Methylation of the *SEPT9_v2* promoter as a novel marker for the detection of circulating tumor DNA in breast cancer patients

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Abstract. The aim of the present study was to evaluate the promoter methylation status of *SEPT9_v2* in breast cancer and to detect this methylated gene in circulating tumor DNA (ctDNA) in plasma. Bisulfite sequencing was performed with a next generation sequencer. Methylation of the *SEPT9_v2* promoter was found in 67% (8/12) of breast cancer cell lines and 53% (10/19) of breast tumor tissue, but not in normal breast tissue (0/19). A clear inverse correlation was observed between the expression of *SEPT9_v2* mRNA and the methylation index (MI) both in cell lines and breast cancer tissues. The MI of *SEPT9_v2* was significantly higher in non-basal subtype of breast cancer (13.0%, n=84) than in basal subtype (3.0%, n=23) (P<0.0001). Methylated *SEPT9_v2* ctDNA in plasma was detected in 11% (9/82) of primary breast cancer patients and 52% (26/50) of metastatic breast cancer patients, but not in the healthy controls (0/51). These results indicate that *SEPT9_v2* promoter hypermethylation, which silences the expression of *SEPT9_v2* mRNA, is observed in a significant proportion of breast tumors, and that methylated *SEPT9_v2* may serve as a novel tumor marker for breast cancer.

Introduction

Methylation of tumor suppressor gene promoters, one of the most common events in various types of cancers, is generally tumor-specific (1,2). Methylation of circulating tumor DNA (ctDNA) can be detected in the plasma or serum of breast cancer patients (3-9); thus, methylated ctDNA is a promising cancer biomarker (2). Several genes including *GSTP1*, *RASSF1A* and *RARβ2* are methylated in breast tumor tissue (3,10,11), and the methylation of these genes can be detected in ctDNA of breast cancer patients (3,4,6). Although several studies have investigated the clinical application of these methylated ctDNAs as prognostic indicators (4,5) or monitoring markers of systemic therapy (7, 8), a more sensitive and specific methylated ctDNA marker is needed.

SEPT9 belongs to a family of GTP-binding proteins recognized as components of the cytoskeleton. These proteins are involved in several cellular processes including membrane trafficking, cytokinesis, angiogenesis, and cell proliferation (12,13). *SEPT9* consists of at least seven transcripts with diverse functions (13,14). Some of these transcripts possess tumor suppressor functions, while others have oncogenic properties (15-18). *SEPT9_v2* has been shown to be epigenetically modified in colorectal cancer, and an assay for detecting methylated *SEPT9_v2* ctDNA in plasma has been developed and validated for clinical use as a marker for the early detection of colorectal cancer (19-21). Recently, a more sensitive and specific (48 and 92%, respectively) assay for methylated *SEPT9_v2* has been developed for colorectal cancer screening (22). *SEPT9* methylation can be also detected in other types of cancers, including breast cancer (23), suggesting that the assay for methylated *SEPT9_v2* in plasma may be useful in breast cancer patients.

The aim of the present study was to determine whether methylation of the *SEPT9_v2* promoter was associated with the expression of this gene in breast cancer cells. In addition, we sought to clarify the clinicopathological characteristics of breast tumors containing methylated *SEPT9_v2*. We analyzed the methylation of the *SEPT9_v2* promoter using next generation sequencing (NGS), which provides a quantitative methylation index (MI) within a broad CpG area. Lastly, we examined whether methylated *SEPT9_v2* ctDNA can be detected in the plasma of breast cancer patients, and explored its utility as a novel blood biomarker for breast cancer diagnosis.

Materials and methods

Patients and breast tumor samples

Study I. Nineteen pairs of tumor and normal tissues were obtained at surgery between 2001 and 2004 from primary breast cancer (PBC) patients who received no preoperative chemotherapy or hormonal therapy. The clinicopathological characteristics of these patients are summarized in Table I. Normal tissue samples were obtained from the quadrant not

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harboring cancer. Tissue samples were snap-frozen in liquid nitrogen and kept at -80˚C until use.

Study II. Tumor samples from stage II or III PBC patients (n=107) were retrospectively included in the present study. These patients had been treated at Osaka University Hospital between 2004 and 2009 with neoadjuvant chemotherapy (NAC) consisting of paclitaxel (80 mg/m^2) weekly for 12 cycles followed by 5-fluorouracil (500 mg/m^2), epirubicin (75 mg/m^2) and cyclophosphamide (500 mg/m^2) every three weeks for four cycles. Each patient underwent vacuum-assisted biopsy of the tumors, and tumor samples were snap-frozen in liquid nitrogen and kept at -80˚C until use. Histological grade, estrogen receptor (ER), progesterone receptor (PR) and HER2 status were determined as previously described (11). Ki67 was defined as 'high' when ≥20% of tumor cells was immunohistochemically positive (clone; MIB-1). A pathological complete response (pCR) was defined as no evidence of invasive cancer components in the breast irrespective of axilla lymph node metastases. Intrinsic subtypes were determined by DNA microarray using the PAM50 method (24,25). The clinicopathological characteristics of these patients are summarized in Table II.

Study III. For the measurement of methylated *SEPT9_v2* ctDNA in plasma, 2 ml plasma samples were obtained from healthy controls (n=51), stage II or III PBC patients (n=82) and metastatic breast cancer (MBC) patients (n=50) at Osaka University Hospital and Osaka Police Hospital between 2012 and 2014. Among these patients, frozen tumor tissues or formalin-fixed paraffin-embedded (FFPE) tumor tissues were available from 49 PBC and 25 MBC patients. These tumor tissues were subjected to the *SEPT9_v2* methylation assay. These studies were approved by the Institutional Review Board for Clinical Research, Osaka University Graduate School of Medicine and the Osaka Police Hospital Ethical Committee. Informed consent was obtained from each patient before sampling.

DNA extraction and sodium bisulfite treatment. Total DNA from cell lines was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and total DNA from snap-frozen breast tissue was extracted using the DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA). For DNA extraction from FFPE tumor tissues, three to five 10-μm sections/tumor were cut from the FFPE tumor tissues, and the tumor area was dissected with a scalpel under a stereoscopic assistance. Total DNA from the paraffin sections was extracted using the QIAamp DNA FFPE kit (Qiagen). For the laser captured microdissection (LCM), a 10 μm section of the FFPE tumor tissues was mounted onto a polyethylene napthalate membrane slide (Leica Microsystems GmbH, Wetzlar, Germany), and the epithelium or stroma was separately collected with the laser microdissection system LMD7000 (Leica) (26) and DNA was extracted using the QIAamp DNA Micro kit (Qiagen). One microgram of genomic DNA was subjected to sodium bisulfite treatment with the EpiTect® Bisulfite kit (Qiagen). Plasma DNA was extracted using the QIAamp® Circulating Nucleic Acid kit (Qiagen) from a 2 ml plasma sample and subjected to sodium bisulfite treatment as previously described (4).

Table I. Clinicopathological characteristics of breast tumors (study I).

Characteristics	No. of patients	%
All cases	19	
Age (years)		
<50	9	52.6
≥50	10	47.4
Menopausal status		
Pre	10	52.6
Post	9	47.4
Tumor size (cm)		
<2	5	26.3
≥2	14	73.7
Lymph node metastasis		
Negative	13	68.4
Positive	6	31.6
Histological type		
IDC	18	94.7
Special type	1	5.26
Histological grade		
1,2	13	68.4
3	6	31.6
ER		
Negative	7	36.8
Positive	12	63.2
PR		
Negative	10	52.6
Positive	9	47.4
HER2		
Negative	12	63.2
Positive	7	36.8
Ki67		
Low	5	26.3
High	1	5.26
Unknown	13	68.4
Subtype (IHC)[a]		
Luminal A	9	47.4
Luminal B	3	15.8
HER2	4	21.1
Triple negative	3	15.8

IDC, invasive ductal carcinoma; IHC immunohistochemistry; [a]luminal A, ER and/or PR-positive and HER2-negative; luminal B, ER and/or PR-positive and HER2-positive; HER2, ER and PR-negative and HER2-positive; triple negative, ER, PR and HER2-negative; ER, estrogen receptor; PR, progesterone receptor.

Quantitative SEPT9_v2 promoter methylation analysis using NGS and methylation-specific polymerase chain reaction. The NGS methylation assay was performed with the GS Junior system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Data were

Table II. Comparison of the *SEPT9_v2* MI with clinicopathological parameters of breast tumors (study II).

Characteristics	No. of pts.	*SEPT9_v2* MI (mean ± SD)	P-value
All cases	107		
Age (years)			0.573
<50	49	10.1±16.1	
≥50	58	11.7±12.8	
Menopausal status			0.657
Pre	51	11.5±15.9	
Post	56	10.3±12.9	
T stage			0.539
T1,2	84	10.3±12.6	
T3, 4	23	13.0±19.8	
Lymph node metastasis			0.486
Negative	30	12.5±15.5	
Positive	77	10.2±13.9	
Stage			0.467
II	88	10.2±12.5	
III	19	13.9±21.2	
Histological type			0.123
IDC	96	10.3±14.7	
Special type	11	15.9±10.2	
ER			<0.001
Negative	42	3.2±5.6	
Positive	65	15.8±16.0	
PR			0.002
Negative	65	7.4±13.3	
Positive	42	16.2±14.5	
HER2			0.592
Negative	76	11.4±13.8	
Positive	31	9.6±15.7	
Subtype (IHC)[a]			<0.001[b]
Luminal A	51	15.8±14.6	
Luminal B	14	16.1±21.0	
HER2	17	4.3±6.3	
Triple negative	25	2.4±5.1	
Subtype (PAM50)			<0.001[b]
Luminal A	29	15.6±13.5	
Luminal B	21	14.3±16.8	
HER2	16	15.2±20.7	
Basal-like	23	3.0±6.7	
Normal-like	18	5.4±5.7	
Histological grade			<0.001
1,2	86	12.7±15.3	
3	21	3.5±5.5	
Ki67			0.116
Low	44	13.6±15.0	
High	62	9.1±13.8	
Unknown	1	0.7	
Pathological response			<0.001
Non-pCR	74	13.4±15.7	
pCR	33	5.2±8.47	

MI, methylation index; pts., patients; IDC invasive ductal carcinoma; IHC immunohistochemistry; [a]luminal A, ER and/or PR-positive and HER2-negative; luminal B, ER and/or PR-positive and HER2-positive; HER2, ER and PR-negative and HER2-positive; triple negative, ER, PR and HER2-negative; [b]Kruskal-Wallis test; ER, estrogen receptor; PR, progesterone receptor.

analyzed using GS Amplicon Variant Analyzer software ver. 2.7 (Roche Diagnostics). The MI was calculated by dividing the number of cytosines by the total reads at each CpG site. NGS primers used for *SEPT9_v2* methylation of frozen tissue or cell lines were designed as follows: forward, 5'-ATTTAGTTGAGTTAGGGGGTTTAGG-3' and reverse, 5'-AACAAACACAAACAACAATAAAAAAAA-3' (NGS primer long, Fig. 1). Among all 57 CpG sites, the average MI of the 11 sites (47-57th CpGl; Fig. 1) showing the most significant difference between cancer and normal tissue was used for the statistical analysis of methylation. NGS primers used for DNA from FFPE specimens were designed as follows: forward, 5'-GGGGGATTTTGTTAGGGGTA-3' and reverse, 5'-AACAAACACAAACAACAATAAAAAAAA -3' (NGS primer short, Fig. 1). The NGS short primer included seven CpG sites, equivalent to 51st-57th CpG (Fig. 1). The cut-off of MI ≥10% was used to define the hypermethylation of *SEPT9_v2* in the breast cancer cell lines and breast tumors according to the previous studies (27-30). For detecting the methylated *SEPT9_v2* in plasma and in the epithelial or stromal cells obtained by LCM, a quantitative methylation-specific polymerase chain reaction (MSP) assay was performed with the protocol modified as previously described (20). The *SEPT9_v2* oligonucleotide sequence including the 20-24th CpG sites (Fig. 1) for detection of methylated *SEPT9_v2* ctDNA in plasma were designed as follows: forward, 5'-AAATAATCCCATCCAACTA-3' and reverse, 5'-GATT-d-spacer-GTTGTTTATTAGTTATTATGT-3' (Fig. 1) (20). The rCpG sequence was used as a reference control as we previously reported (3). The *SEPT9_v2* and rCpG PCR reactions were performed using a 9 μl aliquot of the bisulfite DNA eluate, in a 25 μl total volume using 96-well plates. Methylated *SEPT9_v2* ctDNA in plasma was defined as positive when quantification cycles were <50 for *SEPT9_v2* and <33.7 for rCpG as the loading reference (22).

In situ hybridization for SEPT9_v2 mRNA. The QuantiGene®ViewRNA *In Situ* Hybridization Tissue Assay kit (Affymetrix, Santa Clara, CA, USA) was used according to the manufacturer's protocol. FFPE sections (4 μm) of tumor tissue were incubated for 20 min at 98˚C with a pretreatment

solution followed by protease digestion for 10 min. The *SEPT9_v2*- or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)-specific View RNA™ Probe set (Affymetrix) was hybridized for 3 h. This probe set was designed to hybridize the *SEPT9_v2*-specific sequence with a length of 342 bp, which comprises 25% of the length regularly required for *in situ* hybridization (ISH) (chromosome 17; 77,373,207 to 77,373,548). ISH images were captured by a fluorescent microscope (BZ-9000; Keyence, Osaka, Japan). Signal intensity was semi-quantitatively determined based on the number of cytoplasmic fluorescent dots in five non-overlapping fields at high-power magnification (x400).

Isolation of breast tumor cells by magnetic-activated cell sorting. Breast tumor cells were isolated from the FFPE tumor tissue by magnetic-activated cell sorting (MACS) using the EasySep Human EpCAM Positive Selection Cocktail, the EasySep Human MUC1 Positive Selection Cocktail, and EasySep Magnetic Particles (Stem Cell Technologies, Vancouver, BC, Canada) as previously described (31). Total DNA was extracted from the isolated tumor cells using the QIAamp® DNA FFPE Tissue kit (Qiagen).

Demethylation study with 5-aza-2'-deoxycytidine in cell lines. Twelve breast cancer cell lines (MCF7, ZR75-1, T47D, ZR75-30, MDA-MB-361, BT474, SKBR3, AU565, MDA-MB-453, MDA-MB-231, MDA-MB-468 and BT-20) and one normal breast epithelial cell line (HMEC) were cultured according to ATCC culture guides. For demethylation studies, cultured cells were treated with 10 μmol/l 5-aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA) or with dimethyl sulfoxide as control for 72 h. The medium was changed every 24 h.

RNA extraction and quantitative real-time PCR. Total RNA was isolated from cell lines using TRIzol® reagent (Invitrogen). One microgram of total RNA was reverse-transcribed for single stranded cDNA using random primers and the ReverTra Ace® qPCR RT kit (Toyobo, Osaka, Japan). The reverse-transcription reaction was performed at 65˚C for 5 min and subsequently at 37˚C for 15 min and 98˚C for 5 min. mRNA was quantitated using the LightCycler 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany) at 95˚C (10 min), followed by 50 cycles of 95˚C (15 sec), 60˚C (60 sec) and 1 cycle of 50˚C (10 sec). *SEPT9_v2* and *GAPDH* TaqMan® Gene Expression Assays (assay identification nos. are Hs01107941_m1 and Hs02758991_g1, respectively; Applied Biosystems, Foster City, CA, USA) were used for quantitative real-time PCR. The expression of *SEPT9_v2* was normalized to that of *GAPDH*, and each assay was performed in duplicate. Each 5-aza-2'-deoxycytidine-treated breast cancer cell line was normalized to its control, which was set to a value of 1.

Statistical analyses. JMP statistical software (version 10; SAS Institute, Cary, NC, USA) was used for statistical analyses. Associations between the various parameters and the *SEPT9_v2* MI were evaluated using Student's t-test for two groups or the Kruskal-Wallis test for more than two groups. The paired t-test was used for comparison of frozen cancer and normal tissue MIs in matched-pair samples. Dunnett's test was used for comparison of *SEPT9_v2* MIs in each of the subtypes. Univariate and multivariate analyses of various parameters for their association with pCR were conducted using the logistic regression model. All statistical analyses were two-sided and P-values <0.05 were considered statistically significant.

Results

Methylation of the SEPT9_v2 promoter and its impact on gene expression in breast cancer cell lines. The NGS methylation assay was performed on the *SEPT9_v2* promoter in 12 breast cancer cell lines and a normal human mammary epithelial cell line. A representative NGS result is shown in Fig. 2. The *SEPT9_v2* gene promoter was hypermethylated in eight and hypomethylated in four breast cancer cell lines and normal human mammary epithelial cells (Fig. 3A).

The expression of *SEPT9_v2* mRNA was examined by quantitative real-time PCR using *SEPT9_v2*-specific primers and probes. There was an inverse correlation between the expression of *SEPT9_v2* mRNA and the MI (Pearson's correlation coefficient=-0.987) (Fig. 3A). We then treated eight of these cell lines with a demethylating agent (10 μM 5-aza-2'-deoxycytidine) and compared mRNA expression between treated and untreated cells. Treatment with 5-aza-2'-deoxycytidine induced 20- to 110-fold upregulation of mRNA expression in all four hypermethylated breast cancer cell lines (MCF7, T47D, AU565 and MDA-MB-231) (Fig. 3B). There was no upregulation in the four hypomethylated breast cancer cell lines (MDA-MB-361, BT474, SKBR3 and MDA-MB-468), demonstrating that the *SEPT9_v2* gene was re-expressed by demethylation of its promoter region.

Methylation and expression of SEPT9_v2 in human breast cancer tissues. To study the methylation status of *SEPT9_v2* in human breast cancer and normal breast tissues, the NGS methylation assay was performed using 19 paired tumor and normal tissues (study I). The MI was significantly higher in tumor than normal tissues (median values=10.0 and 1.7%, respectively; P=0.003) (Fig. 4A). The proportion of *SEPT9_v2* tumors that were hypermethylated (MI ≥10%) was 53%.

For more accurate assessment of the cancer cell-specific methylation status, we isolated cells from FFPE tumor tissue by the MACS method. The isolated tumor cells were subjected to the NGS methylation assay. Six tumor tissue samples with a relatively low-MI (<30%) were analyzed since the low-MI was speculated to be due to contamination by normal stromal and inflammatory cells. The MI was clearly high in the tumor cells isolated from the three tumors with a relatively high-MI. However, the MI was low in tumor cells isolated from the remaining three tumors with a very low-MI (Fig. 4B).

Relationship between SEPT9_v2 methylation and clinicopathological characteristics. The NGS methylation assay of *SEPT9_v2* was carried out using the vacuum-assisted biopsy specimens obtained before NAC (study II). The relationship between the extent of *SEPT9_v2* methylation and various clinicopathological parameters including response to NAC was examined (Table II). *SEPT9_v2* hypermethylation (MI ≥10%) was observed in 37% (40/107) of the specimens. Hypermethylation was significantly associated

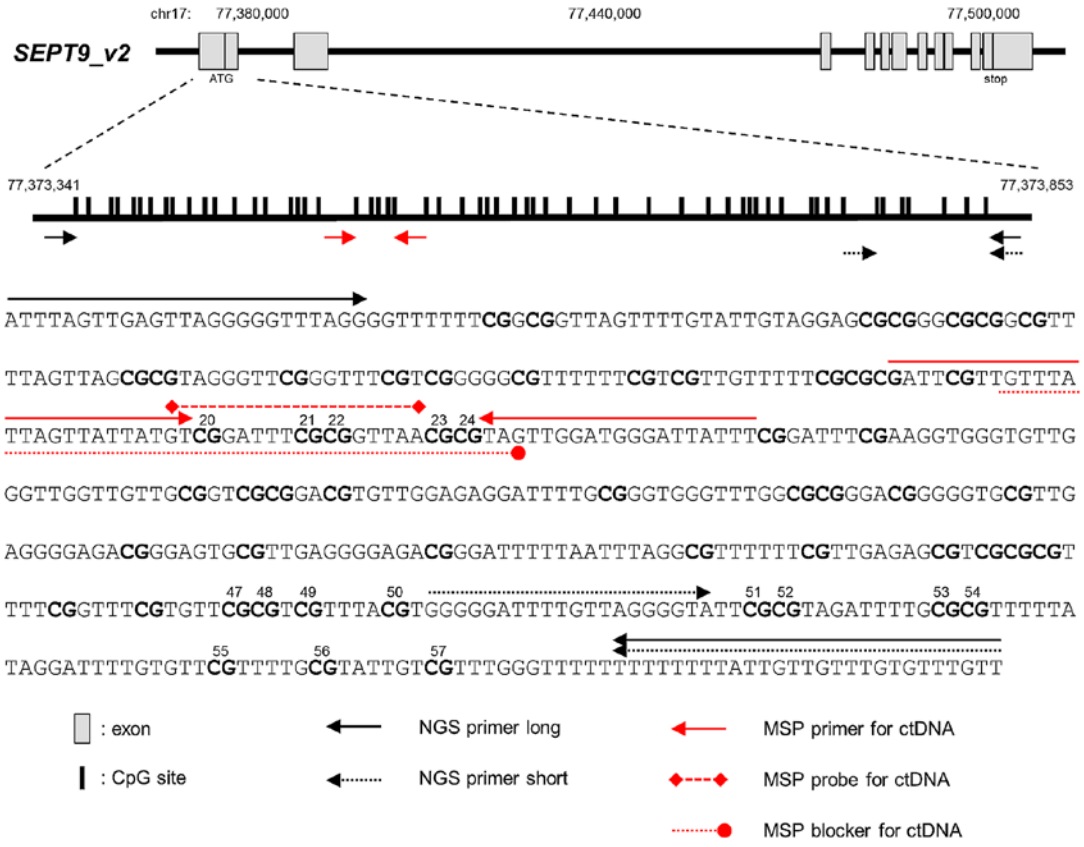

Figure 1. Primer design for DNA methylation analysis of *SEPT9_v2* by NGS and for detection of methylated *SEPT9_v2* ctDNA by real-time PCR. Two NGS primer sets were designed for methylation analysis of *SEPT9_v2*; 'NGS primer long' for DNA from cell lines and frozen tissues, and 'NGS primer short' for DNA from FFPE tissues. Methylated *SEPT9_v2* ctDNA in plasma was detected by real-time PCR using the indicated primers, probes and blocker. The blocker has a C3 spacer in the 3'-end to prevent extension.

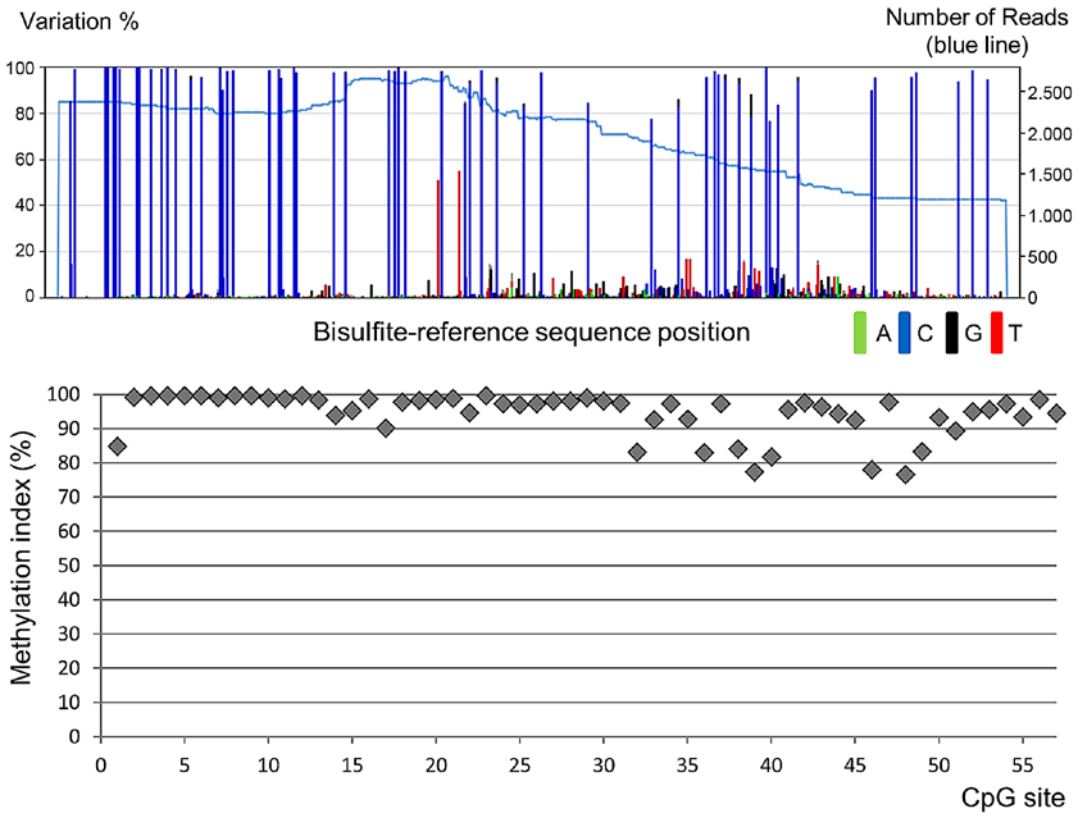

Figure 2. A representative result of *SEPT9_v2* methylation analysis with NGS. The methylation status of 57 CpG sites was analyzed by means of NGS using NGS primer long. The MI of each CpG site was shown by blue bars (cytosine) (upper panel) and diamond symbols (lower panel).

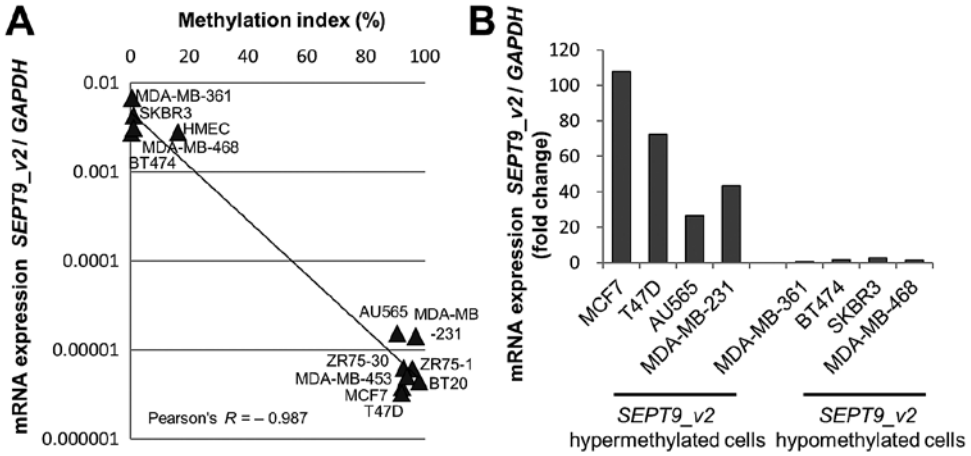

Figure 3. *SEPT9_v2* MI and mRNA expression in 13 breast cancer cell lines. (A) Correlation between the *SEPT9_v2* MI and mRNA expression. The MI of *SEPT9_v2* of 12 breast cancer cell lines and one normal breast epithelial cell line (HMEC) were determined by the NGS-based methylation assay and the correlation between the *SEPT9_v2* MI and mRNA expression is shown. (B) *SEPT9_v2* mRNA expression was evaluated before and after 5-aza-2'-deoxycytidine treatment in eight breast cancer cell lines and the fold-changes in *SEPT9_v2* mRNA expression after 5-aza-2'-deoxycytidine treatment are represented.

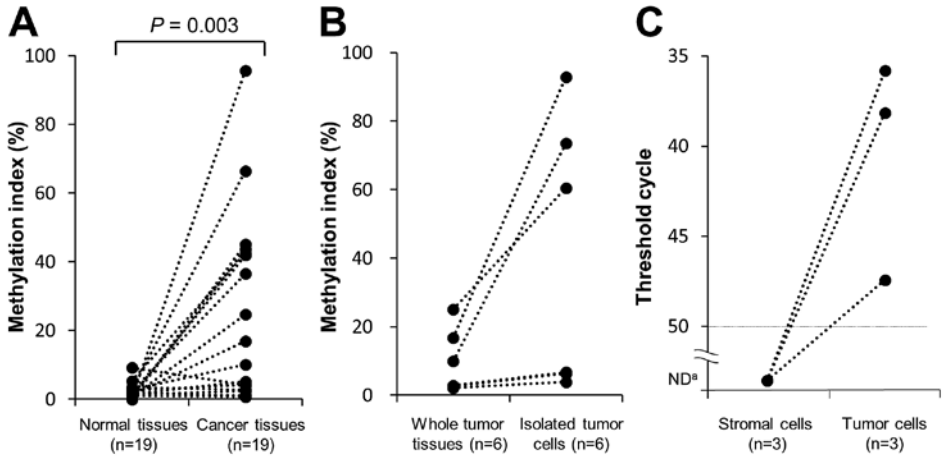

Figure 4. Methylation status of *SEPT9_v2* in breast cancer and normal breast tissues. (A) The *SEPT9_v2* MI was compared between 19 paired normal and cancerous breast tissues. (B) The *SEPT9_v2* MI was assessed using isolated tumor cells by the MACS method and compared with non-isolated whole breast cancer tissues. (C) The *SEPT9_v2* methylation level was assessed with MSP and compared between the epithelial cells and stromal cells separately collected by LCM from primary breast cancer tissues. [a], not detectable.

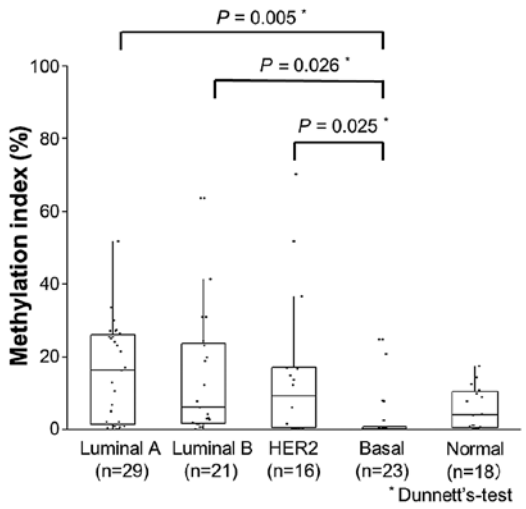

Figure 5. MI of *SEPT9_v2* in 107 breast cancer tumors. Breast cancer tumors (107) were classified into the five intrinsic subtypes by PAM50 (luminal A, luminal B, HER2, basal-like, normal breast-like) and the MI of *SEPT9_v2* was compared according to the intrinsic subtypes. *Dunnett's test.

with hormone receptor positivity, low histological grade and non-pCR (Table II). The MI was significantly lower in basal type tumors (3.0%) than in luminal A (P=0.005), luminal B (P=0.026) or HER2 type (P=0.025) cancers (Fig. 5).

To determine whether methylation was related to gene expression, we performed ISH of *SEPT9_v2* and *GAPDH* mRNA in the *SEPT9_v2* hypermethylated (n=10) and hypomethylated tumors (n=10). *GAPDH* mRNA was expressed in both the *SEPT9_v2* hypermethylated and hypomethylated tumors (Fig. 6B). In contrast, ISH signals of *SEPT9_v2* mRNA in tumor cells were negative or barely detectable in all 10 hypermethylated tumors, but were clearly detectable in eight of 10 hypomethylated tumors (Fig. 6A).

Relationship between SEPT9_v2 methylation and the response to NAC. Clinicopathological parameters were evaluated by univariate analysis for their association with pCR (Table III). Age, Ki67, ER, PR, HER2 and *SEPT9_v2* methylation status were significantly associated with pCR.

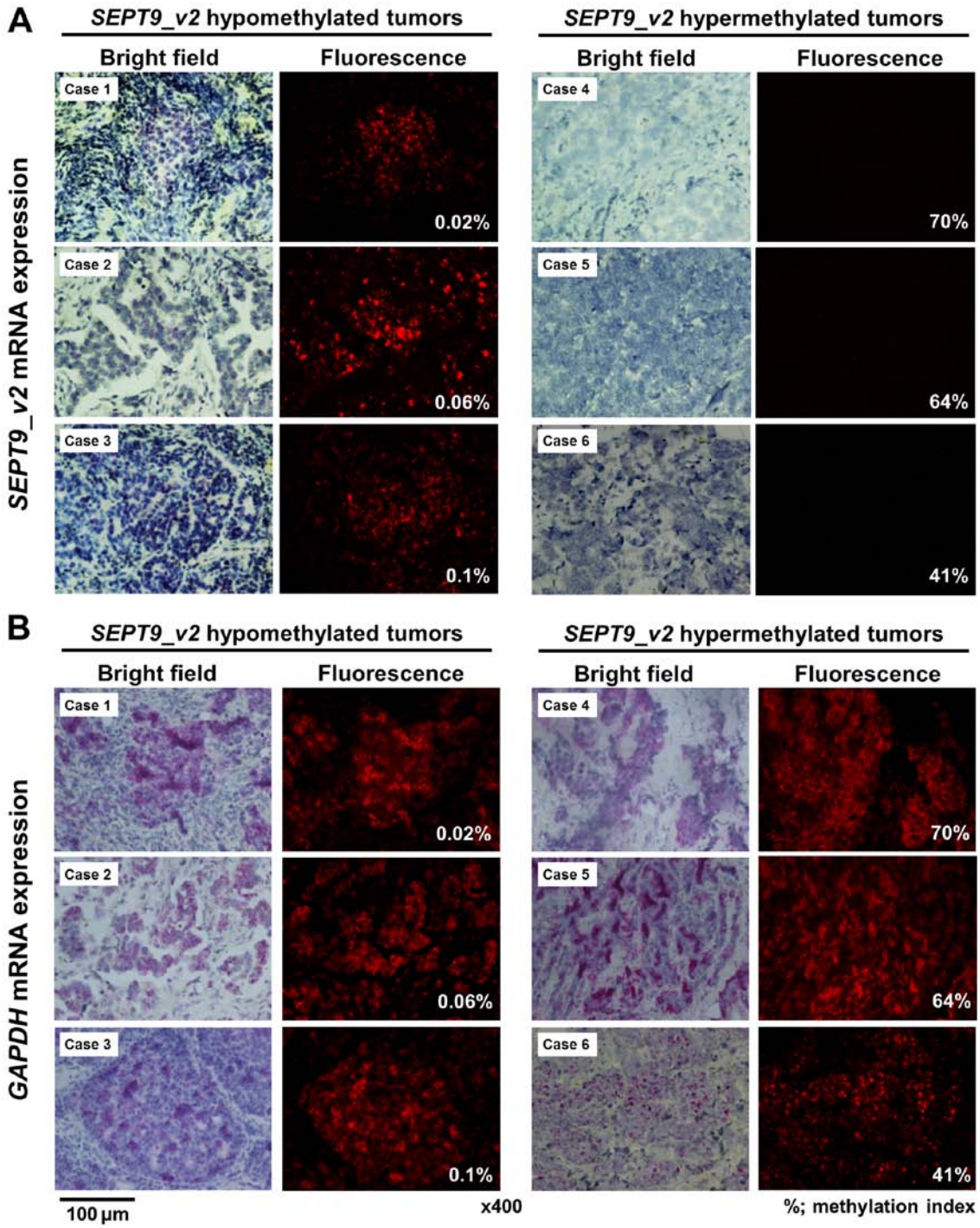

Figure 6. Representative ISH results of *SEPT9_v2* and *GAPDH* mRNA expression in breast cancer tissues. (A) ISH for *SEPT9_v2* mRNA and (B) *GAPDH* mRNA was conducted in *SEPT9_v2* hypomethylated or hypermethylated breast cancer tissues. Microscopic images were obtained by light and fluorescence microscopy. The MI of *SEPT9_v2* in each tumor is expressed as a percentage.

Multivariate analysis showed that ER, but not *SEPT9_v2* methylation, was a significant and independent predictor for pCR. The *SEPT9_v2* methylation status of breast tumors before and after NAC was also investigated in 20 patients with residual tumors (non-pCR) after NAC including 10 patients with *SEPT9_v2* hypermethylated tumors and 10 patients with hypomethylated tumors before NAC. The methylation status of the residual tumors was completely the same as that of the tumors before NAC, i.e., all of 10 *SEPT9_v2* hypermethylated tumors before NAC showed hypermethylation in the residual tumors after NAC and all of 10 *SEPT9_v2* hypomethylated tumors before NAC showed hypomethylation in the residual tumors after NAC (data not shown).

To further investigate the effect of *SEPT9_v2* on chemosensitivity, we carried out a knockdown assay with *SEPT9_v2* siRNA for MDA-MB-468 cells, in which *SEPT9_v2* is highly expressed and sensitive to both paclitaxel and epirubicin. *SEPT9_v2* siRNA specifically decreased the expression of *SEPT9_v2* in MDA-MB-468 cells (Fig. 7A) and the IC_{50} value

Table III. Univariate and multivariate analysis of clinicopathological parameters for pCR.

	Univariate analysis			Multivariate analysis		
Characteristics	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Age (years) (≥50 vs. <50)	3.14	1.32-7.98	0.009	2.74	0.98-8.10	0.054
T stage (T1,2 vs. T3,4)	1.80	0.64-5.90	0.274			
Lymph node status (positive vs. negative)	1.68	0.66-4.68	0.286			
Ki67 (positive vs. negative)	3.04	1.25-8.02	0.013	1.49	0.48-4.68	0.489
ER (negative vs. positive)	10.5	4.16-29.0	<0.001	7.97	2.15-39.40	0.001
PR (negative vs. positive)	5.60	2.09-17.9	<0.001	0.73	0.12-3.58	0.697
HER2 (positive vs. negative)	2.47	1.02-5.99	0.044	1.74	0.62-4.88	0.291
SEPT9_v2 methylation (<10 vs. ≥10%)	2.99	1.20-8.25	0.018	1.61	0.48-5.48	0.440

CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

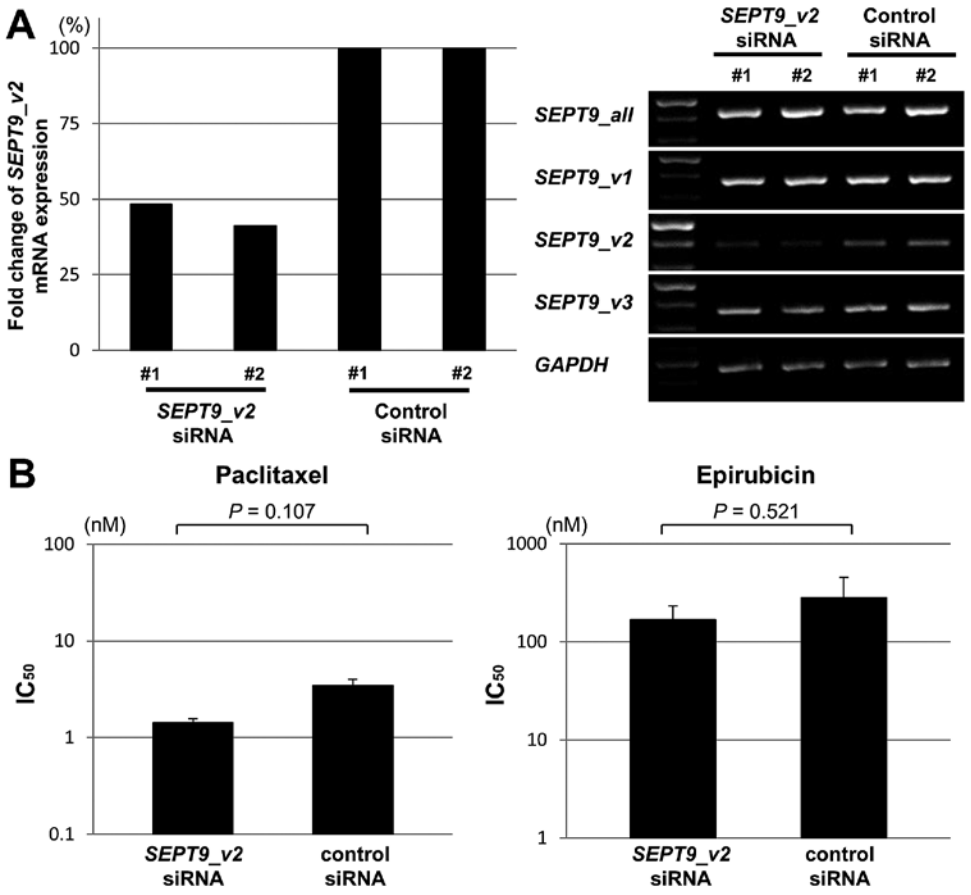

Figure 7. The knockdown effect of *SEPT9_v2* mRNA on chemosensitivity of breast cancer cells. (A) The knockdown effect of *SEPT9_v2* siRNA in MDA-MB-468 cells were assessed with real-time PCR (left) and RT-PCR (right). SEPT9_v1 and SEPT9_v3 were evaluated with RT-PCR to ascertain the specificity of *SEPT9_v2* siRNA (right). (B) The IC_{50} value of paclitaxel and epirubicin was compared between *SEPT9_v2* siRNA-treated MDA-MB-468 and the control cells.

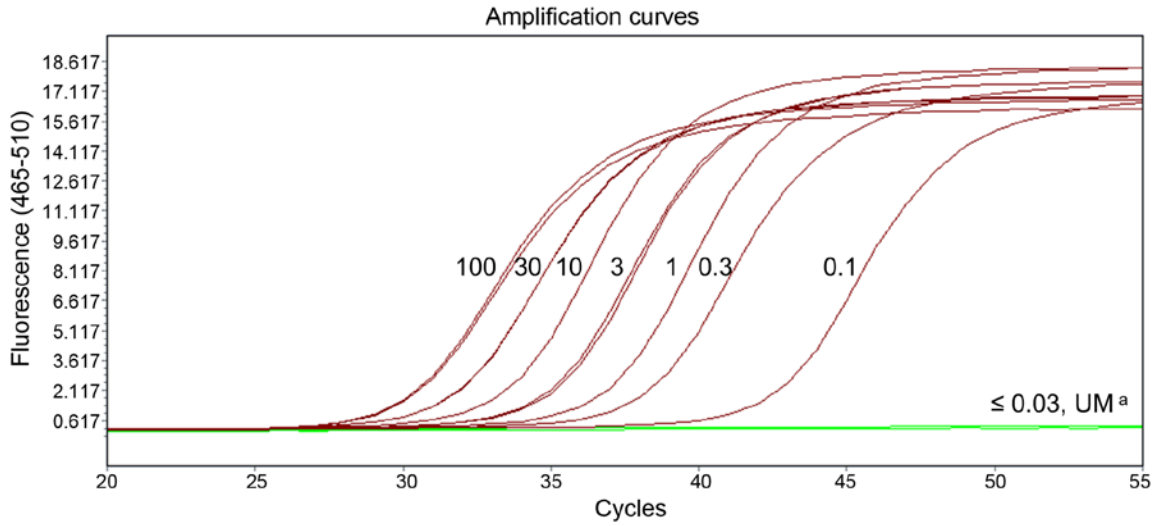

Figure 8. The amplification curves of a real-time PCR assay for *SEPT9_v2* promoter methylation. The amplification curves of the eight standards were obtained by diluting the methylated human control DNA to 100, 30, 10, 3, 1, 0.3, 0.1 and 0.03 ng/well. [a], Unmethylated DNA.

Table IV. Detection sensitivity of methylated *SEPT9_v2* in plasma of primary and metastatic breast cancer patients (study III).

		Methylated *SEPT9_v2* in plasma	
	Total	Positive No. (%)	Negative No. (%)
Healthy control	51	0 (0)	51 (100)
PBC patients	82	9 (11)	73 (89)
MBC patients	50	26 (52)	24 (48)
PBC + MBC patients[a] with	74		
SEPT9_v2 hypermethylated tumors	50	17 (34)	33 (66)[b]
SEPT9_v2 hypomethylated tumors	24	2 (8)	22 (92)

PBC; primary breast cancer; MBC, metastatic breast cancer; [a]primary tumor tissues were available from 49 PBC patients and 25 MBC patients for *SEPT9_v2* methylation assay; [b]P<0.05; PBC, primary breast cancer; MBC, metastatic breast cancer.

for both paclitaxel and epirubicin was not significantly different between *SEPT9_v2* siRNA-treated and control cells (Fig. 7B).

Detection of methylated SEPT9_v2 ctDNA in breast cancer patients. The presence of methylated *SEPT9_v2* ctDNA was assessed in plasma from 82 PBC and 50 MBC patients, and 51 healthy controls (study III). An amplification curve of the eight standards was obtained by diluting the methylated human control DNA to 100, 30, 10, 3, 1, 0.3, 0.1 and 0.03 ng/well (Fig. 8). The limit of detection for methylated *SEPT9_v2* DNA was 0.1 ng/well. Methylated *SEPT9_v2* ctDNA was detected in 11% (9/82) of PBC patients, and 52% (26/50) of MBC patients, but not in any of the healthy controls (Table IV). In addition, the methylation status of *SEPT9_v2* was investigated in the primary tumors available from PBC patients (n=49) and MBC patients (n=25). *SEPT9_v2* hypermethylation in the primary tumors was found in 67% (33/49) of PBC patients and 68% (17/25) of MBC patients. Methylated *SEPT9_v2* ctDNA was significantly (P<0.05) more frequently observed in the PBC and MBC patients with *SEPT9_v2* hypermethylated tumors [34% (17/50)] than those with *SEPT9_v2* hypomethylated tumors [8% (2/24)] (Table IV), indicating a positive correlation of *SEPT9_v2* hypermethylation in primary tumors with the presence of *SEPT9_v2* ctDNA. However, in order to confirm that *SEPT9_v2* methylated ctDNA actually originated from tumor cells, tumor cells and stromal cells were separately collected by means of LCM and subjected to the methylation assay in three patients positive for *SEPT9_v2* ctDNA. In all three patients, *SEPT9_v2* methylation was detectable only in tumor cells but not in the stromal cells (Fig. 4C).

Discussion

In the present study, *SEPT9_v2* hypermethylation was observed in 8 (67%) of the 12 breast cancer cell lines and 53% of breast tumor tissues examined (study I), but not in a normal human mammary epithelial cell line or normal breast tissues. The lower MI in tumor tissues compared to breast cancer cell lines may have resulted from the contamination of tumor tissue by normal stromal or inflammatory cells, since the tumor cells isolated by the MACS method showed higher MIs than the tumor tissue from which they were derived and since *SEPT9_v2* methylation was observed in tumor cells but not in stromal cells separately obtained by LCM. These results clearly indicate that tumor cells actually harbor methylated *SEPT9_v2*.

We found that the expression of *SEPT9_v2* mRNA was inversely correlated with the *SEPT9_v2* MI in breast cancer cell lines, and that treatment of *SEPT9_v2* hypermethylated breast cancer cell lines with a demethylating reagent resulted in the reactivation of *SEPT9_v2* mRNA expression. These results

clearly demonstrate that *SEPT9_v2* expression is epigenetically regulated by promoter methylation in breast cancer cell lines, consistent with the studies on colorectal cancers (32). In addition, we confirmed such epigenetic regulation in breast tumor tissue using ISH to demonstrate that *SEPT9_v2* mRNA expression was silenced in *SEPT9_v2* hypermethylated tumors.

The *SEPT9_v2* MI was significantly lower in basal type tumors than in other intrinsic tumor subtypes. This is consistent with the study that basal type tumors are globally hypomethylated as compared with other subtypes (33). Although *SEPT9_v2* hypermethylation was significantly associated with non-pCR, this does not necessarily mean that such hypermethylation plays a significant role in chemotherapy resistance. Multivariate analysis failed to demonstrate that *SEPT9_v2* hypermethylation was an independent predictor for non-pCR. In addition, we observed that knockdown of *SEPT9_v2* mRNA by siRNA had no effect on chemosensitivity even though the potential involvement of *SEPT9_V1* and *SEPT9_V4* in chemoresistance has been reported (34-36). However, the methylation status of the residual tumors after NAC was completely the same as that of the tumors before NAC, indicating that chemotherapy did not affect the *SEPT9_v2* methylation status. Thus, it is possible that *SEPT9_v2* hypermethylation is indirectly associated with non-pCR via its strong association with the ER, which is a well-established predictor for non-pCR (37-39). Taken together, *SEPT9_v2* is unlikely to play a significant role in chemoresistance, and is not a clinically useful predictor for non-pCR.

Methylated *SEPT9_v2* ctDNA appears to be one of the most successful markers for the early detection of colorectal cancer. Since a recent study showed that this ctDNA can be detected in lung cancer patients (40), its clinical utility for other types of cancers needs to be clarified. The present study revealed that methylated *SEPT9_v2* ctDNA was detectable in 11% of PBC patients and 52% of MBC patients and that it was significantly more frequently observed in the PBC/MBC patients with hypermethylated tumors than those with hypomethylated tumors. However, the sensitivity of this methylated ctDNA for PBC and MBC was lower than that for primary and metastatic colorectal cancer (45 and 77%, respectively) (22). This lower sensitivity can be explained by the lower proportion of methylated *SEPT9_v2* breast cancer [50% (100/200), all tumors combined in the present study] compared to colorectal cancer (82%) (41). Another potential explanation is the different methods of assay that were used. The methylated *SEPT9_v2* ctDNA detection kit, known as Epi proColon® 2.0 (Epigenomics AG, Berlin, Germany), can detect 14 pg/ml of this ctDNA. This high sensitivity is achieved by a triplicate assay using a greater volume of plasma (3.5 ml). The present study was carried out by a single assay using less plasma (2 ml) since our retrospective samples contained insufficient volumes.

In conclusion, although methylation of many other genes has been reported in breast cancer (3-9), methylation status of *SEPT9_v2* and its correlation with mRNA expression has yet to be studied in human breast cancers. Then, we analyzed this issue, and were able to show that hypermethylation of *SEPT9_v2* was seen in a high proportion of breast tumors and that its methylation was clearly correlated with the silencing of *SEPT9_v2* mRNA expression. However, we could show for the first time that *SEPT9_v2* methylated ctDNA was detectable in a significant proportion of PBC and MBC patients, suggesting a possibility that *SEPT9_v2* methylated ctDNA may serve as a novel tumor marker. Our present observation needs to be further investigated in a future study including a larger number of patients.

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