Quantitative Analysis of Tumor-derived Methylated *RUNX3* Sequences in the Serum of Gastric Cancer Patients

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Abstract. *Purpose and Experimental Design: Using real-time quantitative methylation-specific PCR (RTQ-MSP), methylated RUNX3 sequences were quantified and the fractional concentrations of circulating tumor DNA in serum were determined, along with peripheral blood cells collected preoperatively, intraoperatively and postoperatively from 65 patients with gastric cancer. Results: RTQ-MSP was sufficiently sensitive to detect RUNX3 methylation. Quantitative MSP data were expressed in terms of the methylation index, which was defined as the relative amount of methylated RUNX3 sequences divided by the concentration of methylated actin. High levels of methylated RUNX3 sequences were detected in the peripheral circulation of 29% (19 of 65) of the gastric cancer patients. The RUNX3 methylation index was concordant with cancer stage, histology, lymphatic and vascular invasion, and was more sensitive than carcinoembryonic antigen (CEA) as a biomarker. Twenty-nine percent (19 out of 65) of preoperative serum samples had methylated RUNX3 sequences, ranging from 5.2 to 1625955 (median quantity=43 m-index, sensitivity 95.5%, specificity 62.5%, AUC 0.8651). After surgical resection, the median RUNX3 methylation index in serum significantly decreased. These results demonstrate the clinical usefulness and effectiveness of peripheral blood RTQ-MSP for detecting and monitoring gastric cancer after treatment. Furthermore, 5 out of the 30 preoperative control samples of benign disease (cases of panperitonitis due to acute appendicitis or cholecystitis) showed transient RUNX3 methylation which decreased after the operation in accordance with recovery. Conclusion: Quantification of epigenetic changes in serum RUNX3 methylation using RTQ-MSP is useful for the detection and monitoring of gastric cancer.*

Tumor-derived epigenetic changes in serum are potential molecular markers for numerous types of cancer (1-6). Tumor progression involves the transcriptional repression of tumor suppressor genes, DNA repair genes, and metastasis inhibitor genes including *p16, ECAD,* and *RARβ* (4-6). Detection of aberrant DNA methylation is important in understanding the involvement of tumor suppressor genes in carcinogenesis, and would form a novel molecular basis for cancer diagnosis (7-11).

The role of *RUNX3* in gastric carcinogenesis through the disruption of normal gastric mucosal development and differentiation has been previously described, and *RUNX3* was reported to be a novel tumor suppressor of gastric cancer (12). *RUNX3* is inactivated through promoter hyper-methylation and is thought to be involved in the transcriptional regulation of several genes involved in cancer progression and the metastasis of established gastric cancer. In the same report, the down-regulation of *RUNX3* was also investigated, showing progressive silencing of the gene according to cancer stage, especially in stage IV, where a large proportion of cases showed silencing of *RUNX3* (12, 13). Furthermore, it has been reported that *RUNX3* promoter methylation is involved in many types of cancer (14-20).

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Abbreviations: MSP, Methylation-specific PCR; RTQ-MSP, real-time quantitative methylation-specific PCR.

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Key Words: Gastric cancer, *RUNX3*, methylation.

Recently, frequent *RUNX3* promoter hypermethylation has also been detected in serum from patients with gastric, breast and bladder cancer (21). In this study, *RUNX3* real-time quantitative methylation-specific PCR (RTQ-MSP) was developed for the analysis of CpG sites in promoter regions whose hypermethylation promotes transcriptional silencing. RTQ-MSP data were in complete agreement with conventional MSP results (21); however, RTQ-MSP is much more rapid, accurate and amenable to large-scale cancer screening. This method has the combined advantages of MSP and real-time PCR. Additionally, with RTQ-MSP, the diagnostic potential of circulating tumor DNA can be maximized and further the understanding of the biology of this phenomenon.

RTQ-MSP provides quantitative data for methylation analysis which should allow its widespread use in clinical practice and in the assessment of the efficacy of cancer treatments. In this investigation, the concentration of tumor-derived methylated *RUNX3* sequences was investigated in serum from gastric cancer patients before, during and after surgical resection, and then its clinical importance for the early detection and diagnosis of gastric cancer recurrence was assessed.

Materials and Methods

Gastric cancer patients and control samples. With informed consent and ethics approval, 65 peripheral blood samples and 45 surgically removed tumors were collected prospectively from gastric cancer patients. The corresponding surrounding mucosal tissues were also obtained for methylation analysis. The diagnosis of gastric cancer was confirmed histologically in all cases. A total of 65 preoperative and 43 postoperative serum samples were obtained from gastric cancer patients. As controls, serum samples were collected from 50 non-gastric cancer patients with benign disease and from healthy volunteers.

DNA extraction from tissue and serum. DNA was extracted from gastric cancer and the surrounding mucosa using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) as previously described (12). Peripheral blood samples were centrifuged at 3000 × *g*, and plasma and serum samples were carefully collected from EDTA-containing tubes and plain tubes, respectively. DNA was extracted from 400 μL of serum using the QIAamp Blood Kit (Qiagen).

Bisulfite conversion of DNA. Bisulfite Conversion of DNA was performed as previously reported (21). Briefly, 2 μg of DNA were diluted in 50 μL dH_2O and 5.5 μL 2 M NaOH were added. After incubation of the mixture at 37˚C for 10 min, 30 μL freshly prepared 10 mM hydroquinone (Sigma, St. Louis, MO, USA) and 520 μL 3 M sodium bisulfite (pH 5.0) were added. The mixture was incubated at 50˚C for 16 h, and the DNA was purified with Wizard DNA Purification Resin (Promega, Pittsburgh, PA, USA). The DNA pellet was dissolved in 20 μL H_2O.

Development of RTQ-MSP. RTQ-MSP is based on continuous optical monitoring during fluorogenic PCR. One fluorescent dye serves as a reporter (6-carboxylfuorescein, FAM), and its emission spectrum is quenched by a second fluorescent dye (6-carbocytetremethylrhodamine, TAMRA). During the extension phase of PCR, the 5' to 3' exonuclease activity of the Taq DNA polymerase cleaves the reporter from the probe, thus releasing it from the quencher, resulting in an increase in fluorescence emission at 518 nm.

Quantitative real-time two-step MSP system was developed as shown in Figure 1. In the first step PCR reaction, a cocktail of gene-specific primer pairs is used to co-amplify DNA for genes independent of their DNA methylation status. In the second step, quantitative real-time PCR is performed with gene-specific primers, using the DNA template derived from the end product of reaction 1 (RXN 1) (diluted 1:5–1:10^4). DNA was analyzed in separate wells, using methylation status-specific primers (forward, reverse and probe, conjugated with the FAM label and TAMRA quencher).

Two real-time MSP systems were developed; the first for quantifying bisulfite-converted methylated *RUNX3* sequences and *actin* sequences, used as an internal control. Bisulfite-modified DNA was amplified using specifically designed primers for the methylate sequence. The sense and antisense primers for the methylated sequence were *RUNX3* MF (5'-TTATTAGAGGG TGGGGCGGAT CGC-3') and *RUNX3* MR (5'-GACCCCGAA CCGCGACCGTAA-3'), respectively.

The sense and antisense primers for the methylated sequence in second PCR were *RUNX3* MF2 (5'-GGTATAGTTTCGCGCGGGT-3') and *RUNX3* MR2 (5'-TATTCTCGCCCATCTTACCGC-3'), respectively, in conjunction with the fluorogenic probe RUNX3 [5'-(FAM)-AGTAGTATGGAGTCGGCGGCGGG-(TAMRA)-3']. All of the fluorogenic probes were custom-synthesized by Perkin-Elmer Applied Biosystems (Foster City, CA, USA). The forward and reverse primer sequences for the *actin* system were F (5'-TTATT AGAGGGTGGGGCGGATCGC-3') and R (5'-TTATTAGAGGGT GGGGCGGATCGC-3'), respectively. The fluorogenic probe actin system was [5'-(FAM)-CGTCGGGTTAGCGAGGTTTCGTA GCGG-(TAMRA)-3'].

Fluorogenic PCR was set up in a reaction volume of 50 μL with components supplied in the TaqMan PCR Core Reagent Kit (Perkin-Elmer Applied Biosystems). Each reaction contained of 100 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, 300 nM each amplification primer; 25 nM corresponding fluorogenic probe; 200 μM each dATP, dCTP and dGTP; 400 μM dUTP; and 1.25 units of AmpliTaq Gold polymerase. Bisulfite-converted DNA (1 μL) from serum or tissue was used per RTQ-MSP assay.

DNA amplification was carried out in a 0.2 mL reaction plate format in an Applied Biosystems 5700 Sequence Detector. Thermal cycling was initiated with a denaturation step of 12 min at 95˚C. The subsequent thermal profile for *RUNX3 M* RTQ-MSP was 95˚C for 15 s, 55˚C for 30 s and 72˚C for 1 min. Data obtained after 30 and 45 cycles of amplification were analyzed for tissue and serum samples, respectively. Multiple negative water blanks were included in each analysis. Amplification data, collected by the 5700 Sequence Detector and stored in a Macintosh computer (Apple Computer, Cupertino, CA, USA), were then analyzed using the Sequence Detection System software (Version 1.6.3) developed by Applied Biosystems.

A calibration curve was run in parallel with each analysis. A human cancer cell line, MKN28, previously shown to have *RUNX3* methylation by conventional MSP, was used for constructing the calibration curve for *RUNX3* RTQ-MSP. To

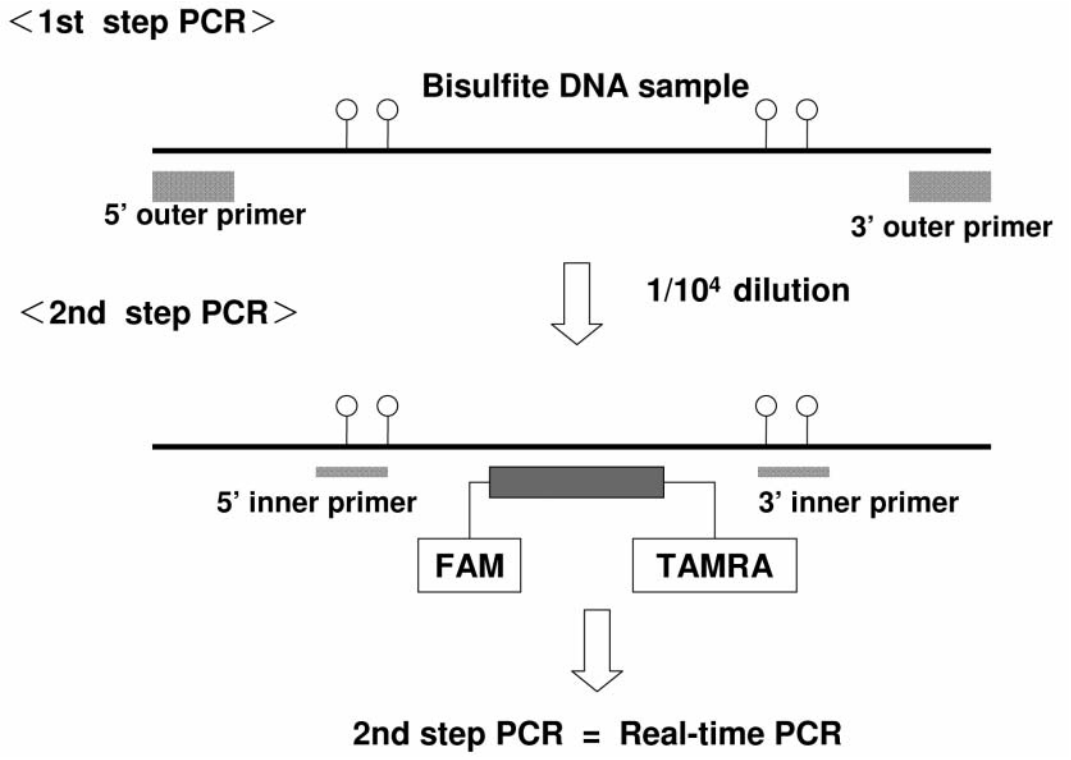

Figure 1. *Scheme for quantitative methylation-specific PCR of RUNX3. Quantitative real-time MSP system was developed as shown in this schema. In the first step PCR reaction, gene-specific primer pairs are used to amplify DNA for genes independent of their DNA methylation status. In the second step, quantitative real-time PCR is performed with gene-specific primers, using the DNA template derived from the end product of reaction 1 (RXN 1) (diluted 1:5–1:10^4). DNA was analyzed in separate wells, using methylation status-specific primers. Methylation index=1000 × [no. of copies of M/no. of copies of actin], as determined by the absolute quantification method computed against a standard curve.*

determine the dynamic range of RTQ-MSP, samples containing serial dilutions of MKN28 DNA were analyzed using the *RUNX3* system. The system was sufficiently sensitive to detect down to 5 ng of methylated *RUNX3* sequences.

The threshold cycle of *RUNX3 M* RTQ-MSP was inversely proportional to the input target quantity for PCR (on a logarithmic scale). The reproducibility of bisulfite conversion followed by RTQ-MSP was tested by performing replicate bisulfite conversions of MKN28-derived DNA (5 fg to 500 ng), followed by *RUNX3* RTQ-MSP.

The methylation index in a sample was calculated according to the ratio of M/*actin* ×1000, where M is the concentration of methylated *RUNX3* sequences measured by *RUNX3 M* RTQ-MSP, and *actin* is the relative amount of *actin* sequences measured by RTQ-MSP after bisulfite conversion. ROC analysis was performed to determine the limit of the positive and negative cut-off values.

Serum collection and tumor marker assay. Serum samples were collected on the day of surgery or postoperatively. Serum carcinoembryonic antigen (CEA) levels were determined by immunoradiometric assay using CIS RIA MoAb-Centocor kits (CIS International, Gif-sur-Yvette, France). Cut-off values for normal CEA serum assay were set at 5 ng/mL, as indicated in the instructions supplied with the kits.

Statistical analyses. Statistical analyses were carried out using SigmaStat 2.03 and JMP software. Group differences were analyzed using Fisher's exact test and *t*-test. $P<0.05$ was considered to indicate statistical significance. Analyses were performed using JMP5 for Windows software (SAS Institute, Inc., Cary, NC, USA).

Results

LightCycler validation of RTQ-MSP of RUNX3 methylation. A scheme for the quantitative methylation-specific PCR of *RUNX3* is shown in Figure 1. Real-time fluorescence PCR monitoring with Applied Biosystems 5700 Sequence Detector using hybridization probes allowed for rapid and sensitive detection of *RUNX3* methylation from patient samples. With this method 5 fg to 500 ng of MKN28-derived DNA (containing methylated *RUNX3* sequences) per 1 μg of human placental DNA (containing unmethylated *RUNX3*), could be quantified as shown in Figure 2.

Quantification of *RUNX3* methylation was assessed by determination of the crossover point (Ct), *i.e.* the cycle when fluorescence of a given sample rose above the background level to yield the maximal slope with respect to log-linear amplification. Figure 2 illustrates a standard curve constructed

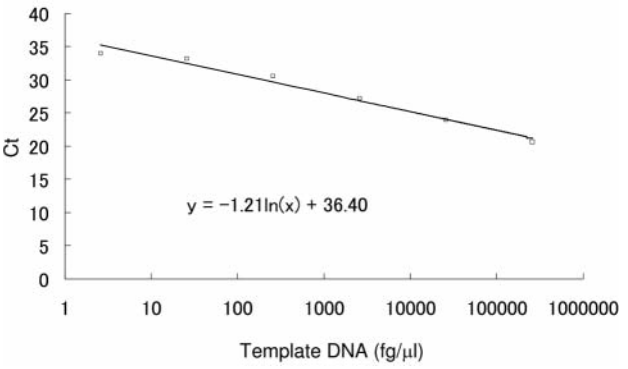

Figure 2. *Calibration curve for RUNX3 methylation estimation constructed from data for external controls by plotting the crossover points (Ct) against the log values (template DNA). Relative RUNX3 methylation values in patient samples were calculated with reference to this curve.*

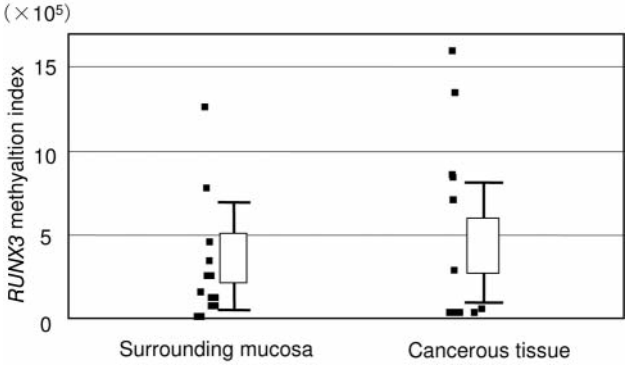

Figure 3. *RUNX3-methylation index in gastric cancer tissues and surrounding mucosa. RUNX3 methylation was significantly higher in primary gastric cancer than in the surrounding mucosa of these tumors (p<0.01).*

by plotting the log number of serially diluted MKN28-derived DNA against their respective Cts. *RUNX3* methylation values for patient samples with unknown methylation index were calculated with reference to the calibration curve.

RUNX3 methylation in primary gastric cancer and surrounding mucosa. Aberrant *RUNX3* promoter methylation was found in 91% (41 of 45) of surgically resected gastric carcinomas using RUNX3 RTQ-MSP. *RUNX3* methylation was higher in primary gastric cancer than in the mucosa surrounding these tumors, but not significantly so; some of the corresponding surrounding mucosa had high levels of methylated *RUNX3* sequences (Figure 3). The difference in methylation between tumor tissue and surrounding mucosa was not significant in poorly differentiated adenocarcinomas, nor in well differentiated adenocarcinomas (data not shown).

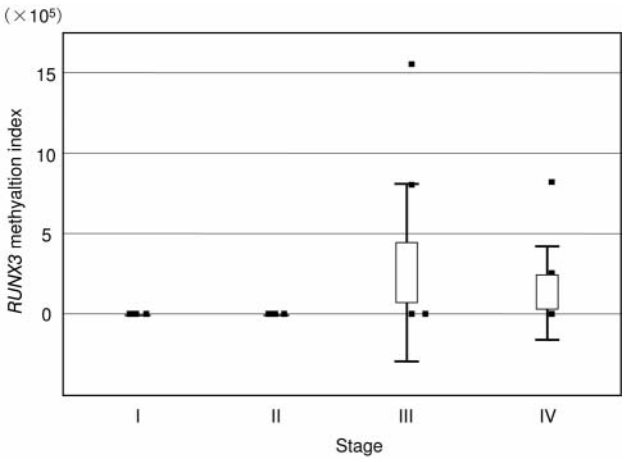

Figure 4. *Relationship between cancer stage and RUNX3 methylation index in serum DNA of gastric cancer patients. RUNX3 methylation index correlated with cancer stage (p<0.01). The cut-off value was calculated by ROC curve analysis as 1.543. Quantities of methylated RUNX3 were detected in the serum of 29% (19 of 65) of the gastric cancers patients.*

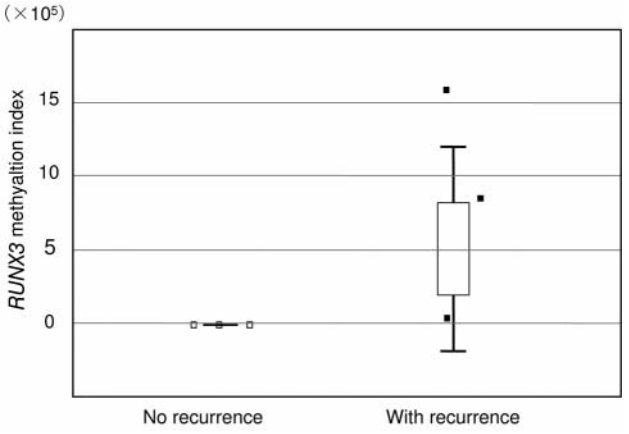

Figure 5. *RUNX3 methylation index in serum DNA measured by real-time RT-PCR in gastric cancer patients with and without cancer recurrence. The RUNX3 methylation values in serum DNA from metastasis-positive patients were significantly higher than in those from metastasis-negative patients (p<0.001).*

RUNX3 methylation index in serum from gastric cancer patients and clinicopathological factors. The value for *RUNX3* methylation was determined as the *RUNX3* methylation/*actin* ratio. The average *RUNX3* methylation/*β-actin* ratio ($\times 10^3$) by stage classification was: stage I, 70±13; stage II, 138±93; stage III, 261833±36613; stage IV, 137285±24139 (average±SD). The results showed that the *RUNX3* methylation/*actin* ($\times 10^3$) index correlates with cancer stage as shown in Figure 4. A summary of *RUNX3*

Table I. *Clinicopathologic findings and serum RUNX3 methylation in patients with gastric cancer.*

	Case number	CEA	*RUNX3* methylation level	*P*-value
Gender				0.7724
Male	37	5.44 (±10.91)	39281 (±15734)	
Female	28	1.98 (±2.29)	140961 (±40174)	
Tumor depth				0.0035
T1	20	1.98 (±1.44)	34 (±55)	
T2	24	2.71 (±1.76)	378 (±998)	
T3	21	7.77 (±14.8)	246434 (±45843)	
T4	–	–	–	
Tumor size				0.0191
< 3 cm	20	2.26 (±2.09)	38 (±54)	
3 cm-6 cm	21	2.65 (±1.31)	57794 (±20680)	
> 6 cm	24	6.73 (±13.61)	155724 (±40173)	
Peritoneal metastasis				0.2004
Positive	4	4.67 (±4.41)	279689 (±38233)	
Negative	61	4.06 (±9.06)	63850 (±26837)	
Lymph node metastasis				0.0677
Positive	30	6.55 (±12.56)	172547 (±39985)	
Negative	35	2.07 (±1.46)	250 (±855)	
Histology				0.0489
Differentiated	28	2.56 (±1.91)	311 (±981)	
Undifferentiated	37	5.17 (±11.24)	365196 (±35814)	
Vascular invasion				0.0029
Positive	28	7.02 (±13.53)	202986 (±42646)	
Negative	37	2.27 (±1.70)	229 (±810)	
Lymphatic invasion				0.0335
Positive	34	5.93 (±11.82)	115141 (±35151)	
Negative	31	2.10 (±1.52)	38509 (±17104)	
Stage				0.0129
I	28	2.34 (±1.69)	70 (±13)	
II	19	2.23 (±2.09)	138 (±93)	
III	14	2.23 (±1.28)	261833 (±36613)	
IV	13	12.21 (±18.31)	137285 (±24139)	

*$p<0.05$, significant difference.

methylation and the clinicopathological factors in gastric cancer patients is shown in Table I. *RUNX3* methylation correlated with differentiation, depth of invasion, lymphatic invasion and tumor size.

No significant levels of *RUNX3* methylation were detected in most of the peripheral blood lymphocytes from healthy volunteers. However, a transient increase in *RUNX3* methylation was observed in serum samples from five postoperative cases (cholecystolithiasis, acute appendicitis and perforation of duodenal ulcer). Preoperative as well as postoperative serum *RUNX3* methylation levels and underlying diseases are shown in Table II.

Methylated *RUNX3* was detected in serum from 29% (19 of 65) of the gastric cancer patients with tumoral *RUNX3* methylation. The *RUNX3-M* score has a sensitivity of 95.5%, specificity of 62.5% and AUC of 0.8651 using the optimal cut-off point (1.543) as determined by ROC curve analysis (Tables I and II, Figure 3).

Table II. *Transient increase of serum RUNX3 methylation level in patients with benign disease.*

Case no.	Age (years)	Underlying disease	Methylation level (preoperative)	Methylation level (postoperative)
1	30	Acute appendicitis	3422.1	20.8
2	36	Acute appendicitis	5425.9	4.3
3	55	Acute cholecystitis	22106.2	110.0
4	72	Acute cholecystitis	391.5	15.6
5	75	Duodenal ulcer perforation	4524.5	5.2

*Serum RUNX3 methylation level in patients with benign disease were estimated before and after operation.

Quantification of methylated RUNX3 index in preoperative and postoperative serum samples. Sixty-five serum samples from gastric cancer patients were analyzed using *RUNX3* RTQ-MSP. Twenty nine percent (19 of 65) of the preoperative serum samples had methylated *RUNX3* sequences, corresponding to 5.2 to 1625955 (median quantity=43 m-index, n=19; Table I). No significant differences were observed in the concentrations of methylated *RUNX3* between plasma samples (n=49) and serum samples (n=65) collected preoperatively from the gastric cancer patients (p=0.286; Mann-Whitney rank-sum test, data not shown).

The median methylation index in postoperative serum was 12-fold lower than the preoperative median methylation index for the gastric cancer patients. In the prospective longitudinal study of patients possessing *RUNX3* methylation in serum samples, which were collected both preoperatively and postoperatively, the *RUNX3* methylation indices decreased significantly after surgical resection in 83% of the gastric cancer patients (p=0.03; Wilcoxon test); however, 6% (4 out of 65) of cases showed no change in methylation (data not shown).

Relationship between cancer recurrence, CEA and serum RUNX3 methylation. For CEA analysis, serum was available for 65 patients of the test set. Using the recommended cut-off level for CEA of 5 ng/mL, it was found that positivity for CEA did not correlate with serum methylation of *RUNX3*. In cases with liver metastases, *RUNX3* methylation increased in 6 out of 10 cases. CEA was able to detect recurrence (distant metastases) in only 2 out of 10 cases. Serum *RUNX3* methylation was therefore a more sensitive indicator of cancer recurrence than CEA. Liver metastases and lung metastases were detected using *RUNX3* RTQ-MSP, however, peritoneal metastases were not detected (data not shown).

Discussion

Gastric cancer is the most common malignancy of the gastrointestinal tract in Japanese and certain South-east Asian populations, and the second most common cause of cancer-related deaths in the world (23-25). The most frequently used tumor markers are the CEA and the CA19-9 antigen, and previous studies relating to serum markers for gastric cancer have indicated that a combination of CEA and CA19-9 is the most informative method of cancer detection (26, 27). However, there are problems with the sensitivities and specificities of these methods and few patients have elevated levels of these antigens. Many types of tumor markers have been reported but a small number of these markers are suitable for early diagnosis or detection of cancer recurrence. Therefore, a good marker is of great importance for determining the right form of therapy and for optimal surveillance of these patients.

Aberrant hypermethylation of CpG islands is a hallmark of cancer (1-6). Their clinical applications and prognostic value have been investigated in many types of cancer (1-6, 28, 29). *RUNX3* is a novel tumor suppressor of gastric cancers and this investigation extends previous work which indicated that aberrant *RUNX3* promoter methylation is a common phenomenon in gastric cancer as well as various other types of cancer (14-20). Furthermore, it has been reported that conventional MSP can also detect serum DNA methylation of *RUNX3* in various types of cancer (21). Highly sensitive RTQ-MSP was able to quantitatively measure circulating, tumor-derived, methylated *RUNX3* sequences in a significant proportion of gastric cancer patients with tumoral *RUNX3* methylation, as shown in this study. In addition, the high specificity of the assay was proven by the absence of methylated *RUNX3* sequences in the serum of most of the controls.

For the first time, the concentration of circulating tumor-derived DNA has been determined in gastric cancer patients using RTQ-MSP. The results revealed a correlation between cancer stage, lymphatic invasion, vascular invasion and the *RUNX3* methylation index. This was completely concordant with conventional MSP data as described previously (21). Using ROC analysis, it was found that the *RUNX3* methylation index provided optimum sensitivity (95.5%) and specificity (62.5%) for predicting disease recurrence during the first 6 months postsurgery, compared with a sensitivity of 6% for CEA (26, 27). It was observed that positivity for CEA did not always correlate with methylation of *RUNX3* in serum. When the cut-off value for CEA was set at 5 ng/mL, the CEA positive rate was 4.2% and the *RUNX3* positive rate was 29%. Some cases showed high serum *RUNX3* methylation without an increase in CEA, suggesting that serum *RUNX3* methylation is more sensitive than CEA for the detection of distant metastasis of gastric cancer; however, peritoneal metastasis could not be detected with this method. The methylation index was seen to rise with progression through the various cancer stages; therefore *RUNX3* methylation may be useful as a novel marker for gastric cancer as an independent prognostic factor. Although the data are limited due to small patient numbers in the subgroup, they provide additional evidence for the potential of serum DNA methylation as a prognostic indicator in gastric cancer. It is possible that *RUNX3* methylation may be useful as a tumor marker in conjunction with CEA. A larger cohort of gastric cancer patients will be required to consolidate this finding.

Improvement in sensitivity and specificity will be critical for the molecular diagnosis of cancer. In this study the majority of postoperative serum samples were *RUNX3* methylation-negative and only 3 out of 28 control samples had a *RUNX3* methylation index of more than 1.543. The *RUNX3* methylation indices of the 3 methylation-positive postoperative serum samples were generally lower than those detected preoperatively in the majority of the gastric cancer patients with serum methylation changes. The *RUNX3* methylation detected postoperatively might possibly be derived from apoptotic or necrotic tumor cells, or could be due to aging or inflammation as supported by the absence of metastasis or tumor recurrence in these patients with a median disease-free interval of 22.3 months (data not shown). Methylation could also occur as a result of panperitonitis due to acute appendicitis or gallbladder perforation in cholecystitis.

Using *RUNX3* as a candidate gene in RTQ-MSP increased the methylation detection frequency up to 29%. Although a larger study is essential for further evaluation, these results demonstrate the usefulness of *RUNX3* in combination with other tumor markers. It has been shown that down-regulation of *RUNX3* increases in the early stages of gastric cancer progression from 40% to 90%. Thus RTQ-MSP for RUNX3 may also prove to be a powerful diagnostic and prognostic tool for the detection of cancer recurrence. These investigations clearly indicate that there is scope for further studies into the utility of *RUNX3* as a promising diagnostic and prognostic marker, as well as for its use in clinical applications in the future.

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Received January 7, 2009
Revised March 17, 2009
Accepted April 15, 2009