported that patients with methylation of *Dkk-3*, a *Wnt/b-Catenin* signaling antagonist, had a significantly shorter survival than patients with no methylation, in a study of primary gastric cancers (18). There have been many studies on the relationship between the methylation of tumor-suppressor genes and the response to chemotherapy in gastric cancer. Satoh *et al* reported that *CHFR* gene expression was frequently silenced by methylation, and that gastric cancer cells not expressing *CHFR*, lacked a mitotic checkpoint and were highly susceptible to microtubule inhibitors, such as taxans (9). Napieralski *et al* reported the relationship between neo-adjuvant chemotherapy and the methylation of six genes (*MGMT*, *LOX*, *p16*, *14-3-3σ*, *E-cadherin*, *HPPI*) in 54 gastric cancer patients. A trend toward shorter survival and lower response rate was observed in patients with two or more methylated genes than in those with no methylation, or the methylation of one gene (19). Our previous study also showed that the methylation of *DAPK* and *TMS1* correlated

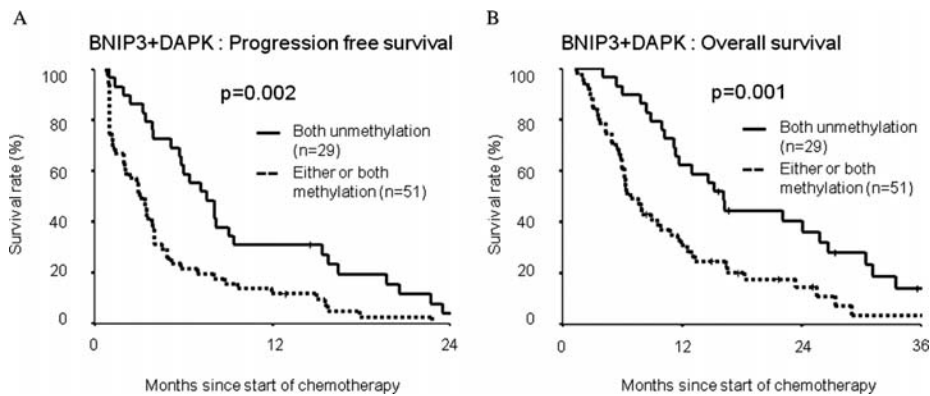


Figure 5. Analysis of two genes together for PFS and OS. PFS (A) and OS (B) of patients with methylation of either, or both genes were significantly shorter than those of patients with no methylation ($p=0.002$, $p=0.001$).



r response rate and shorter OS in 43 gastric cancer treated with fluoropyrimidine-based therapy (20).

There have been many studies on demethylating agents in various cancers (21,22). The combination of demethylating agents and DNA-damaging agents, such as fluoropyrimidine and irinotecan, has been shown to produce marked suppression in tumor growth and good prognosis (7,23). From this perspective, further evidence is required regarding the association of the methylation of tumor-suppressor genes with response to chemotherapy and prognosis in gastric cancer.

Apoptosis is the process of programmed cell death that occurs in multicellular organisms, and it is important for the cellular response to genotoxic drug-induced damage (24). The major actions of chemotherapeutic drugs are exerted via the activation of apoptosis (25). In cancer cells, the apoptotic pathway has been disrupted at several points during the carcinogenesis process (25,26). Considerable evidence has accrued regarding the effect of genetic and epigenetic alterations of these pathways on drug sensitivity.

BNIP3 is a pro-apoptotic member of the *Bcl-2* family (27). The expression of *BNIP3* is induced by hypoxia, such as that which occurs during cardiac ischemia and in the hypoxic regions of tumors, and it acts against pro-survival proteins, including *Bcl-2* and *Bcl-xl* (28-31). The methylation of *BNIP3* has been reported in many cancers, such as colorectal, breast and pancreatic cancers (32). Murai *et al* also reported that the aberrant hypermethylation of *BNIP3* was detected in 66% of primary colorectal and in 49% of primary gastric cancers, but not in normal tissue. Furthermore, the *BNIP3* methylation correlated with the down-regulation of its expression (33). Akada *et al* reported that the chemoresistance to gemcitabine was associated with the decreased expression of *BNIP3* in pancreatic cancer. The reduced expression of *BNIP3* increased resistance to gemcitabine and 5-FU and significantly decreased patient survival in pancreatic cancer (34). Our study also shows that gastric cancer patients with *BNIP3* methylation have lower chemosensitivity and shorter prognosis compared to patients without methylation.

DAPK, a Ca^{2+} /calmodulin-dependent enzyme with serine/threonine kinase activity, has been defined as a tumor suppressor and has been shown to be part of a *p53*-dependent apoptotic pathway that operates via *p19^{ARF}* (35,36). *DAPK* functions as a regulator of apoptosis and induces programmed cell death. It has been demonstrated that the loss of *DAPK* expression is caused by the methylation of its promoter in many cancers (37). The methylation of *DAPK* has been reported to be associated with poor disease-specific survival rates in patients with stage I non-small cell lung carcinoma (38). The study by Fischer *et al* showed that when combining *DAPK*, *RAR β* and *RASSF1A* methylation, patients with double or triple methylations had a worse prognosis than the group with only one or no methylated gene in malignant mesothelioma (39).

In our study, the frequency of methylation of the two apoptosis-related genes, *BNIP3* and *DAPK*, was 39 and 42%, respectively. The methylation status of *BNIP3* or *DAPK* was not associated with the clinicopathological features in gastric cancer. However, *DAPK* was significantly associated with

the response rate and PFS, and *BNIP3* was significantly associated with OS. On the analysis of the two genes together, patients with methylation of either gene had a lower response to chemotherapy and poorer prognosis than patients with no methylation. The combined impact of *BNIP3* and *DAPK* is greater than that of an individual gene alone in all factors.

These results suggest that the inactivation of the apoptotic pathway caused by the methylation of apoptosis-related genes interferes with the response to chemotherapy. Moreover, the chemoresistance produced by the methylation of apoptosis-related genes, causes poor prognosis in gastric cancer.

Thus, the present study shows that the methylation of the apoptosis-related genes causes resistance to chemotherapy and worsens prognosis in patients with metastatic or recurrent gastric cancer. The methylation of *BNIP3* or *DAPK* can predict chemosensitivity and prognosis in gastric cancer.

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