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PRECLINICAL STUDY

PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients

Chiya Oshiro · Naofumi Kagara · Yasuto Naoi · Masafumi Shimoda · Atsushi Shimomura · Naomi Maruyama · Kenzo Shimazu · Seung Jin Kim · Shinzaburo Noguchi

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Abstract We attempted to develop a highly sensitive and specific method for the detection of circulating tumor DNA (ctDNA) using a digital PCR (dPCR) assay for *PIK3CA* mutations (i.e., H1047R, E545K, and E542K) in primary breast cancer patients. The sensitivity of the dPCR assay for the mutant alleles was examined using cell lines with *PIK3CA* mutations and proved to be 0.01 %. Serum samples were collected pre-operatively from 313 stage I–III breast cancer patients, of whom 110 were found to have *PIK3CA* mutant tumors. The serum samples from these patients with *PIK3CA* mutant tumors were subjected to the dPCR assay, and 25 (22.7 %) were found to be positive. No *PIK3CA* mutant ctDNA was detected in the serum samples of 50 healthy women and 30 breast cancer patients with *PIK3CA* non-mutant tumors. The patients with *PIK3CA* mutant ctDNA were dichotomized into mutant ctDNA-high ($ctDNA^{high}$) and ctDNA-low ($ctDNA^{low}$) groups based on the median. The $ctDNA^{high}$ patients exhibited significantly shorter recurrence-free survival (RFS; $P = 0.0002$) and overall survival rates (OS; $P = 0.0048$) compared to those exhibited by the combined $ctDNA^{low}$ patient and ctDNA-free patient group. Multivariate analysis revealed that $ctDNA^{high}$ status significantly predicted poor RFS and OS and did so independently of conventional histological parameters. These results suggest that dPCR is a highly sensitive and specific method for the detection of *PIK3CA* mutant ctDNA and that $ctDNA^{high}$ but not $ctDNA^{low}$ status is a significant and independent prognostic factor for primary breast cancer patients.

Keywords Breast cancer · *PIK3CA* mutation · ctDNA · dPCR · Prognosis

Introduction

Cell-free DNA (cfDNA) is composed of the fragments of cellular nucleic acids that exist in the circulating blood. One type of cfDNA is composed of tumor-derived cfDNA that harbors tumor-specific alterations, including genetic or epigenetic changes, and are known as circulating tumor DNA (ctDNA) [1, 2]. ctDNA is thought to be released from cancer cells by necrosis, apoptosis, or secretion [3, 4]. In general, patients with cancer have higher levels of cfDNA than do healthy individuals, and the fractions of ctDNA within the cfDNA vary widely between 0.01 % and more than 90 % according to the tumor burden [5]. Due to fragmentation and a short half-life (1–2 h) [6], it has been difficult to detect ctDNA in early cancer patients, and most studies of ctDNA have been limited to metastatic or advanced cancer patients with greater amounts of ctDNA, and fewer studies have been performed in early cancer patients. However, the recent advances in the molecular-based technologies, including high sensitivity (<0.01 %) digital PCR (dPCR), have enabled the detection of rare ctDNA and thus its application to early cancer patients [1, 2, 7].

*PIK3C*A mutations are some of the most common genetic alterations in breast cancers and are detected in 20–40 % of these cancers [8, 9]. *PIK3CA* mutations occurs

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C. Oshiro · N. Kagara (✉) · Y. Naoi · M. Shimoda · A. Shimomura · N. Maruyama · K. Shimazu · S. J. Kim · S. Noguchi
Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, 2-2-E10 Yamadaoka, Suita-shi, Osaka 565-0871, Japan
e-mail: kagaran@onsurg.med.osaka-u.ac.jp

predominantly in exons 9 and 20, which are called "hot spots" and include H1047R, E545K, and E542K; these three mutations account for 70–80 % of *PIK3CA* mutations in breast cancers [8]. These hot spot mutations are known to modulate the activity of the PI3K signaling pathway, which regulates cell growth, motility, and other important cellular functions [10, 11]. Given that ctDNA carries tumor-derived DNA alterations, the detection of *PIK3CA* mutations in cfDNA with a highly sensitive assay is expected to be useful in the early detection of recurrence following surgery and in the monitoring of tumor response to systemic therapy in patients with metastatic disease and to replace conventional tumor markers such as CEA and CA15-3 [12, 13].

It has been reported that *PIK3CA* mutations are detectable in the cfDNA in approximately 80 % of metastatic breast cancer patients whose tumors harbor *PIK3CA* mutations and that *PIK3CA* mutations reflect responses to systemic therapies more accurately than CEA or CA15-3 [1, 5, 12]. Very recently, the successful detection of *PIK3CA* mutations in the cfDNA of early breast cancer patients has been reported [14], but the clinicopathological characteristics and prognostic significance of breast tumors with *PIK3CA* mutations in the cfDNA have not been documented [14, 15]. Therefore, in the present report, we investigated whether *PIK3CA* mutations could be detected in the cfDNA of early breast cancer patients using dPCR and the prognostic relevance of the presence of such mutations.

Materials and methods

Patients and sample collection

Three hundred and thirteen primary breast cancer patients (stage I–III) who had received no neoadjuvant systemic therapy and had undergone mastectomy or breast-conserving surgery followed by radiation therapy to the breast at Osaka University Hospital between June 2000 and November 2009 were retrospectively included in this study. Blood samples were collected from each patient prior to surgery and centrifuged at 3000 rpm for 10 min at 4 °C within 2 h after venipuncture, and then serum samples were stored at −80 °C until use. Samples were centrifuged again before DNA extraction to remove debris. The median follow-up period after surgery was 79 months (range 1–133). Twelve patients received no adjuvant systemic therapy, 128 received adjuvant hormonal therapy, 63 received adjuvant chemotherapy, and 110 received adjuvant chemo-hormonal therapy essentially according to the recommendations of the St. Gallen consensus conference [16–19]. The clinicopathological characteristics of these patients are shown in Table 1. The serum tumor markers CEA and CA15-3 were also measured before surgery and defined as positive when they exceeded 5.0 ng/ml and 30.0 U/ml, respectively. A tumor sample was obtained from each patient at surgery, snap frozen in liquid nitrogen and stored at −80 °C until use. Serum samples from 50 healthy women (14 premenopausal and 36 postmenopausal) were used as controls. Informed consent for participation in the study was obtained from each patient and healthy woman.

DNA extraction from tumor samples and real-time PCR

DNA was extracted from frozen tumor tissues with the DNeasy Blood & Tissue Kit® (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. TaqMan®-based real-time PCR analysis was conducted using a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) to detect the three "hot spot" *PIK3CA* mutations (H1047R, E545K and E542K). Custom TaqMan® primers and probes were designed for the three *PIK3CA* mutations as shown in Supplementary Table S2.

DNA extraction from the serum samples and digital PCR (dPCR)

DNA was extracted from 500 µl of serum using the QIAamp Circulating Nucleic Acid Kit® (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted into 50 µl of AVE buffer and stored at −20 °C until further processing. dPCR was performed using a QuantStudio™3D digital PCR system (Life Technologies, Carlsbad, CA) to detect the three *PIK3CA* mutations. The primers and probes shown in Supplementary Table S2 were used. For the dPCR, 9 µl of template DNA was mixed with 1 µl of 20× TaqMan® Assay primer/probe mix and 10 µl of 2× QuantStudio™ 3D Digital PCR Master Mix (Life Technologies) according to the manufacturer's instructions. Fifteen-microliter aliquots of the PCR solutions were then loaded into QuantStudio™ 3D Digital PCR 20 K chips, and the PCR reaction was performed. The thermal cycler protocol was as follows: 10 min at 96 °C, 39 cycles at 60 °C for 2 min, 98 °C for 30 s, and 60 °C for 1 min. All samples were analyzed in a single assay for each mutation. The data were analyzed with the QuantStudio™3D AnalysisSuite™ v1.1.3 (Life Technologies, Carlsbad, CA) for mutation search and quantification of the DNA copies in the serum. The mutant allele fraction (MAF, %) was defined as the proportion of mutant DNA copies relative to the sum of mutant and wild-type DNA copies obtained by dPCR. The samples were defined as positive for mutations ($ctDNA^{positive}$) when one

Table 1 Relationships between clinicopathological parameters and *PIK3CA* mutations

Characteristics	Total	Mutation status (tumor)		*P*	Total[a]	Mutation status (serum)		*P*
		PIK3CA wt	*PIK3CA* mt			*PIK3CA* wt	*PIK3CA* mt	
All cases	313	203	110		110	85	25	
Age (years)								
≦50	121	85	36		36	27	9	
>50	192	118	74	0.113	74	58	16	0.692
Tumor size (clinical)								
≦20 mm	146	93	53		53	42	11	
20 mm < ≦50 mm	161	107	54		54	40	14	
50 mm<	6	3	3	0.654	3	3	0	0.518
Nodal status (pathological)								
Negative	182	125	57		57	48	9	
Positive	131	78	53	0.095	53	37	16	0.072
Histological grade								
1	92	53	39		39	34	5	
2 + 3	221	150	71	0.083	71	51	20	0.066
ER								
Negative	93	69	24		24	15	9	
Positive	220	134	86	0.024	86	70	16	0.051
PR								
Negative	123	93	30		30	19	11	
Positive	190	110	80	0.001	80	66	14	0.033
HER2								
Negative[b]	237	146	91		91	72	19	
Positive[c]	76	57	19	0.033	19	13	6	0.368
ly								
Negative	259	170	89		89	69	20	
Positive	54	33	21	0.526	21	16	5	1.000
v								
Negative	171	113	58		58	42	16	
Positive	142	90	52	0.618	52	43	9	0.199
CEA/CA15-3								
Negative	294	189	105		105	80	25	
Positive	19	14	5	0.406	5	5	0	0.586

wt wild-type, *mt* mutant type, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a] Total patients with *PIK3CA* mutant tumors

[b] IHC 0, 1 + or 2 + (FISH−)

[c] IHC 3 + or 2 + (FISH+)

or more mutant alleles were detected per assay and defined as negative (ctDNA^{negative}) when no mutant alleles were detected.

Immunohistochemical (IHC) examination

ER and PR were defined as positive when 10 % or more of the tumor cells were stained by immunohistochemistry (ER: clone 6F11; PR: clone 16; Ventana Japan K.K. and SRL Inc. Tokyo, Japan). Human epidermal growth factor receptor 2 (HER2) was identified immunohistochemically (anti-human c-erbB-2 polyclonal antibody; Nichirei Biosciences, Tokyo, Japan) or by means of fluorescent in situ hybridization (FISH) using the PathVysion HER2 DNA probe kits (SRL Inc. Tokyo, Japan). For the FISH scoring, the ratio of the HER2 gene signals to the chromosome 17 centromere signals was calculated for each of the specimens. When a tumor exhibited +3 immunohistostaining or

a FISH ratio $\geqq$2.0, it was considered HER2-positive. The histological grade was determined according to the Scarff-Bloom-Richardson grading system [20].

Statistical analyses

The associations between the various clinicopathological parameters and the *PIK3CA* mutations were evaluated with Chi square or Fisher's exact tests. Recurrence-free survival (RFS) rates and overall survival (OS) rates were calculated with the Kaplan–Meier method and evaluated with the log-rank test. Univariate and multivariate analyses of the various parameters for the predictions of recurrence and death were conducted with the Cox proportional hazards model. For all statistical tests, differences were considered significant at $P < 0.05$.

Results

Detection sensitivity of *PIK3CA* mutation by dPCR

First, we evaluated the detection sensitivity of *PIK3CA* mutant alleles by dPCR using the genomic DNA extracted from T47D breast cancer cell lines that were known to harbor the H1047R mutation. The H1047R-mutant DNA from the T47D cells was mixed at different mutant allele concentrations (i.e., 100, 10, 1, 0.1, 0.01, 0.001, and 0 %) with wild-type DNA that was extracted from the peripheral blood mononuclear cells of the healthy women. Our dPCR assay was highly sensitive to detect at least two copies of mutant alleles within 20,000 copies of wild-type alleles (sensitivity = 0.01 %) (Fig. 1a), and the mutant allele frequencies measured by dPCR were well correlated with the expected mutant allele frequencies (Pearson's r = 1.000). Representative dPCR plots for the H1047R mutation are shown in Fig. 1b. Next, the sensitivities for the other two *PIK3CA* mutations were also evaluated using genomic DNA from MCF-7 cells with the E545K mutation and BT-483 cells with the E542K mutation. The detection sensitivities for the E545K and E542K mutations were also found to be 0.01 % (Supplementary Fig. S1).

PIK3CA mutations in breast tumors

The three *PIK3CA* hot spot mutations were screened in 313 primary breast tumors with real-time PCR, and *PIK3CA* mutations were identified in 110 (35.1 %) of the tumors; specifically, 79 H1047R mutations, 19 E545K mutations, and 12 E542K mutations were found. None of the patients carried multiple *PIK3CA* mutations. The clinicopathological characteristics of the *PIK3CA* mutant tumors are shown in Table 1. The *PIK3CA* mutant tumors were significantly more likely to be ER-positive ($P = 0.024$), PR-positive ($P = 0.001$), and HER2-negative ($P = 0.033$).

PIK3CA mutations in the cfDNA

We next performed the dPCR assay for the *PIK3CA* mutations in the cfDNA from the 110 patients with *PIK3CA* mutant tumors. Twenty-five patients (22.7 %) were ctDNA^{positive}, specifically, 21 carried H1047R, 3 carried E545K, and 1 carried E542K. The median copy number and MAF of the *PIK3CA* in the ctDNA^{positive} cases were 29 copies/ml (range 13–2500; Fig. 2a) and 0.37 % (range 0.04–22.00; Supplementary Table S1), respectively. To investigate the specificity of the dPCR assay, serum samples from 50 healthy women and 30 breast cancer patients with *PIK3CA* non-mutant tumors were assayed for three *PIK3CA* mutations by dPCR. No mutant ctDNA was detected in any of these samples (Fig. 2a).

PIK3CA mutant DNA and patient prognosis

The clinicopathological characteristics of the ctDNA^{positive} and ctDNA^{negative} patients are shown in Table 1. The ctDNA^{positive} patients were significantly ($P = 0.033$) more likely to have PR-negative tumors. No other parameters were significantly different between the two groups.

The ctDNA^{positive} patients exhibited significantly (RFS; $P = 0.0029$, OS; $P = 0.0283$) lower RFS (92 % vs. 68 %) and OS rates (96 % vs. 84 %) than did the ctDNA^{negative} patients (Fig. 3a, b). Because there was a trend for recurrences to be more frequently observed in the patients with greater copy numbers of mutant alleles (Fig. 2b; Supplementary Table S1), the ctDNA^{positive} patients were dichotomized into those with high (ctDNA^{high}) and low (ctDNA^{low}) *PIK3CA* mutant ctDNA counts based on the median copy number (29 copies) of mutant alleles. The ctDNA^{high} patients exhibited significantly worse RFS and OS rates than did the ctDNA^{negative} patients (RFS; $P = 0.0001$, OS; $P = 0.0032$), while the RFS and OS rates were not significantly different between the ctDNA^{low} and ctDNA^{negative} patients (RFS; $P = 0.3950$, OS; $P = 0.4886$) (Fig. 3c, d). Next, the ctDNA^{low} and ctDNA^{negative} patients were combined for the following analysis (ctDNA$^{low+negative}$). The ctDNA^{high} patients exhibited significantly worse RFS and OS rates than did the combined ctDNA$^{low+negative}$ patients (PFS; $P = 0.0002$, OS; $P = 0.0048$; Fig. 3e, f). The multivariate analysis revealed that ctDNA^{high} was a significant prognostic factor of RFS ($P = 0.005$, HR = 4.783; Table 2) and OS ($P = 0.128$, HR = 3.917; Table 3) independently of the other parameters. Forest plot analyses revealed that the

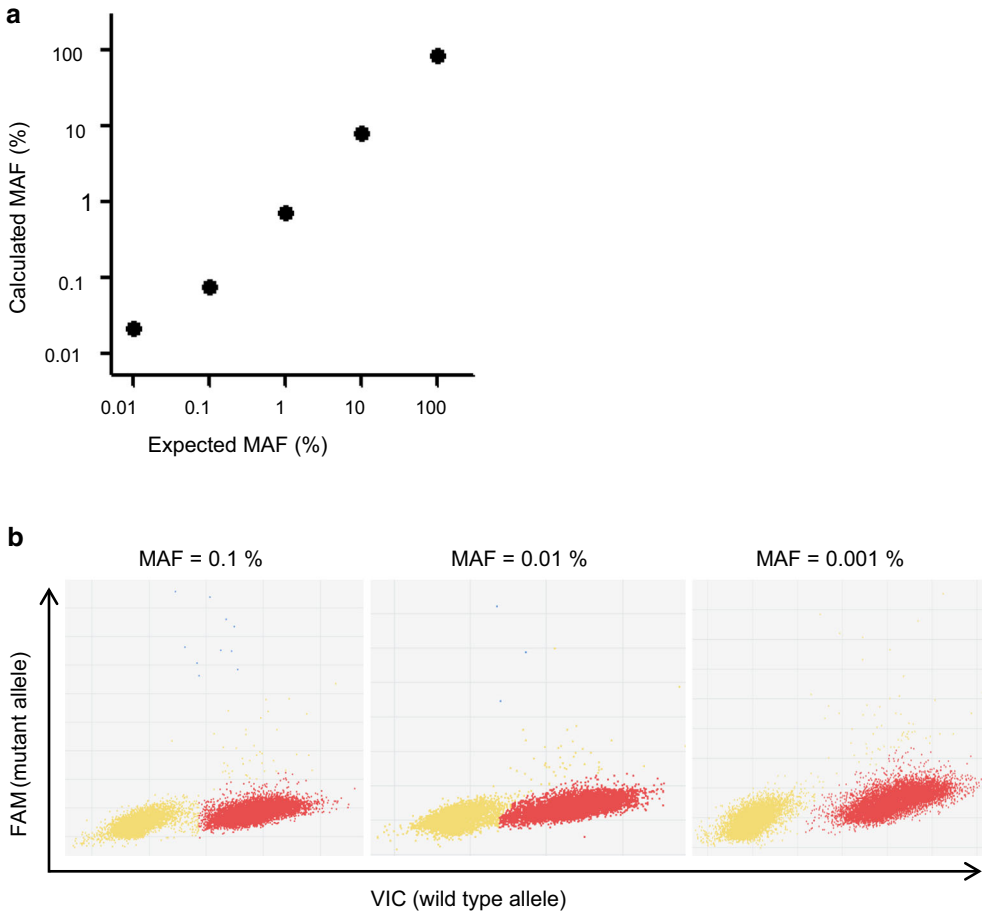

Fig. 1 **a** Detection sensitivity of digital PCR is shown. Genomic DNA from T47D cell line carrying *PIK3CA* H1047R mutation was mixed at different mutant allele concentrations ranging from 0.001 to 100 % with wild-type genomic DNA from peripheral blood mononuclear cells. Mutant alleles were detectable at concentrations of 0.01 %, and the mutant allele fraction (MAF) (%) measured by dPCR were well correlated with expected mutant allele fraction (MAF) (Pearson's r = 1.000). **b** Representative views of digital PCR plots (mutant samples). Data from sample chips are displayed in a scatter plot based on color of FAM and VIC events. *Red*, *blue*, and *yellow plots* mean wild-type alleles, mutant alleles, and no amplifications, respectively

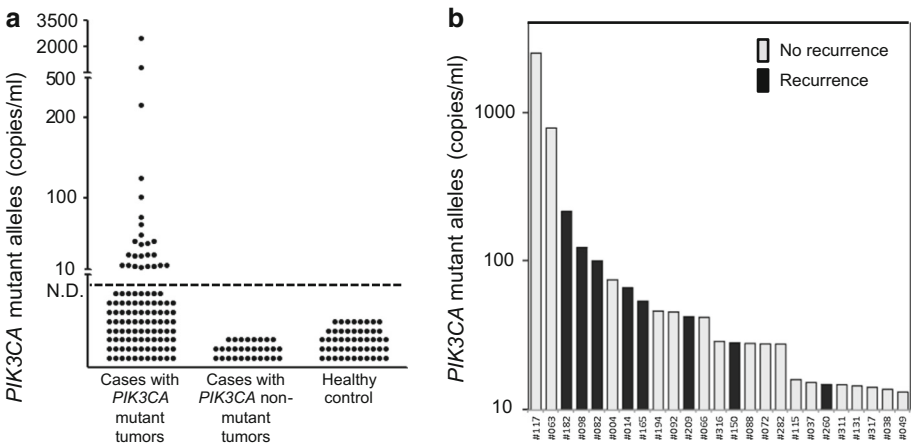

Fig. 2 **a** Copy numbers of *PIK3CA* mutant alleles (copies/ml) in serum samples from the 110 patients with *PIK3CA* mutant tumors are shown. No mutant ctDNA was detected in 50 healthy women and 30 breast cancer patients. *ND* not detected. **b** Mutant allele copy numbers of 25 ctDNA^{positive} cases are shown according to the recurrent status. *Black* and *gray bars* represent recurrent and non-recurrent cases, respectively

prognostic significance of the ctDNA^{high} status was independent of the type of adjuvant therapy (Supplementary Fig. S2). Only two (1.8 %) and three (2.7 %) patients were CEA- and CA15-3-positive, respectively, and neither of these statuses exhibited a significant correlation with patient prognosis (Supplementary Fig. S3).

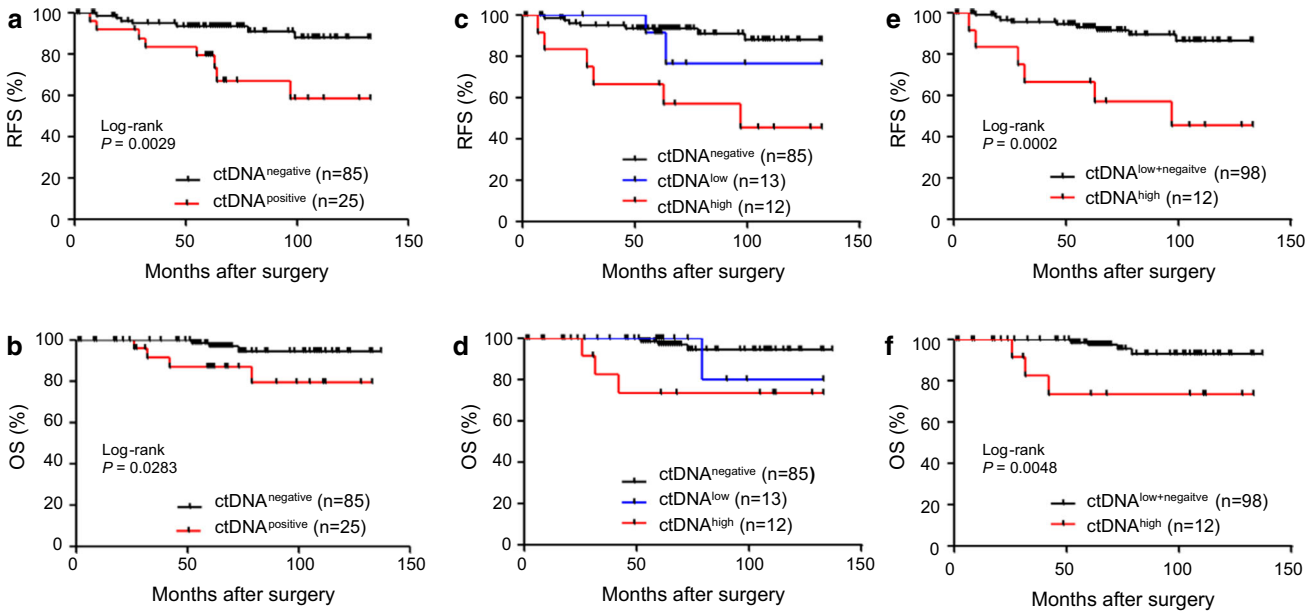

Fig. 3 Kaplan–Meier plots of RFS (**a**, **c**, **e**) and OS (**b**, **d**, **f**) rates according to the levels of *PIK3CA* mutant ctDNA are shown. Comparison between $ctDNA^{positive}$ and $ctDNA^{negative}$ (**a**, **b**), between $ctDNA^{high}$, $ctDNA^{low}$, and $ctDNA^{negative}$ (**c**, **d**) and between $ctDNA^{high}$ and $ctDNA^{low+negative}$ (**e**, **f**) are represented

Table 2 Univariate and multivariate analysis of various parameters associated with prognosis (RFS)

	Univariate			Multivariate		
	Hazard ratio	95 % CI	*P*	Hazard ratio	95 % CI	*P*
Age (≦50 vs > 50)	1.703	0.537–5.394	0.366			
Tumor size (>2 cm vs ≦2 cm)	1.820	0.622–5.327	0.274			
Nodal status (positive vs negative)	0.956	0.344–2.662	0.932			
Histological grade (3 vs 1, 2)	3.802	0.857–16.857	0.079			
ER (positive vs negative)	0.351	0.125–0.988	0.047	0.513	0.172–1.526	0.230
PR (positive vs negative)	0.367	0.133–1.013	0.053			
HER2 (positive vs negative)	2.488	0.849–7.285	0.096			
ly (positive vs negative)	2.098	0.717–6.144	0.176			
v (positive vs negative)	1.235	0.443–3.443	0.686			
CEA/CA15-3 (positive vs negative)	1.417	0.186–10.783	0.736			
ctDNA ($ctDNA^{high}$ vs $ctDNA^{low+negative}$)	5.786	2.057–16.272	0.001	4.783	1.610–14.206	0.005

CI confidence interval, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

Discussion

In the present study, we first investigated whether the dPCR assay was sufficiently sensitive to detect a rare *PIK3CA* mutation in cfDNA. Our experiments using DNA from the several cell lines that harbored different *PIK3CA* mutations revealed that our dPCR assay was sensitive enough to detect as few as two copies of mutant alleles within 20,000 copies of wild-type alleles, representing the sensitivity of 0.01 % for each of the three *PIK3CA* hotspot mutations. Additionally, a high specificity (100 %) was confirmed by the findings that no *PIK3CA* mutations were observed in any of the serum samples from the 50 healthy women and the 30 breast cancer patients with *PIK3CA* non-mutated tumors. These results indicate that the dPCR assay was highly sensitive and specific for the detection of *PIK3CA* mutation.

We detected the *PIK3CA* mutant ctDNA in 25 patients (22.7 %) who had *PIK3CA* mutant tumors. We found that *PIK3CA* mutant ctDNA was more likely to be detected in

Table 3 Univariate and multivariate analysis of various parameters associated with prognosis (OS)

	Univariate			Multivariate		
	Hazard ratio	95 % CI	*P*	Hazard ratio	95 % CI	*P*
Age (≦50 vs > 50)	3.671	0.439–30.718	0.230			
Tumor size (> 2 cm vs ≦2 cm)	5.451	0.656–45.295	0.116			
Nodal status (positive vs negative)	0.578	0.128–2.606	0.476			
Histological grade (3 vs 1, 2)	41.145	0.066–25,837.56	0.258			
ER (positive vs negative)	0.189	0.042–0.848	0.030	1.340	0.169–10.655	0.782
PR (positive vs negative)	0.135	0.026–0.697	0.017	0.165	0.020–1.357	0.094
HER2 (positive vs negative)	6.069	1.358–27.124	0.018	2.514	0.389–16.255	0.333
ly (positive vs negative)	0.621	0.075-5.164	0.659			
v (positive vs negative)	1.972	0.438–8.887	0.166			
ctDNA ($ctDNA^{high}$ vs $ctDNA^{low+negative}$)	6.502	1.452–29.121	0.014	3.917	0.675–22.741	0.128

CI confidence interval, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

the patients with ER-negative ($P = 0.051$) or PR-negative ($P = 0.033$) tumors, and these findings are consistent with our previous report found that *GSTP1, RASSF1A, and RARb*-methylated ctDNA are more likely to be detected in ER-negative tumors [21]. Additionally, the *PIK3CA* mutant ctDNA tended to be associated with high histological grade tumors ($P = 0.066$) but not tumor size or lymph node status. These results indicate that the presence of *PIK3CA* mutant ctDNA reflects the biologically more aggressive phenotype of breast tumors rather than the tumor size itself. Accordingly, Becker S et al. reported that ER-negative tumors are more likely to release CTCs into the blood, and CTCs are known to correlate with ctDNA [22].

We have shown that the patients with *PIK3CA* mutant ctDNA exhibited significantly worse RFS and OS rates than did those without this ctDNA; interestingly, the prognoses of the $ctDNA^{high}$ patients but not the $ctDNA^{low}$ patients were significantly worse than those without the ctDNA. Multivariate analysis showed that high copy number of mutant alleles was a significant prognostic factor that was independent of the conventional parameters. These results suggest the possibility that the level of ctDNA might be important for the prediction of patient prognosis, but the optimal cut-off value remains to be established. We also analyzed the data based on relative mutant allele fraction (MAF) (%), but MAF (%) was not significantly associated with recurrence risk in a MAF-dependent manner unlike mutant copy number in serum (copy/ml) (Supplementary Fig. S4). Although both copy number per ml and MAF (%) are used in recent reports [12, 13], absolute amount of ctDNA in serum seems to reflect the tumor burden in patients more precisely than MAF which is dependent on wild-type alleles susceptible to various biological conditions. Our data suggest that the detection of *PIK3CA* mutations in the cfDNA with dPCR would be clinically useful for the prediction of the prognoses of primary breast cancer patients. In contrast, only 1.8 and 2.7 % of the patients were positive for the conventional tumor markers CEA and CA15-3, respectively, and these markers did not show any association with either DFS or OS, which indicates the superiority of *PIK3CA* mutant ctDNA to CEA and CA15-3 as a tumor marker and prognostic factor.

Recently, Beaver et al. [14] and Turner et al. [23] have reported the higher detection rates (93 and 75 %, respectively) of *PIK3CA* mutant ctDNA than our study (22.7 %) in primary breast cancer patients. This seems to be mostly explained by the difference of dPCR platform. They used droplet-dPCR which can analyze a significantly greater number of DNA molecules than our dPCR platform. In addition, Beaver et al. [14] performed "pre-amplification" in order to increase the detection sensitivity. The difference in the assay samples, i.e., serum or plasma, might be another reason for a lower detection rate in our study. Plasma is currently known to be more suitable for ctDNA study because plasma contains fewer wild-type alleles than serum. Unfortunately, we had only serum samples available for this retrospective study on prognosis since there was no consensus for samples to be used for ctDNA studies when we started collection of the samples. Besides, more advanced stage patients (i.e., node-positive or T3/T4) were included in Turner et al' study [23] than our study, i.e., 91 % (100/110) were stage I or II patients. All these differences mentioned above seem to explain the lower detection rate of *PIK3CA* mutant ctDNA in our study. Nonetheless, we could show that $ctDNA^{high}$ patients, but not $ctDNA^{low}$ patients, had a significantly worse prognosis than $ctDNA^{negative}$ patients, suggesting that very high sensitivity might not be needed, at least, in the prediction of prognosis in early-stage breast cancer patients.

The molecular aberrations of the PI3K pathway, including *PIK3CA* somatic mutations, have important implications as therapeutic targets in breast cancer. Patients harboring such aberrations are thought to be more sensitive to PI3K/AKT/mTOR pathway inhibitors [24] and resistant to anti-HER2 and endocrine therapy [25]. A prospective study is currently investigating targeted regimens that are more tailored to individual metastatic breast cancer patients due to the identification of genomic alterations (SAFIR-01 study) [26]. However, in this trial, serious adverse events related to tumor biopsy have been reported in 4 patients (1 %), suggesting the need for safer and less invasive methods to identify genomic alterations in cancer patients. Mutational analysis of *PIK3CA* in cfDNA from metastatic breast cancer patients, i.e., the so-called liquid biopsy, is one promising option for avoiding such risks, and molecular screening of patients with ctDNA might increase the precision of personalized therapies.

In conclusion, we have shown that dPCR is a highly sensitive and specific method for detecting *PIK3CA* mutant ctDNA, 22.7 % of primary breast cancer patients are positive for this ctDNA, and ctDNA^{high} but not ctDNA^{low} status is a significant prognostic factor that is independent of the other conventional prognostic factors. We also suggest that *PIK3CA* mutant ctDNA is a better tumor marker than CEA and CA15-3. Our preliminary results require validation in future studies.

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Conflict of interest The authors of this study have no conflicts of interest and no financial disclosures to make.

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