



# Methylated septin 9 gene for noninvasive diagnosis and therapy monitoring of breast cancer

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**Background:** Blood-based biomarkers specific for breast cancer is urgently demanded to facilitate the screening and reduce breast cancer-related deaths. This study evaluated the potential of methylated septin 9 gene (*mSEPT9*) as a biomarker for breast cancer.

**Methods:** A total of 86 breast biopsy samples were collected from 59 patients with breast cancer and from 27 patients with benign breast tumor. Two hundred and seven plasma samples were collected from 80 patients with breast cancer, 27 patients with benign breast tumor patients, and 100 healthy volunteers. *mSEPT9* was detected using the Epi proColon 2.0 CE test. Additionally, 40 tissue cases of the tumor samples were analysed by real time PCR for *SEPT9\_v2* mRNA expression and by immunohistochemical staining for *SEPT9* protein expression. Serum CEA, CK19 and CA15-3 concentrations were analyzed using an Electrochemiluminescence Immunoassay Kit.

**Results:** The sensitivity of plasma *mSEPT9* for detecting breast cancer was 26.3% with a specificity of 97.6%. The plasma *mSEPT9*-positive rate was higher in patients with later stage lymph node metastasis (P=0.018), intravascular tumor thrombus (P=0.035), ki67-positive (P=0.033) and p53-positive (P=0.044). Despite its limitation in sensitivity, conversion of plasma *mSEPT9* to negative status in *mSEPT9*-positive breast cancer patients following cancer treatment was correlated with favorable responses, while persistent positivity was associated with poor responses. *mSEPT9* performed comparably with CK19 (P>0.05) and better than CEA (P=0.002) and CA15-3 (P<0.001) in sensitivity. *mSEPT9* was detected in 83.1% (49/59) of the breast cancer tissues, whereas 8.0% (2/25) of the benign breast tumor tissues were *mSEPT9* positive (P<0.001). The positivity of *mSEPT9* in breast cancer tissue was associated with tumor size of >2 cm (P=0.026) and with invasion of adipose tissue (P=0.015).

**Conclusions:** Our results indicate that *mSEPT9* test has the potential to become a valuable tool in the diagnosis and treatment monitoring of breast cancer for Chinese patients.

**Keywords:** Breast cancer; diagnosis; methylation; septin 9; treatment monitoring

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## Introduction

Breast cancer is one of the most common cancers and a leading cause of cancer-related deaths in women worldwide (1). Screening tests enabling early detection of breast tumors facilitate cancer treatment at early stages, thereby improving patient prognosis and clinical outcomes. Currently available approaches for breast cancer diagnosis include imaging techniques and biomarkers tests (2). Breast ultrasound and molybdenum target radiography are generally less sensitive, thus often lead to false negative results for early-stage cancer patients (3). Magnetic resonance imaging (MRI) has high sensitivity in detecting early stage cancers; however, the specificity of breast MRI is limited and questionable (4,5). Despite common use of imaging approaches in clinical practice, tumor markers have been of great interest to physicians, which allow to detect early disease and help screening in a cost-effective manner. Several tumor markers have been recommended for breast cancer diagnosis, including carcinoembryonic antigen (CEA), estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Nevertheless, use of these markers for screening and diagnosis has been hindered and limited due to the low sensitivity and lack of specificity (6). Thus, there is an urgent need to develop novel, more sensitive, and ideally noninvasive diagnostic biomarkers to maximize the number of women who participate in screening and to reduce breast cancer-related deaths. Despite the intensive research efforts, currently there is no reliable test that can prognosticate the efficacy of the therapies shortly after the treatments.

Circulating tumor DNA (ctDNA) has been proposed as a screening tool in breast cancer screening tests (7). Because breast cancers develop as the result of accumulation of genetic and epigenetic changes (8), and molecular markers concerning genetic and epigenetic alterations have been evaluated (9). DNA methylation is considered as a major tumor-associated epigenetic change (10), since methylation of tumor suppressor genes can result in gene silencing and thus potentially sustain cancerogenesis (11). For this reason, differences in the methylation status of tumor DNA have been evaluated in breast tumors (12). Septin 9 (*SEPT9*) is a member of the SEPTIN family involved in cytokinesis and cytoskeletal organization (13), and alterations of *SEPT9* gene have been linked to multiple cancers (14). However, only few studies so far have evaluated the methylation of the *SEPT9* promoter region as a biomarker for breast cancer detection (15,16).

In the present study, we examined the methylation status of *SEPT9\_v2* in both plasma samples and tumor tissues collected from Chinese breast tumor patients (malignant and benign tumor). Subsequently, we analyzed the correlation between methylated septin 9 (*mSEPT9*) status and clinicopathological characteristics as well as molecular classifications of breast cancer patients. We also compared the power of plasma *mSEPT9* as a diagnostic biomarker with several often used tumor biomarkers including serum CEA, cytokeratin 19 fragments (CK19 or CYFRA 21-1) and carbohydrate antigen 15-3 (CA15-3). Finally, the application of the plasma *mSEPT9* as a follow-up biomarker after breast cancer treatment was evaluated.

## Methods

### *Tissues and plasma specimens*

A total of 86 breast tumor tissue samples (59 breast cancer and 27 benign breast tumor patients) confirmed by pathologic examinations were obtained from the needle breast biopsy collections of Liaocheng People's Hospital between August 2016 and June 2017. No statistic differences were detected in either age or menopausal status (*Table S1*). These patients had no prior history of cancer-related surgery, chemotherapy, or hormonal therapy. The clinicopathological characteristics and molecular classifications of these patients are summarized in *Tables 1,2*. Tissue samples were snap-frozen in liquid nitrogen and kept at  $-196^{\circ}\text{C}$  until analyses.

A total of 207 plasma samples collected from 80 patients with breast cancer, 27 patients with benign breast tumor, and 100 healthy volunteers. There were no statistic differences among these cohorts in either age or menopausal status (*Table S2*). The healthy volunteers enrolled as controls had no diagnosed neoplasm in their mammary gland or other organs. The clinicopathological parameters and molecular classifications of these cohorts are summarized in *Tables 3,4*. Twenty-one follow-up specimens were obtained from breast cancer patients who had been treated with neoadjuvant chemotherapy and surgical treatment.

Clinical and pathologic data of the breast cancer patients were collected from the electronic medical records. The TNM cancer staging (T: size or direct extent of the primary tumor; N: degree of spread to regional lymph nodes; M: presence of distant metastasis) was assigned based on the seventh edition of the Cancer Staging Manual of the American Joint Committee on Cancer (17). The efficacy

**Table 1** Relationship between tissue *mSEPT9* status and clinicopathological parameters

Characteristics	N	<i>mSEPT9</i> , n (%)	P value
All cases	59	49 (83.1)	
Age (years)			NS <sup>&amp;</sup>
≤50	29	23 (79.3)	
>50	30	26 (86.7)	
Menopausal status			NS <sup>&amp;</sup>
Pre	29	23 (79.3)	
Post	30	26 (86.7)	
Tumor size (cm)			0.026 <sup>*,&amp;</sup>
≤2	17	11 (64.7)	
>2	42	38 (90.5)	
Lymph node metastasis			
Negative	22	18 (81.8)	NS <sup>&amp;</sup>
Positive	37	31 (83.8)	
N0	22	18 (81.8)	NS <sup>†,&amp;</sup>
N1	29	23 (79.3)	
N2	2	2 (100.0)	
N3	6	6 (100.0)	
N0 + N1	51	41 (80.4)	NS <sup>‡,&amp;</sup>
N2 + N3	8	8 (100.0)	
Histological type			NS <sup>&amp;</sup>
IDC	52	43 (82.7)	
Non-IDC	7	6 (85.7)	
Histological grade			NS <sup>&amp;</sup>
1+2	30	25 (83.3)	
3	24	20 (83.3)	
Unknown	5	4 (80.0)	
TNM stage			NS <sup>§,&amp;</sup>
0	2	2 (100.0)	
I	7	5 (71.4)	
II	40	33 (82.5)	
III	9	8 (88.9)	
IV	2	1 (50.0)	

**Table 1** (continued)

**Table 1** (continued)

Characteristics	N	<i>mSEPT9</i> , n (%)	P value
Invasion into fat			
Negative	5	2 (40.0)	0.015 <sup>#,&amp;</sup>
Positive	37	34 (91.9)	
Unknown	17	13 (76.5)	
Intravascular tumor thrombus			NS <sup>&amp;</sup>
Negative	25	21 (84.0)	
Positive	18	16 (88.9)	
Unknown	16	12 (75.0)	
Invasion into nerve			NS <sup>&amp;</sup>
Negative	31	27 (87.1)	
Positive	13	11 (83.3)	
Unknown	15	11 (25.0)	

<sup>\*</sup>, comparison of *mSEPT9* positive rates between tumor size; <sup>†</sup>, comparison of *mSEPT9* positive rates among the four lymph node metastasis groups; <sup>‡</sup>, comparison of *mSEPT9* positive rates between early stage (N0+N1) and late stage (N2+N3) lymph node metastasis groups; <sup>§</sup>, comparison of *mSEPT9* positive rates among the five stages; <sup>#</sup>, comparison of *mSEPT9* positive rates between invasion into fat-negative and -positive groups; <sup>&</sup>, analysis of the comparison by Fisher's exact test. IDC, invasive ductal carcinoma; NS, non-significant.

of chemotherapy was evaluated by Miller-Payne grade (MP) classification and response evaluation criteria in solid tumors (RECIST) (18,19).

#### *Analysis of mSEPT9 in tissues and plasma*

To test the *mSEPT9* in biopsy tissues, DNA was extracted from the tissues using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. Briefly, the samples were first lysed using proteinase K and then loaded onto DNeasymini spin columns. The DNA was centrifuged, washed, and then eluted using elution buffer. All DNeasy-purified DNA samples had A260/A280 ratios of 1.7–1.9, and spectrometry scans showed a symmetric peak at 260 nm.

DNA in plasma was extracted using the EpiproColon Plasma Quick Kit (Epigenomics AG, Berlin, Germany).

**Table 2** Relationship between tissue *mSEPT9* status and molecular classifications

Characteristics	N	<i>mSEPT9</i> , n (%)	P value
All cases	59	49 (83.1)	
ER			NS*
Negative	11	9 (81.8)	
Positive	46	39 (84.8)	
Unknown	2	1 (50.0)	
PR			NS*
Negative	16	13 (81.3)	
Positive	41	35 (85.4)	
Unknown	2	1 (50.0)	
HER2			NS*
Negative	29	24 (82.8)	
Positive	16	14 (87.5)	
Unknown	14	11 (78.6)	
Ki-67			NS*
<14%	9	8 (85.7)	
≥14%	50	41 (82.0)	
Subtype (IHC)			NS* <sup>#</sup>
Luminal A	6	5 (83.3)	
Luminal B	40	34 (85.0)	
HER2	5	5 (100.0)	
Triple negative	6	4 (66.7)	
Unknown	2	1 (50.0)	
p53			NS*
Negative	15	12 (80.0)	
Positive	40	35 (87.5)	
Unknown	4	2 (50.0)	

\*, analysis of the comparison by Fisher's exact test; <sup>#</sup>, comparison of *mSEPT9* positive rates among the four subtypes. IHC, immunohistochemistry; ER, estrogen receptor; PR, progesterone receptor; NS, non-significant; 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.

**Table 3** Relationship between plasma *mSEPT9* status and clinicopathological parameters

Characteristics	N	<i>mSEPT9</i> , n (%)	P value
All cases	80	21 (26.3)	
Age (years)			NS <sup>#</sup>
≤50	40	11 (30.0)	
>50	40	10 (25.0)	
Menopausal status			NS <sup>#</sup>
Pre	41	10 (24.4)	
Post	39	11 (28.2)	
Tumor size (cm)			NS <sup>#</sup>
≤2	27	7 (7.4)	
>2	53	14 (26.4)	
Lymph node metastasis			
Negative	32	6 (18.8)	NS <sup>#</sup>
Positive	48	15 (31.3)	
N0	32	6 (18.8)	0.071* <sup>,&amp;</sup>
N1	38	9 (23.7)	
N2	3	2 (66.7)	
N3	7	4 (57.1)	
N0 + N1	70	15 (21.4)	0.018 <sup>†,&amp;</sup>
N2 + N3	10	6 (60.0)	
Histological type			NS <sup>&amp;</sup>
IDC	72	20 (27.8)	
Non-IDC	8	1 (12.5)	
Histological grade			NS <sup>#</sup>
1+2	42	12 (28.6)	
3	30	9 (30.0)	
Unknown	8	0	
TNM stage			0.029 <sup>†,&amp;</sup>
0	3	0	
I	11	2 (18.2)	
II	53	11 (20.8)	
III	11	7 (63.6)	
IV	2	1 (50.0)	

**Table 3** (continued)

**Table 3** (continued)

Characteristics	N	mSEPT9, n (%)	P value
Invasion into fat			NS <sup>&amp;</sup>
Negative	11	2 (18.2)	
Positive	49	15 (30.6)	
Unknown	20	4 (20.0)	
Intravascular tumor thrombus			0.035 <sup>§, #</sup>
Negative	39	6 (15.4)	
Positive	23	10 (43.5)	
Unknown	18	6 (33.3)	
Invasion into nerve			NS <sup>#</sup>
Negative	44	10 (22.7)	
Positive	18	7 (38.9)	
Unknown	18	4 (22.2)	

\*, comparison of mSEPT9 positive rates among the four lymph node metastasis groups; †, comparison of mSEPT9 positive rates between early stage (N0+N1) and late stage (N2+N3) lymph node metastasis groups; ‡, comparison of mSEPT9 positive rates among the five TNM stages; §, comparison of mSEPT9 positive rates in intravascular tumor thrombus-positive and -negative groups; #, analysis of the comparison by Chi-square test; &, analysis of the comparison by Fisher's exact test. IDC, invasive ductal carcinoma; NS, non-significant.

To evaluate *SEPT9* methylation, 10 mL peripheral blood samples were collected with 10 mL K<sub>2</sub> EDTA anticoagulant tubes (BD-Plymouth, Oxford, UK). The blood samples were stored at 2 to 8 °C for up to 24 h prior to plasma preparation. Each sample was centrifuged for 12 min at 2,500 rpm at room temperature. Plasma was transferred to a clean 15 mL polypropylene centrifuge tube with conical bottom. The sample was centrifuged again for 12 min at 2,500 rpm. Plasma was transferred into a labeled cryovial and stored at -80 °C. To purify circulating DNA, 3.5 mL plasma was mixed with an equal volume of lysis buffer, and the mixture was then incubated at 15 to 30 °C for 10 min. Then, 90 µL Epi proColon Magnetic Beads (freshly suspended) and 2.5 mL of Absolute Ethanol (for molecular biology, ≥99.5%) were added into the plasma. The mixture was subsequently incubated on a rotator at 15 to 30 °C for 45 min at 15 rpm. The magnetic beads were then washed and DNA eluted into 100 µL elution buffer. This procedure usually yields DNA concentrations at a range between 5.1 to 16.3 ng/mL from plasma samples and 40.2 to

**Table 4** Comparison of the plasma *mSEPT9* status with molecular classifications of breast tumor

Characteristics	N	mSEPT9, n (%)	P value
All cases	80	21 (26.3)	
ER			NS <sup>#</sup>
Negative	15	4 (26.7)	
Positive	63	17 (27.0)	
Unknown	2	0	
PR			NS <sup>§</sup>
Negative	23	7 (30.4)	
Positive	54	14 (25.9)	
Unknown	3	0	
HER2			NS <sup>§</sup>
Negative	44	11 (25.0)	
Positive	20	7 (35.0)	
Unknown	16	3 (18.8)	
Ki-67			0.033 <sup>*, #</sup>
<14%	17	1 (5.9)	
≥14%	63	20 (31.7)	
Subtype (IHC)			NS <sup>§, #</sup>
Luminal A	11	1 (9.0)	
Luminal B	52	16 (30.8)	
HER2	5	2 (40.0)	
Triple negative	9	2 (22.2)	
Unknown	3	0	
p53			0.044 <sup>†, #</sup>
Negative	20	2 (10.0)	
Positive	57	19 (33.3)	
Unknown	3	0	

\*, comparison of *mSEPT9* positive rates between Ki-67 <14% and ≥14% groups; †, comparison of *mSEPT9* positive rates among the four subtype; ‡, comparison of *mSEPT9* positive rates between p53-negative and -positive groups; §, analysis of the comparison by Chi-square test; #, analysis of the comparison by Fisher's exact test. NS, non-significant.

709.7 ng/mL from tissue samples.

The purified DNA was then mixed with 150 µL bisulfite solution and 25 µL Epi proColon Protection Buffer, and then incubated for 45 min at 80 °C. The converted DNA (bisulfite-modified DNA, designated as bisDNA

hereafter) was then analyzed by duplex real-time PCR using methylation-specific primers on a 7500 real-time PCR system (Applied Biosystems, California, America) with simultaneous detection of both the methylated *SEPT9* DNA and the internal control *ACTB* ( $\beta$ -actin) DNA (20). The sequences of primers, blocker, and probes for *SEPT9* detection used in methylation-specific PCR amplification were as follows: forward primer, 5'-CCCACCAACCATCATAT-3'; reverse primer, 5'-GTAGTAGTTAGTTTATTTATTTT-3'; blocker, 5'-CATCATATCAAACCCACAATCAACACACAAC-3'; probe 1, 5'-GTTTCGAAATGATTTTATTTAGTTGC-3'; probe 2, 5'-CGTTGATCGCGGGGTTTC-3' (GenBank Gene ID: 10801) (21). The thermocycling program was as follows: denaturing at 94 °C for 20 min; 45 cycles of 93 °C for 30 s followed by 55.5 °C for 35 sec and 62 °C for 35 s; and cooling at 40 °C for 5 s.

#### *Analysis of SEPT9\_v2 mRNA in tissues*

Total RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections (4  $\mu$ m) using RNeasy FFPE Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Then the cDNA was synthesized using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Tli RNaseH Plus) kit (Takara, Tokyo, JAP) according to the manufacturer's instructions. SYBR-Green real-time quantitative PCR analyses for mRNAs of *SEPT9\_v2* and internal control ( $\beta$ -actin) were performed on a 7500 real-time PCR system (Applied Biosystems, California, America) using the following parameters: 95 °C (30 s), followed by 40 cycles of 95 °C (5 s) and 60 °C (30 s).

#### *Immunohistochemistry (IHC) Staining of SEPT9 protein*

Tissue samples were fixed in 10% formaldehyde and embedded in paraffin. Serial 5- $\mu$ m sections were cut, deparaffinized in xylene, and hydrated through an ethanol series. The sections were then heated at 100 °C for 30 min in citrate buffer (pH 6.0) in a microwave oven to retrieve antigens. After quenching endogenous peroxidase with 3% hydrogen peroxide for 15 min at room temperature, non-specific binding was blocked by incubating the slides with SPlink Detection Kits (ZSGB-BIO, Beijing, China) for 30 min, followed by incubation of the sections with a rabbit polyclonal anti-*SEPT9* (1:100) antibody (Abnova, Taipei, Taiwan) at 4 °C overnight.

For IHC, the sections were washed three times with

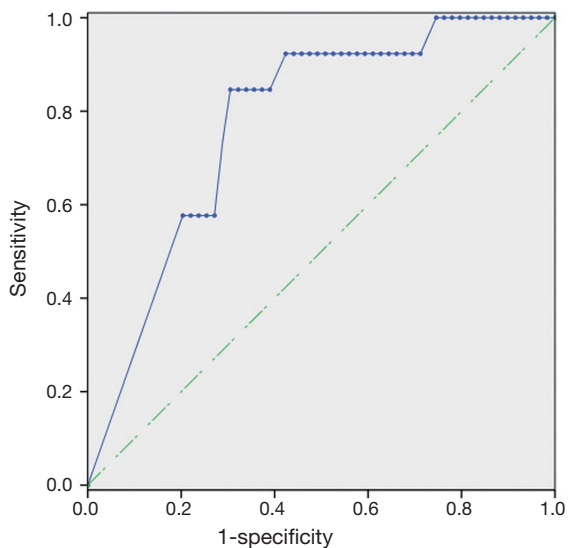
PBS, and then incubated for 1 h with a HRP-conjugated Goat anti-rabbit secondary antibody (Epigentek, Wuhan, China) diluted at a ratio of 1:100. The color on the slides was developed for 2 min using a DAB Chromogen Substrate Kit (Solarbio, Beijing, China), and sections were then counter stained with hematoxylin staining to identify cell nuclei. A scoring system was assigned to evaluate the *SEPT9* protein expression levels: 0 for negative staining, +1 for weak staining, +2 for moderate staining, and +3 for strong staining.

#### *Determination of the status of ER, PR, HER2, Ki67 and p53*

The status of ER, PR, HER2, Ki-67 as well as the p53 index were determined by IHC method. The antibodies used were as follows: anti-ER (SP1) rabbit monoclonal antibody (Roche Diagnostic GmbH, Mannheim, Germany), anti-PR (1E2) rabbit monoclonal antibody (Roche Diagnostic GmbH), anti-HER2 (4B5) rabbit monoclonal antibody (Roche Diagnostic GmbH), anti-Ki67 (MIB-1) mouse monoclonal antibody (Maxim, Fuzhou, China), and anti-p53 (DO-7) mouse monoclonal antibody (Maxim). The procedures of IHC were as described above.

Interpretation standard: ER and PR were considered as positive when stained nuclei constituted  $\geq 1\%$  of total nuclei, according to the guideline recommendations (22). The Ki67 scores were expressed as the percentage of positively staining cells among the total number of invasive cells in the scored area (23). Neoplastic lesions in each p53-immunostained slide were confirmed using the corresponding hematoxylin and eosin stained specimens (24). Briefly, HER2 staining in tumor tissues were categorized into four grades (0, 1, 2, and 3), and tumors with scores of 3+ grades were considered to be positive according to the guideline recommendations (25). When HER2-IHC was 2+, HER2-FISH (fluorescence *in situ* hybridization) assay was performed according to the latest ASCO/CAP guideline.

FISH assays were carried out on paraffinized tissue sections (4- $\mu$ m) using the PathVysion HER-2 DNA Probe Kit (Abbott Laboratories, Des Plaines, USA). HER2 positivity is defined according to American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) recommendations (25). Sixty cells were routinely scored for HER2 signal (red) and CEP17 signal (green). The mean HER2 and CEP17 signals were used to calculate the final FISH ratio.



**Figure 1** Receiver operating characteristic curve of *mSEPT9* for discriminating breast cancer from benign breast tumor in tissues. Receiver operating characteristic curve analysis was performed by SPSS18.0 to evaluate the diagnostic performance of *mSEPT9* as a biomarker for differentiating breast cancer from benign breast tumor samples.

**Table 5** *SEPT9* methylation status in tissues

Characteristics	N	Tissue <i>mSEPT9</i>	P value
Breast cancer	59	49 (83.1%)	
Benign breast tumor	25	2 (8.0%)	<0.001*

\*, comparison of *mSEPT9* positive rates between breast cancer and benign breast tumor by Fisher's exact test.

### Measurements of CEA, CK19 and CA15-3

Serum CEA, CK19 and CA15-3 concentrations were analyzed using an Electrochemiluminescence Immunoassay Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. They were defined as positive when detection results were greater than the following values: 5 U/mL (CEA), 30 U/mL (CA15-3) and 3.3 U/mL (CK19), respectively.

### Statistical analyses

All statistical analyses were performed using the SPSS18 software (SPSS, Chicago, America). The quantitative data was expressed as mean  $\pm$  standard deviation (SD) and

analyzed using *t*-test. Chi-square test or Fisher's exact test (when appropriate) was applied to evaluate the correlation between clinicopathological features and *mSEPT9* status. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of *mSEPT9* as a biomarker for differentiating breast cancer from benign breast tumor samples (26). Discriminative power was assessed by the area under a receiver operating characteristic curve (AUC). Cut-off value was determined by the optimal Youden's index (sensitivity + specificity - 1). The sensitivity and specificity of the *SEPT9* test were calculated as follows: sensitivity = number of true positives/total number of sick individuals in population; specificity = number of true negatives/total number of well individuals in population. 95% CI was calculated according to the efficient-score method (corrected for continuity) (27).  $P < 0.05$  was considered statistically significant.

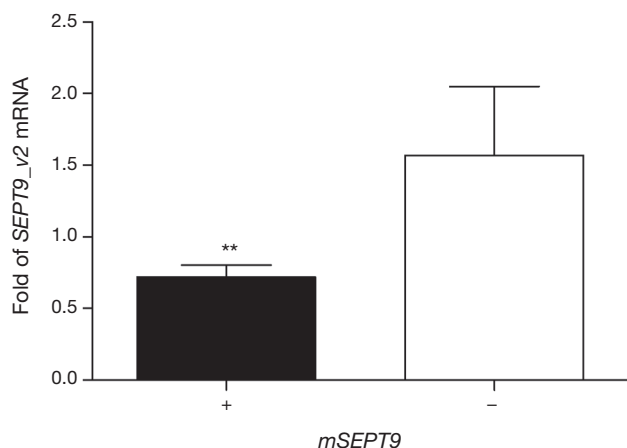
## Results

### *SEPT9* methylation in breast cancer tissues

As plotted in *Figure 1*, ROC curve analysis was performed to evaluate the diagnostic performance of *mSEPT9* as a biomarker for differentiating breast cancer from benign breast tumor tissue samples. The calculated AUC was 0.777 and cut-off value of *mSEPT9* was 37.6 cycles. As shown in *Table 5*, *mSEPT9* was detected in the tissues of 49 of 59 breast cancer patients (83.1%) and 2 of 25 (8.0%) benign breast tumor patients (two samples were excluded due to the cycle numbers of ACTB  $>37.6$ ) ( $P < 0.001$ ). The sensitivity and specificity of *mSEPT9* for breast cancer detection were 83.1% (95% CI, 71.5–90.5%) and 92.0% (95% CI, 75.0–97.8%), respectively (*Table S3*).

### Expression of *SEPT9\_v2* mRNA and *SEPT9* protein in tissues

Total RNA was isolated from the biopsy tissues of 40 cases of breast tumors and reverse transcribed. Real-time quantitative PCR assays were carried out to evaluate the levels of *SEPT9\_v2* mRNA. Expression of *SEPT9\_v2* mRNA was lower in *mSEPT9*-positive breast cancer tissues than in *mSEPT9*-negative benign breast tumor tissues (*Figure 2*). These biopsy tissues were also analyzed by IHC staining for *SEPT9* protein. The *SEPT9* was highly expressed in majority of them *mSEPT9*-negative tumors (IHC grades +2 and +3: 16/20), while either no expressions



**Figure 2** Expression of *SEPT9\_v2* mRNA in *mSEPT9*-positive and *mSEPT9*-negative tumors. Total RNA was isolated from 20 *mSEPT9*-positive and *mSEPT9*-negative formalin-fixed, paraffin-embedded breast cancer sections. The *SEPT9\_v2* mRNA levels in these tissues were assessed by RT-qPCR. \*\* $P < 0.01$ .

or low level expressions of *SEPT9* were observed in majority of the *mSEPT9*-positive tumors (0 and +1: 14/20) (Figure 3, Table S4).

#### Relationship between tissue *mSEPT9* and clinicopathological parameters of breast cancer patients

We investigated the associations between *mSEPT9* status in breast cancer tissues and clinicopathological parameters of the breast cancer patients. As presented in Table 1, breast cancer cases with tumor size  $> 2$  cm showed a significantly higher positive rate of *mSEPT9* than those with tumor size  $\leq 2$  cm (90.5% vs. 64.7%,  $P = 0.026$ ). Compared with breast cancer cases with negative fat invasion, the subjects with positive fat invasion showed a higher positive rate of *mSEPT9* with statistical significance (91.9% vs. 40.0%,  $P = 0.015$ ). However, no association was found between *mSEPT9* and lymph node metastasis, histological type, histological grade, subtype, stage, intravascular tumor thrombus or nerve tissue invasion ( $P > 0.05$ ).

We next sought to examine the associations between *mSEPT9* positive rate and the molecular classifications in breast cancer tissues. But unfortunately, no associations between *mSEPT9* and ER, PR, HER2, Ki67, or p53 status of the tumors were found (Table 2) ( $P > 0.05$ ).

#### Methylated *SEPT9* genomic DNA in plasma

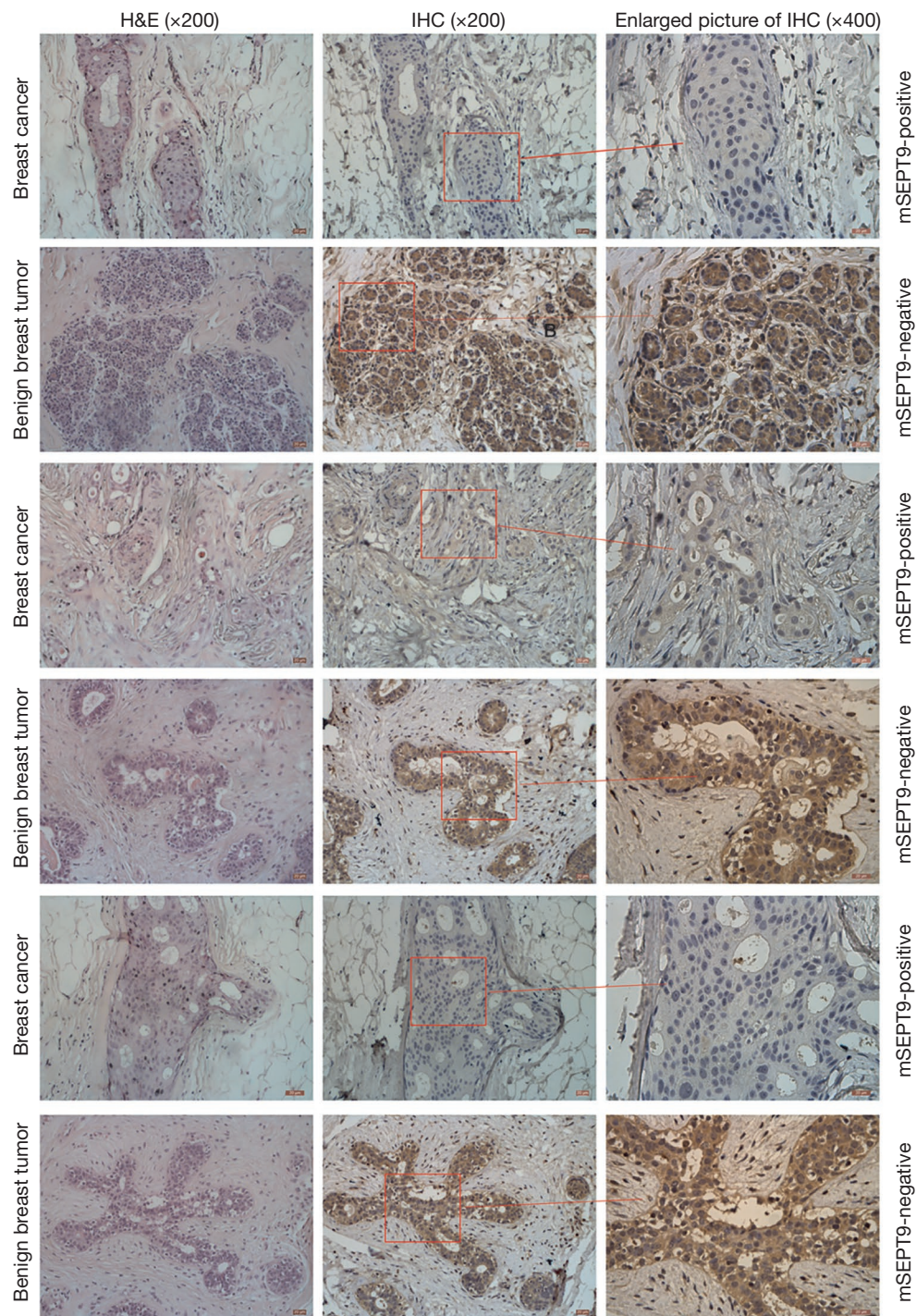
Having researched the status of *mSEPT9* in breast cancer tissues, we next examined the methylation status of *SEPT9* genomic DNA in the plasma of breast cancer patients. The cut-off value of qPCR for plasma was set at amplification cycles of 40.2, with the pre-requisition of positive ACTB detection before amplification cycles of 32.1 (Figure 4). Plasma *mSEPT9* was identified in 21 of 80 (26.3%) breast cancer subjects and 1 of 27 (3.7%) benign breast tumor patients, indicating a significant increase of *mSEPT9* in breast cancer patients compared with benign breast tumor patients ( $P = 0.012$ ) (Table 6). As expected, plasma *mSEPT9* was detected in only 2 of 100 (2.0%) healthy control subjects, showing a significantly lower positive rate than those in breast cancer cases ( $P < 0.001$ ) (Table 6). The sensitivity and specificity of the plasma *mSEPT9* test for breast cancer detection were 26.3% (95% CI, 17.9–36.8%) and 97.6% (95% CI, 93.28–99.19%), respectively (Table S3).

#### Relationship between plasma *mSEPT9* status and clinicopathological parameters of breast cancer

As shown in Table 3, among the 80 patients with breast cancer, the positive rate of plasma *mSEPT9* was 0% (0/3) for stage 0, 18.2% (2/11) for stage I, 20.8% (11/53) for stage II, 63.6% (7/11) for stage III, and 50% (1/2) for stage IV in breast cancer patients, suggesting a positive association between *mSEPT9* positivity rates and advanced tumor stages ( $P = 0.029$ ). The positive rate of plasma *mSEPT9* was 18.8% in stage N0, 23.7% in stage N1, 66.7% in stage N2, and 57.1% in stage N3 breast cancers, respectively, although the differences among the groups were not statistically significant, likely due to the low case numbers of patients with advanced lymph node metastasis. However, when the breast cancer patients were grouped into two lymph node metastasis stage sets [early stage (N0 and N1) and late stage (N2 and N3)], the plasma *mSEPT9* positivity rates became significantly different between the two stage sets, with greater plasma *mSEPT9* positivity rate in the later stage than in the early stage ( $P = 0.018$ ). Whereas the breast cancer patients with fat or nerve invasion did not show a significant increase in *mSEPT9* positivity, patients with intravascular tumor thrombus did show a greater *mSEPT9* positive rate than patients without intravascular tumor thrombus ( $P = 0.035$ ).

We also attempted to detect potential associations between plasma *mSEPT9* positivity and common molecular

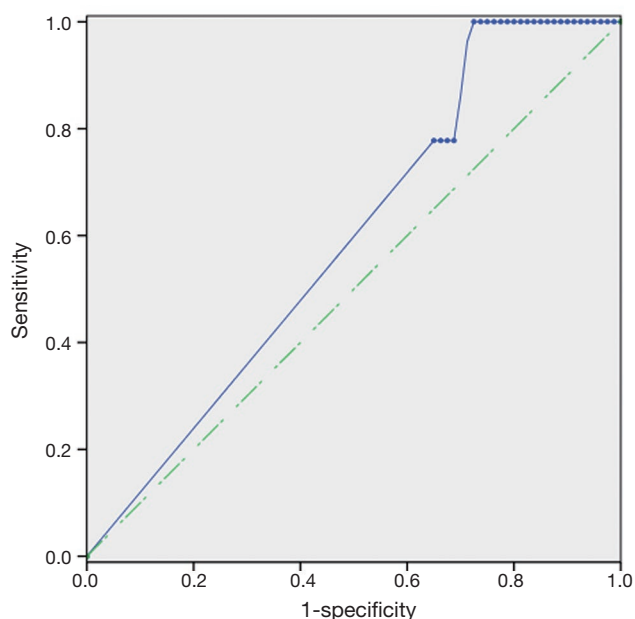




**Figure 3** *SEPT9* protein levels in benign and malignant breast tumors. Breast biopsy samples were fixed and sectioned, then subjected to IHC and H&E staining.

classifications of breast cancer. As shown in *Table 4*, there was a significant difference in the positivity of plasma *mSEPT9* between patients with higher Ki67 scores ( $\geq 14\%$ ) than those

with lower Ki67 scores ( $< 14\%$ ) ( $P=0.033$ ). Additionally, the positive rate of plasma *mSEPT9* was higher in p53-positive patients than those with p53-negative tumors ( $P=0.044$ ).



**Figure 4** Receiver operating characteristic curve of *mSEPT9* for discriminating breast cancer from benign breast tumor in plasma. Receiver operating characteristic curve analysis was performed by SPSS18.0 to evaluate the diagnostic performance of *mSEPT9* as a biomarker for differentiating breast cancer from benign breast tumor samples.

**Table 6** *SEPT9* methylation status in plasma

Characteristics	N	Plasma <i>mSEPT9</i>	P value
Breast cancer	80	21 (26.3%)	
Benign breast tumor	27	1 (3.7%)	0.012*
Healthy controls	100	2 (2.0%)	<0.001 <sup>†</sup>

\*, comparison of *mSEPT9* positive rates between breast cancer and benign breast tumor by Fisher's exact test; <sup>†</sup>, comparison of *mSEPT9* positive rates between breast cancer and non-breast cancer groups (benign breast tumor and healthy controls) by Fisher's exact test.

However, no associations were found between plasma *mSEPT9* positivity and ER, PR and HER2 status.

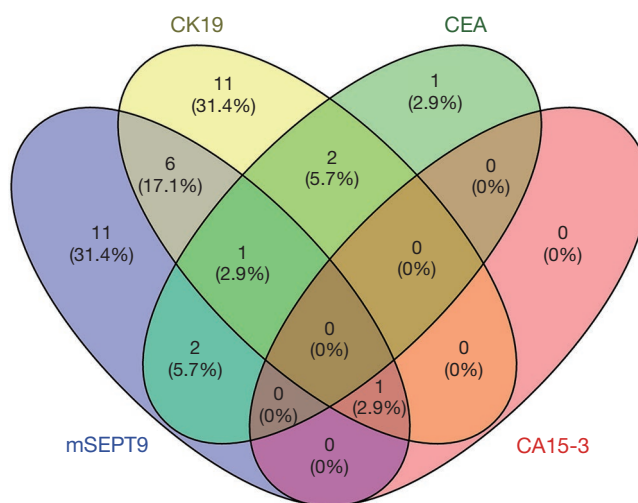
#### Comparison of plasma *mSEPT9* with CEA, CK19, and CA15-3 test

CEA, CK19 and CA15-3 are three most commonly used serum-based breast cancer biomarkers, but they all represent limited sensitivity and specificity (28,29). Coincidentally, for the breast cancer cases already diagnosed by pathological

**Table 7** The status of *mSEPT9* and serum biomarkers (CEA, CK19, CA15-3) for breast cancer test

Characteristics	Total number	Positive number	Positive rate (%)	P value
<i>mSEPT9</i>	80	21	26.3	
CEA	79	6	7.6	0.002* <sup>§</sup>
CK19	79	21	26.6	NS* <sup>§</sup>
CA15-3	79	1	1.3	<0.001* <sup>#</sup>

\*, compared to *mSEPT9* positive rate. <sup>§</sup>, analysis of the comparison by Chi-square test; <sup>#</sup>, analysis of the comparison by Fisher's exact test. NS, non-significant.



**Figure 5** Venn diagram depicting the positivity of the four plasma biomarkers in 79 breast cancer cases. The test results for plasma *mSEPT9*, CEA, CK19, and CA15-3 summarized in the Venn graphs. The graph were drawn using the web-based Venn graphic (<http://bioinfo.cnbc.csic.es/tools/venny/>).

analysis, the positive rates of the CEA, CK19, and CA15-3 tests on plasma samples were 7.6%, 26.6%, and 1.3%, respectively (Table 7). The performances of *mSEPT9*, CEA, CK19, and CA15-3 test for the diagnosis of breast cancer were shown graphically in Figure 5. These results suggest that there were very limited overlaps between these biomarkers, and *mSEPT9* performed comparably with CK19 and better than CEA and CA15-3 in sensitivity.

#### Elevated levels of plasma *mSEPT9* were decreased in breast cancer patients undergoing anti-cancer treatments

We also analyzed the correlation of plasma *mSEPT9* and

**Table 8** Plasma *mSEPT9* status before and after treatment

<i>mSEPT9</i>	MP grade			P value*	RECIST		
	<3	≥3	NA		SD + PD	CR + PR	P value <sup>†</sup>
Positive to negative	0	17	0	0.001	0	17	<0.001
Positive to positive	3	0	1		4	0	

\*, comparison of *mSEPT9* positive rates between MP grade <3 and ≥3 groups by Fisher's exact test; <sup>†</sup>, comparison of *mSEPT9* positive rates between CR + PR and SD + PD groups by Fisher's exact test. MP grade, Miller-Payne grade; RECIST, response evaluation criteria in solid tumors; SD, stable disease; PD, progressive disease; CR, complete response; PR, partial response.

anti-cancer treatment in 21 patients whose plasma samples were *mSEPT9* positive. For this group of patients, plasma specimens were obtained 14 days after the latest treatment. Of the 21 patients, positive to negative conversion was observed in 17 patients (81.0%) (Tables 8,S5). Remarkably, these 17 patients also showed favorable responses to the neoadjuvant chemotherapy, while the remaining 4 patients with persistent plasma *mSEPT9* positivity had poor responses after standardized chemotherapy, as verified by the low MP grades (grades 1 and 2; The MP grade is not available for one patient) and persistent or increased tumor sizes.

## Discussion

In the present study, the diagnostic value of *mSEPT9* in detecting breast cancer as a noninvasive plasma screening marker for breast cancer was evaluated. We found that *mSEPT9* was detected in 83.1% of the breast cancer tissues with a specificity of 92.0%, indicating a remarkable sensitivity as a biomarker for the detection of breast cancer in Chinese women (Tables 5,S3). Because the vast majority of the cancer tissues were *mSEPT9* positive, it is not a surprise that sample *mSEPT9* positivity was not affected by other clinicopathological parameters or molecular classifications, with exception of tumor size and invasion into fat. As a non-invasive blood breast cancer biomarker, the plasma *mSEPT9* assay, in the present setting, still suffers a shortcoming of low sensitivity (26.3%), although the specificity of the *mSEPT9* test was 97.6%, which is high for breast cancer detection. Our finding is consistent with a previous study showing a relatively low sensitivity of 11% in patients with breast cancer (15). While the sensitivity of plasma *mSEPT9* assay is comparable to CK19 in the detection of breast cancer, the sensitivity is still inadequate as a biomarker for primary screening for breast cancer in women at this stage. A possible explanation for the difference in positivity between tissue and plasma samples

is that tissue biopsy sample contains relatively high DNA that is used as template for analysis. Conceivably, early stage breast cancer may release very small amount of DNA in peripheral blood, below the limit of *mSEPT9* detection in the current setting, thus making the plasma *mSEPT9* assay appear less sensitive. Supporting this speculation is the observation that *mSEPT9* positivity was significantly higher in large tumors than in small ones. This is also consistent with the observation that patients with breast cancer of advanced stages, worse lymph node metastasis, intravascular tumor thrombus, higher Ki67 indexes, and p53 protein positivity were more likely to be plasma *mSEPT9*-positive. It has been shown that in most cancers, the p53 gene is mutated, giving rise to a stable mutant protein whose accumulation is regarded as a hallmark of cancer cells (30). Thus p53 positivity is another indication of more advanced malignant cancer. It is suggested that patients with locally advanced and metastatic cancer are likely to have more tumor cells in circulation leading to more tumor DNA plasma. If lower amounts of ctDNA in the blood are the cause of lower sensitivity of the plasma *mSEPT9* test, optimization of the *mSEPT9* assay could improve the sensitivity. As our *mSEPT9* assay relies on real-time PCR to determine the positivity, relaxing the stringency of the cut-off value by increasing the threshold cycle number of real-time PCR will allow the detection of lower amounts of *mSEPT9* DNA. Additionally, lowering the annealing temperature or redesigning of primers covering lower levels of methylation may also significantly increase the sensitivity of the assay methodology. Future studies will focus the fine-tuning of the assay conditions to improve the sensitivity of the *mSEPT9* assay for plasma DNA.

In addition to diagnostic value, we found that persistent plasma *mSEPT9* positivity after cancer treatment, detected in the current assay setting, was correlated nicely with progressive or stable disease whereas *mSEPT9* negative conversion was associated with partial or complete response. It is suggested that the *mSEPT9* assay in its present form

can already serve as a reliable biomarker for evaluation of the efficacy of the cancer treatments for breast cancer patients with *mSEPT9*-positive. Moreover, high positive detection rate of plasma *mSEPT9* for breast cancer makes *mSEPT9* test an ideal modality to distinguish malignancy of tumor at early stages. Of 21 plasma *mSEPT9*-positive patients before treatments, three patients who completed treatments were found *mSEPT9* positive with distant metastases. A possible explanation is that a micro-metastasis was too small to be revealed on imaging study leading to a misjudgment of data interpretation. Given these, use of plasma *mSEPT9* as a marker in breast cancer monitoring is advantageous as simplicity and non-radioisotopes.

Despite the intensive quest for a set of noninvasive reliable biomarkers for breast cancer, there appears to have a long way to go before such goal is finally accomplished. While as a biomarker, *mSEPT9* is on par with CK19 and far better than CEA and CA15-3 in sensitivity in our hands, a great effort is still required to improve its sensitivity prior to its application as a screening tool for breast cancer in Chinese women. The lower sensitivity and limited overlap between CEA, CK19, and CA15-3 suggest that these biomarkers are unlikely to be useful in cancer screening in the Chinese population.

Our study found that *mSEPT9* detection by real-time PCR at the current setting is sensitive to detect breast cancer in biopsy samples. In its current form the *mSEPT9* test can be used to evaluate the clinical outcome of cancer treatment for *mSEPT9*-positive patients. Future studies are needed to improve the sensitivity of the *mSEPT9* test to make it a low cost, reliable, and highly sensitive assay for primary screening of breast cancer.

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### Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2018.05.24>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Liaocheng People's Hospital (IRB approval number: 2016031). Written informed consents were obtained from all study participants.

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## Supplementary

**Table S1** Differences between breast cancer and benign breast tumor cohorts on age and menopausal status

Characteristics	Age (years)			Menopausal status		
	≤50	>50	P	Pre	Post	P
Breast cancer	40	40	NS**†	41	39	NS†‡
Benign breast tumor	16	11		14	13	

\*, comparing differences between the two cohorts on age; †, comparing differences between the two cohorts on menopausal status; ‡, analysis of the comparison by Chi-square test. NS, non-significant.

**Table S2** Differences among the three cohorts on age and menopausal status

Characteristics	Age (years)			Menopausal status		
	≤50	>50	P	Pre	Post	P
Breast cancer	29	30	NS**†	29	30	NS†‡
Benign breast tumor	16	11		14	13	
Healthy volunteers	46	54		51	49	

\*, comparison of differences among the three cohorts on age; †, comparison of differences among the three cohorts on menopausal status; ‡, analysis of the comparison by Chi-square test. NS, non-significant.

**Table S3** Sensitivity and specificity of the *mSEPT9* test for detecting breast cancer

Specimen	<i>mSEPT9</i> *		<i>mSEPT9</i> †	
	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Plasma	26.3% (17.9–36.8%)	96.3% (81.7–99.3%)	26.3% (17.9–36.8%)	97.6% (93.3–99.2%)
Tissue	83.1% (71.5–90.5%)	92.0% (75.0–97.8%)	NA	NA

\*, breast cancer group versus benign breast tumor group; †, breast cancer group versus non-breast cancer group (benign breast tumor and healthy controls). NA, not applicable.

**Table S4** *SEPT9* immunohistochemistry scores in *mSEPT9*-negative and -positive breast cancer samples

Score	<i>mSEPT9</i> -negative breast cancer (n=20)	<i>mSEPT9</i> -positive breast cancer (n=20)
0	1	2
+1	3	12
+2	5	4
+3	11	2

**Table S5** Plasma *mSEPT9* as a follow-up biomarker for breast cancer in treatment monitoring

No. ID	Therapy		<i>mSEPT9</i>		MP grade	RECIST
	Surgery	Chemotherapy	Pretreatment	Posttreatment		
1	×	√	Positive	Positive	NA	PD
2	√	√	Positive	Positive	1	PD
3	√	√	Positive	Positive	2	SD
4	√	√	Positive	Positive	2	SD
5	√	√	Positive	Negative	5	CR
6	√	√	Positive	Negative	4	PR
7	√	√	Positive	Negative	3	PR
8	√	√	Positive	Negative	3	PR
9	√	√	Positive	Negative	3	PR
10	√	√	Positive	Negative	3	PR
11	√	√	Positive	Negative	3	PR
12	√	√	Positive	Negative	3	PR
13	√	√	Positive	Negative	3	PR
14	√	√	Positive	Negative	3	PR
15	√	√	Positive	Negative	3	PR
16	√	√	Positive	Negative	3	PR
17	√	√	Positive	Negative	3	PR
18	√	√	Positive	Negative	3	PR
19	√	√	Positive	Negative	3	PR
20	√	√	Positive	Negative	3	PR
21	√	√	Positive	Negative	5	CR

MP grade, Miller-Payne grade; RECIST, response evaluation criteria in solid tumors; PD, progressive disease; SD, stable disease; CR, complete response; PR, partial response; NA, not applicable.