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

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Received August 16, 2010; Accepted September 30, 2010

DOI: 10.3892/or.2010.1085

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.

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Key words: Bcl-2/adenovirus E1B 19 kDa-interacting protein 3, death-associated protein kinase, methylation, gastric cancer, chemotherapy

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 fluoropyrimi-dines, 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

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-μm-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 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:

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

	BNIP3 (39%)		*DAPK* (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.

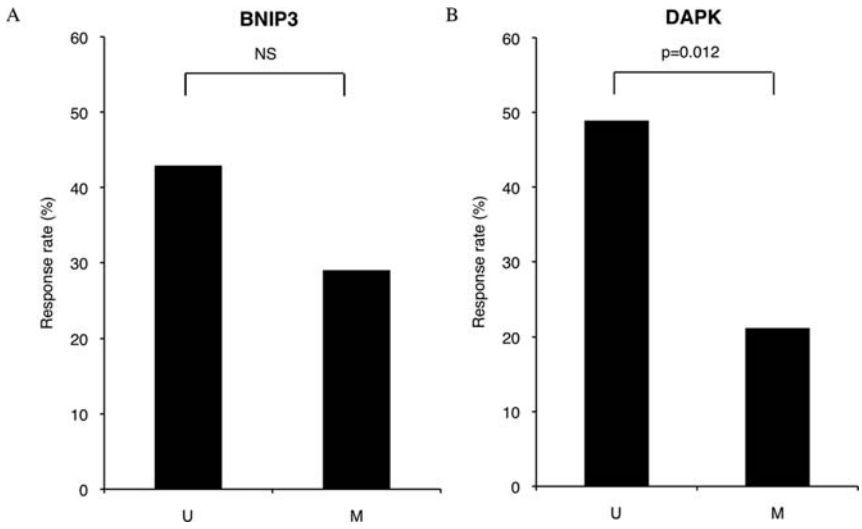


Figure 1. Response rate to chemotherapy with respect to methylation status. (A) The methylation of *BNIP3* led to a lower response rate, although the differences were not significant. (B) The methylation of *DAPK* is significantly associated with lower response rate, as opposed to unmethylation (p=0.012).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

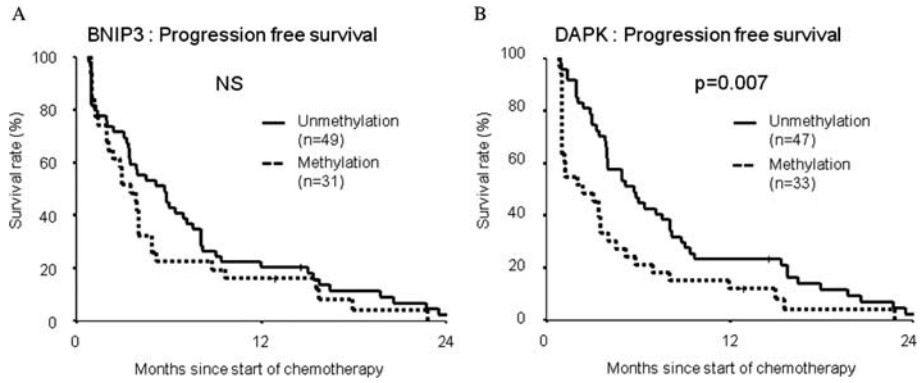


Figure 2. PFS with respect to methylation status. (A) *BNIP3* methylation was not related to PFS. (B) PFS of patients with methylation of *DAPK* was significantly shorter than that of patients without methylation (p=0.007). NS, not significant.

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 GTTGGATTGAGTTAATGTT-3' and reverse, 5'-CA AATCCCTCCCAAACACCAA-3'. PCR was performed in 25-μl 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 μg/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.

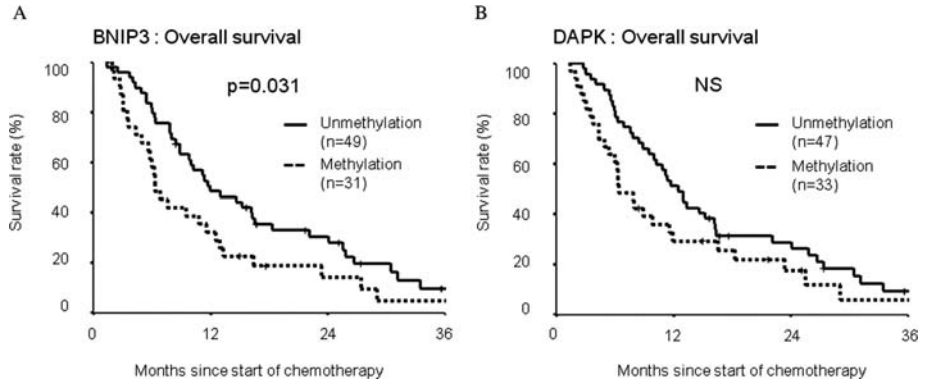
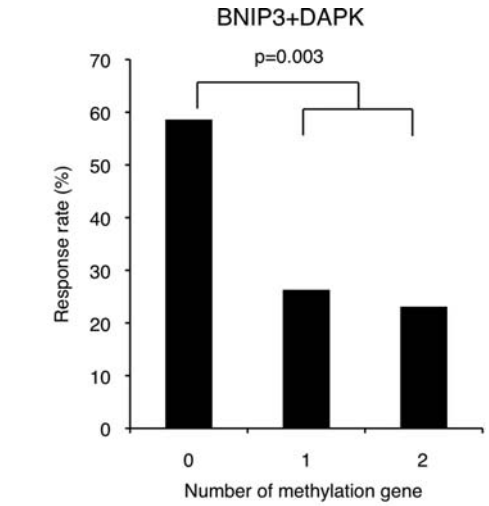


Figure 3. OS with respect to methylation status. (A) OS of patients with methylation of *BNIP3* was shorter than that of patients without methylation (p=0.031). (B) *DAPK* methylation was not related to OS. NS, not significant.



Non responder (n=50)	12	28	10
Responder (n=30)	17	10	3

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*, *HPP1*) 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

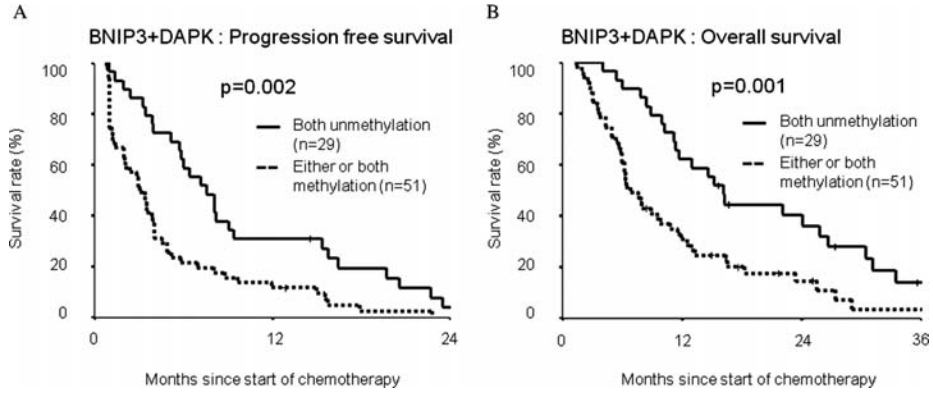


Figure 5. Analysis of two genes together for PFS and OS. PFS (A) and OS (B) of patients with methylation of either, or both genes were significantly shorter than those of patients with no methylation (p=0.002, p=0.001).

with lower response rate and shorter OS in 43 gastric cancer patients 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*, *RARβ* 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.

Acknowledgements

We thank Ms. Aiko Saito for her exceptional technical assistance.

References

1. Hohenberger P and Gretschel S: Gastric cancer. Lancet 362: 305-315, 2003.
2. Dicken BJ, Bigam DL, Cass C, Mackey JR, Joy AA and Hamilton SM: Gastric adenocarcinoma-review and considerations for future directions. Ann Surg 241: 27-39, 2005.
3. Macdonald JS, Smalley SR, Benedetti J, *et al*: Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Eng J Med 345: 725-730, 2001.
4. Van Cutsem E, Moiseyenko VM, Tjulandin S, *et al*: Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 study group. J Clin Oncol 24: 4991-4997, 2006.
5. Koizumi W, Narahara H, Hara T, *et al*: S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. Lancet Oncol 9: 215-221, 2008.
6. Teodoridis JM, Strathdee G and Brown R: Epigenetic silencing mediated by CpG island methylation: potential as a therapeutic target and as a biomarker. Drug Resist Updat 7: 267-278, 2004.
7. Ishiguro M, Iida S, Uetake H, *et al*: Effect of combined therapy with low-dose 5-Aza-2'-deoxycytidine and irinotecan on colon cancer cell line HCT-15. Ann Surg Oncol 14: 1752-1762, 2007.
8. Satoh A, Toyota M, Itoh F, *et al*: DNA methylation and histone deacetylation associated with silencing DAP kinase gene expression in colorectal and gastric cancers. Br J Cancer 86: 1817-1823, 2002.
9. Satoh A, Toyota M, Itoh F, *et al*: Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res 63: 8606-8613, 2003.
10. Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H and Imai K: Inactivation of the 14-3-3 sigma gene is associated with 5' CpG island hypermethylation in human cancers. Cancer Res 60: 4353-4357, 2000.
11. Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T and Hirohashi S: Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci USA 92: 7416-7419, 1995.
12. Iida S, Akiyama Y, Nakajima T, Ichikawa W, Nihei Z, Sugihara K and Yuasa Y: Alterations and hypermethylation of the p14(ARF) gene in gastric cancer. Int J Cancer 87: 654-658, 2000.
13. Belinsky SA, Palmisano WA, Gilliland FD, *et al*: Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. Cancer Res 62: 2370-2377, 2002.

14. Ranzani GN, Luinetti O, Padovan LS, *et al*: p53 gene mutations and protein nuclear accumulation are early events in intestinal type gastric cancer but late events in diffuse type. Cancer Epidemiol Biomarkers Prev 4: 223-231, 1995.
15. Becker KF, Atkinson MJ, Reich U, Becker I, Nekarda H, Siewert JR and Höfler H: *E-cadherin* gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res 54: 3845-3852, 1994.
16. Waki T, Tamura G, Tsuchiya T, Sato K, Nishizuka S and Motoyama T: Promoter methylation status of *E-cadherin*, *hMLH1*, and *p16* genes in nonneoplastic gastric epithelia. Am J Pathol 161: 399-403, 2002.
17. Tamura G: Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. World J Gastroenterol 12: 192-198, 2006.
18. Yu J, Tao Q, Cheng YY, *et al*: Promoter methylation of the Wnt/beta-catenin signaling antagonist Dkk-3 is associated with poor survival in gastric cancer. Cancer 115: 49-60, 2009.
19. Napieralski R, Ott K, Kremer M, *et al*: Methylation of tumor-related genes in neoadjuvant-treated gastric cancer: relation to therapy response and clinicopathologic and molecular features. Clin Cancer Res 13: 5095-5102, 2007.
20. Kato K, Iida S, Uetake H, *et al*: Methylated TMS1 and DAPK genes predict prognosis and response to chemotherapy in gastric cancer. Int J Cancer 122: 603-608, 2008.
21. Issa JP, Garcia-Manero G, Giles FJ, *et al*: Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine in hematopoietic malignancies. Blood 103: 1635-1640, 2004.
22. Miyamoto K and Ushijima T: [DNA methylation and cancer-DNA methylation as a target of cancer chemotherapy]. Gan To Kagaku Ryoho 30: 2021-2029, 2003.
23. Morita S, Iida S, Kato K, Takagi Y, Uetake H and Sugihara K: The synergistic effect of 5-aza-2'-deoxycytidine and 5-fluorouracil on drug-resistant tumors. Oncology 71: 437-445, 2006.
24. Oizumi S, Isobe H, Ogura S, *et al*: Topoisomerase inhibitor-induced apoptosis accompanied by down-regulation of Bcl-2 in human lung cancer cells. Anticancer Res 22: 4029-4037, 2002.
25. Makin G and Dive C: Apoptosis and cancer chemotherapy. Trends Cell Biol 11: 22-26, 2001.
26. Fulda S and Debatin KM: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25: 4798-4811, 2006.
27. Reed JC: Bcl-2 family proteins. Oncogene 17: 3225-3236, 1998.
28. Labi V, Erlacher M, Kiessling S and Villunger A: BH3-only proteins in cell death initiation, malignant disease and anti-cancer therapy. Cell Death Differ 13: 1325-1338, 2006.
29. Strasser A: The role of BH3-only proteins in the immune system. Nat Rev Immunol 5: 189-200, 2005.
30. Willis SN and Adams JM: Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 17: 617-625, 2005.
31. Vande Velde C, Cizeau J, Dubik D, *et al*: BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol Cell Biol 20: 5454-5468, 2000.
32. Mellor HR and Harris AL: The role of hypoxia-inducible BH3-only proteins BNIP3 and BNIP3L in cancer. Cancer Metastasis Rev 26: 553-566, 2007.
33. Murai M, Toyota M, Satoh A, *et al*: Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumors. Br J Cancer 92: 1165-1172, 2005.
34. Akada M, Crnogorac-Jurcevic T, Lattimore S, *et al*: Intrinsic chemoresistance to gemcitabine is associated with decreased expression of BNIP3 in pancreatic cancer. Clin Cancer Res 11: 3094-3101, 2005.
35. Cohen O, Feinstein E and Kimchi A: DAP-kinase is a Ca^{2+}/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. EMBO J 16: 998-1008, 1997.
36. Raveh T, Droguett G, Horwitz MS, DePinho RA and Kimchi A: DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. Nat Cell Biol 3: 1-7, 2001.
37. Toyooka S, Toyooka KO, Miyajima K, *et al*: Epigenetic downregulation of death-associated protein kinase in lung cancers. Clin Cancer Res 9: 3034-3041, 2003.
38. Tang X, Khuri FR, Lee JJ, Kemp BL, Liu D, Hong WK and Mao L: Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. J Natl Cancer Inst 92: 1511-1516, 2000.
39. Fischer JR, Ohnmacht U, Rieger N, *et al*: Promoter methylation of RASSF1A, RARbeta and DAPK predict poor prognosis of patients with malignant mesothelioma. Lung Cancer 54: 109-116, 2006.