Chip-based digital Polymerase Chain Reaction as quantitative technique for the detection of PIK3CA mutations in breast cancer patients

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ARTICLE INFO

Keywords:
Breast neoplasms
PIK3CA
Genetic techniques
Polymerase chain reaction
Mutation
Digital PCR

ABSTRACT

Background: PIK3CA is a gene frequently mutated in breast cancer. With the FDA approval of alpelisib, the evaluation of PIK3CA for activating mutations is becoming routinely. Novel platforms for gene analysis as digital PCR (dPCR) are emerging as a potential replacement for the traditional Sanger sequencing. However, there are still few studies on chip-based dPCR to detect mutations in tumor samples. Thus, this cross-sectional study aimed to assess the sensibility of a chip-based dPCR to detect and quantify PIK3CA mutations and compare its performance with Sanger sequencing.

Materials and Methods: Tumor samples from 57 breast cancer patients (22 pre-treatment samples, 32 tumors after neoadjuvant chemotherapy, and three lymph nodes) were collected and analyzed by Sanger sequencing and dPCR for the three PIK3CA most relevant mutations (p.E545K, p. H1047R, and p. H1047L). Digital PCR sensitivity, specificity, and overall performance were estimated by contingency tables, receptor operator characteristic (ROC), and area under the curve (AUC). Association of PIK3CA mutations with clinicopathological variables was conducted.

Results: Sanger sequencing identified PIK3CA mutations in six patients (10.5%), two with p. H1047R, and four with p. 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, p. H1047R mutation detected by dPCR showed a significant association with breast cancer phenotype (p = 0.019) and lymphatic nodes infiltration (p = 0.046).

Conclusions: 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.

1. Introduction

The PIK3CA gene located in chromosome 3q26.3 and encodes the p110α isoform, part of the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway [1], which converts phosphatidylinositol 4-5 biphosphate (PIP2) to phosphatidylinositol 3-4-5 triphosphate (PIP3). Consequently, it activates multiple downstream signaling pathways involved in cellular growth, motility, apoptosis, and differentiation [2]. In contrast, this activity is regulated by the phosphatase 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 [3, 4].

Since its discovery in 2004 [5], researchers have had special attention to PIK3CA mutations as they are considered to have a potential use as prognostic and predictive factors. PIK3CA mutations are common in breast cancer as described by The Cancer Genetic Atlas (TCGA) with a frequency of 36% [6]. Although this gene has 23 exons, pathological mutations are clustered in two hot spots in exon 9 (p.E542K and p. E545K) and exon 20 (p.H1047R and p. H1047L) [5]. According to the TCGA breast cancer data in the eBioPortal server, p. E545K (40%) and

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https://doi.org/10.1016/j.heliyon.2022.e11396
Received 29 March 2022; Received in revised form 15 September 2022; Accepted 31 October 2022
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p. H1047R (20%) are the most frequent somatic mutations in the PIK3CA gene of breast tumors [6].

Several authors have reported that these PIK3CA mutations confer an improved prognosis [7, 8, 9]. Nonetheless, this data is still controversial as other authors have suggested that these PIK3CA mutations are on the contrary associated with a negative prognosis [4, 10, 11, 12, 13] and current therapy resistance (hormone and anti-HER2 therapy) [14, 15, 16, 17, 18]. Despite that, with the FDA approval of alpelisib in 2019 after the phase-III trial SOLAR-1 [19], evaluation of PIK3CA is becoming routine. This evaluation is traditionally performed with Sanger sequencing [20, 21, 22] or, more recently, using Next Generation Sequencing [6]. However, Sanger sequencing is known to have low sensitivity and NGS applications could be expensive for analyzing a unique gene.

Nevertheless, new technologies have emerged promising better performance, enabling a quantitative and more sensitive gene analysis. One of these technologies is the "digital Polymerase Chain Reaction" (dPCR), which performs an absolute quantification of specific nucleic acid sequences by first dividing a sample into numerous partitions into chambers or droplets (depending on the platform), individually containing at least one target molecule. Then, it performs simultaneous endpoint qPCRs and counts the partitions in a binary style (0 negative and 1 positive) where a fluorescence signal has been produced with a posterior Poisson analysis [23, 24, 25]. In contrast with other PCR techniques such as qPCR, dPCR does not require standard curves calibration. Also, it enhances the effective concentration of the objective nucleic acids and decreases the background effect of abundant molecules over rare or low frequent targets [26]. Therefore, it is capable to detect theoretically up to 0.02% mutant allele in an overwhelming background of the normal allele [27]. Consequently, this technique has a great potential in individualized medicine as well as in cancer research to detect mutant cells at very low frequency [28].

Several studies have already used dPCR technology for mutation analysis with promising results [29, 30, 31, 32]. Most of them have commonly used Droplet Digital PCR (ddPCR), which is a water-oil droplet partition-based platform that fractionates the sample in several droplet reactions [32]. More recently, a novel chip-based platform has been introduced, Quantstudio™ 3D Digital PCR chip system, which offers a highly precise and sensitivity absolute quantification. The chip offers a similar number of partitions that ddPCR but with the same size, a simpler workflow [25], and a relatively low cost. However, there are still few studies using this new technique to detect mutations in tumor samples. Therefore, we conducted a cross-sectional study to determine the sensitivity and specificity of the Quantstudio 3D Digital PCR chip system to detect PIK3CA mutations in breast cancer patients, in comparison with Sanger sequencing, as well as an analysis between patient clinicopathological features and PIK3CA mutational status detected by dPCR.

2. Materials and methods

2.1. Study population

Tumor samples were obtained from breast cancer patients diagnosed at the Instituto Nacional de Enfermedades Neoplasicas (INEN) in Lima-Peru and analyzed in a core Laboratory (Centro de Genética y Biología Molecular) at Universidad de San Martín de Porres. Samples consisted of 22 tumors pre-treatment, 32 tumors after neoadjuvant chemotherapy, and three lymph nodes. The inclusion criteria were based on patient characteristics. We included every patient hospitalized in the breast service who was older than 18 years old and had their tumor sample available in the pathology department. Information regarding demographic and clinicopathological characteristics was obtained from clinical records. Breast cancer phenotypes were classified based on the St. Gallen International Expert Consensus from 2011 [33].

2.2. Ethical considerations

Patients were enrolled prospectively in the study between April and August of 2017 after signing the proper informed 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 (Protocol INEN 17–27).

2.3. Sample processing and DNA extraction

An experienced pathologist reviewed hematoxylin and eosin-stained slides from tumor/biopsy FFPE blocks to confirm and delimitate the area with at least 80% of neoplastic cells. These delimited 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. DNA Concentration and purity were determined using NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific, Boston, MA, USA) (Table S1). The median time between the FFPE processing and DNA extraction was 112 days (range: 78–637 days).

2.4. PIK3CA mutations analysis

Three mutations in the PIK3CA gene (p.E545K, p. H1047R, and p. H1047L) were assessed using the QuantStudio 3D Digital PCR System (ThermoFisher Scientific, Boston, MA, USA) (Catalog number in Table S2) and Sanger Sequencing. For digital PCR, 1.5 μl of sample DNA was mixed with 0.75 μl of 20x TaqMan Assay, 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 Quantstudio™ 3D Digital PCR Chip Loader (ThermoFisher Scientific, Boston, MA, USA). The Cycling conditions for exon 20 mutations (p.H1047R and p. H1047L) were an initial denaturation at 96 °C for 10 min followed by 39 cycles of 60 °C for 2 min, 30 s at 98 °C and a final stage of 2 min at 60 °C for the extension. While for exon 9 mutation (p.E545K) were an initial denaturation at 96 °C for 10 min followed by 40 cycles of 52 °C for 2 min, 30 s at 98 °C and a final stage of 10 min at 72 °C for a final extension [34]. All samples were then set at 22 °C for at least 20 min. 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 automatically calculated 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 (RFU) for the FAM channel on all the samples based on our most representative positive cases for p. H1047R (Figure 1A), p. E545K (Figure 1C), p. H1047L (Figure 1E), and their respective negative cases (Figure 1B, D and F). Additionally, a quality threshold of 0.5 was established. Digital PCR assays were performed following the digital MIQE guidelines (Table S3) [35].

2.5. Calibration process for dPCR mutation detection

To reduce potential technically based false positives, we evaluated the minimal mutation burden detected by dPCR. The Total mutant allele frequency (TMAF) is the proportion of mutant alleles over the sum of wild and mutant alleles. We considered a patient to be positive for a mutation when she had at least one blue dot (FAM channel) and a percentage of TMAF higher than 0.23%. This cutoff was established by a dilution assay, where we mixed a control positive case (per mutation) at different concentrations (1%, 0.1% and 0.01%) with DNA from a healthy control. Then Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated based on the standard deviation of the response and the slope [5]. The standard error of the y-intercepts from the regression line was used as standard deviation for LOD and LOQ calculation.
2.6. Sanger Sequencing

For Sanger sequencing, we used the ABI PRIMS 3500 (Applied Biosystems™, Foster City, CA, USA). Conventional PCR was carried out for exon 9 (p.E545K) and exon 20 (p.H1047R and p. H1047L). Primers used are presented in Table S4 while cycling conditions for exon 9 in Table S5 and exon 20 in Table S6. 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 60 °C for at least 10 min 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).

2.7. Statistical analysis

To calculate the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) 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 Receiver Operator Characteristic (ROC) curve of dPCR against Sanger sequencing and calculate the area under the curve (AUC). Test qualification according to the AUC can be from non-discriminatory (0.5 or below) to outstanding (more than 0.9) [36]. In addition, we performed the chi-square test and Fisher’s exact test, when necessary, to assess which clinicopathological feature was related to PIK3CA p. E545K and p. H1047R mutational status. The residuals from the chi-square analysis were used as post-hoc to determine the direction of the association among levels. The 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 analyses, we calculated 95% confidence intervals (CI) and considered two-sided P-value 0.05 as the statistically significant threshold.

3. Results

3.1. Patients characteristics

Overall, 57 eligible patients were included in the study. Demographic and clinicopathological characteristics are presented in Table 1.

3.2. PIK3CA mutation status

We analyzed 22 pre-treatment samples (28.1%), 32 (56.1%) residual tumors (after neoadjuvant chemotherapy), and three (5.3%) lymph nodes infiltrating tumors. Mean DNA concentration 32.31 ng/μL ± 29.07 and purity 1.85 ± 0.55. Tumor DNA samples were analyzed by dPCR and Sanger sequencing. By dPCR, 25 patients (43.9%) showed to have at least one mutation. Mean copies per partition, the total volume of partition, and partition number are shown in Table 2. From the three evaluated mutations, p. E545K was the most frequent with 18 cases (31.6%), followed by p. H1047R in 11 cases (19.3%), and p. H1047L in 3 cases (5.3%). Examples of positive and negative cases can be seen in Figure 1. Furthermore, seven patients presented the coexistence of two mutations, from which four had p. H1047R and p. E545K, and three p. H1047R and p. H1047L. On the other hand, regarding p. H1047R, p. H1047L and p. E545K mutations exclusively, Sanger sequencing identified six mutated cases (10.5%) from which four (7%) was p. E545K (Figure 2A) and two (3.5%) p. H1047R (Figure 2B). Neither p. H1047L nor mutation coexistence was identified by Sanger sequencing (Table S7).

3.3. Dilution and intra-rater assay

In order to define the lowest mutant frequency to be detected by QuantStudio 3D digital PCR system, we performed a dilution assay. One
Table 1. Patient demographic and clinicopathological features (N = 57).

| Patient Feature          | N (%) |
|--------------------------|-------|
| Age                      |       |
| ≤50                      | 22 (38,6) |
| >50                      | 35 (61,4) |
| Stage                    |       |
| 0                        | 1 (1,8) |
| I                        | 3 (5,3) |
| II                       | 16 (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) |
| 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.

Table 2. dPCR quantification characteristics.

| Template | Mutation   | Mean copies per partition (nL median) | Partition volume (nL) | Total partition number (median) | Total volume of partitions measure μL (median) |
|----------|------------|---------------------------------------|-----------------------|---------------------------------|-----------------------------------------------|
| Tumor DNA | p.H1047R   | 0,039                                 | 0,755                 | 17084                           | 12,9                                          |
|          | p.E545K    | 0,05                                  |                       | 17065                           | 12,88                                         |
|          | p.H1047L   | 0,045                                 |                       | 17080                           | 12,9                                          |

Discussion

Digital PCR is a relatively new technique that enables mutation analysis of low-rate target molecules due to its partition properties and Poisson analysis [26]. In this study we compared the QuantStudio 3D Digital PCR chip system against Sanger sequencing, as the 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 (p.E545K, p. H1047R, and p. H1047L). Although our results are concordant with the overall PIK3CA mutations prevalence in breast cancer estimated by Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) studies reported in TGCA database (36%) [6]. However, this inconsistency can be explained by the difference in sensitivity between dPCR and TCGA techniques. Furthermore, Sanger sequencing identified only 10.5% of mutated tumors, which is low compared with dPCR, but similar to previous studies taking into consideration only p. E545K, p. H1047R, and p. H1047L mutations [37, 38].

In terms of performance, sensitivity and specificity, dPCR showed excellent results as compared to Sanger sequencing. Nevertheless, dPCR sensitivity had a broad CI which ranges from 53 to 100% and a low PPV. This issue can be due to the number of copies per μL in the sample as case 14, with a mutation rate of...
11.66% for p. E545K, showed to have 229 mutant copies/μL, compared to 216 and 6.5 copies/μL from patient 27 and 33 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.

Individually, p. E545K was the most frequent mutation, followed by p. H1047R and p. H1047L in both techniques which contrast with previous data, where p. H1047R mutation represents approximately 50% of PIK3CA total mutation rate and p. E545K up to 20% [40]. Nevertheless, it is important to note that most of the p. E545K cases detected by dPCR have a mutation rate lower than 1%, which might have been underestimated in previous studies [7, 37, 38, 41]. Additionally, dPCR detected seven patients with mutation coexistence, which were not identified by Sanger sequencing. Mutation coexistence has been reported previously [27, 42, 43] and may reflect breast cancer intra-tumoral heterogeneity, which has been suggested to have an important role in tumor progression and treatment [44]. Furthermore, it is suggested that therapeutic failures and metastases may be due to the outgrowth of the resistant sub-clones present before the beginning of the treatment and underestimated by standard techniques as shown by recent studies [45, 46]. For this reason, it is important to develop more sensitive techniques, such as dPCR, capable to detect potential resistant sub-clones and guarantee the practice of individualized medicine.

According to previous reports, tumors produce several neoplastic lineages with different mutational profiles [47, 48]. Furthermore, the tumor microenvironment becomes a scenario for the conservation of the most resistant variants, which can be observed clearly in a single-cell approach [49]. However, an economically viable and rapid test is needed to decide on new therapeutic options when previous treatments have failed. In this way, Sanger sequencing is usually responsible for some decisions to change cancer treatment protocols despite the lack of sensitivity being their most known limitation [50]. In this study, we evaluated the sensitivity and specificity capabilities of dPCR (Tables S8 to S11). Herein, we determined that dPCR can reliably detect until 0.1% diluted mutation. In the breast cancer samples of this study, the minimum percentage of mutation detected by dPCR was equal to 0.24% (Table S7, patient 15), confirming that dPCR can detect mutations present in samples with more sensitivity than Sanger sequencing.

In a clinical and biological approach, the application of dPCR to evaluate the PIK3CA gene would allow early detection of some resistant tumor lineages in a patient, allowing changing their clinical protocol. On the other hand, mutations with low concentrations could be taken up by other tumor populations in the same patient, reducing the possibility of treatment resistance [51]. Unfortunately, one of the limitations of our study is related to the lack of follow-up information on these patients. Consequently, we do not have enough data to define a reasonable threshold between the two scenarios mentioned above. We believe that our findings will stimulate further research on dPCR for approximating their features to clinical routine.

Moreover, we found a significant association of p. H1047R mutation with HER2 phenotype and lymph nodes infiltration. Even though previous studies have not reported a correlation or association between this mutation and HER2 phenotype, its presence is associated with an overall worse survival in breast cancer patients and therapy resistance [52, 53]. Besides, animal studies have shown an acceleration on tumor progression as well as an enhancement on metastasis potential in mice with tumors expressing both p. H1047R mutations and HER2 over-expression [18, 54] suggesting a synergic effect. This may also explain the association with lymph node infiltration. However, as this analysis was exploratory other studies should be performed to sustain and expand these results.

Finally, with the FDA approval of alpelisib in 2019 after the phase-III trial SOLAR-1 [19], evaluation of PIK3CA is becoming a routine; consequently, dPCR might be a potential technique to identify patients that could be benefited with alpelisib. Hence, future studies with larger

![Figure 2. PIK3CA mutation profile detected by Digital PCR and Sanger sequencing for p. E545K (A) and p. H1047R (B) variants. For each relevant mutation, we compared the frequency of the minor allele (obtained by dPCR) with the classification (altered or unaltered) supported by Sanger sequencing. For both mutations, samples with low-rate mutations were described as unaltered by Sanger sequencing.](image)

![Figure 3. Receiver operator characteristics (ROC) curve for PIK3CA mutations detected by dPCR. Digital PCR sensitivity showed to be 100%, while the specificity was 84.2%. Furthermore, ROC analysis showed an AUC of 0.998.](image)
samples should be performed to validate and standardize this technique for clinical purposes.

5. Conclusions

Our study has shown a good performance of chip-based dPCR, compared with Sanger sequencing, in the detection of three PIK3CA mutations with high sensitivity and specificity values. Moreover, due to the detection of low-rate mutations and mutation coexistence 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 extend our results.

Declarations

**Author contribution statement**

Stefano Giannoni-Luza: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Oscar Acosta: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Alexis Murillo Carrasco, Pierina Danos: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Jose Cotrina Concha, Henry Guerra Miller, Alfredo Aguilar, Ricardo Fujita: Contributed reagents, materials, analysis tools or data; Wrote the paper.
Joseph A. Pinto, Jhajaira M. Araujo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
José Buleje: Conceived and designed the experiments; Wrote the paper.

Funding statement
Dr José Buleje was supported by Universidad de San Martín de Porres (E10012016038), Programa Nacional de Innovación para la Competitividad y Productividad - Innovate Perú (138-PNIP-PIAP-2015) and Oncosadul - AUNA.

Data availability statement
Data included in article/ supp. material/referenced in article.

Declaration of interest's statement
The authors declare no conflict of interest.

Additional information
Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e11396.

Acknowledgements
The authors are grateful to the patients for participating in this study.

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