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Article

Chip-Based Digital PCR improves the detection of low-rate *PIK3CA* mutations in Breast Cancer Patients

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Simple Summary: *PIK3CA* mutations are frequent mutations in breast cancer and are considered as potential predictive and prognosis factors. Novel platforms for gene analysis as digital PCR (dPCR) are emerging as a potential replacement for traditional Sanger sequencing. However, there are still few studies on dPCR. Thus, this cross-sectional study aimed to assess the sensibility of dPCR to detect and quantify *PIK3CA* mutations in breast cancer patients and compare its performance with Sanger sequencing. Our findings describe dPCR as a technique able to detect low-rate *PIK3CA* mutations. It represents tumor subpopulations not usually detected by Sanger sequencing, which could induce treatment changes at clinical routine.

Abstract: *PIK3CA* is a gene usually mutated in breast cancer and has an important role in tumor progression and treatment. Therefore, there is required a technique to detect low-rate *PIK3CA* mutations improving the clinical conduct. This study aimed to compare chip-based dPCR and Sanger sequencing to detect *PIK3CA* mutations in breast cancer patients. Fifty-seven tumor samples from breast cancer patients were collected and analyzed by Sanger sequencing and dPCR for *PIK3CA* mutations (E545K, H1047R, and H1047L). Digital PCR sensitivity, specificity, and overall performance were estimated by contingency tables, receptor operator characteristic (ROC), and area under the curve (AUC). Sanger sequencing identified PIK3CA mutations in six patients (10.5%), two with H1047R, and four with E545K. Digital PCR confirmed those mutations and identified 19 additional patients with at least one mutation. Comparison between dPCR and Sanger sequencing showed a sensitivity of 100% (95% CI 53-100%), and a specificity of 84.2% (95% CI 83 - 84.2%). Besides, H1047R mutation showed a significant association with breast cancer phenotype (p =0.019) and lymphatic node infiltration (p =0.046). Digital PCR showed a high sensitivity to detect mutations in tumor samples and it might be capable to detect low-rate mutations and tumor subpopulations not detected by Sanger sequencing.

Keywords: Breast cancer; PIK3CA; digital PCR; Sanger sequencing

1. Introduction

PIK3CA mutations are common in breast cancer as described by The Cancer Genetic Atlas (TGCA) with a frequency of 36% [1]. Although this gene has 23 exons, their mutations are clustered in two hot spots in exon 09 (E542K and E545K) and exon 20 (H1047R and H1047L) [2]. The *PIK3CA* gene is located in chromosome 3q26.3 and encode the p110α isoform, part of the phosphatidilinositol 3-kinase (PI3K)/AKT/mTOR pathway [3], which converts phosphatidylinositol 4-5 biphosphate (PIP2) to phosphatidylinositol 3-4-5 triphosphate (PIP3). Consequently, it activates multiple down-stream signaling pathways involved in cellular growth, motility, apoptosis and differentiation [4,5]. In contrast, this

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activity is regulated by the phosphate and tensin homolog (PTEN) which converts back PIP3 into PIP2. Hence a mutation that makes a hyperactive PIK3CA or an underactive PTEN, will end to overstimulate the PI3K/AKT/mTOR pathway leading to an oncogenic behavior [5,6].

Since its discovery in 2004 [2], researchers have had a special attention to *PIK3CA* mutations as they considered them to have a potential use as prognostic and predictive factors. In addition, several authors have reported a significant association among *PIK3CA* mutations and positive prognostic factors [7-9], as well as a prolonged relapse free survival [10]. Nonetheless, this data is still controversial [11] as other authors have suggested that *PIK3CA* mutation are for the contrary associated with a negative prognosis (6,10) and current therapy resistance (hormone and anti-HER2 therapy) [12,13].

Traditionally, gene analysis in tumor samples have been conducted by Sanger sequencing, despite of its limitations [14-16]. Nevertheless, new technologies have emerged promising a better performance, enabling a quantitative and more sensitive gene analysis.

One of these technologies is the "digital Polymerase Chain Reaction" (dPCR) technology, which performs an absolute quantification of specific nucleic acid sequences by first dividing a sample in several individual partitions in chambers or droplets (depending on the platform), so at least one target molecule is contained in a partition. Then it performs simultaneous endpoint PCRs and count in a binary style (0 negative and 1 positive) the partitions where a fluorescence signal has been produced with a posterior Poisson analysis [17-19]. In contrast with other PCR techniques such as qPCR, dPCR does not require standard curves calibration and due to partition, it enhance the effective concentration of the objective nucleic acids and decreases the background effect of abundant molecules over rare or low frequent targets [20]. Thus, it is capable to detect until a theoretical 0.1% mutant allele frequency. Consequently, this technique has a great potential in personalized medicine as well as in cancer research [21].

There are several studies that have already used dPCR technology for mutation analysis with optimistic results [22-25]. Even though, most of them have commonly used Droplet Digital PCR (ddPCR), which is a water-oil droplet based platform that fractionate the sample in several droplet reactions [25], there is a novel chip-based platform, QuantStudio 3D Digital PCR System, which offers a highly precise and sensitivity absolute quantification with a similar quantity of partitions as ddPCR but with a simpler workflow [19] and a relatively low cost. However, there are still few studies using this new technique. Therefore, we designed a cross-sectional study, to determine the sensitivity of QuantStudio 3D Digital PCR system to detect *PIK3CA* mutations in breast cancer patients, in comparison with Sanger sequencing, as well as an analysis between patient clinicpathological features and *PIK3CA* mutational status detected by dPCR.

2. Materials and Methods

2.1. Study Population

Tumor/biopsy samples were obtained from patients with breast cancer diagnosed at the Instituto Nacional de Enfermedades Neoplásicas (INEN) in Lima-Peru and analyzed in a core Laboratory (Centro de Genética y Biología Molecular) at Universidad de San Martin de Porres. Patients were enrolled prospectively in the study between April and August of 2017 after signing the proper in-formed consent. The study protocol and informed consent were approved by Universidad de San Martín de Porres IRB (IRB00003251-FWA0015320) and the Protocols Review Committee from INEN. Biopsy samples were obtained when patient´s tumor was not available. Additional information regarding demographic and clinicopathological information was obtained from clinical records. Breast cancer phenotypes were classified based on the St. Gallen International Expert Consensus from 2011 [26].

2.2. Sample Processing and DNA Extraction

For each case, hematoxylin and eosin-stained slides from tumor/biopsy FFPE blocks were reviewed by a pathologist to confirm and delimitate the area with neoplastic cells. These delimitated areas were then localized in the FFPE blocks and cut in eight slices of 4mm thick. Genomic DNA extraction was performed according to the GeneJet FFPE DNA purification Kit (ThermoFisher Scientific, Boston, MA, USA) protocol. Tumor DNA was eluted in 80 µL of the given elution buffer and stored at -20°C. Concentration and purity of DNA were determined using NanoDropTM Lite Spectrophotometer (ThermoFisher Scientific, Boston, MA, USA). The median time between the FFPE processing and DNA extraction was 112 days.

2.3. PIK3CA Mutations Analysis

Three mutations in the PIK3CA gene (E545K, H1047R and H1047L) were assessed using QuantStudio 3D Digital PCR System (ThermoFisher Scientific, Boston, MA, USA) and Sanger Sequencing. For digital PCR, 1.5 µL of sample DNA was mixed with 0.75 µL of 20x TaqMan Assay (Table S1), plus 7.5 µL of QuantStudio 3D Master Mix 2X and 5.25 µL of water. The total mixture of 15 µL was then loaded in the QuantStudio 3D Digital PCR 20k Chips by the QuantStudioTM 3D Digital PCR Chip Loader. The Cycling conditions for exon 20 mutations (H1047R and H1047L) were an initial denaturation at 96ºC for 10 minutes followed by 39 cycles of 60ºC for two minutes, 30 seconds at 98°C and a final stage of two minutes at 60°C for extension. While for exon 9 mutation (E545K) were an initial denaturation at 96ºC for 10 minutes followed by 40 cycles of 52ºC for two minutes, 30 seconds at 98°C and a final stage of 10 minutes at 72°C for a final extension [27]. All samples were then left at 22° C for at least 20 minutes. Results were analyzed by QuantStudio 3D Analysis Suite™ Cloud Software (ThermoFisher Scientific, Boston, MA) based on the Poisson plus algorithm (v.4.4.10). The software calculated automatically the thresholds for FAM (mutant alleles) and VIC (wild type alleles) signals. However, to homogenize the results, reduce false positives and avoid observer bias we established a fixed threshold of 6000 relative fluorescence units for FAM on all the samples based on our most representative positive cases (Figure S1). Additionally, a quality threshold of 0.5 was established. Calibration process for dPCR can be found in supplementary material. Digital PCR assays were performed following the digital MIQE guidelines [28] (Figure S2, Table S11).

For Sanger sequencing, we used the ABI PRIMS 3500 (Applied Biosystems™, Foster City, CA, USA). Conventional PCR was carried on for exon 9 (E545K) and for exon 20 (H1047R and H1047L). Primers used and cycling conditions are presented in supplementary material (Table S2, S3 and S4). Before sequencing, samples were purified according to GeneJet PCR Purification Kit (ThermoFisher Scientific, Boston, MA, USA) protocol with the exception that we preheated the elution buffer at 60ºC for at least 10 minutes to increase DNA concentration. DNA was then eluted in 35 µL of the elution buffer supplied. Two independent readers analyzed the sequences and compared them with the reference sequence: NC_0000003.12 (GRCh38).

2.4. Statistical Analysis

To calculate the sensitivity, specificity, predictive positive value (PPV) and predictive negative value (PNV) of dPCR, we compared the 171 essays performed by dPCR against their respective results from Sanger sequencing. Each patient had three essays (one for each mutation) in both dPCR and Sanger sequencing. The analysis was performed with contingency tables from JavaStat (http://statpages.info/ctab2x2.html). Whereas, we used STATA v15.1 for the other statistical analysis. We estimate the Receptor Operator Characteristic (ROC) curve of dPCR against Sanger sequencing and calculate the area under the curve (AUC). The test qualification according the AUC curve might be from non-discriminatory (0.5 or minus) to outstanding (over 0.9) [29]. Additionally, we performed the chi-

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square test and Fisher exact test (when needed) to assess which clinicpathological feature were related to the mutational status of PIK3CA E545K and H1047R. Intra-rater analysis was estimated by Intraclass Correlation Coefficient (ICC) based on a single-rating (k=1), consistency of agreement, 2-way mixed-effects model. For all the analysis, we calculated 95% confident intervals (CI) and considered two-sided P value 0.05 as statistically significant threshold.

3. Results

3.1. Patients Characteristics

Sixty-nine patients accepted to participate in the study by signing the informed consent. However, one patient decided to quit and 11 were excluded because it was not possible to obtain their tumor and biopsy sample. A total of 57 patients were finally eligible for the study. Demographic and clin-ic-pathological characteristics are resume in Table 1. From them, 16 samples were primary tumor (28.1%), 32 (56.1%) residual tumors (had received neoadjuvancy), six were biopsies (10.5%) and three (5.3%) belonged to lymph nodes.

Table 1. Patient Demographic and Clinic-pathological Features (N=57)

Patient Feature	N (%)
Previous Neoadyuvancy	
Yes	38 (66.7)
No	19 (33.3)
Age	
≤ 50	22 (38.6)
>50	35 (61.4)
Stage	
0	1 (1.8)
I	3 (5.3)
II	10 (17.5)
III	37 (64.9)
IV	5 (8.8)
No evaluable	1 (1.8)
Histological Type	
Ductal	46 (80.7)
Lobular	4 (7.0)
Other	7 (12.3)
HER-2 Receptor	
Positive	19 (33.3)
Negative	34 (59.7)
No evaluable	4 (7.0)
Estrogen Receptor	
Positive	37 (64.9)
Negative	19 (33.3)
No evaluable	1 (1.8)
Progesterone Receptor	
Positive	32 (56.1)

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Negative	24 (42.1)
No evaluable	1 (1.8)
Immunophenotype	
Luminal A like	9 (15.8)
Luminal B like	28 (49.1)
HER-2 (+)	10 (17.5)
Basal-like	8 (14.0)
No evaluable	2 (3.5)
Lymph Node	
Positive	40 (70.2)
Negative	17 (29.8)
Systemic Metastasis	
Positive	5 (8.8)
Negative	52 (91.2)
Hormonal Status	
Pre-menopause	13 (22.8)
Post-menopause	44 (77.2)
Region of Birth	
Lima	19 (33.33)
Pacific coast	20 (35.09)
Andes	13 (22.81)
Amazon	5 (8.77)
Ki-67	
≥14	41 (71.93)
<14	10 (17.54)
No evaluable	6 (10.53)

Note: Percentage may not sum 100% due to rounding.

3.2. PIK3CA Mutation Status

Tumor DNA samples were analyzed by dPCR. Twenty-five patients (43.9%) showed to have at least one mutation. Mean copies per partition, total volume of partition and partition number are shown in supplementary material (Table S5). From the three mutations, E545K was the most frequent with 18 cases (31.6%), followed by H1047R 11 cases (19.3%) and H1047L 3 cases (5.3%). Examples of positive and negative cases can be seen in supplementary material (Figure S1). Furthermore, seven patients presented coexistence of two mutations, from which four had H1047R and E545K, and three H1047R and H1047L. On the other hand, regarding H1047R, H1047L and E545K mutations exclusively, Sanger sequencing identified six mutated cases (10.5%) from which four (7%) were E545K and two (3.5%) H1047R. Neither H1047L nor mutation coexistence were identified by Sanger sequencing (Table 2). Dilution and intra-rater assay can be found in the supplementary material (Figure S2, Tables S6-S10).

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Table 2. *PI3KCA* Mutations detected by Digital PCR and Sanger sequencing

Patient	Digital PCR	FAM Copies/µL	Sanger sequencing
01	E545K (0.44%)	2.93	WT
02	H1047R (57.94%)	2364.8	H1047R
	E545K (0.50%)	10.75	WT
03	H1047R (4.34%)	12.2	WT
	E545K (0.50%)	2.31	WT
04	WT	-	WT
05	WT	-	WT
06	WT	-	WT
07	WT	-	WT
08	WT	-	WT
09	WT	-	WT
10	WT	-	WT
11	WT	-	WT
12	H1047R (0.35%)	3.1	WT
	E545K (0.89%)	10.14	WT
13	E545K (0.29%)	0.74	WT
14	E545K (11.66%)	229.0	E545K
15	H1047R (0.24%)	1.6	WT
	E545K (0.44%)	2.13	WT
16	WT	-	WT
17	WT	-	WT
18	E545K (0.28%)	1.39	WT
19	E545K (54.60%)	3263.90	E545K
20	WT	-	WT
21	WT	-	WT
22	H1047R (41.17%)	358.93	H1047R
	H1047L (0.30%)	1.57	WT
23	E545K (0.25%)	0.23	WT
24	E545K (0.30%)	7.01	WT
25	WT	-	WT
26	WT	-	WT
27	H1047R (14.11%)	216.90	WT
28	H1047R (0.24%)	9.67	WT
	H1047L (0.85%)	4.79	WT
29	WT	-	WT
30	WT	-	WT
31	WT	-	WT
32	WT	-	WT
33	H1047R (12.48%)	6.5	WT
34	E545K (0.30%)	0.81	WT
35	WT	-	WT

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36	WT	-	WT
37	H1047R (9.64%)	32.76	WT
38	WT	-	WT
39	WT	-	WT
40	E545K (6.39%)	85.81	WT
41	E545K (2.25%)	15.88	WT
42	WT	-	WT
43	WT	-	WT
44	WT	-	WT
45	WT	-	WT
46	WT	-	WT
47	E545K (34.85%)	1567.30	E545K
48	E545K (10.05%)	53.96	WT
49	WT	-	WT
50	WT	-	WT
51	WT	-	WT
52	E545K (17.66%)	4630.4	E545K
53	H1047R (11.42%)	76.06	WT
54	H1047R (2.67%)	17.61	WT
	H1047L (5.13%)	36.63	WT
55	WT	-	WT
56	WT	-	WT
57	E545K (0.24%)	4.77	WT

WT: Wild type.

3.3. Sensitivity and Specificity Analysis

For sensitivity and specificity analysis, Sanger sequencing was used as gold standard against dPCR. Digital PCR sensitivity showed to be 100% (95 CI 53-100%), while the specificity 84.2% (95% CI 83 - 84.2%), PPV 18.8% (95% CI 9- 18%) and PNV 100% (95% CI 98-100%). Furthermore, ROC analysis (Figure 1) showed an AUC of 0.998 (95% CI 0.978-1.00).

3.4. PIK3CA Mutations and Patient´s Clinic-pathological Features

No statistical significance was found between clinic-pathological features and overall PI3KCA mutational status (Table 3). Additionally, mutational status was assessed individually by mutation (E545K and H1047R) showing a significant association between H1047R and Breast cancer Phenotype (p=0.019) and lymph node infiltration (p=0.046). Further, post hoc analysis through Pearson X^2 test determined the association between H1047R and HER2 breast cancer phenotype (residuals 2.234, p=0.026).

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Table 3. Association among *PIK3CA* mutations´ and Clinic-pathological Features

CLINICAL FEATURES		N=57 (%)	E545K M= 18	*p*	N=57 (%)	H1047R M=11	*p*	N=57 (%)	ANY Mutation M=25	*p*
Age (years)	≤ 50	15 (38.46)	7(38.89)	0.975	17 (36.96)	5 (45.45)	0.603	11(34.38)	11 (44)	0.459
	>50	24 (61.54)	11(61.11)		29 (63.04)	6 (54.55)		21 (65.63)	14 (56)	
Stage	In situ	1 (2.63)	0 (0)	0.823	1 (2.22)	0 (0)	0.638	1 (3.23)	0 (0)	0.677
	I	3 (7.89)	0 (0)		2 (4.44)	1 (9.09)		2 (6.45)	1 (4)	
	II	6 (15.79)	4 (22.22)		7 (15.56)	3 (27.27)		4 (12.9)	6 (24)	
	III	25 (65.79)	12 (66.67)		31 (68.89)	6 (54.55)		22 (70.97)	15 (60)	
	IV	3 (7.89)	2 (11.11)		4 (8.89)	1 (9.09)		2 (6.45)	3 (12)	
Histological type	Ductal	30 (76.92)	16 (88.89)	0.428	39 (84.78)	7 (63.64)	0.124	27 (84.38)	19 (76)	0.695
	Lobular	4 (10.26)	0 (0)		2 (4.35)	2 (18.18)		2 (6.25)	2 (8)	
	Others	5 (12.82)	2 (11.11)		5 (10.87)	2 (18.18)		3 (9.38)	4 (16)	
Region	Lima	11(28.21)	8 (44.44)	0.709	15 (32.61)	4 (36.36)	0.605	10 (31.25)	9 (36)	0.781
	Pacific coast	15 (38.46)	5 (27.78)		17 (36.96)	3 (27.27)		13 (40.63)	7 (28)	
	Andes	9 (23.08)	4 (22.22)		11 (23.91)	2 (18.18)		7 (21.88)	6 (24)	
	Amazon	4 (10.26)	1 (5.56)		3 (6.52)	2 (18.18)		2 (6.25)	3 (12)	
Immunophenotype	Basal-like	5 (13.51)	3 (16.67)	0.622	8 (17.39)	0 (0)	**0.019**	5 (15.63)	3 (13.04)	0.307
	Luminal A	5(13.51)	4 (22.22)		6 (13.04)	3 (33.33)		3 (9.38)	6 (26.09)	
	Luminal B	21 (56.76)	7 (38.89)		26 (56.52)	2 (22.22)		19 (59.38)	9 (39.13)	
	HER-2 +	6 (16.22)	4 (22.22)		6 (13.04)	4 (44.44)*		5 (15.63)	5 (21.74)	
	No eval.	.	.		.	.				
HER-2	Positive	13 (37.14)	6 (33.33)	0.784	14 (31.82)	5 (55.56)	0.255	11 (36.67)	8 (34.78)	0.887
	Negative	22 (62.86)	12 (66.67)		30 (68.18)	4 (44.44)		19 (63.33)	15 (65.22)	

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Estrogen receptor	Positive	26 (68.42)	11 (61.11)	0.589	31 (67.39)	6 (60)	0.720	21 (65.63)	16 (66.67)	0.935
	Negative	12 (31.58)	7 (38.89)		15 (32.61)	4 (40)		11 (34.38)	8 (33.33)	
Progesterone receptor	Positive	24 (63.16)	8 (44.44)	0.186	27 (58.7)	5 (50)	0.615	19 (59.38)	13 (54.17)	0.697
	Negative	14 (36.84)	10 (55.56)		19 (41.4)	5 (50)		13 (40.63)	11 (45.83)	
Lymph nodes infiltration	Positive	27 (69.23)	13 (72.22)	0.819	35 (76.09)	5 (45.45)	**0.046**	24 (75)	16 (64)	0.368
	Negative	12(30.77)	5 (27.78)		11 (23.91)	6 (54.55)		8 (25)	9 (36)	
Ki-67	<14	5 (14.71)	5 (29.41)	0.212	7 (16.67)	3 (33.33)	0.353	3 (10.34)	7 (31.82)	0.079
	>=14	29 (85.29)	12 (70.59)		35 (83.33)	6 (66.67)		26 (89.66)	15 (68.18)	
Systemic metastasis	Positive	3 (7.69)	2 (11.11)	0.646	4 (8.70)	1 (9.09)	1.000	2 (6.25)	3 (12)	0.645
	Negative	36 (92.31)	16 (88.89)		42 (91.30)	10 (90.91)		30 (93.75)	22 (88)	
Menstrual status	Pre-menopause	10 (25.64)	3 (16.67)	0.520	11 (23.91)	2 (18.18)	1.000	8 (25)	5 (20)	0.655
	Post-menopause	29 (74.36)	15 (83.33)		35 (76.09)	9 (81.09)		24 (75)	20 (80)	
Neoadjuvancy	No	14 (35.90)	5 (27.78)	0.546	14 (30.43)	5 (45.45)	0.342	10(31.25)	9 (36)	0.706
	Yes	25 (64.1)	13 (72.22)		32 (69.57)	6 (54.55)		22 (68.75)	16 (64)	

The analysis was performed with the Chi-square or Fisher's exact test. The numbers highlighted in bold indicate significant differences ($p < 0.05$).

M: mutations. * Significantly associated under X^2 residual evaluation.

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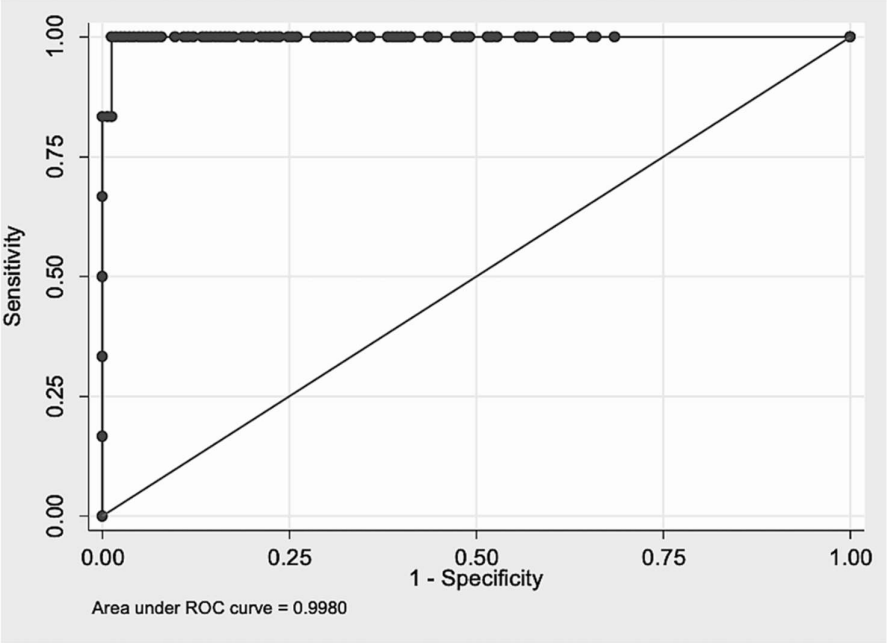

Figure 1. Receiver operator characteristics (ROC) curve for PIK3CA mutations detected by dPCR.

4. Discussion

Digital PCR is a relatively new technique that enable mutation analysis of low rate target molecules due to its partition properties and Poisson analysis [20]. In this study we compared the QuantStudio 3D digital PCR system against Sanger sequencing, as gold standard, to assess its sensitivity, specificity and overall performance in the detection of three PIK3CA mutations in breast cancer tumors. In our study, dPCR revealed a prevalence of 43.9% of three PIK3CA mutations exclusively (E545K, H1047R and H1047L). Even though, our results are concordant with the one reported by Beaver et al. in a similar platform (ddPCR) [30], it differs in almost 8% with the overall PI3KCA mutations prevalence in breast cancer estimated by Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) studies reported in TGCA database (36%) [1]. However, this incon-sistency can be explained by the difference on sensitivity between dPCR and TGCA techniques. Furthermore, Sanger sequencing identified only 10.5% of mutated tumors, which is low compared with dPCR, but similar to previous studies taking in consideration only E545K, H1047R and H1047L mutations [31,32].

In terms of performance, sensitivity and specificity, dPCR showed excellent results as compared to Sanger sequencing. Nevertheless, dPCR sensitivity had a broad CI which range from 53 to 100% and a low PPV. These might be explained by the 19 cases that Sanger sequencing was not able to detect as previous studies have reported a detection limit of 15-30% for Sanger sequencing [15,16]. In our study, Sanger sequencing was able to detect until a mutation rate of 11.66% according to dPCR. However, it draws attention cases 34 and 42 which were not detected by Sanger sequencing in spite of a mutation rate for H1047R of 14.11% and 12.48% respectively by dPCR. This issue can be due to the number of copies per µL in the sample as case 18, with a mutation rate of 11.66% for E545K, showed to have 229 mutant copies/µL, compared to 216 and 6.5 copies/µL from patient 34 and 42 respectively; suggesting the importance of not only the proportion of mutant alleles over the wild types but also the overall number of copies within the sample for Sanger sequencing analysis.

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Individually, E545K was the most frequent mutation, followed by H1047R and H1047L in both techniques which contrast with previous data, where H1047R mutation represent approximately 50% of *PIK3CA* total mutation rate and E545K up to 20% [33]. Nevertheless, it is important to note that most of E545K cases detected by dPCR have a mutation rate lower than 1%, which might have been underestimated in previous studies [8,31,32,34]. Additionally, dPCR detected seven patients with mutation coexistence, not identified by Sanger sequencing. This phenomenon has been reported previously [30,35,36] and may reflects the intra-heterogeneous characteristics from breast cancer tumors and has been suggested to have an important role in tumor progression and treatment [37]. Further, it is suggested that therapy failures and metastasis may be due to the outgrowth of resistant sub-clones present before the beginning of the treatment and underestimated by standard techniques as shown by recent studies [38,39]. Because of this, resembles the importance on developing more sensitive techniques dPCR capable to detect potential resistant sub-clones and guaranty a personalized medicine.

Furthermore, we found a significant association of HER2 phenotype and lymph nodes infiltration with H1407R mutation. Event though, previous studies haven't reported a correlation or association between this mutation and HER2 phenotype, its presence is associated with a worse overall survival in breast cancer patients and therapy resistance [40,41]. Besides, animal studies have shown an acceleration on tumor progression as well as an enhancement on metastasis potential in mice with tumors expressing both H1047R mutations and HER2 overexpression [42,43] suggesting a synergic effect. This same way may also explain the association with lymph node infiltration. However, this analysis was mainly exploratory and may lack of enough power. Further considerations should be taken into account in future studies.

5. Conclusions

Our study has shown a good performance of dPCR, compared with Sanger sequencing, in the detection of three *PIK3CA* mutations with high sensitivity and specificity values. Besides, due to the detection of low rate mutations and mutation co-existence that Sanger sequencing could not detect, dPCR has the potential to become an important tool for gene analysis and personalized medicine. Thus, future studies with larger populations should be performed to confirm and extent our results.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Examples of positive and negative samples for each analyzed mutations, Figure S2: Three examples from the dilution assays´ samples, Table S1: Catalog number of QuantStudio TM 3D Digital PCR Products, Table S2: Primers used for conventional PCR. Table S3: Cycling conditions PIK3CA primers exon 20, Table S4: Cycling conditions PIK3CA primers exon 09, Table S5: Mean λ, partition volume, total partition number and total volume of part, Table S6: PIK3CA H1047R mutations dilution assay by dPCR, Table S7: PIK3CA H1047L mutations dilution assay by dPCR, Table S8: PIK3CA E545K mutations dilution assay by dPCR, Table S9: PIK3CA mutations dilution assay by dPCR, Table S10: Inter-rater assay results from mutant allele frequency, Table S11: digital MIQE checklist for authors, reviewers and editors.

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