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PIK3CA mutation profiling in patients with breast cancer, using a highly sensitive detection system

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PIK3CA mutations are common activating mutations associated with breast cancer (occurring in 20–30% of all cases) and are potent predictive markers for responses to PI3K inhibitors. Thus, it is important to develop sensitive methods to detect these mutations. We established a novel detection method using a quenching probe (QP) system to identify *PIK3CA* mutations, using DNA from 309 breast cancer tissues. In a developmental cohort, we determined the optimal detection threshold of the QP system with human tumor DNA from 119 freshly frozen tumor samples. We found a 96% concordance rate with the QP system between DNA from 26 matching fresh-frozen specimens and formalin-fixed paraffin-embedded (FFPE) specimens from the same patients, and known *PIK3CA* mutation status in the developmental cohort. In a validation cohort, we evaluated whether the threshold for judging mutations using the QP system with frozen specimen-derived DNA was applicable with FFPE-derived DNA. In the validation cohort, 30 DNA samples from 190 FFPE-derived DNA samples with known *PIK3CA* mutation status were analyzed by direct sequencing (DS) and droplet digital PCR, in a blinded manner. The sensitivity and specificity of the droplet digital PCR results were 100% and 100% (QP system), and 60% and 100% (DS), respectively. We also analyzed the relationship between clinical outcomes and the *PIK3CA* mutational status of 309 breast cancer samples, including the developmental cohort and validation cohort samples. Multivariate analysis suggested that *PIK3CA* mutations, especially H1047R, were prognostic factors of relapse-free survival. Our novel detection system could be more useful than DS for detecting clinical *PIK3CA* mutations.

KEYWORDS
breast cancer, highly sensitive, *PIK3CA* mutation, quenching probe system, survival

1 | INTRODUCTION

Activation of the PI3K pathway in breast cancer is observed in approximately 70% of all cases. The gene encoding the catalytic α subunit of PI3K (*PIK3CA*) is commonly activated in breast cancer,

Abbreviations: CI, confidence interval; ddPCR, droplet digital PCR; DS, direct sequencing; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; HER2, human epidermal growth factor receptor-2; HR, hazard ratio; OS, overall survival; PgR, progesterone receptor; *PIK3CA*, catalytic subunit α of PI3K; QP, quenching probe; QProbe, quenching probe; RFS, relapse-free survival; TNBC, triple-negative breast cancer.

and *PIK3CA* mutations occur in 20–30% of patients with breast cancer.[1,2] Previous studies have shown that *PIK3CA* mutations in breast cancer often present as high-frequency subclonal mutations.[3–5] In a report comparing the frequency of *PIK3CA* mutations between primary tumors and metastatic lesions in untreated patients with metastatic breast cancer, a high concordance rate of 70% was observed.[6] Furthermore, an investigation of the concordance rate of *PIK3CA* mutations in primary tumors and recurrent lesions after relapse[7,8] suggested the involvement of selection by treatment, with a concordance rate of approximately 90%. Thus, it is certain that *PIK3CA* mutations are important for the survival of patients with breast cancer. Three *PIK3CA* mutational hot spots (E542K, G1624A and E545K, and G1633A) occur in two helical domains corresponding to exon 9, and mutations in the catalytic domain (H1047R and A3140G; corresponding to exon 20) account for 70–80% of all *PIK3CA* mutations detected in breast cancer.[9,10] However, the prognostic impact of these mutations might differ between breast cancer subtypes. A previous report shows that *PIK3CA* mutations might be good prognostic factors for hormone receptor-positive breast cancer.[11] However, *PIK3CA* mutations could have a more negative impact on patient survival than WT (non mutant type) *PIK3CA*, especially in HER2-positive tumors.

In the metastatic setting, *PIK3CA* mutations correlated with poor response to trastuzumab and survival time,[12] whereas in the adjuvant setting, *PIK3CA* mutations correlated strongly with poor disease-free survival and OS.[13,14] In the neoadjuvant setting (e.g. in the NeoALTTO, GeparQuattro, GeparQuinto, and GeparSixto trials), *PIK3CA* mutations correlated strongly with poor pathological responses.[15,16] Moreover, data from recent clinical trials suggest that *PIK3CA* mutations are potent predictive markers for responses to PI3K inhibitors.[17] Thus, a simple and accurate system for detecting *PIK3CA* mutations is important for determining appropriate therapy.

Among the various methods used for detecting *PIK3CA* mutations, DS constitutes the standard procedure for mutational analysis with breast cancer samples. The low detection-sensitivity limit (20–50%) and the risk of contamination while handling post-PCR products are the main disadvantages.[18]

The QP system was originally developed for detecting single nucleotide polymorphisms, such as those in *CYP2C19*.[19] The QP system provides high specificity; additionally, it facilitates the design of shorter probes that do not require long amplicons.[20] Moreover, the QP system can detect DNA mutations within 90 min. To detect *PIK3CA* mutations, the original area thresholds (E542K, 13.2; E545K, 3.3; and H1047R, 16.5) were predetermined by carrying out multiple measurements with purified human genomic DNA lacking *PIK3CA* mutations. Evaluating plasmid samples without any mutations indicated the presence of *PIK3CA* mutations with 100% sensitivity and specificity. Thus, a sample containing 40 ng of pure plasmid DNA, including DNA with ≥3% mutations, is required to generate positive results. The QP system was useful for detecting *PIK3CA* mutations; however, the validity of the *PIK3CA* mutational analysis data obtained using this system in human breast cancer tissue has not yet been confirmed.

Therefore, in this study, we developed the first system for detecting *PIK3CA* mutation hot spots (E542K, E545K, and H1047R) in human breast cancer using a modified version of the QP system.

2 | MATERIALS AND METHODS

2.1 | Samples and DNA extraction

We used a two cohorts to develop the QP system for human samples (Fig. 1). We used 309 samples from patients with breast cancer (119 freshly frozen tumor samples and 190 FFPE samples) provided by the National Cancer Center Biobank of Japan (Tokyo, Japan).

For the developmental cohort, we utilized 119 freshly frozen tumor surgical specimens preserved between May 2009 and December 2012. We extracted tumor DNA using the QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan).

For the validation cohort, we utilized 190 FFPE surgical or biopsy tissues from primary tumor samples preserved between November 1979 and February 2015. We extracted the tumor DNA using the QIAamp DNA FFPE Tissue Kit (Qiagen).

2.2 | Quenching probe system analysis and threshold setting for detecting *PIK3CA* mutations

To detect *PIK3CA* mutations, we used the QP system, which incorporates the i-densy IS-5320 genetic analysis system (ARKRAY, Kyoto, Japan).[21] DNA samples (≥10 ng/μL, ≥4 μL) were prepared for analysis with the QP system. *PIK3CA* mutation analysis was based on previous reports.[20,22,23] A mixture of *PIK3CA* mutation-specific QProbes and amplification primers for each target sequence in exon 9 or 20 was placed in specified wells of an i-densy Pack UNIVERSAL (ARKRAY) containing DNA polymerase and other reagents necessary for PCR. The presence of *PIK3CA* mutations (E542K, E545K, or H1047R) in the amplified sequences was determined by monitoring the fluorescence intensity of the QProbes (Nippon Steel Kankyo Engineering, Tokyo, Japan) complementary to E542K, E545K, or H1047R mutations, which were labeled with BODIPY FL, Pacific Blue, or TAMRA, respectively. The probe sequences were as follows: E542K probe, 5′-CTC TCT **A**AA ATC ACT GAG C-3′; E545K probe, 5′-CTC TCT GAA ATC ACT **A**AG C-3′; and H1047R probe, 5′-CCA TGA **C**GT GCA TCA TT-3′, where the bold underlined characters indicate the mutated bases. The sequences of the exon 19 primers, which yielded a 106-bp amplicon, were as follows: forward, 5′-GAA CAG CTC AAA GCA ATT TCT ACA CGA G-3′; reverse, 5′-CAG AGA ATY TCC ATT TTA GCA CTT ACY TGT GAC, where "Y" indicates C or T. The sequences of the exon 20 primers, which yielded a 96-bp amplicon, were as follows: forward, 5′-GAG GCT TTG GAG TAT TTC ATG AAA CAA ATG-3′; and reverse, 5′-GCA TGC TGT TTA ATT GTG TGG AAG ATC CAA TC-3′.

Polymerase chain reaction was carried out as follows: 95°C for 1 min, followed by 50 cycles of 1 s at 95°C and 30 s at 58°C. After PCR, melting-curve analysis of the products was undertaken as follows: 1 s at 95°C, 1 min at 40°C, and 1°C steps with a hold of 3 s

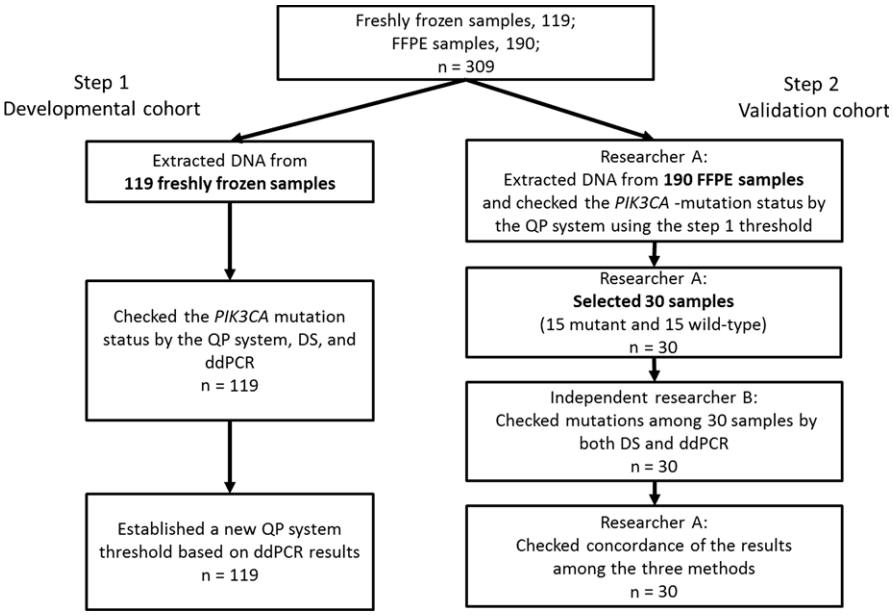

FIG. 1 Schematic representation of the developmental and validation cohorts of Japanese patients with breast cancer, to assess the utility of the quenching probe (QP) for *PIK3CA* mutation profiling. ddPCR, droplet digital PCR; DS, direct sequencing; FFPE, formalin-fixed paraffin-embedded

at each step from 40°C to 75°C. The results were analyzed using Area-Analyzing software (ARKRAY), which calculates peak areas. The measurement principle of QProbes was previously described.[21] As mentioned above, QProbes were designed to be perfectly complementary to the *PIK3CA* mutated sequences. At each optical detection for BODIPY FL, Pacific Blue, and TAMRA, the peaks of WT E542, E545, and H1047 were observed at approximately 48°C. In contrast, the peaks for mutated E542K, E545K, and H1047R were observed at approximately 57°C. For the mutant peaks, the temperature range of the analysis area for the WT peak was 40–52°C on the low-temperature side and 54–60°C on the high-temperature side. The area-threshold values of the mutation hot spots (E542K, E545K, and H1047R) were set to 13.2, 3.3, and 16.5, respectively, after multiple measurements of WT human genomic DNA (Roche/Sigma-Aldrich, Tokyo, Japan). Samples were considered mutation-positive in cases where the area value of the high-temperature side was wider than the threshold value.

2.3 | Direct sequencing analysis of *PIK3CA* mutations

Exons 9 and 20 were amplified by PCR using the following primers: exon 9 forward, 5′-GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC GAC AAA GAA CAG CTC AAA GCA A-3′ and exon 9 reverse, 5′-ACA TGC TGA GAT CAG CCA AA-3′; and exon 20 forward, 5′-GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC TGA GCA AGA GGC TTT GGA GT-3′ and exon 20 reverse, 5′-GGT CTT TGC CTG CTG AGA GT-3′. The resulting amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Direct sequencing was carried out by Eurofins Genomics (Tokyo, Japan). We extracted tumor-derived DNA from 26 paired freshly frozen and FFPE samples from the same patients, and mutations were detected using the QP system.

2.4 | Droplet digital PCR analysis of *PIK3CA* mutations

Tumor tissue DNA was analyzed by ddPCR on a QX200 droplet digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA), using PrimePCR for ddPCR Assays (Bio-Rad Laboratories) along with *PIK3CA* E542K, E545K, and *PIK3CA* H1047R mutation controls. Total DNA (1 ng) containing serially diluted mutant DNA was combined with a solution containing 1× ddPCR Supermix for Probes (no dUTP), and 1× mutant (FAM-labeled) and WT (HEX-labeled) PrimePCR ddPCR assay reagents in a final volume of 20 μL. The mixture was compartmentalized into approximately 20 000 oil droplets using a QX200 droplet generator. The emulsified PCR mix was transferred to a 96-well plate and PCR amplified under the following conditions: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 55°C for 1 min, a 10-min incubation at 98°C, and a final hold step at 4°C. After amplification, the PCR plate was transferred to a droplet reader where the droplets were streamed in single file past an optical detector and counted. Data analysis was carried out using the QuantaSoft version 1.7.4.0917 software (Hercules, CA, USA), and the target concentration was calculated as the number of copies/reaction. The amplitude thresholds of the E542K, E545K, and H1047R hot spots were ch1:4395 ch2:2937, ch1:1993 ch2:2201, and ch1:3726 ch2:1700, respectively. The thresholds were determined by measuring the WT HDx FFPE reference standards (Horizon Diagnostics, Cambridge, UK), and the mutational threshold was 1%. Compared to ddPCR, the method for determining the threshold of reliable frequency detection differs with the equipment used and is not established. In contrast, the threshold of reagent-induced false positives was assumed to be 0.5% of the mutation frequency. Therefore, the threshold at which mutation positivity can be reliably evaluated by ddPCR was set to 1%.

2.5 | Developmental cohort

The developmental cohort was created to confirm whether the positive threshold value obtained in experiments using the pure plasmid could be reproduced with DNA derived from patient specimens. In addition, the purpose of this cohort was to determine the positive threshold of the new QP system in cases where differences were observed between the threshold values obtained from plasmid and patient sample DNA. We checked the *PIK3CA* mutation status by DS and the QP system. After determining the concordance between both methods, we rechecked the discordant samples by ddPCR. Based on these results, the optimal new QP system thresholds were determined.

2.6 | Validation cohort

The second step involved in the creation of a validation cohort, in which the validity of the new threshold determined in the first step (developmental cohort), was confirmed. The *PIK3CA* mutation status was ascertained with the QP system, using the validated thresholds determined using the developmental cohort. Subsequently, we blinded the mutational information of 15 mutant and WT samples, which were tested by an independent researcher using DS and ddPCR. Finally, the concordance among the results of the three methodologies was ascertained, according to a previous report.[24]

2.7 | Clinical outcome analysis based on *PIK3CA* mutation information

For the outcome analysis, we utilized 64 additional FFPE tissues sampled between November 1979 and February 2015 from the National Cancer Center Biobank of Japan, as well as extracted tumor DNA, using the QIAamp DNA FFPE Tissue Kit (Qiagen).

We analyzed differences in outcomes between patients with and without *PIK3CA* mutations using the 309-patient dataset. Proportional differences for categorical variables were evaluated by the χ^2-test. Kaplan–Meier analysis was used to analyze RFS after surgery and OS. Relapse-free survival was defined as the time between the day of surgery and disease progression, or the day of last follow-up. Overall survival was measured from the day of surgery to the day of death, or the day of last follow-up. Multivariate analysis was performed using the Cox hazard model to detect significant prognostic factors for RFS and OS. The clinically relevant covariates (age >52 years, initial stage, hormone receptor status, HER2 status, and *PIK3CA* helical-domain and kinase-domain mutations) were included in a multiple Cox proportional hazards model. We used JMP software, version 11 (SAS Institute, Cary, CA, USA) for all statistical analyses. Values of $P < 0.05$ indicated statistically significant differences. This study protocol was approved by the National Cancer Center Institutional Review Board (No. 2014-092). Written informed consent was not obtained from the patients because of the retrospective nature of this study. The outline of the research plan, which was approved by the ethics review committee, is published on the hospital's website.

2.8 | Histopathological evaluation and immunohistochemistry

Histopathological assessment of histological grades and immunohistochemical staining for estrogen receptor, progesterone receptor, and HER2 was carried out as described previously.[25]

3 | RESULTS

3.1 | Developmental cohort

The results of the *PIK3CA* mutation analysis, as determined by the QP system and DS, are shown in Table 1. Fourteen discordant samples (12 samples with QP system-positive and DS-negative results, and two with QP system-negative and DS-positive results) were identified among the 119 developmental-cohort samples.

We retested the 14 discordant samples by ddPCR, including one that showed both E542K and H1047R mutations. The ddPCR results indicated that 13 samples were concordant with the QP system and one was discordant with DS (E542K) (Table S1). Based on these results, we obtained validated QP system thresholds of 17.1 for E542K, 10.2 for E545K, and 10.2 for H1047R. Although one sample was negative for *PIK3CA* mutations by the QP system, it was positive by DS and ddPCR, with the latter showing a 0.085% mutation frequency (Fig. S1). We concluded that this discordant sample was a false positive because of the low proportion of mutations.

We extracted tumor DNA from matching 26 freshly frozen and FFPE samples from the same patients, and mutations were measured using the QP system. The concordance rates were 100% (26/26) for E542K and E545K, and 96.2 (25/26) for H1047R. One FFPE sample showed positive result for the H1047R mutation, although a negative result was obtained in the corresponding freshly frozen sample. As the result did not change after re-examination, we considered the possibility of heterogeneity. The results are shown in Table S2.

3.2 | Validation cohort

We analyzed 126 FFPE-derived DNA samples (validation cohort) using the QP system, of which 34 samples (27.4%) had *PIK3CA* mutations. We masked the mutational information for 15 different *PIK3CA* mutation-positive DNA samples (three E542K, four E545K, and eight H1047R) and 15 mutation-negative samples from this cohort, and sent the samples to an independent researcher (Marifu Yamagishi, ARKRAY) for ddPCR and DS analyses.

TABLE 1 Results of *PIK3CA* mutation analysis obtained with the developmental cohort of breast cancer patients (n = 119), using the quenching probe (QP) system and direct sequencing

		QP system	
		Positive, *n* (%)	**Negative, *n* (%)**
Direct sequencing	Positive, *n* (%)	26 (22)	2 (2)
	Negative, *n* (%)	12[†] (10)	79 (66)

[†]One sample had both E542K and H1047R mutations.

Tables 2–7 show the mutational results obtained with the QP system (by the primary researcher) and with other detection methods (by the independent researcher). Tables 2–7 also show the sensitivity, specificity, and concordance rate between the QP system and DS method, when compared with from the ddPCR results. The sensitivities of the QP system and DS were 100% and 60%, respectively. The concordance rates of the QP system and DS were 100% and 80%, respectively. These observations revealed that the mutational results of the QP system were completely concordant with the ddPCR results for DNA samples with mutant frequencies >0.3%, whereas the DS method had a higher false-negative rate than the QP system. These data confirmed the validated threshold (E542K, 17.1; E545K, 10.2; H1047R, 10.2) used for testing DNA from FFPE tissues using the QP system.

While detecting point mutations in DNA extracted from tumors, we found that the DNA quantity was more important than the quality for detecting mutations. We accurately determined the amount of DNA present in each sample. The median amount of FFPE-derived DNA was 35.4 ng/μL (range, 0.23–610), and the median amount of DNA derived from freshly frozen specimens was 78.4 ng/μL (range, 1.71–464). All samples with concentrations <10 ng/μL were tested after evaporation and concentration to >10 ng/μL.

3.3 | Clinical outcome analysis

We detected *PIK3CA* mutations in 65 additional FFPE samples using the QP system and retrospectively analyzed 309 samples for outcome analysis. *PIK3CA* mutations were identified in 105 samples (33%), of which 18 had the E542K mutation, 19 had the E545K mutation, and 71 had the H1047R mutation. Both E545K and H1047R were detected in two samples. Table 8 shows patient characteristics for the 309 donors and the distribution of patients who were negative or positive for *PIK3CA* somatic mutations. Statistical analysis indicated that lobular histology was more frequent among patients carrying *PIK3CA* mutations ($P = 0.049$). Table 9 shows the distribution of each *PIK3CA* hot spot mutation based on

TABLE 2 E542K mutation results, sensitivity, specificity, and concordance rate of the quenching probe (QP) system based on droplet digital PCR (ddPCR) in the validation cohort

		Mutation results of ddPCR (n = 30)		
		E542K	Without E542K	Total
QP system	E542K	3	0	3
	Without E542K	0	27	27
Total		3	27	30

Concordance rate, 100%; sensitivity, 100%; specificity, 100%.

TABLE 3 E542K mutation results, sensitivity, specificity, and concordance rate of the direct sequencing (DS) method with the validation cohort of breast cancer patients (n = 30), based on droplet digital PCR (ddPCR) results

		Mutation results of ddPCR (n = 30)		
		E542K	Without E542K	Total
DS method	E542K	0	0	0
	Without E542K	3	27	30
Total		3	27	30

Concordance rate, 90%; sensitivity, 0%; specificity, 100%.

TABLE 4 E545K mutation results, sensitivity, specificity, and concordance rate of the quenching probe (QP) system with the validation cohort of breast cancer patients (n = 30), based on droplet digital PCR (ddPCR) results

		Mutation results of ddPCR (n = 30)		
		E545K	Without E545K	Total
QP system	E545K	4	0	4
	Without E545K	0	26	26
Total		4	26	30

Concordance rate, 100%; sensitivity, 100%; specificity, 100%.

TABLE 5 E545K mutation results, sensitivity, specificity, and concordance rate of the direct sequencing (DS) method with the validation cohort of breast cancer patients (n = 30), based on droplet digital PCR (ddPCR) results

		Mutation results of ddPCR (n = 30)		
		E545K	Without E545K	Total
DS method	E545K	3	0	3
	Without E545K	1	26	27
Total		4	26	30

Concordance rate, 97%; sensitivity, 75%; specificity, 100%.

TABLE 6 H1047R mutation results, sensitivity, specificity, and concordance rate of the quenching probe (QP) system with the validation cohort of breast cancer patients (n = 30), based on droplet digital PCR (ddPCR) results

		Mutation results of ddPCR (n = 30)		
		H1047R	Without H1047R	Total
QP system	H1047R	8	0	8
	Without H1047R	0	22	22
Total		8	22	30

Concordance rate, 100%; sensitivity, 100%; specificity, 100%.

TABLE 7 H1047R mutation results, sensitivity, specificity, and concordance rate of direct sequencing (DS) method with the validation cohort of breast cancer patients (n = 30), based on droplet digital PCR (ddPCR) results

		Mutation results of ddPCR (n = 30)		
		H1047R	Without H1047R	Total
DS method	H1047R	6	0	6
	Without H1047R	2	22	24
Total		8	22	30

Concordance rate, 93%; sensitivity, 75%; specificity, 100%.

TABLE 8 Characteristics of breast cancer patients according to *PIK3CA* mutation status (n = 309)

Variable	*PIK3CA* mutant n = 105 (%)	*PIK3CA* WT n = 204 (%)	Total n = 309 (%)	*P*-value[†]
Median age, years (range)	53 (29–90)	51 (22–84)	52 (22–90)	0.070
Initial stage				
I	27 (26)	45 (22)	72 (24)	0.920
II	43 (41)	83 (41)	126 (41)	
III	23 (22)	50 (25)	73 (23)	
IV	10 (9)	18 (8)	28 (9)	
ND	2 (2)	8 (4)	10 (3)	
Histology				
Ductal	94 (90)	189 (92)	283 (92)	0.037*
Lobular	7 (6)	4 (2)	11 (3)	
ND	3 (4)	12 (6)	15 (5)	
Grade				
1	9 (10)	21 (11)	30 (11)	0.160
2	57 (54)	90 (44)	147 (47)	
3	32 (30)	83 (40)	115 (37)	
ND	7 (6)	10 (5)	17 (5)	
LVI				
Positive	40 (37)	89 (50)	129 (40)	0.300
Negative	45 (42)	76 (37)	121 (38)	
ND	10 (21)	39 (23)	49 (22)	
HR				
Positive	88 (84)	175 (85)	263 (85)	0.730
Negative	17 (16)	29 (15)	46 (15)	
HER2				
Positive	18 (17)	36 (18)	54 (18)	0.910
Negative	87 (83)	168 (82)	255 (82)	
Surgical procedure				
Bp	38 (36)	71 (34)	109 (34)	0.720
Bt	57 (53)	117 (55)	174 (54)	
Not accepted	10 (11)	16 (11)	26 (12)	
Treatment for perioperative disease (n = 276)				
Neoadjuvant therapy	24 (26)	49 (24)	73 (23)	0.810
Adjuvant CTx	42 (40)	90 (44)	132 (43)	0.630
Adjuvant hormone	69 (66)	148 (72)	218 (71)	0.350
No treatment	11 (12)	17 (9)	28 (9)	0.470
Treatment for advanced disease (n = 174)				
Chemotherapy	32 (30)	61 (30)	93 (30)	0.570
Hormone therapy	51 (49)	89 (44)	140 (45)	0.970

*Statistically significant.

[†]Mutant *PIK3CA* *versus* WT *PIK3CA*.

Bp, partial mastectomy; Bt, total mastectomy; CTx, chemotherapy; HER2, human epidermal growth factor receptor-2; HR, hormone receptor; LVI, lymphovascular invasion; ND, no data.

TABLE 9 Number of patients with *PIK3CA* mutations among all breast cancer subtypes (n = 309)

Mutation	HR+ HER2− n = 235 (%)	HR+ HER2+ n = 28 (%)	HR− HER2+ n = 25 (%)	TNBC n = 21 (%)	*P*-value
E542K	16 (7)	1 (4)	0 (0)	1 (5)	0.51
E545K	14[†] (5)	4 (14)	0 (0)	2 (10)	0.14
H1047R	53[†] (23)	3 (11)	9 (36)	5 (24)	0.18
PIK3CA mutation total	81[†] (34)	8 (29)	9 (36)	8 (38)	0.90

[†]Two patients found to have both the E545K and H1047R mutations.

+, positive; −, negative; HER2, human epidermal growth factor receptor-2; HR, hormone receptor; TNBC, triple-negative breast cancer.

the breast cancer subtype. No biased *PIK3CA* mutation distribution was detected in any breast cancer subtype.

At the time of analysis (December 12, 2016), the median follow-up time was 60 months, considering the death of 43 of 309 patients (14%) and relapse of 146 of 276 patients (53%) who had accepted surgical procedures for early breast cancer. Survival analysis showed that patients with *PIK3CA* mutations had better RFS than those with wild-type *PIK3CA* (P = .028). More detailed analysis of the *PIK3CA* mutation hot spots demonstrated that the *PIK3CA* kinase-domain (H1047R) mutation was a statistically significant good prognostic factor for RFS (P = 0.0098), whereas the *PIK3CA* helical-domain mutation was not (E542K, P = 0.93; E545K, P = 0.32) (Fig. 2). Multivariate analysis of the total subtype cohort indicated that the initial low stage and the *PIK3CA* kinase-domain mutation were prognostic factors. Moreover, multivariate analysis of the luminal subtype revealed that an initial low stage (P = 0.0014; HR = 1.99; 95% CI, 1.31–2.98), age <52 years at diagnosis (P = 0.027; HR = 1.56; 95% CI, 1.05–2.32), and *PIK3CA* mutations in the kinase domain (P = 0.017; HR = 0.55; 95% CI, 0.32–0.90) were good prognostic factors (Table S3a,b). Moreover, the *PIK3CA* mutation status was not related to prognosis based on the OS (Fig. S2). Multivariate analysis showed that an initial low stage was the sole favorable prognostic factor of OS in all cohorts (P < 0.0001; HR = 3.75; 95% CI, 2.07–6.99) and in the luminal cohort (P < 0.0001; HR = 4.11; 95% CI, 2.10–8.37) (Table S3c,d).

4 | DISCUSSION

We established a novel, fully automated QP system for detecting E542K, E545K, and H1047R *PIK3CA* mutations in patients with breast cancer. Several prior reports showed that patients with *PIK3CA* mutations, especially those with HER2-positive tumors, might have poorer survival or reduced response to anti-HER2 therapy than those with WT *PIK3CA*.[12–17] Moreover, recent clinical trials suggest that *PIK3CA* mutations are potent predictive markers for responses to PI3K inhibitors.[17] Therefore, a system for easy detection of *PIK3CA* mutations is urgently required.

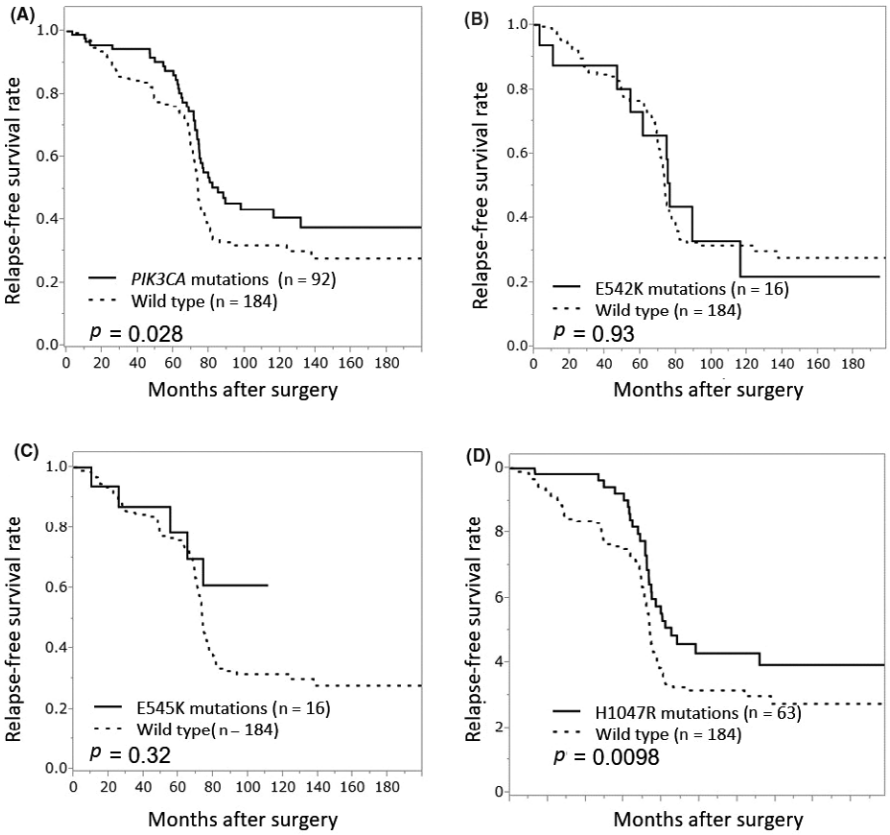

FIG. 2 Relapse-free survival (RFS) curves according to the *PIK3CA* status in 276 Japanese patients with early breast cancer. (A) Overall RFS for *PIK3CA* mutation-positive *versus* WT patients. (B–D) RFS for patients with E542K, E545K, and H1047R *PIK3CA* mutations. Panels (A) and (D) show that patients with *PIK3CA* mutations, especially H1047R, had a favorable RFS compared to patients with WT *PIK3CA*

Our system compared favorably with existing systems, yielding results with patient breast cancer tumor specimens that were identical to those obtained by DS. Using the validated threshold, we detected *PIK3CA* mutations in DNA from FFPE tissues by the QP system, with a sensitivity comparable to that obtained for ≥3% mutant plasmid alleles, which was superior to that of the DS method. In addition, the QP system does not require complicated operation or a high level of expertise and is faster than the DS and ddPCR methods (Fig. S3). Rare mutations can be detected more easily and rapidly using this method than by next-generation sequencing because the QP system is specialized for detecting point mutations and is, therefore, more suitable for clinical use.

The frequency of *PIK3CA* mutations in our cohort was 33%. As shown in Table 9, the frequency of *PIK3CA* mutations was approximately 30–40% in all subtypes, and no statistical difference in terms of the mutation frequency was found between the subtypes. Although the mutation frequencies varied by the breast cancer or intrinsic subtypes,[1,9,11,26,27] our results are consistent with those of previous reports, as most samples in our cohort were from hormone receptor-positive breast cancers.[9,28] Moreover, the mutation frequency in TNBC was 8.3–25% in these reports. In contrast, the TNBC mutation frequency of our cohort was 8/21 (38%), which is slightly higher than those in previous reports. However, as another study undertaken in our institution revealed a TNBC mutation frequency of 35% (26/75),[29] we suggest that the Japanese population might have a higher TNBC *PIK3CA* mutation frequency than other populations.

PIK3CA mutations, especially in the kinase domain (H1047R), were found to be a good prognostic factor for RFS, but not OS. These results might have been found because 85% of the breast cancer tissues were hormone receptor-positive in our study, and prior research indicated that the *PIK3CA* kinase-domain mutation is a favorable prognostic factor in hormone receptor-positive breast cancer.[11]

Notably, an epidermal growth factor receptor mutation has been detected in blood samples from patients with non-small-cell lung cancer by the QP system.[20] Thus, in the future, we might be able to detect *PIK3CA* mutations using the QP system in liquid biopsy assays.

This study has certain limitations. The sample-storage period varied in our National Cancer Center Biobank. As we evaluated point mutations using DNA extracted from tumors and assumed that DNA quantity was more important than its quality, we accurately evaluated the amount of DNA for each sample. Moreover, all samples were tested at DNA concentrations of >10 ng/μL. In addition, prognostic analysis between the four subtypes could not be carried out because the specimens used in our study were biased toward hormone receptor-positive, HER2-negative samples. We undertook multivariate analysis only in hormone receptor-positive cases because of the small sample size of the other subtypes.

In conclusion, we established the QP system for detecting *PIK3CA* mutations in a Japanese cohort. The mutation distribution, especially that of TNBC, in breast cancer subtypes in Japan differed from those of other countries. Additionally, a favorable prognostic impact of the *PIK3CA* kinase-domain mutation on RFS was suggested in this cohort. Thus, the QP system constitutes a highly sensitive

and convenient method for *PIK3CA* mutation detection that could be clinically useful for breast cancer characterization and prognosis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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