Promoter methylation of TRIM9 as a marker for detection of circulating tumor DNA in breast cancer patients

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Abstract
The aim of the present study was to investigate the promoter methylation status of TRIM9 in breast cancer and to determine the presence of TRIM9-methylated circulating tumor DNA (ctDNA) in plasma. Bisulfite sequencing with a next generation sequencer showed TRIM9 promoter methylation in 92 % (11/12) of breast cancer cell lines (BCCs) and 68 % (13/19) of breast tumor tissues but not in any normal breast tissues (0/19). Methylation ratio of TRIM9 was significantly lower in basal type (9 %, n = 23) than luminal A (69 %, n = 29, P = 0.0003). Quantitative RT-PCR of BCCs disclosed an inverse correlation between TRIM9 mRNA expression and methylation ratio. TRIM9 methylated ctDNA in plasma was detected in 18 % (10/56) of metastatic breast cancer patients but not in any of 60 healthy controls. These results indicate that TRIM9 promoter hypermethylation, which suppresses TRIM9 mRNA expression, occurs in a significant proportion of breast tumors, and that TRIM9-methylated ctDNA thus may serve as a tumor marker for breast cancer.

Keywords: Breast cancer, TRIM9, Methylation, Biomarker

Background
The promoter methylation of tumor suppressor genes is one of the most common events in carcinogenesis and has been detected in various malignant diseases including breast cancer. Recent studies have also revealed that tumor-specific gene methylation can be detected in the circulating tumor DNA (ctDNA) of cancer patients and methylated ctDNA is considered to be a promising biomarker. Several genes including GSTPI (Glutathione S-transferase P1), RASSF1A (Ras association domain family 1A), and RARβ2 (Retinoic acid receptor β2) have been identified as methylated genes in breast cancer (Yamamoto et al. 2012; Arai et al. 2006; Miyake et al. 2012) but each of these markers is not always specific to breast cancer and several markers have been used in various combinations. Therefore, the need has arisen for methylation markers which are more specific to breast cancer.

TRIM9 belongs to the TRIM (tripartite motif-containing protein) family which has been identified as an ubiquitin ligase (E3) and plays important roles in various cellular processes (Berti et al. 2002). The TRIM family consists of over 70 members, several of which, i.e., TRIM8, 13, 19, 24, 25, 27, 28, 29, 31, 32, 33, 40 and 69, are known to be involved in oncogenesis or tumor progression by affecting specific signal pathways such as RARs and p53 (Hatakeyama 2011). Specifically for breast cancers, TRIM 24, 25 and 27 have been shown to be significant for breast cancer prognosis, such as facilitation of the ubiquitination of estrogen receptors or HER2 gene amplification (Hatakeyama 2011; Tsai et al. 2010; Chambon et al. 2011; Suzuki et al. 2005; Cao et al. 1996). TRIM9 protein is known as a brain-specific E3 ligase expressed in the human brain neurons and associated with neurological disorders such as Parkinson’s disease, Alzheimer’s disease, epilepsy and stroke (Tanji et al. 2010; Winkle et al. 2014; Shi et al. 2014). However, there have been no reports on possible correlation between TRIM9 and carcinogenesis. Using the Illumina Human Methylation 450 database, we found TRIM9 is specifically methylated in breast cancer.
tissues. The aim of the present study was therefore first to investigate whether methylation of TRIM9 promoter is associated with its gene expression in breast cancer cells, and second to clarify the clinicopathological characteristics of TRIM9 methylated breast tumors. We analyzed the methylation of TRIM9 promoter by means of next generation sequencing (NGS), which yields a quantitative methylation ratio within a broad CpG area. Lastly, we examined whether TRIM9 methylated ctDNA can be detected in plasma of breast cancer patients and explored its utility as a novel blood biomarker for breast cancer diagnosis.

Methods

Extraction of targeted gene

We used a common methylation database, Illumina Human Methylation 450, provided by the Cancer Genome Atlas (TCGA) Data Portal, National Cancer Institute, Washington, D.C., USA (http://cancergenome.nih.gov/) to find 90 cases which included methylation data of both primary breast carcinoma and normal breast tissue (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm?mode=ApplyFilter&showMatrix=tissue&platformType=N&platformSubType=N&platform=42). We downloaded the β-score calculated from about 485,000 CpG sites of 90 paired cancerous and non-cancerous breast tissues, and 547 probes met all of three criteria for inclusion in our study, that is, methylation ratio in cancer tissues >45 %, methylation ratio in normal tissues <5 %, and area under the ROC curve >0.85. Next, we used the t test to compare the methylation status of breast cancer and other cancers. Finally, ten probes showing the highest methylation ratio specific to breast cancers qualified as candidates, and among these we decided to target TRIM9, since this was the only probe as yet not known to be associated with breast cancers.

Patients and breast tumor samples

Study I

Nineteen pairs of tumor tissues and normal tissues were obtained at surgery between 2001 and 2004 from primary breast cancer patients who had received no preoperative chemotherapy or hormonal therapy. The clinicopathological characteristics of these patients are summarized in Additional file 1: Table S1. Normal tissues were obtained from a quadrant other than the one harboring cancer. Tissue samples were snap frozen in liquid nitrogen and kept at −80 °C until use.

Study II

Stage II or III primary breast cancer patients (n = 107), who had been treated with neoadjuvant chemotherapy (NAC) consisting of paclitaxel (80 mg/m²) weekly for 12 cycles followed by 5-FU (500 mg/m²), epirubicin (75 mg/m²) and cyclophosphamide (500 mg/m²) every 3 weeks for four cycles at Osaka University Hospital between 2004 and 2009, were retrospectively included in this study. Each patient underwent vacuum assisted biopsy (VAB) of the tumors, and the tumor samples were snap frozen in liquid nitrogen and kept at −80 °C until use. Histological grade, ER, PR, and HER2 status were determined as described in a previous report of ours (Miyake et al. 2012). Ki67 was classified as “high” when >20 % of tumor cells were immunohistochemically positive (clone; MIB-1). Pathological complete response (pCR) was defined as no evidence of invasive cancer components in breast irrespective of any axilla lymph nodes metastases. Intrinsic subtypes were determined by means of DNA microarray using the PAM50 method as previously described (Naoi et al. 2011; Parker et al. 2009). The clinicopathological characteristics of these patients are summarized in Table 1. These studies were approved by the Ethical Review Board of Osaka University Hospital and the Research Ethics Committee of Osaka University, and 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 the breast tissues was extracted using the DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). 1 μg of genomic DNA was then subjected to sodium bisulfite treatment with the EpiTect® Bisulfite Kit (QIA-GEN), and the QIAamp® Circulating Nucleic Acid Kit (QIAGEN) was used to extract plasma DNA from a 2 ml plasma sample, which was then subjected to sodium bisulfite treatment as previously described (Fujita et al. 2012).

Quantitative TRIM9 promoter methylation analysis using NGS

The NGS methylation assay was performed with the GS Junior system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions, and data was analyzed with GS Amplicon Variant Analyzer (AVA) software (version 2.7; Roche Diagnostics). The methylation index (MI) was calculated by dividing the number of cytosines by that of the total reads at each CpG site. NGS primers used for TRIM9 methylation of frozen tissues or cell lines were designed as follows: forward 5′-TGTTTGGAGTAAATATTGAGATT-3′, reverse 5′-ACAATAAAACATTCTCTTCCCT-3′ (long primer; Fig. 1). The average methylation ratio of 12 of the 26 CpG sites (6th–17th CpG), which showed the most significant difference between cancer and normal tissues,
was used for methylation analysis. NGS primers used for DNA from formalin-fixed paraffin embedded (FFPE) specimens were designed as follows: forward 5′-AGTTTAGTTAGGTGTTTTGGGAGGT-3′, reverse 5′-ACATTAATCAAAATCTATAACCCCTTC-3′ (short primer; Fig. 1). The NGS short primer included 7 CpG sites, corresponding to 6th–12th CpG.

In situ hybridization (ISH) for TRIM9 mRNA and immunohistochemical staining (IHC) for TRIM9 protein

The QuantiGene® ViewRNA ISH Tissue Assay kit (Affymetrix, Santa Clara, CA, USA) was used according to the manufacturer’s protocol. FFPE Sections (4 μm) of tumor tissues were incubated at 98 °C with a pretreatment solution for 20 min, followed by protease digestion for 10 min. The TRIM9-specific View RNA™ Probe set (Affymetrix) was hybridized for 2 h. A TRIM9 specific probe set was then designed to hybridize the common sequence of TRIM9_v1 and TRIM9_v2 (1319 bp). ISH images were obtained under fluorescent microscopy (BZ9000; 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 (×400).

Formalin-fixed paraffin Sections (3 μm) of the tumor tissues were obtained for immunohistochemical staining with rabbit anti-TRIM9 polyclonal antibody (ProteinTech Group, Inc., Chicago, IL, USA) at a dilution of 1:400 according to a previously described method for ER, PR and Ki-67, with a slight modification in that antigen retrieval was accomplished by incubating at 98 °C in citrate buffer (pH 9.0) for 40 min (Shimomura et al. 2009;

### Table 1 Comparison of TRIM9 methylation ratio with various clinicopathological parameters of breast tumors

| Characteristics | Total | TRIM9 Methylation ratio mean ± SE | P value* |
|-----------------|-------|---------------------------------|----------|
| All cases       | 107   |                                 |          |
| Age (years)     |       |                                 |          |
| <50             | 49    | 11.2 ± 1.54 0.052               |          |
| ≥50             | 58    | 7.44 ± 1.10                  |          |
| Menopausal status |     |                                 |          |
| Pre             | 51    | 10.7 ± 1.51 0.117               |          |
| Post            | 56    | 7.73 ± 1.14                  |          |
| Tumor size      |       |                                 |          |
| T1+2            | 84    | 8.97 ± 1.04 0.709               |          |
| T3+4            | 23    | 9.83 ± 2.19                  |          |
| Lymph node metastasis | |                                 |          |
| Negative        | 30    | 10.9 ± 2.27 0.338               |          |
| Positive        | 77    | 8.48 ± 0.96                  |          |
| Stage           |       |                                 |          |
| II              | 88    | 9.02 ± 1.02 0.768               |          |
| III             | 19    | 9.75 ± 2.50                  |          |
| Histological type |     |                                 |          |
| IDC             | 97    | 8.66 ± 0.99 0.104               |          |
| ILC             | 10    | 13.9 ± 2.68                  |          |
| Estrogen receptor |    |                                 |          |
| Negative        | 42    | 3.47 ± 0.56 <0.0001           |          |
| Positive        | 65    | 12.8 ± 1.32                  |          |
| Progesterone receptor | |                                 |          |
| Negative        | 65    | 6.93 ± 1.01 0.005               |          |
| Positive        | 42    | 12.6 ± 1.70                  |          |
| HER2 receptor   |       |                                 |          |
| Negative        | 76    | 9.07 ± 1.10 0.893               |          |
| Positive        | 31    | 9.35 ± 1.85                  |          |
| TNBC            |       |                                 |          |
| No              | 82    | 11.2 ± 1.13 <0.0001           |          |
| Yes             | 25    | 2.39 ± 0.32                  |          |
| Subtype (IHC)   |       |                                 |          |
| LumA            | 51    | 12.3 ± 1.42 <0.0001           |          |
| LumB            | 14    | 14.6 ± 3.40                  |          |
| HER2            | 17    | 5.06 ± 1.21                  |          |
| TN              | 25    | 2.39 ± 0.32                  |          |
| Subtype (PAM50) |       |                                 |          |
| LumA            | 29    | 13.7 ± 1.89 <0.0001           |          |
| LumB            | 21    | 10.1 ± 2.24                  |          |
| HER2            | 16    | 9.90 ± 2.65                  |          |
| Basal-like       | 23    | 3.22 ± 0.84                  |          |
| Normal-like     | 18    | 7.70 ± 2.24                  |          |
| Histological grade |     |                                 |          |
| 1+2             | 86    | 9.89 ± 1.09 0.112               |          |
| 3               | 21    | 6.12 ± 1.64                  |          |
| Ki67            |       |                                 |          |
| Low (<20 %)     | 44    | 10.2 ± 1.31 0.366               |          |

### Table 1 continued

| Characteristics | Total | TRIM9 Methylation ratio mean ± SE | P value* |
|-----------------|-------|---------------------------------|----------|
| Clinical response |     |                                 |          |
| No CR           | 70    | 8.62 ± 1.13 0.434               |          |
| CR              | 37    | 10.2 ± 1.70                  |          |
| Histological response | |                                 |          |
| Grade 1, 2a     | 58    | 11.4 ± 1.37 0.006               |          |
| Grade 2b, 3     | 49    | 6.45 ± 1.17                  |          |
| Pathological response | |                                 |          |
| No pCR          | 74    | 11.0 ± 1.20 0.001               |          |
| PCR             | 33    | 5.02 ± 1.15                  |          |
| Recurrence      |       |                                 |          |
| No              | 90    | 9.88 ± 1.08 0.007               |          |
| Yes             | 17    | 5.30 ± 1.12                  |          |

* t test

were obtained under fluorescent microscopy (BZ9000; 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 (×400).

Formalin-fixed paraffin Sections (3 μm) of the tumor tissues were obtained for immunohistochemical staining with rabbit anti-TRIM9 polyclonal antibody (ProteinTech Group, Inc., Chicago, IL, USA) at a dilution of 1:400 according to a previously described method for ER, PR and Ki-67, with a slight modification in that antigen retrieval was accomplished by incubating at 98 °C in citrate buffer (pH 9.0) for 40 min (Shimomura et al. 2009;
Immunohistochemical staining for TRIM9 was classified as 3+ (strongly positive), 2+ (intermediately positive), 1+ (weakly positive) or 0 (negative). The sections were counterstained with hematoxylin.

Isolation of breast tumor cells by magnetic-activated cell sorting (MACS)

Breast tumor cells were separated from the FFPE tumor tissues with the magnetic-activated cell sorting (MACS) method 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 (Otani et al. 2014). Total DNA was extracted from these isolated tumor cells using the QIAamp® DNA FFPE Tissue Kit (QIAGEN).

Demethylation study of cell lines using 5-aza-2’-deoxycytidine

Twelve breast cancer cell lines (BCCs) and one normal breast cell line were cultured under the conditions shown in Additional file 2: Table S2. For demethylation studies, the cultured cells were treated with 10 μmol/L 5-aza-2’-deoxycytidine (5-aza; Sigma-Aldrich, St Louis, MO, USA) or with dimethylsulfoxide (DMSO) as control for 72 h, with the medium changed every 24 h.

RNA extraction and real-time qRT-PCR

Total RNA was isolated from cell lines using TRIzol® reagent (Invitrogen), and 1 μg of total RNA was reverse-transcribed for single strand cDNA, using random primers and the ReverTra Ace® qPCR RT kit (Toyobo, Osaka, Japan). Reverse-transcription reaction was performed first at 65 °C for 5 min and then at 37 °C for 15 min and at 98 °C for 5 min. Quantitative mRNA expression was measured using the Light Cycler 480 Real-time PCR System (Roche Applied Science, Mannheim, Germany) at 95 °C (10 min), followed by 50 cycles at 95 °C (15 s) and at 60 °C (60 s), and 1 cycle at 50 °C (10 s). TRIM9 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan® Gene Expression Assays (assay identification numbers: Hs00364838_m1 and Hs02758991_g1. Applied Biosystems, Foster City, CA, USA) were used for the real time qPCR assay. The expression of TRIM9 was normalized to that of GAPDH, and each assay was performed in duplicate. For the 5-aza treated BCCs, each treated cell line was normalized to the value of its control, which was set at 1.
Measurement of TRIM9 methylated ctDNA in plasma

Two ml of plasma samples was obtained from healthy controls (n = 60) and from metastatic breast cancer (MBC) patients (n = 56), 41 cases of recurrent and 15 of primary advanced breast cancer, before they had been treated at Osaka Police Hospital or Osaka University Hospital between 2012 and 2014. TRIM9 methylated ctDNA in plasma was measured by using quantitative methylation-specific PCR (MSP) with the TRIM9 short primer (Fig. 1). The double-dye probe, including TRIM9 8th–10th CpG sites (5′-TCGTGGAAGGAATGTTATATATTAC-3′; Fig. 1) for detection of TRIM9 methylated ctDNA in plasma, is shown in Fig. 1. 9 µl aliquot each of the bisulfite DNA, eluted for a total PCR reaction volume of 20 µl, was placed in 96-well plates for the TRIM9 PCR reactions. TRIM9 methylated ctDNA in plasma was classified as positive when quantification cycles were less than 50 cycles for TRIM9.

Statistical analysis

The JMP statistical software package (version 11.2.1; SAS Institute, Cary, NC, USA) was used for statistical analyses. Association between the various parameters and TRIM9 methylation ratio was evaluated using the 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 MI in matched-pair samples. The Tukey test was used for comparison of the TRIM9 methylation ratio for each subtype. The univariate and multivariate analysis of various parameters for the association with pCR were performed with the logistic regression model. All statistical analyses were two-sided and P values <0.05 were considered to be statistically significant.

Results

Promoter methylation of TRIM9 and its impact on gene expression in BCCs

To study the methylation status of TRIM9, we performed an NGS methylation assay of the TRIM9 promoter in 12 BCCs and a normal breast cell line (HMEC). The methylation ratio of the TRIM9 gene promoter varied greatly from 10.3 to 92.6 % in 11 of the BCCs and was relatively hypomethylated in the HMEC cells (Fig. 2a; Additional file 3: Table S3).

We next used TRIM9-specific primers and probes to investigate the TRIM9 mRNA expression by qRT-PCR. An inverse correlation between the TRIM9 mRNA expression and methylation ratio was clearly observed (Pearson’s correlation coefficient: −0.753) (Fig. 2a). We then treated eight of these cell lines with a demethylating reagent (10 µM 5-aza) and compared the mRNA expression of the treated and untreated cells. 5-aza treatment induced a 16- to 110-fold up-regulation of mRNA expression in all four hypermethylated BCCs (MDA-MB-453, BT474, SKBR3 and MDA-MB-361), while no up-regulation was detected in any of the four hypomethylated BCCs (MDA-MB-468, ZR75-30, T47D and MDA-MB-231), demonstrating that the TRIM9 gene was re-expressed by the demethylation of its promoter region (Fig. 2b).

Methylation and expression of TRIM9 in human breast cancer tissues

To study the methylation status of TRIM9 in human breast cancer and normal breast tissues, we performed an NGS methylation assay using the 19 paired tumor and normal tissues (study I). The methylation ratio was significantly higher for the tumor tissues than the
normal tissues (median values, 19 and 1.8 %, respectively, \( P = 0.00067 \); Fig. 3a). The ratio of TRIM9 hypermethylated tumors (methylation ratio \( \geq 8.2 \% \)) was 68 %.

For a more accurate assessment of the cancer cell-specific methylation status, we isolated the tumor cells from the FFPE tumor tissues with the MACS method, and the isolated tumor cells were then subjected to an NGS methylation assay. Five tumor tissues with a low methylation ratio (<25 %) were further analyzed since it was thought the low methylation ratio of some of them was due to contamination by the normal stromal and inflammatory cells. Methylation ratios increased in the tumor cells isolated from whole tumor tissues (Fig. 3b).

Relationship between TRIM9 methylation and clinicopathological characteristics

An NGS methylation assay of TRIM9 was performed using the biopsy specimens obtained before NAC (study II) to examine the relationship between TRIM9 methylation and the various clinicopathological parameters including response to NAC (Table 1). TRIM9 hypermethylation (methylation ratio \( \geq 8.2 \% \)) was observed in 40 % (43/107) of the specimens. TRIM9 hypermethylation was significantly associated with ER positivity, PR positivity, low histological grade and no pCR (Table 1). Furthermore, the methylation ratio was significantly lower for basal type (9 %) than for luminal A type (\( P = 0.0007 \); Fig. 4).

Next, to determine whether methylation is related to gene expression, we subjected the TRIM9 hypermethylated (\( n = 10 \)) and hypomethylated tumors (\( n = 10 \)) to ISH and IHC and found that neither ISH signals nor IHC scores in tumor cells were significantly associated with methylation ratios (Fig. 5).

Relationship between TRIM9 methylation and response to NAC

The clinicopathological parameters were assessed by means of univariate analysis for their association with pCR (Table 2). Age, Ki67, ER, PR, HER2, and TRIM9 methylation were found to be significantly associated with pCR. The multivariate analysis showed that only ER, but not TRIM9 methylation, was a significant and independent predictor for pCR.

Detection of TRIM9-methylated ctDNA in MBC patients

TRIM9-methylated ctDNA in plasma of 56 MBC patients and 60 healthy controls was assayed by using MSP. An amplification curve of the eight standards was obtained by diluting the methylated human control DNA (diluted to 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 ng/ml plasma). The limit of detection for methylated TRIM9 DNA was 0.1 ng/ml in plasma. TRIM9 methylated ctDNA was detected in 18 % (10/56) of MBC patients but not in any of the healthy controls. Primary breast tumor tissues for determination of TRIM9 methylation status were available for 27 of the 56 cancer patients, and TRIM9 methylated ctDNA was detected in 44 % (4/9) of the MBC patients with TRIM9 hypermethylated tumors but in only 6 % (1/18) of the MBC patients with TRIM9 hypomethylated tumors (Table 3).

![Fig. 3](image-url) Methylxation status of TRIM9 in breast cancer and normal breast tissues. a Comparison of TRIM9 methylation index for 19 paired normal breast and cancer tissues. b Comparison of TRIM9 methylation index for whole breast cancer tissues and tumor cells isolated with the MACS method.
Discussion

For this study, we selected the TRIM9 gene as a breast cancer specific methylation marker by referring to the methylation array database and observed TRIM9 hypermethylation in 92% (11/12) of the BCCs and 68% (13/19) of breast tumor tissues but not in any of the normal breast epithelial cell line (HMEC) cells or normal breast tissues. Several methylation markers for breast cancers have been investigated, such as GSTP1, RASSF1A and RARβ2, and hypermethylation of these genes has been reported as, respectively, 17–48%, 43–90% and 26–78% in breast cancer tissues and as 2–3%, 3–8% and 0% in normal breast tissues (Yamamoto et al. 2012; Jung et al. 2013; Hagrass et al. 2014; Pirouzpanah et al. 2015), indicating the equally high sensitivity and specificity of TRIM9 as a methylation marker for breast cancer. Although methylation of these other genes has reportedly been detected in other types of cancers than breast

![Fig. 4](image-url) Methylation status of TRIM9 in 107 breast cancer tissues. Breast tumors were classified into five intrinsic subtypes (luminal A, luminal B, HER2, basal-like, normal breast-like) by PAM50 for comparison of their methylation index of TRIM9. *Tukey’s test

![Fig. 5](image-url) Association between TRIM9 mRNA expressions obtained with ISH and IHC analysis and methylation ratios in breast cancer tissues. TRIM9 hypermethylated or hypomethylated breast cancer tissues were subjected to ISH (a) and IHC (b) for TRIM9 mRNA. Each immunoreactivity was one of four scores (0, 1+, 2+ and 3+). ISH, in situ hybridization; IHC, immunohistochemistry

| Characteristics | Univariate analysis | Multivariate analysis |
|-----------------|---------------------|----------------------|
|                 | Odds ratio | 95% CI   | P value | Odds ratio | 95% CI   | P value |
| Age (≥50 vs <50) | 3.13       | 1.29–7.66 | 0.0091 | 2.24       | 0.80–6.56 | 0.1236 |
| T stage (T1+2 vs T3+4) | 1.80       | 0.61–5.35 | 0.2743 |           |          |        |
| Lymph node status (positive vs negative) | 1.68       | 0.64–4.41 | 0.2858 |           |          |        |
| ER (negative vs positive) | 10.5       | 4.00–27.4 | <0.0001 | 5.14       | 1.23–27.2 | 0.0240 |
| Pgr (negative vs positive) | 5.60       | 1.95–16.1 | 0.0004 | 0.98       | 0.17–4.86 | 0.9823 |
| HER2 (positive vs negative) | 2.47       | 1.03–5.94 | 0.0440 | 1.98       | 0.69–5.76 | 0.2002 |
| TRIM9 methylation (<8.2 vs ≥8.2 %) | 10.6       | 2.96–37.6 | <0.0001 | 3.96       | 0.97–20.1 | 0.0545 |

CI: confidence interval
Table 3: Sensitivity for detection of methylated TRIM9 in plasma of Stage IV and metastatic breast cancer patients

|                         | Total | Methylated TRIM9 in plasma |       |       |
|-------------------------|-------|---------------------------|-------|-------|
|                         |       |                           | Positive | Negative |
|                         | No.   | (%)                       | No.     | (%)     |
| Healthy control         | 60    | 0 (0)                     | 60 (100) |         |
| MBC patients (total)    | 56    | 10 (18)                   | 46 (82)  |         |
| MBC patients with TRIM9 hypermethylated tumors | 9     | 4 (44)                    | 5 (56)   |         |
| MBC patients with TRIM9 hypomethylated tumors | 18    | 1 (6)                     | 17 (94)  |         |

MBC: metastatic breast cancer

Our study detected TRIM9 methylated ctDNA in only 18% (10/56) of MBC patients. However, this sensitivity was as high as 44% (4/9) when only the MBC patients with TRIM9 methylated tumors were taken into consideration, but it was only 5.6% (1/18) for those without TRIM9 methylated tumors. Previous studies have reported that aberrant promoter methylation in serum DNA of MBC patients was 18–25% for GSTP1 (Yamamoto et al. 2012; Müller et al. 2003; Sharma et al. 2010), 33–39% for RASSF1A (Yamamoto et al. 2012; Müller et al. 2003; Kim et al. 2010) and 20–87% for RARβ2 (Yamamoto et al. 2012; Sharma et al. 2010; Kim et al. 2010). Although methylated TRIM9 in blood was less sensitive than the existing methylation markers, the specificity was 100% which was superior to that of any other genes (2–10% for GSTP1, 0–10% for RASSF1A and 5–6% for RARβ2) (Yamamoto et al. 2012; Müller et al. 2003; Kim et al. 2010). TRIM9-methylated ctDNA may thus be a potential tumor marker and might work better in combination with other blood biomarkers for breast cancers to compensate for its lower sensitivity. However, the number of patients in our study was limited and further prospective studies are needed to verify our findings.

Conclusions

We found that TRIM9 promoter hypermethylation occurred in 68% of breast tumors but not in normal breast tissues. Methylated TRIM9 was detected in the plasma from 44% of metastatic breast cancer patients with TRIM9 methylated tumors. Although the regulatory mechanism of TRIM9 gene expression and its biological functions remain unclear, our preliminary results suggest that methylated TRIM9 may serve as a novel blood biomarker specific to breast cancer patients.
Additional files

Additional file 1. Clinopathological characteristics of breast tumors used for comparison of TRIM9 methylation index for paired tumor and normal breast tissues.

Additional file 2. Breast cell lines used in this study.

Additional file 3. TRIM9 methylation index and mRNA expression in breast cancer cell lines.

Abbreviations
ER: Estrogen receptor; PR: Progesterone receptor; HR2: Human epidermal growth factor receptor 2; CEA: Carcinoembryonic antigen; CA15-3: Carbohydrate antigen 15-3.

Authors’ contributions
NK and SN participated in the design of the study. SM acquired the data and performed the statistical analysis. TT and KS contributed the immunohistochemical staining. YN advised the statistical analysis. MS, SK conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no conflict of interest.

Ethical standards
The study complied with the current laws of Japan.

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