Promoter methylation status of *DAP-kinase* and *RUNX3* genes in neoplastic and non-neoplastic gastric epithelia

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Silencing of tumor suppressor and tumor-related genes by hypermethylation at promoter CpG islands is frequently found in human tumors, including gastric cancer. Promoter methylation is not restricted to cancer cells, and is also present in non-neoplastic cells as an age-related tissue-specific phenomenon. To clarify the physiological consequence of *DAP-kinase* and *RUNX3* age-related methylation in gastric epithelia, we investigated the promoter methylation status of these genes in both neoplastic and non-neoplastic gastric epithelia obtained at autopsy and surgery, as well as in 10 gastric cancer cell lines. Methylation of *DAP-kinase* and *RUNX3* was detected in 10% (1/10) and 70% (7/10) of the cell lines, respectively, and was almost concordant with their expression status. Among autopsy samples, methylation of these genes was not seen in non-neoplastic gastric epithelia from persons who were aged 22 years and below (0%; 0/4). *DAP-kinase* was methylated in 87% (13/15) of non-neoplastic gastric epithelia of persons who were aged 45 years or older, while *RUNX3* methylation in non-neoplastic gastric epithelia was restricted to individuals who were aged 77 years or older. Among samples obtained from patients with stomach cancer, methylation was observed in both the neoplastic and the corresponding non-neoplastic gastric epithelia; 43% (40/93) and 73% (68/93) for *DAP-kinase*, and 45% (42/93) and 8% (7/93) for *RUNX3*, respectively. Frequencies of *DAP-kinase* and *RUNX3* methylation differed significantly in non-neoplastic gastric epithelia (*P*<0.01), although those in gastric cancers were almost the same. *RUNX3* methylation is mostly cancer-specific, except for very old individuals, and therefore may be a possible molecular diagnostic marker and malignancy predictor. (Cancer Sci 2003; 94: 360–364)

DNA methylation has an essential regulatory function in mammalian development, suppressing gene activity by changing chromatin structure.[1, 2] It has become apparent that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to coding region mutation for the inactivation of tumor suppressor or tumor-related genes, and is therefore involved in tumorigenesis.[3, 4] Promoter hypermethylation of tumor suppressor and tumor-related genes has been reported in various types of human tumor.[4] In gastric cancer, *E-cadherin*, *hMLH1* and *p16* promoter methylation with loss of expression is frequently present, and ultimately results in characteristic biological features.[5–8] Promoter methylation is not cancer-specific; for example, estrogen receptor (*ER*) methylation was shown to increase during aging in normal human colonic mucosa.[9] It was hypothesized that the cells of origin of colonic tumors were the ones in which the *ER* gene became hypermethylated.[9] Promoter methylation of other genes, such as *IGF2*, *MYOD1*, *N33*, *PAX6* and *Versican*, also increases with age in the normal colon.[10, 11] While the mechanism of age-related methylation is not known, it is clear that age-related methylation affects only a subset of genes, suggesting a gene-specific susceptibility to this process.[12] Furthermore, there are considerable tissue-specific differences in the extent of age-related methylation.[11] Several factors may modulate age-related methylation, such as exogenous carcinogens, endogenously generated reactive oxygen species, and genetic differences in individual susceptibility.[12] We have recently reported that methylation of *E-cadherin*, *hMLH1* and *p16* was present in non-neoplastic gastric epithelia at variable frequencies, and that the significance of detecting methylation in non-neoplastic gastric epithelia differed among genes.[13, 14]

Death-associated protein kinase (DAP kinase, 160 kDa in size) is a Ca^{2+} calmodulin-regulated serine/threonine kinase that participates in several apoptotic systems initiated by interferon-γ, tumor necrosis factor (TNF)-α, activated Fas, and detachment from the extracellular matrix.[15] Loss of *DAP-kinase* expression associated with its promoter methylation has been reported in various carcinomas, including gastric cancer.[16, 17] The *RUNX3* gene, one of three mammalian runt-related genes, is a recently identified tumor suppressor gene that frequently shows loss of expression due to hemizygous deletion and hypermethylation in gastric cancer.[18, 19] To clarify the physiological consequences of *DAP-kinase* and *RUNX3* promoter methylation in gastric epithelia, the methylation status of these genes was investigated in neoplastic and non-neoplastic gastric epithelia obtained at autopsy and surgery. Gastric cancer cell lines were utilized to confirm the association of methylation status and expression of these genes.

Materials and Methods

Cell lines. Ten gastric cancer cell lines with different histologies were cultured under appropriate conditions in our laboratory; MKN1, an adenosquamous cell carcinoma; MKN7, a well-differentiated adenocarcinoma; MKN28 and MKN74, moderately differentiated adenocarcinomas; MKN45 and KWS-I, poorly differentiated adenocarcinomas; KATO-III, a signet ring cell carcinoma; ECC10 and ECC12, endocrine cell carcinomas; and TSG11, a hepatoid carcinoma. Genomic DNA and mRNA were extracted using standard procedures.

Autopsy samples. The autopsies consisted of 13 males and 6 females, ranging in age from 0.7 to 87 years (56 years on average). Non-neoplastic mucosal tissue samples were obtained from the upper, middle and lower portions of the stomach, as well as from the esophagus, duodenum, jejunum, ileum, colon, rectum, liver, pancreas, lung and kidney at autopsies.

Gastric cancer patients and surgical specimens. Ninety-three gastric cancer samples and matching non-neoplastic gastric tissues were obtained at surgery from 93 patients (68 males and 25 fe-
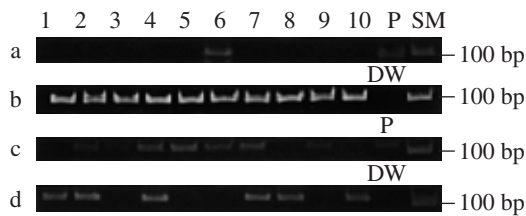
Fig. 1. Methylation-specific PCR (MSP) of gastric cancer cell lines. a. Methylated-sequence-specific PCR for *DAP-kinase*. b. Unmethylated-sequence-specific PCR for *DAP-kinase*. c. Methylated-sequence-specific PCR for *RUNX3*. d. Unmethylated-sequence-specific PCR for *RUNX3*. Methylated products are present in lane 6 for *DAP-kinase* (a), and in lanes 2–7 and 9 for *RUNX3* (c). Unmethylated products are present in all lanes for *DAP-kinase* (b). Unmethylated PCR products are present in lanes 1, 2, 4, 7, 8 and 10 for *RUNX3* (d). Lanes: 1, MKN1; 2, MKN7; 3, MKN28; 4, MKN45; 5, MKN74; 6, KATO-III; 7, ECC10; 8, ECC12; 9, KWS-I; 10, TSG11; P, positive control; DW, distilled water; and SM, size marker.
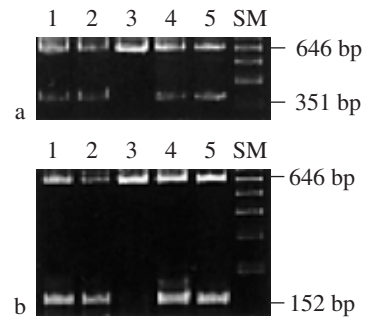

Fig. 3. RT-PCR in gastric cancer cell lines. a. RT-PCR for *DAP-kinase*. b. RT-PCR for *RUNX3*. Expression of *DAP-kinase* (351 bp) is obvious in lanes 1, 2, 4 and 5 (a). Expression of *RUNX3* (152 bp) is evident in lanes 1, 2, 4 and 5 (b). β-Actin (646 bp) serves as an internal control. Lanes: 1, MKN45; 2, KATO-III; 3, KWS-I; 4, ECC12; 5, TSG11; and SM, size marker.
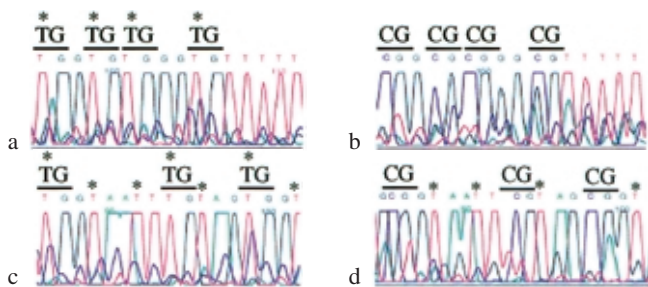

Fig. 2. Sequence analysis of methylated and unmethylated *DAP-kinase* and *RUNX3* alleles. a. Unmethylated *DAP-kinase* promoter of MKN45. b. Methylated *DAP-kinase* promoter of KATO-III. c. Unmethylated *RUNX3* promoter of ECC12. d. Methylated *RUNX3* promoter of ECC10. All CpGs are methylated in b and d, while all CpGs are unmethylated in a and c, resulting in the sequence "TpG" after bisulfite treatment. ∗, converted Ts.

males). The patients ranged in age from 43 to 89 years (average, 66 years). All the patients received a median of 37 months of follow-up care (range, 1–76 months). All samples were snap-frozen and stored at −80°C until processed. The tumors consisted of 33 early and 60 advanced tumors, identified as 52 differentiated and 41 undifferentiated tumor samples by histological examination. Genomic DNA was extracted using standard procedures.

Bisulfite modification and methylation-specific PCR (MSP). Treatment of DNA samples with bisulfite converts all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. The samples were then purified using Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, recovered in ethanol and resuspended in 30 μl of distilled water. Amplification was achieved in a 20-μl reaction volume containing 2 μl of GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA), 1.0 m*M* MgCl₂, 1 μ*M* of each primer, 0.2 m*M* dNTPs, and 1 unit of *Taq* polymerase (Ampli*Taq* Gold DNA Polymerase, PE Applied Biosystems). Hot start PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, followed by a final 7 min extension at 72°C. A positive control and a negative control (distilled water without DNA) were included for each amplification. The PCR products were separated on a 6% non-denaturing polyacrylamide gel.

Primer sequences for MSP were: nucleotides 5 to 28, 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′, and nucleotides 88 to 102, 5′-CCC TCC CAA ACG CCG-3′ (98 bp), for methylated DNA of *DAP-kinase*; nucleotides 2 to 28, 5′-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3′, and nucleotides 87 to 107, 5′-CAA ATC CCT CCC AAA CAC CAA-3′ (106 bp), for unmethylated DNA of *DAP-kinase* (Accession no. X76104); nucleotides 64 917 to 64 940, 5′-ATA ATA GCG GTC GTT AGG GCG TCG-3′, and nucleotides 65 008 to 65 031, 5′-GCT TCT ACT TTC CCG CTT CTC GCG-3′ (115 bp), for methylated DNA of *RUNX3*; and nucleotides 64 917 to 64 940, 5′-ATA ATA GTG GTT GTT AGG GTG TTG-3′, and nucleotides 65 008 to 65 031, 5′-ACT TCT ACT TTC CCA CTT CTC ACA-3′ (115 bp), for unmethylated DNA of *RUNX3* (Accession no. AL023096). All DNA samples were checked for bisulfite modification using the primer set for unmethylated *DAP-kinase*. As a positive control, Sss-I methylase (New England BioLabs, Inc., Beverly, MA) was used to methylate 100 μg of liver tissue-derived DNA obtained from an autopsy sample, and was modified by sodium bisulfite as described above.

Sequence analysis. Methylated and unmethylated PCR products of *DAP-kinase* and *RUNX3* genes in gastric cancer cell lines were sequenced. The PCR products were purified using QIA Quick PCR Purification Kit (QIAGEN, Tokyo). The purified PCR products were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Gel electrophoresis, data collection and analysis were done with a Genetic Analyzer (model 310, PE Applied Biosystems).

Reverse-transcription PCR (RT-PCR). *DAP-kinase* and *RUNX3* transcripts were analyzed by RT-PCR in five (MKN45, KATO-III, ECC12, KWS-I, and TSG11) of the ten gastric cancer cell lines. Isolated RNA from the cell lines was reverse-transcribed and amplified using a ONE-STEP RT-PCR System (Gibco BRL Life Technologies, Gaithersberg, MD). Primer sequences were: sense 5′-GAC CGT GTT CAG GCA GGA-3′ and antisense 5′-TCA GTT GCT TCC TCT TCA GT-3′ (351 bp) for *DAP-kinase* (Accession no. X76104); sense 5′-AGG CAT TGC GCA GCT CAG CGG AGT A-3′ and antisense 5′-TCT GCT CCG TGC TGC CCT CGC ACT G-3′ (152 bp) for *RUNX3* (Accession no. Z38104); and sense 5′-AAA TCT GGC ACC ACA CCT T-3′ and antisense 5′-AGC ACT GTT GGC GTA CAG-3′ for β-actin (646 bp).

Statistical analysis. Statistical comparisons were performed using Fisher's exact test. A value of *P*<0.05 was considered significant. The Kaplan-Meier curve with log rank test and the Breslow-Gehan-Wilcoxon test were used for univariate analysis of event-free survival.

Table 1.   Methylation status of *DAP-kinase* and *RUNX3* in non-neoplastic tissues of autopsies

Gene	Age (y)	Sex[1]	Cause of death[2]	Organs[3]												
				S(U)	S(M)	S(L)	Es	Du	Je	Il	Co	Re	Li	Pa	Lu	Ki
DAP-kinase	0.7	f	COARC		—		—	—	—	—	—	—	—	—	—	—
	8	m	AML	—	—	—	—	—	—	—	—	—	—	—	—	—
	17	m	ALL	—	—	—	—	—	—	—	—	—	—	—	—	—
	22	m	Brain tumor	—	—	—	—	M	M	M	—	M	—	—	—	—
	45	m	Liver cancer	M	M	M	—	—	—	—	M	M	—	—	—	—
	47	m	Brain tumor	—	M	—	—							—	—	—
	49	m	Lung cancer		—		—								—	—
	55	m	Rectal cancer	M	M	M	—		—			M	—	—	—	—
	59	m	Rectal cancer	—	—	—	—	M	M				—	—	—	—
	60	f	Lung cancer	M	M	M	—	—	M	M	M	—	—	—	—	—
	67	m	Pancreas cancer	M	M	M	—	M	M	M	—		—	—	—	—
	68	f	Ovarian cancer	M	M	M	—					—	—	—	M	—
	69	f	Ovarian cancer	M	M	M	—	M	M		—		—		—	—
	77	m	Lung cancer	M	M	M	—	—	M	—	M		—	—	—	—
	78	m	Lung cancer	M	M	M	—	—	—	M	M		—	—	—	—
	78	m	Aneurysm	M	M	M	—	—	—		—		—	—	—	—
	82	m	Parkinson's disease	M	M	M	—	M	M	M	M	M	—	—	—	—
	86	f	Colon cancer		M		M				M	M	—	—	—	—
	87	f	Lung cancer	M	M		—	M	M	M	M		—	—	—	—
RUNX3	0.7	f	COARC		—		—	—	—	—	—	—	—	—	—	—
	8	m	AML	—	—	—	—	—	—	—	—	—	—	—	—	—
	17	m	ALL	—	—	—	—	—	—	—	—	—	—	—	—	—
	22	m	Brain tumor	—	—	—	—	—	—	—	—	—	—	—	—	—
	45	m	Liver cancer	—	—	—	—	—	—	—	—	—	M	—	—	—
	47	m	Brain tumor	—	—	—	—	—	—	—	—	—	—	—	—	—
	49	m	Lung cancer		—		—		—						—	—
	55	m	Rectal cancer	—	—	—	—		—			—	—	—	—	—
	59	m	Rectal cancer	—	—	—	—	—		—		—	—	—	—	—
	60	f	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	67	m	Pancreas cancer	—	—	—	—	M	—	—	—	—	—	—	—	—
	68	f	Ovarian cancer	—	—	—	—					—	—	—	—	—
	69	f	Ovarian cancer	—	—	—	—	—	—		—		—		—	—
	77	m	Lung cancer	—	—	M	—	—	—	—	—	—	—	—	—	—
	78	m	Lung cancer	—	—	M	—	—	—	—	—	—	—	—	—	—
	78	m	Aneurysm	—	—	M	—	—	—		—		—	—	—	—
	82	m	Parkinson's disease	—	—	M	—	—	—	—	—	—	—	—	M	—
	86	f	Colon cancer		—		—				—		—	—	—	—
	87	f	Lung cancer	—	—	M	—	—	—	—	—	—	—	—	—	—

1) f, female; m, male.
2) COARC, coarctation of aorta; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia.
3) S(U), upper third portion of the stomach; S(M), middle third portion of stomach; S(L), lower third portion of stomach; Du, duodenum; Je, jejunum; Il, ileum; Co, colon; Re, rectum; Li, liver; Pa, pancreas; Lu, lung; Ki, kidney.

Results

Methylation status and expression of *DAP-kinase* and *RUNX3* mRNA in gastric cancer cell lines. Among the ten gastric cancer cell lines in which *DAP-kinase* and *RUNX3* promoter methylation was investigated, the presence of methylated *DAP-kinase* promoter alleles was detected only in the KATO-III cell line, together with unmethylated alleles. Methylated alleles of *RUNX3* were detected in seven (MKN7, MKN28, MKN45, MKN74, KATO-III, ECC10, and KWS-I) of the ten cell lines, three (MKN7, MKN45, and ECC10) of which also exhibited unmethylated alleles (Fig. 1). Sequence analysis revealed methylation of all the CpGs in the *DAP-kinase* and *RUNX3* promoters in the methylated PCR products of these cell lines (Fig. 2). Because all ten cell lines exhibited unmethylated *DAP-kinase* alleles (together with methylated alleles in KATO-III), all (MKN45, KATO-III, ECC12, and TSG11) but one (KWS-I) of the cell lines analyzed by RT-PCR expressed a significant amount of *DAP-kinase* mRNA (Fig. 3). For *RUNX3*, the mRNA was not detected in KWS-I, which had a methylated *RUNX3* promoter, but it was expressed in MKN45, ECC12 and TSG11, which contained unmethylated *RUNX3* promoters (MKN45 had both methylated and unmethylated *RUNX3* alleles). KATO-III expressed *RUNX3* mRNA, although the *RUNX3* promoter was methylated in this cell line (Fig. 3).

Methylation status in autopsy samples. Methylation of the *DAP-kinase* promoter was not detected in non-neoplastic gastric epithelia obtained from individuals who were aged 22 years or below (0/4). In contrast, methylated *DAP-kinase* was detected in non-neoplastic gastric epithelia in 87% (13/15) of individuals who were aged 45 years or more (Table 1 and Fig. 4). Methylation of the *RUNX3* promoter was present in non-neoplastic gastric epithelia in 16% (3/19) of the individuals, and all individuals showing methylation of *RUNX3* were aged 77 years or older (Table 1).

Methylation status in neoplastic and non-neoplastic gastric epithelia of gastric cancer patients. Methylation of *DAP-kinase* was present in 43% (40/93) of neoplastic and 73% (68/93) of non-neoplastic gastric epithelia (Fig. 5). *DAP-kinase* methylation was significantly more frequent in undifferentiated tumors than
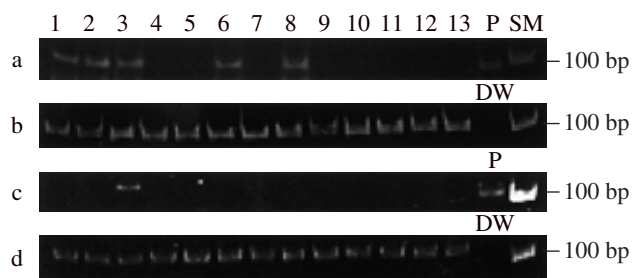
Fig. 4. Methylation-specific PCR (MSP) of autopsy samples (77-year-old male, see Table 1). a. Methylated-sequence-specific PCR for *DAP-kinase*. b. Unmethylated-sequence-specific PCR for *DAP-kinase*. c. Methylated-sequence-specific PCR for *RUNX3*. d. Unmethylated-sequence-specific PCR for *RUNX3*. Methylated PCR products are present in lanes 1–3, 6 and 8 for *DAP-kinase* (a), and in lane 3 for *RUNX3* (c). Unmethylated *DAP-kinase* and *RUNX3* PCR products are present in all the samples (b and d). Lanes: 1, stomach (upper); 2, stomach (middle); 3, stomach (lower); 4, esophagus; 5, duodenum; 6, jejunum; 7, ileum; 8, colon; 9, rectum; 10, liver; 11, pancreas; 12, lung; 13, kidney; P, positive control; DW, distilled water; and SM, size marker.

Fig. 5. Methylation-specific PCR (MSP) of samples from patients with stomach cancer. a. Methylated-sequence-specific PCR for *DAP-kinase*. b. Unmethylated-sequence-specific PCR for *DAP-kinase*. c. Methylated-sequence-specific PCR for *RUNX3*. d. Unmethylated-sequence-specific PCR for *RUNX3*. Methylated PCR products are present in T and N of M105 and M254, in N of M123 and 170, and in T of 174 for *DAP-kinase* (a), in T and N of M123, and in T of M105, 174, 218 and 254 for *RUNX3* (c), unmethylated *DAP-kinase* and *RUNX3* PCR products are present in all the samples (b and d). Lanes: T, tumor DNA; N, normal DNA; P, positive control; DW, distilled water; and SM, size marker.

in differentiated ones ($P<0.05$). Methylation of *RUNX3* was present in 45% (42/93) of neoplastic and 8% (7/93) of non-neoplastic gastric epithelia (Fig. 5). No significant correlation between methylation status of *RUNX3* and clinicopathological characteristics was observed. Incidences of *DAP-kinase* and *RUNX3* methylation differed significantly in non-neoplastic gastric epithelia ($P<0.01$), although those in gastric cancers were almost the same. These results are summarized in Table 2.
Survival analysis of gastric cancer patients. The methylation status of *DAP-kinase* and *RUNX3* genes did not significantly influence event-free survival rates, as analyzed by the Kaplan-Meier curve with log rank test and Breslow-Gehan-Wilcoxon test (data not shown).

Discussion

Among ten gastric cancer cell lines tested for *DAP-kinase* and *RUNX3* methylation in the present study, a methylated *DAP-kinase* promoter was discovered only in the KATO-III cell line. However, the *DAP-kinase* mRNA was expressed, because of the concomitant presence of unmethylated promoter alleles. Seven of the ten cell lines had a methylated *RUNX3* promoter, and its methylation status was consistent with *RUNX3* mRNA

Table 2. Correlation of methylation status and clinicopathological characteristics in gastric cancer

Clinicopathological characteristics (*n*)	Promoter methylation status			
	DAP-kinase		*RUNX3*	
	M (40)[1]	U (53)[1]	M (42)	U (51)
Sex				
Male (68)	27	41 ⎤	33	35 ⎤
Female (25)	13	12 ⎦ NS[2]	9	16 ⎦ NS
Age				
≤50 (11)	6	5 ⎤	4	7 ⎤
50< (78)	31	47 ⎦ NS	37	41 ⎦ NS
Unknown (4)	3	1	1	3
Location				
Upper third (17)	8	9 ⎤	5	12 ⎤
Middle third (33)	13	20 ⎥ NS	17	16 ⎥ NS
Lower third (37)	13	24 ⎦	18	19 ⎦
Unknown (6)	6	0	2	4
Histological type				
Differentiated (52)	17	35 ⎤	21	31 ⎤
Undifferentiated (41)	23	18 ⎦ $P<0.05$	21	20 ⎦ NS
Stage				
Early (33)	16	17 ⎤	19	14 ⎤
Advanced (60)	24	36 ⎦ NS	23	37 ⎦ NS
Lymph node metastasis				
Present (48)	17	31 ⎤	21	27 ⎤
Absent (44)	22	22 ⎦ NS	21	23 ⎦ NS
Unknown (1)	1	0	0	1

1) M, methylated; U, unmethylated.
2) NS, not significant.

expression, except in the case of KATO-III. The inability to detect *DAP-kinase* mRNA in KWS-I with an unmethylated promoter suggests that other mechanisms besides promoter methylation might contribute to the silencing of *DAP-kinase* in this cell line. Our present results regarding *RUNX3* methylation in gastric cancer cell lines are consistent with a previous report,[19] in which loss of *RUNX3* expression was completely in agreement with its promoter methylation status in gastric cancer cell lines. In the present study, we analyzed similar CpG islands to those in the previous study.[19] However, KATO-III expressed *RUNX3* mRNA despite its methylated promoter. Therefore, it is possible that other CpG islands in the *RUNX3* promoter are more critical for silencing of this gene.

In our recent study, we found that *E-cadherin* and *p16* methylation in non-neoplastic cells was apparently age-related and preferentially occurred in the stomach in autopsy samples.[14] On the other hand, autopsy samples indicated that methylation of *hMLH1* was exceptional in any organ from any age group.[14] Thus, susceptibility to age-related methylation differs considerably among genes. In the present study, we found that *DAP-kinase* and *RUNX3* methylation, like that of *E-cadherin* and *p16*, was also age-related. The methylation patterns of *E-cadherin* and *DAP-kinase* in non-neoplastic epithelia are almost the same, and methylation frequently appears at around age 45.[14] For *RUNX3*, methylation was restricted to individuals who were aged 77 years or older. Although the frequencies of methylation of *DAP-kinase* and *RUNX3* in primary gastric cancers were similar, their frequencies in non-neoplastic gastric epithelia differed significantly. Such differences in methylation patterns may correspond to type A (aging-specific) and type C (cancer-specific) methylation previously described for gastric

and colorectal cancers.[20, 21] However, *hMLH1* methylation, which was thought to be cancer-specific,[20] was found to be a common age-related event in normal colonic mucosa, when the entire *hMLH1* promoter ~700-bp region was analyzed.[22] *hMLH1* methylation is partial in normal colonic mucosa and increases with age; it spreads to reach a threshold, and ultimately shuts down protein expression.[22] Therefore, these contradictory results might have been due to analysis of different CpG sites in these studies.[22] In addition, it has recently been reported that *DAP-kinase* methylation was present in virtually every tumor and normal gastric and colorectal sample when the edge of CpG islands was examined, although it turned out as a more infrequent, cancer-specific phenomenon when the central region of the CpG islands was analyzed.[23] Therefore, the significance of the detection of methylated gene might depend on the position of the examined CpG sites. If critical CpG sites for each gene silencing are more precisely analyzed, apparently age-related methylation may prove to be cancer-specific.

We also found that *DAP-kinase* and *RUNX3* methylation occurred most preferentially in non-neoplastic gastric epithelia among non-neoplastic cells of various organs that were obtained at autopsy. The mechanism that underlies this tissue specificity is unknown. The antral location of the stomach is susceptible to methylation of several tumor suppressor and tumor-related genes.[24, 25] Because intestinal metaplasia, especially that of the incomplete type, commonly arises in the antrum and then expands toward the body of the stomach, intestinal metaplasia may predispose to promoter methylation of these genes. Actually, among autopsy samples, *RUNX3* methylation in non-neoplastic gastric epithelia was detected only in the lower portion of the stomachs from very old individuals in the present study.

In conclusion, *RUNX3* methylation is mostly cancer-specific, except for very old individuals, and therefore may be a possible molecular diagnostic marker and malignancy predictor.

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