Spreading of methylation within *RUNX3* CpG island in gastric cancer

Naoyuki Homma,[1,2] Gen Tamura,[1,5] Teiichiro Honda,[1,2] Yutaka Matsumoto,[1] Satoshi Nishizuka,[3,4] Sumio Kawata[2] and Teiichi Motoyama[1]

[1]Departments of Pathology and [2]Gastroenterology, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan, [3]Molecular Therapeutics Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA, and [4]Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, National Cancer Institute at Frederick, Frederick, MD 21702, USA

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***RUNX3* is a novel tumor suppressor gene that is frequently silenced by promoter hypermethylation in gastric cancer. The methylation status of multiple regions within the *RUNX3* promoter CpG island (3478 bp) was examined in gastric cancer cell lines, primary gastric cancers and non-neoplastic gastric mucosa to clarify how methylation spreads within the CpG island. The critical regions for *RUNX3* silencing were evaluated by analysis of cell lines. The most 5′ region of the CpG island was methylated in 90% (9/10) of gastric cancer cell lines, 96% (43/45) of primary gastric cancers and in 96% (43/45) of non-neoplastic gastric mucosa. The frequencies of methylation were less near the transcription start site and were 40% (4/10) in cell lines, 53% (24/45) in primary gastric cancers and 11% (5/45) in non-neoplastic gastric mucosa, where methylation was proven to be critical for gene silencing. Thus, hypermethylation initially occurs at the most 5′ region of the *RUNX3* CpG island and spreads to the transcription start site before ultimately shutting down *RUNX3* mRNA expression. The detection of hypermethylation at multiple regions within the *RUNX3* CpG island may be useful in the diagnosis and risk assessment of gastric cancer. (*Cancer Sci* 2006; 97: 51–56)**

DNA methylation is an epigenetic modification that introduces 5′-methylcytosine, with 70–80% of CpG dinucleotides being heavily methylated in human cells.[1] However, CpG islands are protected from methylation and approximately 60% of human genes are associated with a CpG island.[2] Unmethylated CpG islands may become methylated in cancer cells with resultant loss of gene function.[2] Promoter methylation prevents the binding of methylation-sensitive transcription factors and results in suppression of transcription.[3,4] Recent studies have demonstrated that promoter methylation of tumor suppressor and tumor-related genes occurs not only in tumor cells but may also be evident in non-neoplastic cells during aging or following exposure to certain environmental factors.[5–7]

The human runt-related transcription factors (RUNXs) are an important target of transforming growth factor (TGF)-β superfamily signaling[8] and three different *RUNX* genes have been identified that are human homologs of the Drosophila genes runt and lozenge.[9] *RUNX1* (*PEBP2αB*/*CBFA2*/*AML1*) is required for definitive hematopoiesis[10] and its alteration by chromosomal translocation was observed in approximately 30% of cases of human acute leukemia.[11] *RUNX2* (*PEBP2αA*/*CBFA1*/*AML3*) is essential for osteogenesis and is associated with the human bone disease cleidocranial dysplasia.[12] *RUNX3* (*PEBP2αC*/*CBFA3*/*AML2*) was originally cloned as *AML2* and is localized on chromosome 1p36.1.[13] RUNX3 protein combines with Smads and acts synergistically to regulate various target genes.[8] *RUNX3* has two promoters, and the proximal promoter is almost ubiquitously expressed, including for the stomach, whereas the distal promoter is expressed in a few tissues such as thymus and ovary.[14] Little or no expression of *RUNX3* due to CpG island hypermethylation was observed in gastric cancer,[15] or in carcinoma of the liver, lung, breast, prostate, endometrium or colon.[16] The gastric epithelium of *RUNX3* null mice displays hyperplasia and epithelial proliferation with suppression of TGF-β-induced apoptosis.[15]

In the present study the methylation status of multiple regions of the *RUNX3* promoter CpG island (3478 bp) within the proximal promoter was examined in gastric cancer cell lines, primary gastric cancers and non-neoplastic gastric mucosa to clarify how methylation spreads within the CpG island. The critical regions required for *RUNX3* silencing were evaluated by analysis of cell lines.

Materials and Methods

Gastric cancer cell lines

Ten gastric cancer cell lines derived from tumors with various histologies were used for methylation analysis and included: 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; TSG11, a hepatoid carcinoma; and ECC10 and ECC12, endocrine cell carcinomas. Figure 1 shows the *RUNX3* CpG island and analyzed regions. All cell lines were cultured under appropriate conditions in our laboratory.

[5]To whom correspondence should be addressed.
E-mail: gtamura@med.id.yamagata-u.ac.jp
Abbreviations: 5-aza-dC, 5′-aza-2′-deoxycytidine; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; TGF, transforming growth factor.

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Primary gastric cancers

A total of 45 gastric cancer samples (18 early and 27 advanced stages; 13 differentiated and 32 undifferentiated carcinomas) and corresponding non-neoplastic gastric mucosa were studied. The patients were 28 males and 17 females (mean age 63 years; range 30–85 years). All the samples were obtained by surgical resection and stored at –80°C until examination.

DNA and RNA extraction

DNA was extracted using SepaGene (Sanko-Junyaku, Tokyo, Japan) from the gastric cell lines and gastric cancer samples as well as the corresponding non-neoplastic gastric mucosa. RNA was extracted using Trizol reagent (Gibco BRL Life Technologies, Gaithersburg, MD, USA) from the gastric cell lines.

Bisulfite modification and methylation-specific polymerase chain reaction (PCR)

DNA samples were treated with bisulfite to convert all unmethylated cytosines to uracils whilst leaving methylated cytosines unaffected. Briefly, 2 μg of genomic DNA was denatured by NaOH treatment and modified by sodium bisulfite.[17] The samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, USA), treated with NaOH, recovered in ethanol and resuspended in 30 μL of distilled water. Amplification was carried out in a 20 μL reaction volume containing 2 μL 10 × PCR buffer with 15 mM $MgCl_2$ (Qiagen, Hilden, Germany), 4 μL 5 × Q-Solution (Qiagen), 10 pM of each primer, 0.2 mM dNTPs and 0.75 U Taq polymerase (HotStar Taq DNA polymerase; Qiagen). After the mixture was heated at 95°C for 15 min, PCR was performed in a thermal cycler (GeneAmp 2400; PE Applied Biosystems, Foster City, CA, USA) for 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (58°C for No. 5–8) for 60 s, and extension at 72°C for 60 s, followed by a final 10 min extension at 72°C. A positive control (Sss I-treated DNA) and negative control (distilled water without DNA) were included in each amplification reaction. The PCR products were separated on a 6% non-denaturing polyacrylamide gel. The following primer sets were used (GenBank accession number AL023096) (Fig. 1): 5′-AAC GTT TTC GAG AAG GCG TAG CGC-3′ and 5′- CAC GAT ACA AAC CGA AAC CAT TCG-3′ for methylated *RUNX3* (No. 1; 154 bp); 5′-TTT TAG ATT TTG GGG AAC GAA CGC-3′ and 5′-TAA TAA AAT CTT ACG ACC ACC GTC-3′ for methylated *RUNX3* (No. 2; 161 bp); 5′-TAC GGG ATT TTG CGC GTC GTT TAC-3′ and 5′-AAA AAC TCC CTT CCG CCT ATC CCC-3′ for methylated *RUNX3* (No. 3; 123 bp); 5′-TTG TTT AGA ACG TTC GGG TTT TAC-3′ and 5′-AAA ACG ACT CCC AAT ACG ACG TCA CC-3′ for methylated *RUNX3* (No. 4; 215 bp); 5′-GAT TTC GCG GTC GTA GTT TTA GAA TAA ATT T-3′ and 5′-ACT AAA ACC TCC TCC GCG AAA TAA CGC CTT CC-3′ for methylated *RUNX3* (No. 5; 244 bp); 5′-TAT TCG TTA GGG TTC GTT CGT TGC-3′ and 5′- ACG ACC GCG AAC GAA CTT CGA AAC-3′ for methylated *RUNX3* (No. 6; 201 bp); 5′- ATA ATA GCG GTC GTT AGG GCG TCG-3′ and 5′-GCT TCT ACT TTC CCG CTT CTC GCG-3′ for methylated *RUNX3* (No. 7; 115 bp); 5′-CGG TGC GTA CGA GTT CGT TTG CG-3′ and 5′-GTA AAC CCA AAC ACC AAC CGC CGC TTC A-3′ for methylated *RUNX3* (No. 8; 196 bp); 5′-AGG GCG TAT TTA AAA CGG AAC GTC-3′ and 5′-AAC GCC GAC CCT AAA ACT CCG AAC-3′ for methylated *RUNX3* (No. 9; 164 bp); 5′-GAC GTA TAA TAT TTT CGA GTA AAC-3′ and 5′-ACA CCC CTC CTC CCG CGC CGC TTC-3′ for methylated *RUNX3* (No. 10; 132 bp); 5′- TGG GAA TGT TTT TGA GAA GGT GTA GTG T-3′ and 5′-CAC AAT ACA AAC CAA AAC CAT TCA-3′ for unmethylated *RUNX3* (No. 1; 158 bp); 5′-TTT TAG ATT TTG GGG AAT GAA TGT-3′ and 5′- TAA TAA AAT CTT ACA ACC ACC ATC-3′ for unmethylated *RUNX3* (No. 2; 161 bp); 5′-TAT GGG ATT TTG TGT GTT GTT TAT-3′ and 5′-AAA AAC TCC CTT CCA CCT ATC CCC-3′ for unmethylated *RUNX3* (No. 3; 123 bp); 5′-AAA TTT GTT TAG AAT GTT TGG GTT TTA T-3 and 5′-TAA AAC AAC TCC CAA TAC AAC ATC ACC-3′ for unmethylated *RUNX3* (No. 4; 220 bp); 5′-GAT TTT GTG GTT GTA GTT TTA GAA TAA ATT T-3′ and 5′-ACT AAA ACC TCC TCC ACA AAA TAA CAC CTT CC-3′ for unmethylated *RUNX3* (No. 5; 244 bp); 5′-TAT TTG TTA GGG TTT GTT TGT TGT-3′ and 5′-ACA ACC ACA AAC AAA CTT CAA AAC-3′ for unmethylated *RUNX3* (No. 6; 201 bp); 5′-ATA ATA GTG GTT GTT AGG GTG TTG-3′ and 5′-ACT TCT ACT TTC CCA CTT CTC ACA-3′ for unmethylated *RUNX3* (No. 7; 115 bp); 5′-GTT GGT GTG TAT GAG TTT GTT TGT G-3′ and 5′-CAT AAA CCC AAA CAC CAA CCA CCA CTT CA-3′ for unmethylated *RUNX3* (No. 8; 199 bp); 5′-AGG GTG TAT TTA AAA TGG AAT GTT-3′ and 5′- AAC ACC AAC CCT AAA ACT CCA AAC-3′ for unmethylated *RUNX3* (No. 9; 164 bp); 5′-TGG GAT GTA TAA TAT TTT TGA GTA AAT-3′ and 5′-CCT CCA CCT CCA ACA CCC CTC CTC CCA C-3′ for unmethylated *RUNX3* (No. 10; 147 bp).

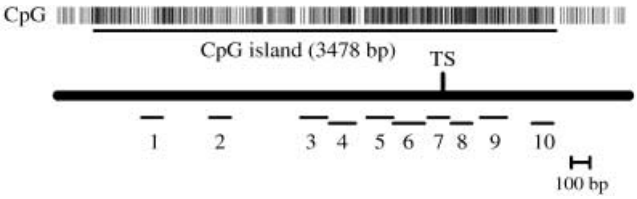

Fig. 1. *RUNX3* CpG island and analyzed regions (No. 1–10). CpG sites are shown as vertical bars. Transcriptional start site (TS) is located within region No. 7.

Reverse transcription PCR (RT-PCR)

RT-PCR was performed in 10 gastric cell lines. Isolated RNA was reverse-transcribed and amplified using a One-Step RT-PCR System (Gibco BRL). The following primer set was used: 5′-AGG CAT TGC GCA GCT CAG CGG AGT A-3′ and 5′-TCT GCT CCG TGC TGC CCT CGC ACT G-3′ for *RUNX3* (152 bp) (GenBank accession numbers Z38104 and Z38105); and 5′-AAA TCT GGC ACC ACA CCT T-3′ and 5′-AGC ACT GTT GGC GTA CAG-3′ for β-actin (646 bp).

5′-aza-2′-deoxycytidine (5-aza-dC) treatment

To investigate the restoration of *RUNX3* mRNA expression, four cell lines (MKN28, MKN74, KATO-III and KWS-1) were incubated for 24 h with 5 μmol/L 5-aza-dC (Sigma, St Louis, MO, USA), and then harvested for RNA extraction and RT-PCR. MKN45, which expresses *RUNX3* mRNA with an unmethylated CpG island, was used as a control.

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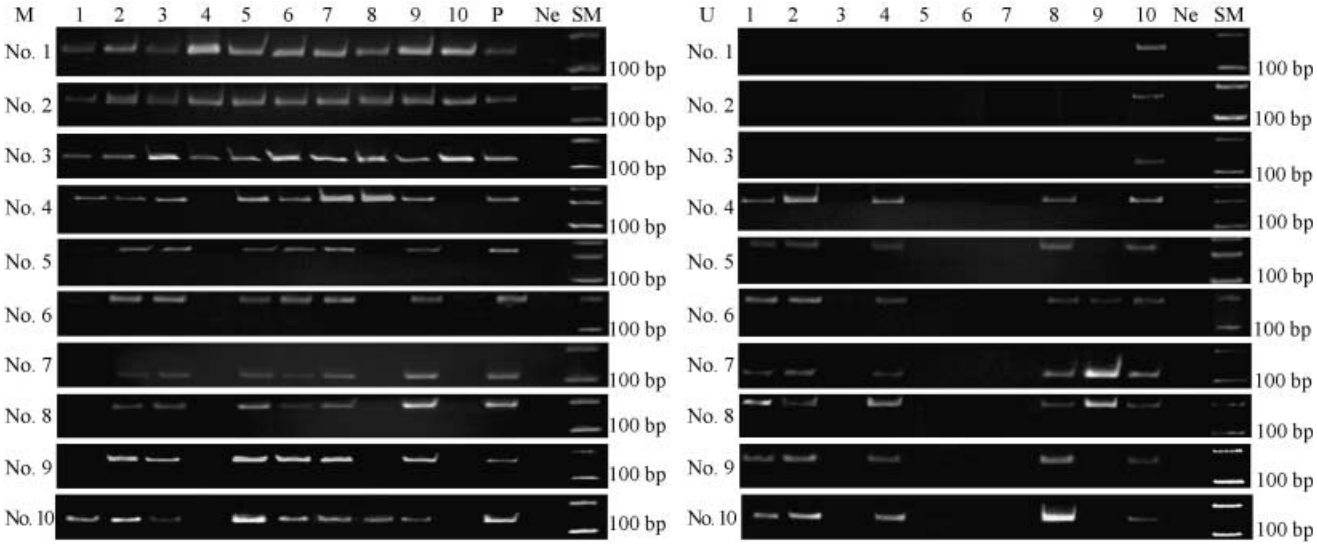

Fig. 2. Representative illustrations of methylation-specific polymerase chain reaction (PCR) at multiple regions (No. 1–10) of the *RUNX3* CpG island in gastric cancer cell lines. Lanes: 1, MKN1; 2, MKN7; 3, MKN28; 4, MKN45; 5, MKN74; 6, KATO-III; 7, KWS-1; 8, TSG11; 9, ECC10; 10, ECC12. M, methylated-sequence-specific PCR for *RUNX3*; Ne, negative control; P, positive control; SM, size marker; U, unmethylated-sequence-specific PCR for *RUNX3*.

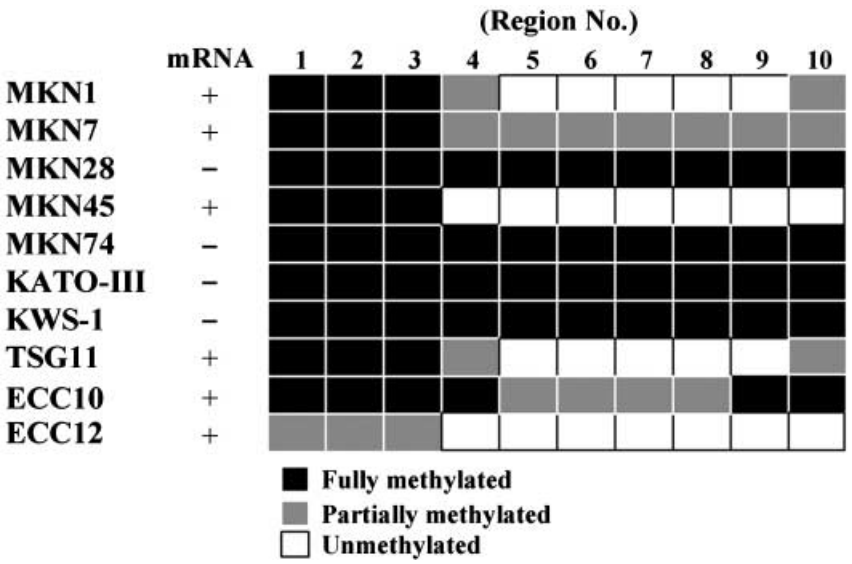

Fig. 3. Summary of methylation and expression status of *RUNX3* in gastric cancer cell lines.

Statistical analysis

Statistical analysis was performed using Fisher's exact probability test and Mann–Whitney's *U*-test. *P* values of less than 0.05 were considered to be statistically significant.

Results

Methylation and expression status of *RUNX3* in gastric cancer cell lines

Four (MKN28, MKN74, KATOIII and KWS-1) of the 10 gastric cancer cell lines were fully methylated at all the regions studied (Figs 2 and 3). These cell lines exhibited a loss of *RUNX3* mRNA expression that was restored following treatment with 5′-aza-dC (Fig. 4). The other six (MKN1, MKN7, MKN45, TSG11, ECC10, ECC12) cell lines were either partially methylated or unmethylated at regions No. 5–8 which spanned the transcription start site, and expressed *RUNX3* mRNA (Figs 2 and 3). The 5′ regions were generally more heavily methylated in all of the cell lines except ECC12 (Figs 2 and 3). Thus, the critical region for *RUNX3* gene silencing lies between regions No. 5–8 spanning the transcription start site.

Methylation status of *RUNX3* in primary gastric cancers and non-neoplastic gastric mucosa

Hypermethylation was detected in various regions of the primary gastric cancers and corresponding non-neoplastic gastric mucosa at the following respective frequencies:

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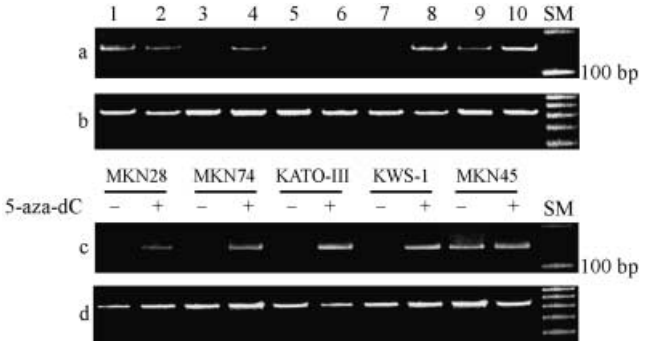

Fig. 4. Results of reverse transcription–polymerase chain reaction (RT-PCR) in gastric cancer cell lines. (a) RT-PCR for *RUNX3* in gastric cancer cell lines before 5′-aza-2′-deoxycytidine (5′-aza-dC) treatment. (b) RT-PCR for *β-actin* in gastric cancer cell lines before 5′-aza-dC treatment. (c) RT-PCR for *RUNX3* in gastric cancer cell lines before and after 5′-aza-dC treatment. (d) RT-PCR for *β-actin* in gastric cancer cell lines before and after 5′-aza-dC treatment. Lanes: 1, MKN1; 2, MKN7; 3, MKN28; 4, MKN45; 5, MKN74; 6, KATO-III; 7, KWS-1; 8, TSG11; 9, ECC10; 10, ECC12. *RUNX3* mRNA is present in lanes 1, 2, 4, 8, 9 and 10 before 5′-aza-dC treatment (a), and *RUNX3* mRNA is restored after 5′-aza-dC treatment (c).

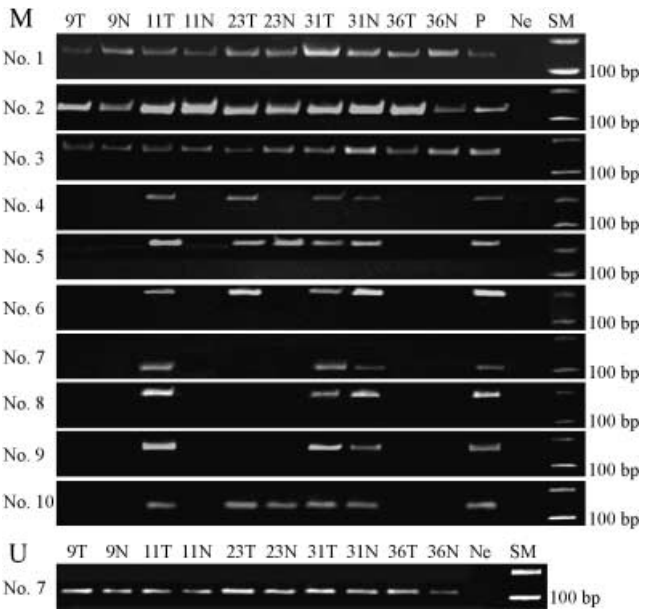

Fig. 5. Representative illustration of methylation-specific polymerase chain reaction (PCR) at multiple regions (No. 1–10) of the *RUNX3* CpG island in primary gastric cancers and corresponding non-neoplastic gastric mucosa. M, methylated-sequence-specific PCR for *RUNX3*; N, non-neoplastic gastric mucosa; Ne, negative control; P, positive control; SM, size marker; T, tumor; U, unmethylated-sequence-specific PCR for *RUNX3*.

95.6% (43/45) and 95.6% (43/45) at No. 1 (the most 5′ region); 95.6% (43/45) and 95.6% (43/45) at No. 2; 93.3% (42/45) and 95.6% (43/45) at No. 3; 71.1% (32/45) and 46.7% (21/45) at No. 4; 68.9% (31/45) and 46.7% (21/45) at No. 5; 60.0% (27/45) and 11.1% (5/45) at No. 6; 53.3% (24/45) and 11.1% (5/45) at No. 7 (spanning the transcription start site); 53.3% (24/45) and 13.3% (6/45) at No. 8; 55.6% (25/45) and 17.8% (8/45) at No. 9 and 68.9% (31/45) and 31.1% (14/45) at No. 10 (Figs 5 and 6). No significant correlation was found between *RUNX3* methylation status and various clinicopathological features of primary gastric cancers including gender, age, tumor location, tumor stage, histological differentiation or the presence of lymph node metastasis, lymphatic permeation or venous permeation (data not shown). *RUNX3* hypermethylation (regions Nos 6–8) in non-neoplastic gastric mucosa was significantly more frequent in older patients ($P < 0.05$; data not shown).

Discussion

Our data suggest that methylation at the regions spanning the transcription start site (Nos 5–8) is critical for loss of *RUNX3* expression. Dense methylation spanning the transcription start site is closely associated with gene silencing, whereas methylation at the 5′ and 3′ outskirt regions, which are more prone to undergo methylation, was not associated with gene silencing. The majority or all CpGs, including those near the transcription start site of *hMLH1*, were methylated in colon cancer cell lines exhibiting loss of hMLH1 expression.[18] In contrast, CpGs near the transcription start site were unmethylated in colon cancer cell lines expressing hMLH1 with only 5′ outskirt regions being methylated.[18] The non-neoplastic colonic mucosa from colon cancer patients whose tumors exhibited microsatellite instability (MSI), exhibited methylation of all CpGs including those near the transcription start site. Methylation at the 5′ outskirt regions only was found in cases where tumors did not exhibit MSI.[19] Thus, methylation initially occurs in the 5′ outskirt regions of CpG islands in non-neoplastic cells and subsequently spreads to involve the transcription start site prior to shutting down gene expression. This pattern of methylation spreading may be common in tumor suppressor and tumor-related genes.[20]

In the present study, we found different methylation frequencies between primary gastric cancers and corresponding non-neoplastic gastric mucosa at the regions spanning the transcription start site, although they were similarly high at the 5′ outskirt regions. In a previous study, we reported that *RUNX3* methylation was observed in 45% (42/93) of primary gastric cancers and 8% (7/93) of non-neoplastic gastric mucosa.[21] Other investigators reported that *RUNX3* methylation was observed in 71% (57/80) of primary gastric cancers and 84% (38/45) of non-neoplastic gastric mucosa,[22] and in 64% (48/75) of primary gastric cancers.[16] These differences in methylation frequency can be explained by the fact that different regions were examined within the *RUNX3* CpG island.

The lower frequency of methylation near the transcription start site suggests that physiological mechanisms exist to protect the regions near the transcription start site from undergoing methylation. *RUNX3* has two promoters and the distal promoter does not contain a CpG island, whereas the proximal promoter has a CpG island that includes binding sites for Sp1.[23] Sp1 elements locate upstream and downstream of the transcription start site and have been shown to protect CpG islands from methylation.[24] Other mechanisms to protect the spreading of methylation may also exist.

RUNX3 methylation in non-neoplastic gastric mucosa was age-related and this is similar to our previous finding in which methylation was observed in people older than 70 years.[7] Age-related methylation in non-neoplastic gastric

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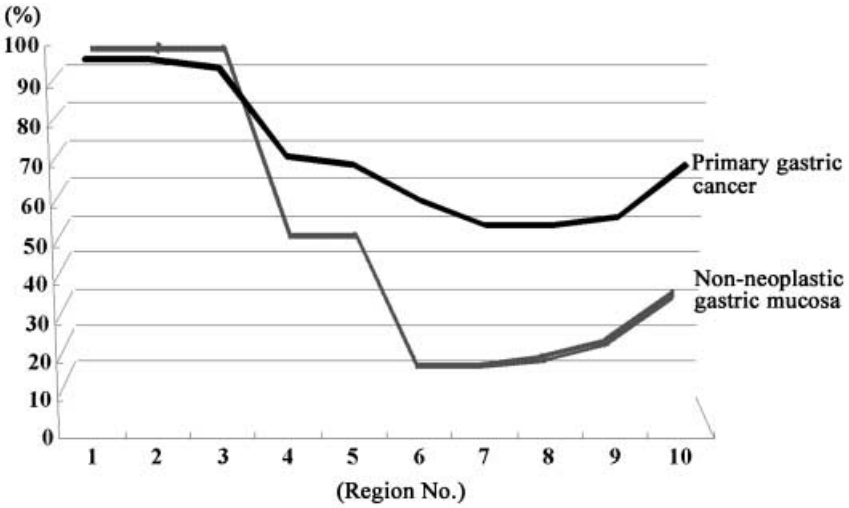

Fig. 6. Percentages of methylation at multiple regions (No. 1–10) of the *RUNX3* CpG island in primary gastric cancer and non-neoplastic gastric mucosa.

mucosa was also reported for *hMLH1* and *p16*.[7] Furthermore, methylation in non-neoplastic gastric mucosa might be accelerated by *Helicobacter pylori* infection.[25] The accumulation of methylation in tumor suppressor and tumor-related genes may constitute a field defect that may predispose to the evolution of gastric cancer.[26] Our present study demonstrates that the significance of methylation detection depends on the genetic region analyzed. Analysis at the 5′ outskirt regions in the CpG island is not informative. In contrast, methylation spanning the transcription start site (Nos 6–8), which is predominantly cancer-specific, can be used as a diagnostic marker of gastric cancer using samples of gastric biopsies, gastric juice and ascites. Furthermore, detection of methylation adjacent to such regions (Nos 4, 5 and 9) may be used as an early harbinger of gastric cancer development.

In conclusion, our present results suggest that methylation at the regions spanning the transcription start site within the *RUNX3* promoter CpG island is critical for gene silencing. In addition, analysis at multiple regions of the *RUNX3* CpG island can be used in the diagnosis and risk assessment of gastric cancer.

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