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ORIGINAL ARTICLE

Comparison of Three Real-Time PCR Assays for the Detection of *PIK3CA* Somatic Mutations in Formalin-Fixed Paraffin Embedded Tissues of Patients with Breast Carcinomas

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Abstract
Breast cancer is the leading cause of cancer-related death in women worldwide. Mutations of the *PIK3CA* gene are found in approximately 25% of breast carcinomas and are reported as activators of the PI3K/AKT/mTOR pathway. This study aims to compare three assays for the somatic mutation detection of *PIK3CA* gene in FFPE tissues of patients with breast cancer. We compared Cobas® *PIK3CA* Mutation Test (Roche Diagnostics, Meylan, France), PCR amplification-refractory mutation system Scorpions® (ARMS) and High-Resolution Melting PCR assay (HRM) for the detection of *PIK3CA* mutations. Discrepant samples were assessed using Next Generation Sequencing (NGS). 46 FFPE breast carcinomas samples of patients treated for breast cancer have been assessed for *PIK3CA* mutations using the three PCR assays. Among the 46 samples, 17 (37.8%), 13 (28.36%) and 19 (41.3%) had a *PIK3CA* mutation, with Cobas®, ARMS and HRM assays respectively. Three different mutations of *PIK3CA* have been detected for one sample. Calculated kappa were 0.95[0.86;1] between Cobas® and HRM, 0.75[0.55;0.95] between Cobas® and ARMS and 0.72[0.51;0.92] between HRM and ARMS. Five samples were found with discrepant results. Our study shows that the Cobas® assay is suitable for *PIK3CA* mutation assessment in patients with breast cancer. HRM assay is also suitable for *PIK3CA* mutation assessment but requires a mutation characterization with a specific assay.

Keywords PIK3CA · Real-time PCR · Breast cancer · PI3K · ARMS · HRM

Introduction

Breast cancer is the most frequent cancer in women worldwide and the leading cause of cancer-related death [1, 2].

Approximately 25% of breast carcinomas carry a *PIK3CA* gene mutation. The *PIK3CA* gene encodes the p110α catalytic subunit of Phosphatidylinositol 3-kinases (PI3K) involved in the PI3K/AKT/mTOR pathway. Mutations occurring on exons 10 and 21 of *PIK3CA* gene can lead to dysregulation or activation of this signaling pathway and also resistance to anti-HER2 therapies. The genotyping of *PIK3CA* has also recently been proven of interest for the prescription of anti-mTOR and/or anti-PI3K therapies in patients with breast carcinomas [3]. Reduced pathological complete responses rates in primary Her2-positive breast cancer were associated with the presence of *PIK3CA* mutations [4] and patients with wild-type *PIK3CA* tumor were of better prognosis in the CLEOPATRA phase III trial [5].

Four hotspot mutations with an oncogenic role and representing more than 90% of the *PIK3CA* variants described in the COSMIC database in patients with breast carcinomas (Catalogue Of Somatic Mutations in Cancer Database, Wellcome Trust Genome Campus, Hinxton, Cambridge; accessed June 2017; http://www.sanger.ac.uk) have been identified: c.1624G > A p.(Glu542Lys), c.1633G > A p.(Glu545Lys) and c.3140A > G p.(His1047Arg), c.3140A > T p.(His1047Leu) [6].

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The detection of *PIK3CA* gene mutations in formalin-fixed paraffin-embedded (FFPE) tumor tissues [3, 7, 8] require robust and sensitive assays. Several methods have been described or are commercially available for the detection of *PIK3CA* mutations [9].

The aim of this study was to compare three different assays for the detection of *PIK3CA* mutations in FFPE tumor samples (Cobas® *PIK3CA* Mutation Test (Roche Diagnostics, Meylan, France), PCR amplification-refractory mutation system Scorpions® (ARMS) and High-Resolution Melting PCR assay (HRM). We finally analyzed samples with discrepant results using Next Generation Sequencing (NGS).

Materials & Methods

Patients and Samples

Forty-six FFPE breast carcinomas samples of patients treated for a breast cancer at the Institut de Cancérologie de Lorraine have been retrospectively collected for this study. All patients gave their informed consent to Institut de Cancérologie de Lorraine for the research of somatic mutations and the use of their biological sample for research purposes. The study has been approved by Institut de Cancérologie de Lorraine scientific board. All methods were performed in accordance with the relevant guidelines and regulations. All patients' data was anonym and de-identified prior to analysis.

All 46 samples were assessed using two allele specific assays (Cobas® *PIK3CA* Mutation Test and PCR ARMS Scorpions®) and high-resolution melting assay (HRM) which is a non-specific real-time PCR assay. Massive bi-parallel pyrosequencing (GS Junior, Roche Diagnostics) was used for the analysis of samples presenting discrepancies with the 3 PCR assays.

DNA Isolation

FFPE tumor specimens were macro-dissected after hematoxylin-eosin slide (HES) analysis by a senior pathologist to evaluate the tumor purity [10]. After macrodissection, paraffin shavings containing the selected area were then collected for genomic DNA isolation (Cobas® DNA Sample preparation kit, Roche Diagnostics). NanoVue Plus™ spectrophotometer (GE Healthcare Life Sciences, Velizy-Villacoublay, France) was used for DNA quantification.

PIK3CA Mutation Detection

All samples have been analyzed using the 3 assays.

Cobas®

Mutations that can be detected by the Cobas® assay are described in Table 1. Twenty nanograms of DNA were amplified in a final volume of 96 µl. The real-time PCR Cobas® *PIK3CA* Mutation Test kit (Roche Diagnostics) uses a pool of primers allowing (target size from 85 to 155 bp), divided into three different mixes for each samples and controls. Assay was finally run using Cobas z480 thermocycler (Roche Diagnostics) according to the manufacturer, the Cobas® assay has a > 95% hit rate for variants with an allele frequency greater than 5%.

Amplification-Refractory Mutation System (ARMS)

ARMS assay use sequence-specific PCR primers that allow the detection of the four most common hotspot mutations of *PIK3CA* (Table 1). ARMS analysis has been assessed as previously described [6, 11] with LightCycler 480 Real-Time PCR system (Roche Diagnostics) in 384-well plates. Briefly, 80 ng of DNA were amplified in a final volume of 20 µl. LightCycler SW v. 1.5.0.39 software was finally used for data analysis. We described that this assay is able to detect variants with a 0.5% allele frequency [6].

High Resolution Melting

HRM PCR is a non-specific real-time PCR assay able to detect all mutations located on exons 10 and 21 of *PIK3CA*. As previously described LightCycler 480 HRM Master kit

Table 1 Detectable *PIK3CA* Mutations by assay

Exon	Mutation	ARMS	HRM	Cobas®
2	c.263G > A p.(Arg88Gln)			X
5	c.1035 T > A p.(Asn345Lys)			X
8	c.1258 T > C p.(Cys420Arg)			X
10	c.1624G > A p.(Glu542Lys)	X	X	X
	c.1634A > C p.(Glu545Ala)		X	X
	c.1635G > T p.(Glu545Asp)	X	X	X
	c.1634A > G p.(Glu545Gly)		X	X
	c.1633G > A p.(Glu545Lys)	X	X	X
	c.1636C > G p.(Gln546Glu)		X	X
	c.1636C > A p.(Gln546Lys)		X	X
	c.1637A > T p.(Gln546Leu)		X	X
	c.1637A > G p.(Gln546Arg)		X	X
21	c.3129G > T p.(Met1043Ile)		X	X
	c.3140A > T p.(His1047Leu)	X	X	X
	c.3140A > G p.(His1047Arg)	X	X	X
	c.3139C > T p.(His1047Tyr)		X	X
	c.3145G > C p.(Gly1049Arg)		X	X

Table 2 Samples characteristics

	Sample DNA	HRM		ARMS		Cobas	
Sample #	concentration (ng/μl)	Test result	Mutation result	Test result	Mutation result	Test result	Mutation result
1	65	Mutation detected	Exon 21	Mutation detected	p.(His1047Arg)	Mutation detected	p.(His1047?)
2	49.5	Mutation detected	Exon 10, Exon 21	Mutation detected	p.(Glu542Lys)	Mutation detected	p.(Glu542Lys), p.(His1047?), p.(His1049Arg)
3	210.5	Mutation detected	Exon 21	Mutation not detected		Mutation detected	p.(His1047?)
4	125.25	Mutation not detected		Mutation not detected		Mutation not detected	
5	126.25	Mutation not detected		Mutation not detected		Mutation not detected	
6	159	Mutation detected	Exon 21	Mutation detected	p.(His1047Leu)	Mutation detected	p.(His1047?)
7	74.5	Mutation detected	Exon 10	Mutation detected	p.(Gly545Lys)	Mutation detected	p.(Glu545?)
8	369.75	Mutation not detected		Mutation not detected		Mutation not detected	
9	192	Mutation not detected		Mutation not detected		Mutation not detected	
10	153	Mutation not detected		Mutation not detected		Mutation not detected	
11	99.5	Mutation not detected		Mutation not detected		Mutation not detected	
12	208	Mutation not detected		Mutation not detected		Mutation not detected	
13	224.75	Mutation detected	Exon 21	Mutation detected	p.(His1047Arg)	Mutation detected	p.(His1047?)
14	138	Mutation detected	Exon 21	Mutation detected	p.(His1047Leu)	Mutation detected	p.(His1047?)
15	446.75	Mutation not detected		Mutation not detected		Mutation not detected	
16	136.5	Mutation not detected		Mutation not detected		Mutation not detected	
17	123.75	Mutation detected	Exon 10	Mutation not detected		Mutation detected	p.(Gln546?)
18	33	Mutation detected	Exon 10	Mutation not detected		Mutation not detected	
19	28.25	Mutation detected	Exon 10	Mutation not detected		Mutation detected	p.(Glu542K)
20	86.25	Mutation detected	Exon 10	Mutation detected	p.(Glu545Lys)	Mutation detected	p.(Glu545?)
21	94.25	Mutation not detected		Mutation not detected		Mutation not detected	
22	428.25	Mutation not detected		Mutation not detected		Mutation not detected	
23	107.25	Mutation not detected		Mutation not detected		Mutation not detected	
24	160.25	Mutation not detected		Mutation not detected		Mutation not detected	
25	53.25	Mutation not detected		Mutation not detected		Mutation not detected	
26	217.25	Mutation detected	Exon 10	Mutation not detected		Mutation detected	p.(Glu545?)
27	148.25	Mutation not detected		Mutation not detected		Mutation not detected	
28	149.25	Mutation not detected		Mutation not detected		Mutation not detected	
29	254.75	Mutation detected	Exon 21	Mutation detected	p.(His1047Leu)	Mutation detected	p.(His1047?)
30	163.25	Mutation detected	Exon 10	Mutation detected	p.(Glu542Lys)	Mutation detected	p.(Glu542Lys)
31	128	Mutation not detected		Mutation not detected		Mutation not detected	
32	114	Mutation not detected		Mutation not detected		Mutation not detected	
33	492	Mutation not detected		Mutation not detected		Mutation not detected	

Table 2 (continued)

	Sample DNA	HRM		ARMS		Cobas		
34	143.75	Mutation not detected		Mutation not detected		Mutation not detected		
35	297.5	Mutation detected	Exon 10	Mutation detected	p.(Glu545Lys)	Invalid	Invalid	
36	120.5	Mutation not detected		Mutation not detected		Mutation not detected		
37	49.75	Mutation detected	Exon 21	Mutation not detected		Mutation detected	p.(His1047?)	
38	80.5	Mutation not detected		Mutation not detected		Mutation not detected		
39	273.75	Mutation not detected		Mutation not detected		Mutation not detected		
40	133.25	Mutation not detected		Mutation not detected		Mutation not detected		
41	105.5	Mutation detected	Exon 10	Mutation detected	p.(Glu542Lys)	Mutation detected	p.(Glu542Lys)	
42	235.25	Mutation not detected		Mutation not detected		Mutation not detected		
43	105.25	Mutation not detected		Mutation not detected		Mutation not detected		
44	138	Mutation not detected		Mutation not detected		Mutation not detected		
45	114	Mutation detected	Exon 21	Mutation detected	p.(His1047Arg)	Mutation detected	p.(His1047?)	
46	139.25	Mutation detected	Exon 10	Mutation detected	p.(Glu542Lys)	Mutation detected	p.(Glu542Lys)	

?: unknown amino acid (Cobas assay is not specific for some mutations)

(Roche Diagnostics, Meylan, France), 384 well plates (Roche Diagnostics) and LightCycler 480 thermocycler (Roche Diagnostics) were used for HRM analysis [6, 12, 13]. Briefly, for each sample, 40 ng of DNA were added to Master Mix (Roche Diagnostics), MgCl2 and specific primers for a final volume of 20 μL. Data were finally analyzed using LightCycler SW v. 1.5.0.39 software (Roche Diagnostics). This assay is able to detect variants with 5% and 10% allele frequencies for exon 10 and 21 respectively.

NGS

Exons 10 and 21 of *PIK3CA* were analyzed using ultra-deep pyrosequencing (Roche Diagnostics). A DNA input of 50 nanograms was used for PCR amplification (High Fidelity PCR System, Roche Diagnostics, Meylan, France) with specific primers (exon 10:

Forward 5'-AATCATCTGTGAATCCAGAGGGG-3′; Reverse 5'-AGGTATGGTAAAAACATGCTGAGA-3′ and exon 21: Forward 5'-TTTGCTCCAAACTGACCAAA CTG-3′; Reverse 5'-TGGAATCCAGAGTGAGCTTTCAT-3′). Primer3Plus online software v.2.3.6 was used for primers design [14] and universal M13 tails. Agencourt AMPure XP beads (Beckman Coulter, SA, Nyon, Switzerland) were used for purification. Quant-it™ PicoGreen dsDNA Assay Kit (Life Technologies, Oregon, USA) was finally used for library quantification. Emulsion PCR (emPCR) was finally assessed as previously described [15]. Amplicon Variant Analyzer software (454 Life Sciences Corp. Roche, Branford, Connecticut, USA) was used for data primary analysis. Sequences were aligned with NM_006218.3 for reference sequence and variant calling was processed. NGS sensitivity has been set to 1% at 1000x depth. A second analysis was finally run (mapping with BWA 0.7.12 (mem algorithm, default parameters) and SAMtools for sorting and indexing). VarScan2 (mpileup2snp algorithm, with filters—min-coverage 100—minreads 20—min-var-freq 0.01—*p* value 0.05) was used for variant calling [15].

Statistical Analysis

Qualitative data were described by frequency and percentage. The Agreement between the three routines were assessed with the Kappa coefficient and its 95% confidence interval. According to the terminology suggested by Landis and Koch [16] a kappa value less than 0 indicates poor agreement, 0 to 0.2 represents slight agreement, 0.2 to 0.4 is fair agreement, 0.4 to 0.6 indicates moderate agreement, 0.6 to 0.8 shows substantial agreement, and 0.8 to 1.0 is almost perfect agreement. SAS software (SAS Institute, Cary, NC 25513; version 9.2) was used to perform statistical analysis [16].

Table 3 Agreement assessment between the three routine assays with Kappa coefficient

	HRM($n = 46$)	ARMS(n = 46)	Cobas® ($n = 45$)	Agreement		
				(Kappa values and 95% confidence interval)		
				Cobas®/HRM (n = 45)	Cobas®/ARMS (n = 45)	HRM /ARMS (n = 46)
Mutation	19 (41.3%)	13 (28.3%)	17 (37. 8%)	0.95 [0.86;1]	0.75 [0.55;0.95]	0.72 [0.51;0.92]
Exon 10	10 (21.7%)	7 (15.2%)	8 (17.8%)	0.93 [0.80;1]	0.76 [0.51;1]	0.73 [0.48;0.97]
Exon 21	*9* (19.6%)	6 (13.0%)	9 (20%)	1	0.76 [0.51;1]	0.76 [0.51;1]

Results

Among the forty-six samples, *PIK3CA* gene mutations have been found in 17 (37.8%), 13 (28.36%) and 19 (41.3%) with Cobas®, HRM and ARMS assays respectively (Table 2). The sample #2 was found to carry 3 mutations of *PIK3CA* with Cobas® assay (p.(Glu542Lys), p.(His1047X) and p.(His1049Arg). Calculated kappa were 0.95[0.86;1] between Cobas® and HRM, 0.75[0.55;0.95] between Cobas® and ARMS and 0.72[0.51;0.92] between HRM and ARMS. For exon 10 mutations, calculated kappa were 0.93[0.80;1] between Cobas® and HRM, 0.76[0.51;1] between Cobas® and ARMS and 0.73[0.48;0.97] between HRM and ARMS. For exon 21 mutations, calculated kappa were 1 between Cobas® and HRM, 0.76[0.51;1] between Cobas® and ARMS and 0.76[0.51;1] between HRM and ARMS. All agreement assessment between the three routine techniques with Kappa coefficient is presented in Table 3. We concluded with an almost perfect agreement between Cobas® and HRM and a substantial agreement between Cobas®/ARMS and HRM/ARMS. Discordant samples were assessed using NGS for the detection of all mutations in exons 10 and 21 as presented in Table 4. Samples #2 and #19 were found to carry p.(Glu542Lys) mutation, sample #17 was found to bear p.(Gln546Lys) mutation, sample #26 p.(Glu545Lys) mutation and samples #3 and #37 p.(His1047Arg) mutation. Samples #18 and #35 were not found to bear any mutation using NGS.

Table 4 Comparison of discordant cases with NGS

Nucleotide change	Protein change	Number of samples	Samples #
c.1624 G > A	p.(Glu542Lys)	2	2 and 19
c.1633 G > A	p.(Glu545Lys)	1	26
c.1636 C > A	p.(Gln546Lys)	1	17
c.3140 A > G	p.(His1047Arg)	2	3 and 37
No mutation	No mutation	2	18 and 35

Discussion

Breast cancer is a heterogeneous disease at the genetic level and a vast choice of treatments is available for patients' management, including targeted therapies. Dysregulation or activation of the PI3K/AKT/mTOR signaling pathway have been associated to resistance to anti-HER2 therapies and a reduction of overall survival in patients with breast carcinomas.

All mutations of *PIK3CA* were detected by the Cobas® assay. The presence of mutations in samples #2, #3, #17, #19, #26 and #37 detected only with the Cobas® assay were confirmed using NGS. NGS also confirmed the absence of *PIK3CA* mutations in samples #18 and #35. Quality threshold has not been reached for sample #35 for Cobas® which explains the "invalid" result for this sample. This "invalid" result is consistent with the high Ct values determined with HRM and ARMS assays, synonym of low DNA quality and explain the false positive results with these two assays. The ARMS assay can detect variants with allele frequencies as low a 0.5%, but is only designed for the detection of the 4 most common *PIK3CA* mutations, which explains that more than 10% (6 out of 46) of the samples were "discrepant by design" and therefore identified as wild-type whereas Cobas®, HRM and NGS assays detected these non-hotspot mutations. Most of discrepant results in this study were assay's design-related false wild-type *PIK3CA*, but no false negative results were found due to a lack of sensitivity of an assay. According to these results, the detection of only 4 hotspot mutations of *PIK3CA* is not sufficient for routine use. Indeed, non-hotspot mutations have been detected here in a highest frequency than described in the COSMIC database.

In our study, Cobas® and HRM assays showed comparable abilities for the detection of *PIK3CA* mutations. In previously published data, sensitivities of both assays have been showed comparable for the detection of *KRAS* mutation in metastatic colorectal cancer [10]. One of the main difference between Cobas® and HRM assays is the ability of the Cobas® assay to characterize the mutation which is relevant with precision medicine context especially for the prescription of PI3K inhibitors or to predict the absence of resistance to anti-Her2 therapies [17–19].

NGS is widely used in routine diagnostic in many laboratories for the mutational status determination of a large panel of genes including *PIK3CA* but this assay is more expensive and requires more hands-on time than real-time PCR (2 to 3 days for NGS and 1 day for PCR). Moreover, NGS assay often requires better DNA quality than specific PCR assays, which justify that real-time PCR still has its place in the landscape of somatic mutations testing.

In conclusion, our data demonstrate that the Cobas® assay is suitable for the assessment of *PIK3CA* mutation in patients with breast carcinomas. HRM assay showed comparable results to Cobas® assay but requires further characterization of the mutation with a specific assay.

Author's Contribution All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

A. Lambert wrote the manuscript, J. Salleron performed statistical analysis, M. Lion, M. Rouyer, N. Lozano collected the data and analyzed the samples. A. Leroux performed histological analysis. J.L. Merlin and A. Harlé (guarantor) wrote the study and analyzed the data.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Ethics Approval and Consent to Participate All patients gave their informed consent to Institut de Cancérologie de Lorraine for the research of somatic mutations and the use of their biological sample for research purposes. The study was approved by Institut de Cancérologie de Lorraine scientific board. All methods were performed in accordance with the relevant guidelines and regulations. All patients' data was anonym and de-identified prior to analysis.

Competing Interests The authors declare to have no competing financial interests.

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