Circulating methylated *HOXA9* tumor DNA as a biomarker for mortality in recurrent breast cancer: An exploratory study

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Abstract. Methylated homeobox A9 (meth-*HOXA9*) circulating tumor DNA may be a relevant biomarker in breast cancer, although its clinical significance remains unknown. The present exploratory study aimed to investigate the association between meth-*HOXA9* and mortality in patients with recurrent breast cancer. The cohort study enrolled 51 patients with breast cancer recurrence from the Department of Oncology, Vejle Hospital, University Hospital of Southern Denmark (Vejle, Denmark). Tissue samples from primary surgery and biopsies, and plasma samples obtained at the time of recurrence were analyzed for meth-*HOXA9* using a methylation-specific droplet digital polymerase chain reaction. Using Cox regression, hazard ratios (HRs) for mortality with 95% confidence intervals (CIs) comparing patients with detectable and undetectable meth-*HOXA9* in both tumor tissue and plasma were estimated. Among the 50 patients with data on tumor tissue meth-*HOXA9*, there was no association between meth-*HOXA9* in the primary tumor and mortality (HR 1.09, 95% CI 0.47-2.52). A total of 34 patients had data on plasma meth-*HOXA9* at the time of recurrence. Detectable plasma meth-*HOXA9* was associated with higher mortality (HR 3.95, 95% CI 1.50-10.37). Among the 20 patients with data on both plasma and metastatic tissue meth-*HOXA9*, meth-*HOXA9* was detectable in 90% of metastases and 65% of plasma samples. In conclusion, detectable plasma meth-*HOXA9* was significantly associated with higher mortality in recurrent breast cancer; therefore, plasma meth-*HOXA9* may prove useful as a prognostic marker in patients with breast cancer.

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Abbreviations: CI, confidence interval; ctDNA, circulating tumor DNA; ER, estrogen receptor; ddPCR, droplet digital polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; HER2, human epidermal growth factor receptor 2; *HOX*, homeobox; IHC, immunohistochemistry; meth-*HOXA9*, methylated homeobox A9; PR, progesterone receptor; ROC, receiver operating characteristic; SISH, silver *in situ* hybridization

Key words: HOXA9, methylation, biomarker, circulating tumor DNA, breast cancer, recurrence

Introduction

Breast cancer is the most commonly diagnosed cancer worldwide and the fifth leading cause of cancer-related deaths (1). In developed countries, more than two-thirds of breast cancers are diagnosed early, resulting in low mortality due to promising treatment options (2). However, there is still room for improvement, especially in late-stage breast cancer (3). Early detection of breast cancer recurrence improves survival but relies mainly on radiological imaging, which requires a particular tumor load to be detectable (4). Recurrence is often confirmed by biopsy, but in a clinical setting, multiregional biopsy sampling is usually impossible, leading to a lack of information on tumor or metastasis heterogeneity, which may be better represented in plasma biomarkers (5). Such biomarkers may have prognostic and treatment monitoring potential, improving early detection of breast cancer recurrence (6). Therefore, the need for minimal invasive biomarkers like circulating tumor DNA (ctDNA) is apparent.

Homeobox (*HOX*) genes are represented in humans as 39 genes in four clusters: *HOXA* (chromosome 7; 11 genes), *HOXB* (chromosome 17; 10 genes), *HOXC* (chromosome 12; 9 genes), and *HOXD* (chromosome 2; 9 genes) (7). *HOX* genes encode transcription factors involved in cell identity, cell division, cell differentiation, and regulation of morphogenesis during embryonic development (8). *HOX* genes, like *HOXA9*, may act as tumor suppressor genes, and their aberrant regulation may

contribute to malignancy (9,10). The gene expression can be altered by methylation of CpG islands. CpG islands are regions of DNA that contain a high frequency of CpG dinucleotides, often found near the promoter regions of genes. In the context of tumor suppressor genes, DNA hypomethylation can lead to the activation of these genes, which can then inhibit cancer development. On the other hand, DNA hypermethylation can silence tumor suppressor genes, leading to a loss of their cancer-preventing function and potentially contributing to cancer development (11). Thus, DNA hypo- or hypermethylation of CpG islands is a general feature of cancer cells and malignant disease, and hypermethylation of tumor suppressor genes significantly contributes to neoplastic transformation. Furthermore, specific genes seem to be methylated at different tumor stages, boding well for usage in early cancer detection or prognostic assessment (12).

Hence, aberrant regulation of the *HOXA9* tumor suppressor gene may contribute to and induce the progression of malignancies. The clinical potential of *HOXA9* as a biomarker for breast cancer is still unclear. Still, previous studies suggest that methylated *HOXA9* (meth-*HOXA9*) not only in tumor tissue but also in the blood may serve as a diagnostic or prognostic marker in different types of cancer. Thus, recent studies suggest that meth-*HOXA9* ctDNA may be a diagnostic or prognostic marker in ovarian and lung cancers (13-18). To our knowledge, no previous studies examined plasma meth-*HOXA9* in breast cancer patients. However, a previous study found a model associated with breast cancer prognosis using tissue meth-*HOXA9* and meth-*HOXA10* (19). Another study found an association between low HOXA9 mRNA levels and reduced relapse-free survival and that HOXA9 significantly predicts death or disease relapse in estrogen-receptor (ER) negative tumors (9). This exploratory study aims to i) examine the association between meth-*HOXA9* in the primary tumor and overall survival, ii) investigate the association between meth-*HOXA9* levels in the blood at the time of recurrence and overall survival, and iii) examine whether elevated meth-*HOXA9* in breast cancer metastatic tissue associates with meth-*HOXA9* in blood samples at the time of recurrence.

Patients and methods

Setting and design. In this cohort study, 51 patients diagnosed with breast cancer recurrence were recruited from the Department of Oncology, Vejle Hospital, University Hospital of Southern Denmark, Denmark, between April 2011 and December 2015. To be eligible for the study, patients had to have histologically confirmed breast cancer recurrence and be at least 18 years old. The patients had previously undergone primary surgical treatment and adjuvant therapy according to national guidelines between February 1993 and October 2013. Tumor tissue samples were obtained during the primary surgery or from a preliminary biopsy. Blood samples and biopsies from metastases were performed before medical treatment of the recurrence. The study followed patients until their death or 31 January 2019; the median follow-up was 95.3 months (range 6.6-311.2) from primary surgery and 23.4 months (range 0.6-93.5) from time of recurrence, respectively. As investigations on the prognostic value of meth-*HOXA9* in breast cancer are limited, the present study was conducted with an explorative approach, and no specified effect size was expected. The study followed the REporting recommendations for tumor MARKer prognostic studies (REMARK) checklist (20).

Analysis of meth-HOXA9

Tissue samples. The tissue specimens were fixed in formalin and embedded in paraffin (FFPE). An experienced pathologist histologically classified the specimens according to the World Health Organization's classification of breast tumors (21). DNA was extracted from the FFPE tissue samples using the Maxwell 16 FFPE Tissue DNA purification kit (cat. no. AS1135; Promega, WI, USA) and subjected to bisulfite conversion using the EZ DNA Methylation-Lightning Kit (cat. no. D5031; Zymo Research Corp., Irvine, CA, USA). The DNA was analyzed with an in-house designed methylation-specific assay for *HOXA9* and albumin normalization assay using the BioRad droplet digital polymerase chain reaction (ddPCR) QX200 system (BioRad, Hercules, CA, USA). Details on thermocycling protocol, primer, and probe sequences are listed in Tables SI and SII, respectively. Human methylated DNA (Zymo Research Corp., Irvine, CA, USA), water, and a lymphocyte DNA pool were included in each round of analyses as positive and negative controls.

To establish the cut-off for a positive result, tissue was obtained from anonymized specimens used for method development and quality control. A receiver operating characteristic (ROC) curve analysis was performed using tissue samples from 50 healthy women undergoing breast reduction surgery and 50 breast cancer patients from an independent cohort. The ROC curve analysis showed that meth-*HOXA9* had 98% (95% confidence interval (CI) 0.96-1.0) diagnostic accuracy in distinguishing malignant from normal breast tissue. The optimal cut-off was established at ≥7.4% with a sensitivity of 90% and a specificity of 98%. This cut-off was used for metastatic tissue samples as well.

Plasma samples. ctDNA was extracted from 100-2,000 μl of plasma using the QIAsymphony DSP Circulating DNA kit (cat. no. 937556; Qiagen, Hilden, Germany). The ctDNA was subjected to bisulfite conversion and ddPCR analysis using an in-house designed methylation-specific assay as described for tissue specimens. The same primer and probe sequences were used for tissue and plasma samples (Tables SI and SII). The limit of blank and cut-off for meth-*HOXA9* plasma samples has previously been determined (16). A positive test was indicated by detecting ≥5 meth-*HOXA9*-containing droplets, and samples with lower values were considered negative. Meth-*HOXA9* was reported as a percentage of total DNA (meth-*HOXA9* copies/albumin copies x100) and as positive/negative.

The DNA isolation and meth-*HOXA9* analysis methodology have previously been described for tissue specimens and plasma samples (14,18,22-24). The meth-*HOXA9* analyses were performed blinded to the study endpoints.

Outcomes and covariates. All-cause mortality was ascertained from patient records by 31 January 2019. Information on age, primary tumor characteristics, treatment, and location of metastases were obtained from patient records. ER and progesterone receptor (PR) status in the primary tumor was defined according to the contemporary Danish Breast Cancer

Group guidelines, with tumors showing ≥10 and ≥1% staining by immunohistochemistry considered positive before and after 1 March 2010, respectively. All metastases with ≥1% staining were considered positive. Human epidermal growth factor receptor 2 (HER2) status in the metastasis biopsy was determined using an immunohistochemical test with scores of 0 or 1+ indicating HER2-negative breast cancer, 2+ indicating a borderline result, and 3+ indicating HER2-positive breast cancer. If a marginal result was obtained (score 2+), the HER2 status was further determined using silver *in situ* hybridization (SISH) to establish a positive or negative HER2 status. SISH was performed using the VENTANA HER2 Dual ISH kit (Roche, Basel, Switzerland). Detailed ER and HER2 assessment criteria are available in Tables SIII and IV; Figs. S1 and S2. Age was handled as a continuous variable, while all other covariates were handled as categorical variables.

Statistical analysis. The primary endpoint was overall survival. Overall survival was calculated from primary surgery and biopsy-verified recurrence to death or 31 January 2019. Kaplan-Meier curves of the plasma meth-*HOXA9* groups were plotted, depicting the absolute mortality risk over time. Multivariate survival analysis was performed using the Cox regression model. The proportional hazards assumption was tested using log-log plots, and the assumption was violated for the analysis of primary tumor meth-*HOXA9* and mortality. The violation was handled by including an exposure-time interaction term, which showed no significant interaction. The survival analyses were adjusted for age. Overall survival was further evaluated in receptor status groups using the log-rank test, stratifying on ER positivity/HER2 negativity, HER2 positivity, and triple-negative status in the primary tumor or metastasis biopsy. Fisher's exact test was used to compare plasma and metastasis meth-*HOXA9* levels.

Sensitivity analyses were performed to test the robustness of the results. In the sensitivity analysis of meth-*HOXA9* in breast cancer tissue and mortality, we excluded patients who received neoadjuvant chemotherapy and repeated the Cox regression analysis. Due to the low sample volume (≤200 μl) in some blood samples, a worst-case scenario sensitivity analysis was performed. In the subgroup analysis, missing data on receptor status was handled using complete case analysis.

Statistical analyses were performed using Stata 17 (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC). The Kaplan-Meier plot was produced using ggplot2 for R 4.1.1 (R Core Team. 2021. R Foundation for Statistical Computing, Vienna, Austria).

Results

Participants. We assessed 68 breast-cancer patients with suspected recurrence and included 51 patients who experienced recurrence between 2011 and 2015 in the study (Fig. 1). The remaining patients were not eligible due to suspected metastases being from other cancers, benign, or inaccessible for biopsy.

Meth-HOXA9 in primary breast-cancer tissue. Table I summarizes the baseline characteristics of 50 breast cancer patients according to meth-*HOXA9* status in the primary tumor. The median age at primary diagnosis was 59 years in patients with detectable meth-*HOXA9* and 56 years in patients with undetectable meth-*HOXA9*. The majority of patients had either grade 1 (24%) or grade 2 (48%) tumors, and tumors that were either ≤20 mm (38%) or >20 ≤50 mm (48%). Nearly 78% of patients had ER-positive/HER2-negative disease, 14% had HER2-positive disease, and 8% had triple-negative disease. Some patient data were missing, with 18% missing tumor grade, 2% missing ER data, 18% missing PR data, and 16% missing HER2 data. The distribution of missing data was not even between the two groups (Table I).

Table II shows the HRs of mortality after the primary operation according to meth-*HOXA9* status in the primary tumor. During the follow-up period, 41 patients died. Median overall survival in patients with detectable and undetectable meth-*HOXA9* was 83.9 and 80.2 months, respectively (log-rank P=0.450). There was no significant difference in mortality between patients with and without detectable meth-*HOXA9* (Table II).

Subgroup analysis. Survival analyses were repeated for the three receptor-status groups: ER-positive/HER2-negative, HER2-positive, and triple-negative. There was no association between tumor meth-*HOXA9* status and mortality in ER-positive/HER2-negative disease (log-rank P=0.476), HER2-positive disease (log-rank P=0.126), or triple-negative disease (log-rank P=0.433) (Tables SV-SVII).

Sensitivity analysis. Six patients received neoadjuvant chemotherapy, which may have affected the meth-*HOXA9* status in the primary tumor. A sensitivity analysis excluding these patients changed the association (HR 1.07; 95% CI 1.01-1.12) (Table SVIII).

Meth-HOXA9 in plasma at the time of breast cancer recurrence. Thirty-four patients had data on meth-*HOXA9* in plasma and were included in the analysis examining the association between plasma meth-*HOXA9* and overall survival. Sixty-two percent of the patients had detectable plasma meth-*HOXA9* at the time of recurrence. The median age for breast cancer recurrence was 63 years in patients with detectable meth-*HOXA9* and 69 years in patients with undetectable meth-*HOXA9*. Most patients had liver metastases (85%), and other metastasis locations included the lungs (21%), lymph nodes (15%), bone (9%), peritoneum (3%), and adrenal gland (3%). Most metastases were ER-positive (77%), and 9% were HER2-positive. In total, one patient (3%) was missing ER data, 16 (47%) were missing PR data, and two (6%) were missing HER2 data. The missing data were unevenly distributed between exposure groups.

During the follow-up period, 26 patients died. Mortality was significantly higher in patients with detectable meth-*HOXA9* (81%) than those without (69%). Median overall survival in patients with detectable and undetectable meth-*HOXA9* was 12.2 and 27.1 months, respectively (log-rank P=0.119, Fig. 2). The age-adjusted HR was 3.95 (95% CI 1.50-10.37) in patients with detectable meth-*HOXA9* (Table III).

Subgroup analysis. Survival analyses were repeated for the three receptor-status groups: ER-positive/HER2-negative, HER2-positive, and triple-negative. Subgroups were based on receptor status in the metastasis. There was no association between plasma meth-*HOXA9* status and mortality in ER-positive/HER2-negative disease (log-rank P=0.180),

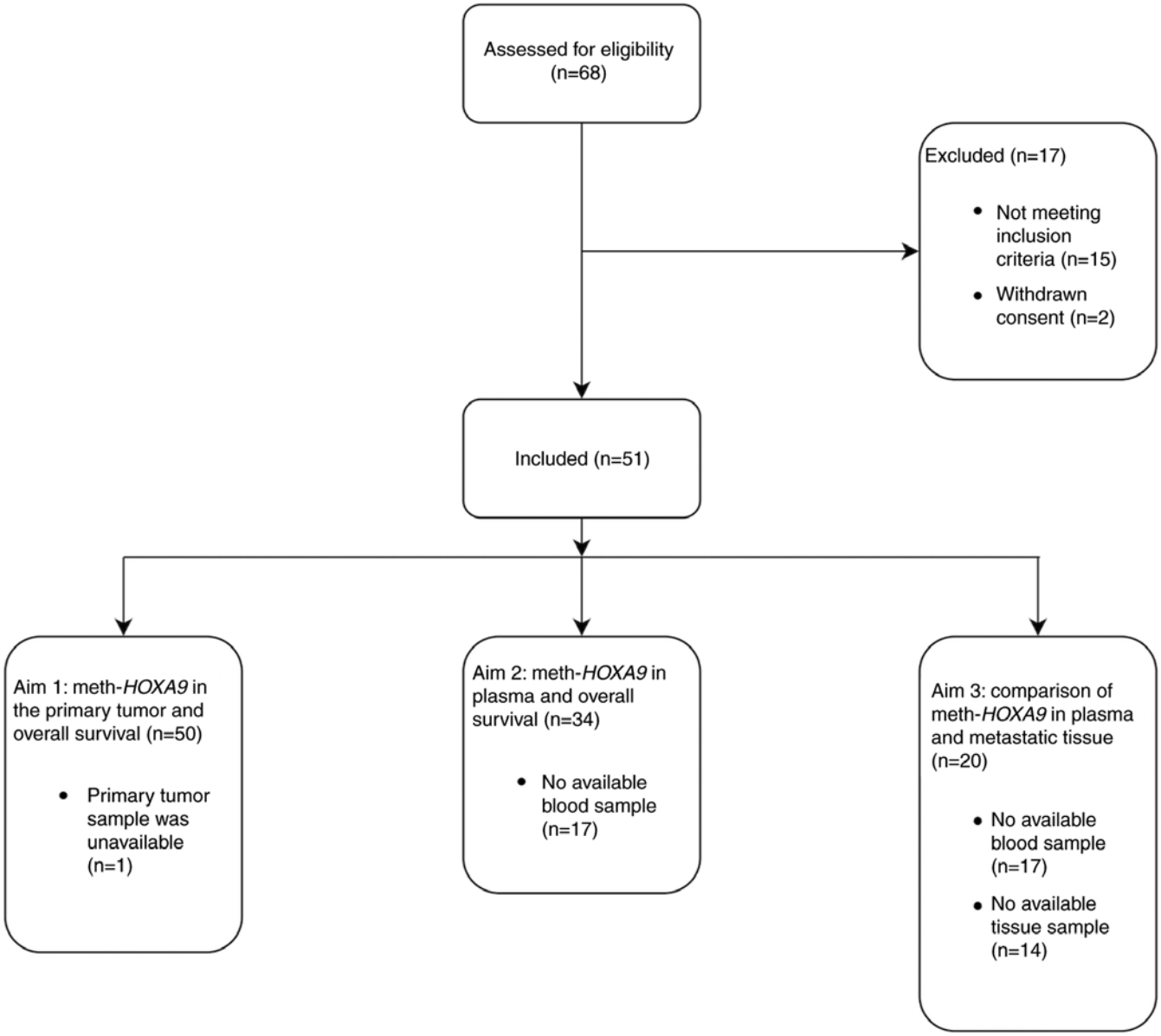

Figure 1. Flow diagram for inclusion of patients. meth-*HOXA9*, methylated homeobox A9.

HER2-positive disease (log-rank P=0.157), or triple-negative disease (log-rank P=0.707) (Tables SIX-SXI).

Sensitivity analysis. We identified two patients with ≤200 µl plasma, which may have resulted in false negative meth-*HOXA9* status. In the worst-case scenario sensitivity analysis, the age-adjusted HR of mortality changed to 2.23 (95% CI 0.92-5.44) (Table SXII).

Meth-HOXA9 in plasma and metastatic tissue at breast cancer recurrence. Only 20 patients had data on meth-*HOXA9* in both plasma and metastatic tissue at the time of breast cancer recurrence. No association was found when comparing plasma and metastatic tissue meth-*HOXA9* levels in these patients (P>0.99). Meth-*HOXA9* was detectable in 90% of metastatic tissue samples, and plasma meth-*HOXA9* was detectable in two-thirds of these patients (Table IV). In a worst-case scenario sensitivity analysis, two patient samples with ≤200 µl plasma were considered false negative, but the association between plasma and metastatic-tissue meth-*HOXA9* did not change (P=0.447) (Table SXIII).

Discussion

In the present long-term cohort study, we aim to explore the association between the methylation of the *HOXA9* gene in the primary tumor and blood samples at the time of recurrence with overall survival in breast cancer patients. Only plasma meth-*HOXA9* was associated with higher mortality after breast cancer recurrence.

We found no association between meth-*HOXA9* in the primary tumor and mortality. However, after a sensitivity analysis excluding six patients who received neoadjuvant chemotherapy, patients with detectable meth-*HOXA9* in the primary tumor had higher mortality than those without detectable meth-*HOXA9*. A previous study showed that neoadjuvant chemotherapy reduces the amount of methylated DNA in breast

Table I. Patient characteristics and meth-*HOXA9* status in breast cancer tissue at baseline.

Characteristic	Meth-*HOXA9* in breast cancer tissue		
	All patients (n=50)	Detectable (n=40)	Undetectable (n=10)
Median age, years	59	59	56
Year of primary surgery			
1990-1995	1 (2.0%)	1 (2.5%)	0 (0.0%)
1996-2000	4 (8.0%)	4 (10.0%)	0 (0.0%)
2001-2005	10 (20.0%)	7 (17.5%)	3 (30.0%)
2006-2010	26 (52.0%)	20 (50.0%)	6 (60.0%)
2011-2015	9 (18.0%)	8 (20.0%)	1 (10.0%)
Primary surgery type			
Breast-conserving	26 (52.0%)	20 (50.0%)	6 (60.0%)
Mastectomy	15 (30.0%)	12 (30.0%)	3 (30.0%)
Primary disseminated	7 (14.0%)	6 (15.0%)	1 (10.0%)
Other	2 (4.0%)	2 (5.0%)	0 (0.0%)
Tumor grade			
Grade 1	12 (24.0%)	11 (27.5%)	1 (10.0%)
Grade 2	24 (48.0%)	15 (37.5%)	9 (90.0%)
Grade 3	5 (10.0%)	5 (12.5%)	0 (0.0%)
Unknown	9 (18.0%)	9 (22.5%)	0 (0.0%)
Tumor size			
T1: ≤20 mm[a]	19 (38.0%)	15 (37.5%)	4 (40.0%)
T2: >20 ≤50 mm	24 (48.0%)	19 (47.5%)	5(50.0%)
T3: >50 mm	3 (6.0%)	3 (7.5%)	0 (0.0%)
T4: Ingrowth/mastitis	4 (8.0%)	3 (7.5%)	1 (10.0%)
Pathological nodal status[b]			
N0: 0	16 (32.0%)	14 (35.0%)	2 (20.0%)
N1: 1-3	18 (36.0%)	12 (30.0%)	6 (60.0%)
N2: 4-9	6 (12.0%)	6 (15.0%)	0 (0.0%)
N3: ≥10[c]	10 (20.0%)	8 (20.0%)	2 (20.0%)
Estrogen receptor status[d]			
Positive	44 (88.0%)	36 (90.0%)	8 (80.0%)
Negative	5 (10.0%)	3 (7.5%)	2 (20.0%)
Unknown	1 (2.0%)	1 (2.5%)	0 (0.0%)
Progesterone receptor status[d]			
Positive	27 (54.0%)	20 (50.0%)	7 (70.0%)
Negative	14 (28.0%)	11 (27.5%)	3 (30.0%)
Unknown	9 (18.0%)	9 (22.5%)	0 (0.0%)
HER2 status[e]			
Positive	7 (14.0%)	5 (12.5%)	2 (20.0%)
Negative	35 (70.0%)	28 (70.0%)	7 (70.0%)
Unknown	8 (16.0%)	7 (17.5%)	1 (10.0%)
Neoadjuvant chemotherapy			
Yes	6 (12.0%)	4 (10.0%)	2 (20.0%)
No	44 (88.0%)	36 (90.0%)	8 (80.0%)
Adjuvant chemotherapy			
Yes	17 (34.0%)	11 (27.5%)	6 (60.0%)
No	33 (66.0%)	29 (72.5%)	4 (40.0%)
Adjuvant trastuzumab			
Yes	5 (10.0%)	3 (7.5%)	2 (20.0%)
No	45 (90.0%)	37 (92.5%)	8 (80.0%)

Table I. Continued.

Characteristic	Meth-*HOXA9* in breast cancer tissue		
	All patients (n=50)	Detectable (n=40)	Undetectable (n=10)
Adjuvant radiation therapy			
Yes	35 (70.0%)	26 (65.0%)	9 (90.0%)
No	15 (30.0%)	14 (35.0%)	1 (10.0%)
Adjuvant endocrine treatment			
None	20 (40.0%)	18 (45.0%)	2 (20.0%)
Tamoxifen	12 (24.0%)	7 (17.5%)	5 (50.0%)
Aromatase inhibitors	10 (20.0%)	9 (22.5%)	1 (10.0%)
Tamoxifen + aromatase inhibitors	8 (16.0%)	6 (15.0%)	2 (20.0%)

[a]One patient had no primary tumor, and tumor size was classified as ≤20 mm. [b]Pathological lymph nodes defined as malignant cells in primary lymph node biopsy, malignant cells in sentinel lymph node preoperatively, or malignant cells in lymph nodes removed during breast cancer surgery. [c]One patient had no pathological nodal status evaluation, and classification was done according to the clinical nodal status cN3. [d]Estrogen and progesterone receptor status in the primary tumor evaluated by IHC. Positive: ≥10% staining before 1 March 2010 and ≥1% after. [e]Human epidermal growth factor receptor 2 status in breast cancer tumor evaluated by IHC and SISH. Positive: IHC 3+ or IHC 2+ and SISH ≥2. Negative: IHC 0 or IHC 1+ or IHC 2+ and SISH <2. SISH, silver *in situ* hybridization; IHC, immunohistochemistry; meth-*HOXA9*, methylated homeobox A9.

Table II. HR with 95% CI of mortality according to breast-cancer tissue meth-*HOXA9* status (n=50).

Status	Mortality, n	Incidence, % (95% CI)	Unadjusted HR (95% CI)	Adjusted[a] HR (95% CI)
Undetectable meth-*HOXA9*	7/10	70.00 (36.83-90.33)	Ref.	Ref.
Detectable meth-*HOXA9*	34/40	85.00 (69.95-93.24)	1.37 (0.60-3.11)	1.09 (0.47-2.52)

[a]Adjusted for age at primary operation. CI, confidence interval; HR, hazard ratio; meth-*HOXA9*, methylated homeobox A9.

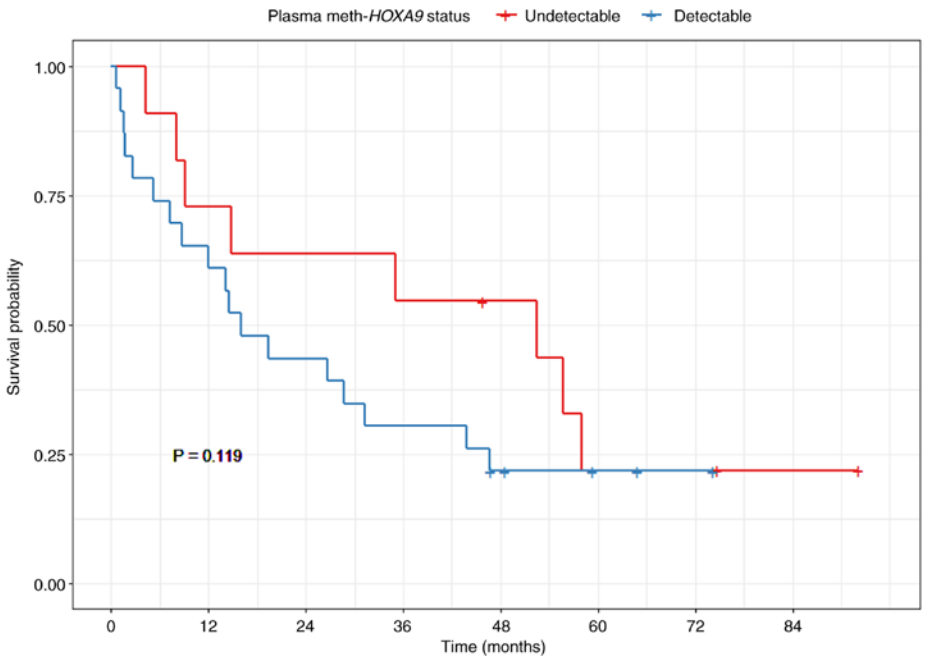

Figure 2. Kaplan-Meier plot showing mortality according to plasma meth-*HOXA9* status in patients with breast cancer recurrence (n=34). meth-*HOXA9*, methylated homeobox A9.

Table III. HR with 95% CI of mortality after breast cancer recurrence according to plasma meth-*HOXA9* status (n=34).

Status	Mortality, n	Incidence, % (95% CI)	Unadjusted HR (95% CI)	Adjusted[a] HR (95% CI)
Undetectable meth-*HOXA9*	9/13	69.23 (38.57-90.91)	Ref.	Ref.
Detectable meth-*HOXA9*	17/21	80.95 (58.09-94.55)	1.90 (0.84-4.33)	3.95 (1.50-10.37)

[a]Adjusted for age at recurrence. CI, confidence interval; HR, hazard ratio; meth-*HOXA9*, methylated homeobox A9.

Table IV. Comparison of metastatic tissue and plasma meth-*HOXA9* using Fisher's exact test (n=20).

	Meth-*HOXA9* in metastatic tissue		
Status	+	-	P-value
Meth-*HOXA9* in plasma +	12 (92%)	1 (8%)	>0.99
Meth-*HOXA9* in plasma -	6 (86%)	1 (14%)	

meth-*HOXA9*, methylated homeobox A9.

tumors (25). We expect most excluded patients to have lower levels of meth-*HOXA9* in the primary tumor and, thus, better survival. Indeed, a higher proportion of patients, who received neoadjuvant chemotherapy, had undetectable meth-*HOXA9* in the primary tumor. Consequently, we see relatively higher mortality in patients with detectable meth-*HOXA9* after adjustment for age. However, the present results should be interpreted with caution because patients were included based on the occurrence of recurrence and the sample size was limited.

The finding that detectable plasma meth-*HOXA9* is associated with increased mortality is supported by a meta-analysis that found that ctDNA was associated with shorter disease-free survival in early and locally advanced or metastatic breast cancer (26). In the sensitivity analysis, where two samples with low plasma volume were considered false negative, the association between plasma meth-*HOXA9* and mortality was insignificant. However, all patients had advanced disease, and we would therefore expect to find high concentrations of meth-*HOXA9* in plasma, even in small samples (27). These results must be validated in another cohort using larger plasma volumes.

There was no association between metastasis and plasma meth-*HOXA9*, which may be caused by the small cohort or the samples' low plasma volume. However, meth-*HOXA9* was detectable in most metastases (90%) and two-thirds of plasma samples. Metastasis heterogeneity may lead to different expression levels of ctDNA in other areas of the same metastasis and between metastases. A previous study showed that the association between metastasis mutations and ctDNA is strong in breast cancer (5). Therefore, plasma meth-*HOXA9* may be valuable in early recurrence detection. In addition, measuring meth-*HOXA9* in plasma is faster and less inconvenient to patients than obtaining a biopsy.

The *HOXA9* gene is present in normal breast tissue and breast cancer (28). Epigenetic modifications such as DNA methylation frequently occur in tumors, and therefore plasma meth-*HOXA9* may qualify as a general marker of ctDNA in breast cancer patients. Other methods to determine ctDNA in breast cancer involve tumor mutation analysis and advanced next-generation sequencing (6). These methods are complicated and expensive in contrast to the method used in the present study. Our study shows that *HOXA9* is present in most metastases, and measuring plasma meth-*HOXA9* in recurrent breast cancer is possible. Plasma meth-*HOXA9* measurement is even possible in smaller sample volumes than previously assumed. Another recent study from our group investigated plasma meth-*HOXA9* in breast cancer patients undergoing neoadjuvant chemotherapy (29). Comparing results from this study to the present study suggests that plasma meth-*HOXA9* is considerably increased at the time of breast cancer recurrence.

A significant limitation of the present study is the small sample size, which may limit the applicability of the results. The limited sample size also restricted the possibility of multivariate analyses including more covariates. Hence, internal and external validation of the results is necessary. Another limitation is the lack of the patients' genetic profiles precluding evaluation of the association between *HOXA9* and prognosis in different underlying gene mutations. Differences in underlying driver gene mutations could potentially influence subsequent methylation of *HOXA9* and thereby the effects of meth-*HOXA9* on patient outcomes. This should be taken into account in future studies. The long follow-up and the detailed method description is a significant strength, which improves the possibility of meaningful external validation. The present analyses were performed at the same laboratory, ensuring uniformity, reproducibility, and minimizing analytical variation. Samples were frozen at -80 degrees until analysis, which should not affect the amount of ctDNA (27).

So far, no ctDNA test has proved helpful in monitoring therapy effectiveness, diagnostics, or screening in a clinical setting (30). However, several ongoing studies investigate the value of ctDNA testing in different cancers. For research purposes, ctDNA has proven helpful in tracking the evolution of endocrine treatment resistance in breast cancer (31). Compared to a histopathological examination of tumor tissue, blood-based ctDNA analysis is significantly less invasive and causes minimal patient inconvenience. ctDNA can easily be repeated during follow-up and even before recurrence is visible using imaging or biopsy procedures. ctDNA's half-life is two hours, allowing us to observe a disease snapshot (32). Finally, ctDNA and, in this

case, meth-*HOXA9* may prove helpful in prognosis prediction after primary surgery (6). ctDNA biomarkers such as meth-*HOXA9* may address the limitations of imaging, such as costs, inter-operator/inter-reader variability, and detection of small tumors/metastases (27). Future studies concerning meth-*HOXA9* in breast cancer should focus on plasma meth-*HOXA9* at the time of diagnosis and the evolution of plasma meth-*HOXA9* during treatment and follow-up. The finding that meth-*HOXA9* is detectable at the time of breast cancer recurrence gives rise to a hypothesis about meth-*HOXA9* as a possible biomarker for early detection of cancer recurrence.

This exploratory study suggests that patients with detectable plasma meth-*HOXA9* at the time of breast cancer recurrence had higher mortality than those with undetectable meth-*HOXA9*. Meth-*HOXA9* is present in most metastases and is detectable in two-thirds of plasma samples at the time of recurrence. Future validation studies are needed to investigate the clinical relevance of plasma meth-*HOXA9* as a prognostic biomarker in breast cancer patients. Further studies are required to examine the potential of plasma meth-*HOXA9* as a biomarker for disease activity and treatment monitoring in breast cancer patients.

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Availability of data and materials

The data generated in the present study are not publicly available because it contains person-sensitive data used under license for the study and is only available with permission from the relevant legal authorities and according to existing regulations but may be requested from the corresponding author.

Authors' contributions

IMK, JSM, SBB and TB conceptualized the study. The methodology was developed by IMK, JSM, RFA, SBB, TB, TFH and TPT. The initial investigation was performed by RFA, TB and TPT. IMK and TB confirm the authenticity of all the raw data. SBB carried out formal analysis of the study data while IMK, JSM, RFA, SBB, TB, TFH and TPT contributed to the interpretation of data. JSM, TB and TFH provided the necessary resources. IMK, SBB and TB curated the data. Visualization and data presentation was performed by SBB. IMK, JSM and TB supervised the project. The project was administered by IMK, and funding was acquired by JSM, TB and TFH. The original draft was written by SBB. All authors reviewed and edited the manuscript, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Regional Committee on Health Research Ethics for Southern Denmark (S-20100081) and the Danish Data Protection Agency (23/7602) approved the study. The study was conducted according to The Declaration of Helsinki. All participants provided written informed consent at inclusion.

Patient consent for publication

All participants provided written informed consent to the publication of anonymized results at inclusion.

Competing interests

The authors declare that they have no competing interests.

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