Analysis of *PIK3CA* exon 9 and 20 mutations in breast cancers using PCR-HRM and PCR-ARMS: Correlation with clinicopathological criteria

ALEXANDRE HARLÉ[1,3], MAËVA LION[1,3], NICOLAS LOZANO[1], MARIE HUSSON[1], VALENTIN HARTER[2], PASCAL GENIN[1,3] and JEAN-LOUIS MERLIN[1,3]

Departments of [1]Biopathology and [2]Biostatistics, Centre Alexis Vautrin, Vandoeuvre-lès-Nancy; [3]Research Center for Automatic Control of Nancy (CRAN), University of Lorraine, Nancy, France

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Abstract. Phosphatidylinositol-3-kinases (PI3K) are essential for cell signaling, proliferation, differentiation and survival. The catalytic subunit of PI3K, encoded by the *PIK3CA* oncogene, is mutated in 18-45% of breast carcinomas. These mutations, involved in tumorigenic processes, activate the PI3K/AKT/mTOR signaling pathway. Resistance to anti-human epidermal growth factor receptor, hormonal or anti-PI3K therapies have been described in breast carcinomas bearing activation of the PI3K signaling pathway. The present study reports the evaluation of *PIK3CA* exon 9 and 20 mutations in 149 invasive breast cancer cases using a validated PCR-high resolution melting assay (PCR-HRM). An amplification refractory mutation system (PCR-ARMS) using allele-specific scorpion primers was used to detect hotspot mutations in exons 9 (c.1624G→A and c.1633G→A) and 20 (c.3140A→G and c.3140A→T) in 118 tumor specimens. No correlation was observed with age at diagnosis, histological type, hormone receptor and HER2 status. *PIK3CA* exon 9 and 20 mutations were found to be related to Scarff-Bloom-Richardson (SBR) grade with a lower rate of mutations and a higher frequency of exon 9 mutations in SBRI and exon 20 mutations in SBRII/III tumors. No difference was observed in the incidence rates of the two different mutations screened for each exon in any subcategory. A statistically significant correlation was found between PCR-HRM and PCR-ARMS (κ=0.845; P<0.001). PCR-ARMS was found to be more sensitive than PCR-HRM (sensitivity 0.5 and 5-10% of mutated DNA, respectively). We propose that PCR-HRM and PCR-ARMS can be combined for the cost-effective routine clinical identification of *PIK3CA* mutations for the purpose of personalizing therapy for invasive breast cancers.

Correspondence to: Dr Alexandre Harlé, Department of Biopathology, Centre Alexis Vautrin, 6 Avenue de Bourgogne, CS 30159, F-54519 Vandoeuvre-lès-Nancy Cedex, France
E-mail: a.harle@nancy.unicancer.fr

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Introduction

Female breast cancer is the most commonly diagnosed cancer with more than one million new cases every year and breast cancer is one of the leading causes of cancer-related death in women (1). The phosphatidylinositol-3-kinase (PI3K) pathway is a major signaling pathway in cells and is involved in essential cell processes such as metabolism, survival, proliferation, growth and motility (2). Dysregulation of the PI3K pathway occurs in a large variety of human cancers (3) and has been proven to be implicated in breast cancer development and progression (4). PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). The phosphatase and tensin homolog (PTEN) antagonizes and negatively regulates PI3K by converting PIP3 to PIP2 (5).

The PI3K/AKT/mTOR pathway appears to have a major role in the response to treatment and in the development of resistance to anticancer drugs. Overactivation of the PI3K pathway downstream of human epidermal growth factor receptors (HER) can be driven by mutations of PI3K, an enzyme from the lipid kinase family involved in cell signaling. These activating mutations occur mainly on p110α, the catalytic subunit of PI3K encoded by the *PIK3CA* (phosphoinositide-3-kinase, catalytic, α polypeptide) gene located on chromosome 3. *PIK3CA* mutations are present in 25% of breast carcinomas, and the most common activating mutations occur on exons 9 and 20 according to the COSMIC database (Catalogue Of Somatic Mutations in Cancer Database, Wellcome Trust Genome Campus, Hinxton, Cambridge; accessed June 2012; http://www.sanger.ac.uk). More precisely, E542K (c.1624G→A, p.Glu542Lys), E545K (c.1633G→A, p.Glu545Lys) in exon 9 and H1047R (c.3140A→G, p.His1047Arg), H1047L (c.3140A→T, p.His1047Leu), represent more than 90% of the mutations encountered in breast carcinomas. These four mutations have been shown to have an oncogenic role in breast cancers (6-9).

Recent studies have shown that PI3K may be implicated in the resistance of breast cancers to anti-estrogen therapy agents (10,11), anti-HER2 tyrosine kinase inhibitor (lapatinib) (12) and anti-HER2 monoclonal antibody (trastuzumab) (5,11,13). Mutations of *PIK3CA* and loss of the PTEN protein are keys

Table I. Relationship between the *PIK3CA* mutation status analyzed using PCR-HRM and main standard clinicopathological and biological characteristics of the breast cancer cases.

		No. of patients (%)		
	Total/class	*PIK3CA* wild-type	*PIK3CA* mutated	P-value[a]
Total	118 (100.0)	88 (72.5)	30 (27.5)	
Age at diagnosis (years)				
≤50	36 (30.5)[f]	26 (72.2)[g]	10 (27.8)	0.873
>50	82 (69.5)	62 (75.6)	20 (24.4)	
Tumor histology				
Ductal	102 (86.4)	73 (71.6)	29 (28.4)	0.137
Lobular	15 (12.7)	14 (93.3)	1 (6.7)	
Others	1 (0.8)	1 (100.0)	0 (0.0)	
Estrogen receptor (ER) α status[b]				
Positive	94 (81.7)	71 (75.5)	23 (24.5)	0.910
Negative	21 (19.3)	15 (71.4)	6 (28.6)	
Progesterone receptor (PR) status[b]				
Positive	74 (64.3)	57 (77.0)	17 (23.0)	0.603
Negative	41 (35.7)	29 (70.7)	12 (29.3)	
HER2 status[b]				
Positive[c]	12 (10.4)	9 (75.0)	3 (25.0)	1.000
Negative	103 (89.6)	77 (74.8)	26 (25.2)	
Hormone receptor (HR) status[b,d]				
Positive	95 (82.3)	72 (75.8)	23 (24.2)	0.796
Negative	20 (27.7)	14 (70.0)	6 (30.0)	
Combined HER and HR status[b]				
$HER2^+/HR^+$	8 (7.0)	7 (87.5)	1 (12.5)	0.575
$HER2^-/HR^+$	87 (7.6)	65 (74.7)	22 (25.3)	
$HER2^+/HR^-$	4 (3.5)	2 (50.0)	2 (50.0)	
$HER2^-/HR^-$	16 (13.9)	12 (75.0)	4 (25.0)	
SBR grade[e]				
I	14 (11.9)	8 (57.1)	6 (42.9)	0.050
II	56 (47.5)	39 (69.6)	17 (30.4)	
III	48 (40.7)	41 (85.4)	7 (14.6)	

[a]χ^2 or Fisher's exact test. [b]Information available for 115 patients. [c]HER2 (3+) by immunohistochemistry or FISH-positive; [d]HR^+, ER^+ and/or PR^+; HR^-, ER^- and PR^-. [e]Scarff-Bloom-Richardson classification. [f]Refers to the total population. [g]Refers to the population of each category.

factors in the development of resistance to these drugs (14-16). Moreover, lapatinib and trastuzumab resistance can occur in HER2-amplified breast cancers bearing a *PIK3CA* mutation (5,14,15). HER2 overexpression in breast cancers is present in 15-25% of tumors (17,18). Approximately 75% of the breast cancers express estrogen receptors and/or progesterone receptors and a relationship between anti-estrogen resistance and activation of the PI3K pathway has recently been found (11). These new findings imply that the PI3K pathway may be an important target for novel targeted therapies.

New anti-PI3K and anti-mTOR drugs are currently under development (19,20) for breast cancer treatment. Activation of the PI3K/AKT pathway and overexpression of PI3K may play a major role in the use of new therapeutic schemes, and identification of *PIK3CA* mutations could be a major biomarker for predicting the response to these new therapies.

In light of these issues, there is a huge interest in developing rapid, reliable and sensitive methods that can be used for clinical routine detection of *PIK3CA* mutations in breast tumors. In the present study, we used a polymerase chain reaction (PCR)-high resolution melting assay (HRM) and a PCR-amplification refractory mutation system (ARMS) to analyze alcohol-formalin-acetic acid (AFA)-fixed paraffin-embedded breast tumor specimens. PCR-HRM is a cost-effective post-PCR method that enables the identification of alterations in single nucleotides, i.e., mutations through the analysis of thermal denaturation of double-stranded DNA. PCR-ARMS is a powerful mutation-specific real-time

Table II. Relationship between *PIK3CA* mutation status analyzed using PCR-ARMS and the main standard clinicopathological and biological characteristics of the breast cancer cases.

		No. of patients (%)		
	Total/class	*PIK3CA* wild-type	*PIK3CA* mutated	P-value[a]
Total	149 (100.0)	122 (81.9)	27 (18.1)	
Age at diagnosis (years)				
≤50	48 (30.5)[f]	36 (72.2)[g]	12 (27.8)	0.202
>50	101 (69.5)	86 (75.6)	15 (24.4)	
Tumor histology				
Ductal	127 (85.2)	102 (71.6)	25 (28.4)	0.521
Lobular	19 (12.8)	17 (93.3)	2 (6.7)	
Others	3 (2.0)	2 (66.7)	1 (33.3)	
Estrogen receptor (ER) α status[b]				
Positive	113 (81.7)	93 (75.5)	20 (24.5)	0.981
Negative	30 (19.3)	24 (71.4)	6 (28.6)	
Progesterone receptor (PR) status[b]				
Positive	88 (64.3)	71 (77.0)	17 (23.0)	0.823
Negative	55 (35.7)	46 (70.7)	9 (29.3)	
HER2 status[b]				
Positive[c]	19 (10.4)	16 (75.0)	3 (25.0)	1.000
Negative	124 (89.6)	101 (74.8)	23 (25.2)	
Hormone receptor (HR) status[b,d]				
Positive	115 (82.3)	95 (75.8)	20 (24.2)	0.823
Negative	28 (27.7)	22 (70.0)	6 (30.0)	
Combined HER and HR status[b]				
$HER2^+/HR^+$	13 (7.0)	12 (87.5)	1 (12.5)	0.593
$HER2^-/HR^+$	102 (7.6)	83 (74.7)	19 (25.3)	
$HER2^+/HR^-$	6 (3.5)	4 (50.0)	2 (50.0)	
$HER2^-/HR^-$	22 (13.9)	18 (75.0)	4 (25.0)	
SBR grade[e]				
I	14 (11.9)	7 (57.1)	7 (42.9)	0.004
II	67 (47.5)	55 (69.6)	12 (30.4)	
III	64 (40.7)	56 (85.4)	8 (14.6)	

[a]χ^2 or Fisher's test. [b]Information available for 143 patients. [c]HER2 (3+) by immunohistochemistry or FISH-positive; [d]HR^+, ER^+ and/or PR^+; HR^-, ER^- and PR^-. [e]Scarff-Bloom-Richardson classification. [f]Refers to the total population. [g]Refers to the population of each category.

PCR-based technique combining ARMS and a bi-functional fluorescent probe/primer molecule (Scorpion).

The present study evaluated the relationship between *PIK3CA* exon 9 and 20 mutations and conventional clinicopathological criteria and compared the sensitivity of the two techniques by examining the correlation of the results achieved using both methods. The final goal was to validate a double technique approach, ensuring a cost-effective, rapid process yielding a high quality level of analysis, according to the recommendations of the French National Cancer Institute (INCa) for clinical routine analysis of mutations in tumors in a treatment-choosing process. In such a context, PCR-HRM could be proposed to determine the *PIK3CA* mutational status yielding binary results (mutated or wild-type) and PCR-ARMS-Scorpion to accurately identify the four main hotspot mutations of *PIK3CA*.

Patients and methods

Population. One hundred and forty-nine invasive breast carcinoma tumor specimens, from patients diagnosed between 2008 and 2009, were retrospectively included in this study. All specimens were collected as AFA-fixed paraffin-embedded tissues from our institutional Biobank. The tumor characteristics of this population were consistent with literature data regarding mean age at diagnosis, histological type (ductal and lobular), Scarff-Bloom-Richardson (SBR) grade as well as hormone receptor and HER2 status (Tables I and II). According to the Biobank procedure of our institute, all women involved in this study were informed that their tumor samples might be used for research purposes and had the opportunity to decline. No opposition was expressed.

DNA extraction. For each tumor specimen, hematoxylin and eosin slide analysis was conducted by a pathologist to ensure a minimum of 20% tumor tissue content as recommended in previous studies (21). Selected areas were macrodissected and 5 10-μm serial sections were cut from each paraffin block and collected in RNAase DNAase-free vials (SafeSeal Microcentrifuge Tubes, Sorenson Biosciences, Salt Lake City, UT, USA). Paraffin was removed by extraction with toluene (VWR BDH Prolabo, Fontenay Sous Bois, France) and centrifuged. DNA isolation was performed using the QIAamp DNA FFPE tissue kit (Qiagen, Courtaboeuf, France) protocol. The pellet was washed with ethanol, centrifuged and resuspended with 180 μl of tissue lysis buffer (ATL buffer; Qiagen) and 20 μl of proteinase K (Qiagen). The sample was then gently mixed, incubated at 56˚C for 1 h and at 90˚C for 1 h under agitation. DNA was extracted with MinElute Columns (Qiagen) as recommended by the manufacturer. The nucleic acids were eluted in a volume of 100 μl. Final concentration of eluates, ranging from 33.6 to 729.0 ng/ml, were suitable for PCR-HRM and PCR-ARMS analyses (Table III). DNA extracts from cell lines bearing E542K (Cal51), E545K (MCF7), H1047R (HCT116) and H1047R (SUM159PT) PI3-kinase mutations were used as positive controls. DNA extracted from the MDA231 cell line was used as a wild-type negative control. DNA quality was controlled using agarose gel electrophoresis with *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as the control housekeeping gene. Only DNA with no degradation was used. DNA concentrations were determined using a Bio-photometer (Eppendorf, Hamburg, Germany).

PCR-HRM. HRM analysis was performed using the LightCycler 480® Real-Time PCR system (Roche Diagnostics, Meylan, France) and the LightCycler 480 HRM Master kit (Roche Diagnostics) in 384-well plates (Roche Diagnostics). Twenty micrograms of DNA was amplified in a final volume of 20 μl. All data and melting curves were analyzed using LightCycler SW v. 1.5.0.39 software (Roche Diagnostics). One mix was prepared for each exon. For each sample, 10 μl of Master Mix (Roche Diagnostics), 2.8 μl of $MgCl_2$ 25 mM, PCR-quality grade water and 1 μl of the primers (forward and reverse) were added. Eighteen microliters of mix was added to each well, and 2 μl of the sample was used for the analysis. One set of primers were used for each of the *PIK3CA* exons 9 and 20. All primers were designed as previously described (22).

PCR-HRM was divided into different phases: a phase of pre-incubation (10 min at 95˚C) was followed by 45 cycles of classic PCR (10 sec at 95˚C, a temperature decrease from 60 to 54˚C by 0.5˚C/cycle in 15 sec and, finally, 10 sec at 72˚C). The PCR phase was followed by the high resolution melting phase which consisted of 1 min at 95˚C, 1 min at 40˚C and a temperature increase by 0.2˚C/sec from 65 to 95˚C. A cooling phase of 1 min at 40˚C was finally performed.

PCR-ARMS. ARMS analysis was performed using the LightCycler 480 Real-Time PCR system (Roche Diagnostics) in 384-well plates. Eighty micrograms of DNA was amplified in a final volume of 20 μl. Data and fluorescence curves were analy`ed using LightCycler SW v. 1.5.0.39 software. All primers were designed as previously described (23). One mix was made for each tested mutation and all samples were proceeded as simplex. For each sample, 0.06 μl of Hot Diamond Taq polymerase (Eurogentec, Angers, France) was added together with 2 μl of reaction buffer 10X (Eurogentec), 3.2 μl of $MgCl_2$ 25 mM (Eurogentec), 0.4 μl of dNTP 10 mM (Eurogentec), PCR-quality water, 0.8 μl of ARMS primers 6.25 μM (Eurogentec) and 0.8 μl of Scorpion primers 6.25 μM (ATD Bio, Southampton, UK). Eighteen microliters of mix was added to each well, and 2 μl of the sample was used for the analysis. The mix for exon 9 mutations contained ARMS control primers for exon 15, specific ARMS primers, respectively, of E542K and E545K mutations and exon 9 and 15 specific Scorpion primers. The mix for exon 20 mutations contained ARMS control primers for exon 15, specific ARMS primers, respectively, of H1047R and H1047L mutations and exon 20 and 15 specific Scorpion primers.

Sensitivity. The sensitivity of PCR-HRM and PCR-ARMS was evaluated by mixing mutated and wild-type DNA from the cell lines at 50, 25, 10, 5, 2, 1 and 0.5% ratios.

Statistical analysis. The significance of the concordance of mutation detection using the two methods was assessed using κ statistics. $\kappa>0.8$ was considered as indicative of significance to conclude that both methods provide similar results. The χ^2 test was also used to compare mutation frequencies with those obtained from the literature. χ^2 and Fisher's exact tests were used to test for differences between classes of patients and tumors based on clinical, pathological and biological characteristics. Limit of statistical significance was set at $P<0.05$.

Results

Mutation analysis using PCR-HRM. One hundred and eighteen specimens were analyzed using PCR-HRM (Table I). *PIK3CA* mutations (exons 9 and 20) were detected in 30 (27.5%) of the specimens. No correlation was found regarding patient age (≤50 or >50 years), ductal or luminal type, estrogen and progesterone status (alone or combined as hormonal receptor status), HER2 status, and for the four subtypes identified as $HER2^+/HR^+$, $HER2^+/HR^-$, $HER2^-/HR^+$ and $HER2^-/HR^-$ (triple negative).

PIK3CA mutations were found to be correlated with SBR grade (P=0.050) with a lower mutation rate noted in the highest grades. Regarding the frequency of exon 9 and 20 mutations, a low rate of exon 9 mutations was observed in SBR grade III tumors (P=0.025) while no difference was observed for the exon 20 mutation rate. Exon 9 mutations were found to be more frequent in SBR grade I tumors while exon 20 mutations were predominantly observed in SBR grade II and III tumors.

Mutation analysis using PCR-ARMS. One hundred and forty-nine specimens were analyzed using PCR-ARMS (Table II). *PIK3CA* mutations (exons 9 and 20) were detected in 27 (18.1%) specimens. No difference was found regarding patient age (≤50 or >50 years), ductal or luminal type, estrogen and progesterone status (alone or combined as hormonal receptor status), HER2 status, and for the four subtypes ($HER2^+/HR^+$, $HER2^+/HR^-$, $HER2^-/HR^+$ and $HER2^-/HR^-$, i.e., triple negative). As with PCR-HRM, *PIK3CA* mutations were found to be related with SBR grade (P=0.004) with a lower mutation rate

Table III. Contingency table of the 102 samples analyzed by combined PCR-HRM and PCR-ARMS assays.

Sample no.	Sample DNA concentration (ng/μl)	PCR-HRM		PCR-ARMS	
		Test result	Mutation result	Test result	Mutation result
1	33.6	Mutation not detected	N/A	Mutation not detected	N/A
2	61.2	Mutation not detected	N/A	Mutation not detected	N/A
3	95.9	Mutation detected	Exon 20	Mutation detected	c.3140A→G
4	64.3	Mutation not detected	N/A	Mutation not detected	N/A
5	105.2	Mutation not detected	N/A	Mutation not detected	N/A
6	164.4	Mutation not detected	N/A	Mutation not detected	N/A
7	71.1	Mutation detected	Exon 9	Mutation detected	c.1624G→A
8	69.8	Mutation not detected	N/A	Mutation not detected	N/A
9	95.6	Mutation not detected	N/A	Mutation not detected	N/A
10	39.9	Mutation not detected	N/A	Mutation not detected	N/A
11	68.7	Mutation not detected	N/A	Mutation not detected	N/A
12	**60.7**	**Mutation detected**	**Exon 9**	**Mutation not detected**	**N/A**
13	62.0	Mutation not detected	N/A	Mutation not detected	N/A
14	108.0	Mutation not detected	N/A	Mutation not detected	N/A
15	**162.7**	**Mutation detected**	**Exon 9**	**Mutation not detected**	**N/A**
16	319.9	Mutation not detected	N/A	Mutation not detected	N/A
17	**221.4**	**Mutation detected**	**Exon 20**	**Mutation not detected**	**N/A**
18	109.7	Mutation not detected	N/A	Mutation not detected	N/A
19	146.3	Mutation not detected	N/A	Mutation not detected	N/A
20	201.3	Mutation not detected	N/A	Mutation not detected	N/A
21	199.3	Mutation not detected	N/A	Mutation not detected	N/A
22	224.7	Mutation not detected	N/A	Mutation not detected	N/A
23	35.5	Mutation not detected	N/A	Mutation not detected	N/A
24	201.2	Mutation not detected	N/A	Mutation not detected	N/A
25	175.8	Mutation detected	Exon 20	Mutation detected	c.31401→T
26	104.6	Mutation not detected	N/A	Mutation not detected	N/A
27	211.4	Mutation not detected	N/A	Mutation not detected	N/A
28	662.0	Mutation detected	Exon 9	Mutation detected	c.1633G→A
29	457.8	Mutation not detected	N/A	Mutation not detected	N/A
30	223.3	Mutation not detected	N/A	Mutation not detected	N/A
31	199.8	Mutation not detected	N/A	Mutation not detected	N/A
32	68.0	Mutation not detected	N/A	Mutation not detected	N/A
33	122.1	Mutation not detected	N/A	Mutation not detected	N/A
34	232.4	Mutation not detected	N/A	Mutation not detected	N/A
35	252.4	Mutation detected	Exon 20	Mutation detected	c.31401→T
36	155.3	Mutation detected	Exon 20	Mutation detected	c.3140A→G
37	75.0	Mutation not detected	N/A	Mutation not detected	N/A
38	486.5	Mutation not detected	N/A	Mutation not detected	N/A
39	164.2	Mutation not detected	N/A	Mutation not detected	N/A
40	204.1	Mutation not detected	N/A	Mutation not detected	N/A
41	**124.4**	**Mutation detected**	**Exon 9**	**Mutation not detected**	**N/A**
42	291.3	Mutation not detected	N/A	Mutation not detected	N/A
43	206.5	Mutation not detected	N/A	Mutation not detected	N/A
44	67.6	Mutation detected	Exon 9	Mutation detected	c.1633G→A
45	96.4	Mutation detected	Exon 20	Mutation detected	c.3140A→G
46	26.9	Mutation detected	Exon 20	Mutation detected	c.31401→T
47	99.5	Mutation not detected	N/A	Mutation not detected	N/A
48	**41.4**	**Mutation detected**	**Exon 9**	**Mutation not detected**	**N/A**
49	127.8	Mutation not detected	N/A	Mutation not detected	N/A
50	184.0	Mutation not detected	N/A	Mutation not detected	N/A
51	86.9	Mutation not detected	N/A	Mutation not detected	N/A
52	499.3	Mutation not detected	N/A	Mutation not detected	N/A

Table III. Continued.

Sample no.	Sample DNA concentration (ng/μl)	PCR-HRM		PCR-ARMS	
		Test result	Mutation result	Test result	Mutation result
53	188.5	Mutation not detected	N/A	Mutation not detected	N/A
54	140.1	Mutation not detected	N/A	Mutation not detected	N/A
55	357.4	Mutation not detected	N/A	Mutation not detected	N/A
56	252.5	Mutation detected	Exon 20	Mutation detected	c.3140A→G
57	211.7	Mutation not detected	N/A	Mutation not detected	N/A
58	213.5	Mutation not detected	N/A	Mutation not detected	N/A
59	230.3	Mutation detected	Exon 20	Mutation detected	c.3140A→G
60	**227.3**	**Mutation detected**	**Exon 9**	**Mutation not detected**	**N/A**
61	729.0	Mutation not detected	N/A	Mutation not detected	N/A
62	144.4	Mutation not detected	N/A	Mutation not detected	N/A
63	202.3	Mutation not detected	N/A	Mutation not detected	N/A
64	45.6	Mutation not detected	N/A	Mutation not detected	N/A
65	154.0	Mutation not detected	N/A	Mutation not detected	N/A
66	301.8	Mutation detected	Exon 20	Mutation detected	c.31401→T
67	178.7	Mutation not detected	N/A	Mutation not detected	N/A
68	151.5	Mutation not detected	N/A	Mutation not detected	N/A
69	543.7	Mutation not detected	N/A	Mutation not detected	N/A
70	70.3	Mutation detected	Exon 9	Mutation detected	c.1624G→A
71	179.8	Mutation not detected	N/A	Mutation not detected	N/A
72	202.1	Mutation not detected	N/A	Mutation not detected	N/A
73	244.9	Mutation detected	Exon 9	Mutation detected	c.1633G→A
74	130.5	Mutation not detected	N/A	Mutation not detected	N/A
75	256.0	Mutation not detected	N/A	Mutation not detected	N/A
76	**90.2**	**Mutation not detected**	**N/A**	**Mutation detected**	**c.1624G→A**
77	302.6	Mutation not detected	N/A	Mutation not detected	N/A
78	138.8	Mutation not detected	N/A	Mutation not detected	N/A
79	117.0	Mutation not detected	N/A	Mutation not detected	N/A
80	130.9	Mutation not detected	N/A	Mutation not detected	N/A
81	120.6	Mutation not detected	N/A	Mutation not detected	N/A
82	149.4	Mutation not detected	N/A	Mutation not detected	N/A
83	221.2	Mutation detected	Exon 20	Mutation detected	c.3140A→G
84	107.8	Mutation detected	Exon 9	Mutation detected	c.1624G→A
85	166.1	Mutation not detected	N/A	Mutation not detected	N/A
86	277.2	Mutation not detected	N/A	Mutation not detected	N/A
87	111.1	Mutation not detected	N/A	Mutation not detected	N/A
88	81.1	Mutation not detected	N/A	Mutation not detected	N/A
89	170.2	Mutation not detected	N/A	Mutation not detected	N/A
90	252.6	Mutation not detected	N/A	Mutation not detected	N/A
91	127.9	Mutation detected	Exon 20	Mutation detected	c.3140A→G
92	102.3	Mutation not detected	N/A	Mutation not detected	N/A
93	111.9	Mutation not detected	N/A	Mutation not detected	N/A
94	306.6	Mutation not detected	N/A	Mutation not detected	N/A
95	96.3	Mutation detected	Exon 20	Mutation detected	c.3140A→G
96	88.7	Mutation not detected	N/A	Mutation not detected	N/A
97	122.6	Mutation detected	Exon 20	Mutation detected	c.3140A→G
98	222.9	Mutation not detected	N/A	Mutation not detected	N/A
99	194.4	Mutation detected	Exon 9	Mutation detected	c.1624G→A
100	44.5	Mutation not detected	N/A	Mutation not detected	N/A
101	185.3	Mutation detected	Exon 20	Mutation netected	c.3140A→G
102	127.3	Mutation detected	Exon 9	Mutation detected	c.1624G→A

Discordant results are highlighted in bold. N/A, not applicable.

Table IV. Frequencies of mutations detected with combined PCR-ARMS and PCR-HRM assays.

Nucleotide change	Protein change	No. of samples	Relative (%)	Total (%)
PCR-ARMS				
Exon 9				
c.1624G→A	E542K	6	26.1	5.9
c.1633G→A	E545K	3	13.0	2.9
Exon 20				
c.3140A→G	H1047R	10	43.5	9.8
c.31401→T	H1047L	4	17.4	3.9
Total		23	100.0	22.5
PCR-HRM				
Exon 9	Not available	13	46.4	12.8
Exon 20	Not available	15	53.6	14.7
Total		28	100.0	27.5

Table V. Summary of the results achieved with combined PCR-HRM and PCR-ARMS assays.

	PCR-HRM		
	Mutated	Wild-type	Total
PCR-ARMS			
Mutated	22	1	23
Wild-type	6	73	79
Total	28	74	102

in the highest grades. Regarding the frequency of exon 9 and 20 mutations, a lower rate of exon 9 and exon 20 mutations was observed in SBR grade III tumors (P=0.009). Again, exon 9 mutations were found to be predominant in SBR grade I tumors (5/7) while exon 20 mutations were predominantly observed in SBR grade II and III tumors (13/20).

Comparative analysis of PCR-HRM and PCR-ARMS. One hundred and two breast tumor samples (Table III) were analyzed using both PCR-HRM and PCR-ARMS. *PIK3CA* mutations were detected (Table IV) in 28 tumors (27.5%) when PCR-HRM was used and 23 (22.5%) when PCR-ARMS was used.

Among the 28 mutated tumors (Table IV), PCR-HRM results showed that 13 (46.4%) carried a mutation on exons 9 and 15 (53.6%) on exon 20.

Among the 23 samples in which a mutation was detected using PCR-ARMS assay (Table IV), 9 (39.1%) carried a mutation in exon 9, identified as c.1624G→A in 6 cases (26.1%) and as c.1633G→A in the 3 others (13.0%). Among the remaining 14 (60.9%) specimens identified as mutated in exon 20, 10 (43.5%) carried the c.3140A→G and 4 (17.4%) the c.3140A→T mutation. No sample was found with mutations in both exons 9 and 20 with any of the assays.

Table VI. Limits of sensitivity of PCR-HRM and PCR-ARMS assays.

Nucleotide change	Protein change	Sensitivity (%)	DNA quantity (ng)
PCR-HRM			
Exon 9	Not available	5	1.0
Exon 20	Not available	10	2.0
PCR-ARMS			
Exon 9			
c.1624G→A	E542K	0.5	0.8
c.1633G→A	E545K	0.5	0.8
Exon 20			
c.3140A→G	H1047R	0.5	0.8
c.31401→T	H1047L	0.5	0.8

Sensitivity. The analytical sensitivity of each assay was evaluated from dilution of DNA extracted from *PIK3CA*-mutated cell lines into DNA from *PIK3CA* wild-type cell lines (MDA 231) from 0.5 to 50%. PCR-HRM was able to discriminate a dilution corresponding to 5% of exon 9 mutated DNA and 10% of exon 20 mutated DNA (Fig. 1, Table VI). PCR-ARMS was able to discriminate a DNA dilution corresponding to 0.5% of c.1624G→A, 0.5% of c.1633G→A, 0.5% of c.3140A→G and 0.5% of c.3140A→T mutated DNA (Fig. 2, Table VI).

Results obtained using PCR-ARMS and PCR-HRM (Table V), were found to be statistically comparable (κ=0.845, P<0.001). Contingency table is shown in Table III.

No statistical difference in the frequencies of *PIK3CA* mutations was found between results achieved with any of the two assays and data from Sanger database.

Discussion

Recent studies have shown that *PIK3CA* mutations play a major role in resistance to trastuzumab or lapatinib (5) or to hormonal therapy (11,13) of breast carcinomas and could represent a potent response predictive marker for PI3-kinase- and mTOR-targeted therapies (24) and be used as a treatment-choosing parameter. *PIK3CA* mutations are mostly located within exons 9 and 20 and four hotspots (c.1624G→A, c.1633G→A, c.3140A→G and c.3140A→T) represent more than 90% of all mutations. Although the prognostic value of *PIK3CA* mutations in breast cancer remains controversial, in a recent evaluation of 2587 breast cancers cases from 12 independent studies, Dumont *et al* (24) reported a more favorable clinical outcome in patients with *PIK3CA* mutated tumors and that improved prognosis may pertain only to patients with a mutation in the kinase domain of p110α and to post-menopausal women with estrogen-positive cancers.

The relative prognostic value of exon 9 vs. 20 mutations also remains controversial. Barbareschi *et al* (25), found that exon 9 mutations have a negative prognostic value while exon 20 mutations were associated with favorable outcome while Lai *et al* (26) reported exon 20 mutations as associated with poor prognosis. Furthermore, Lerma *et al* (27)

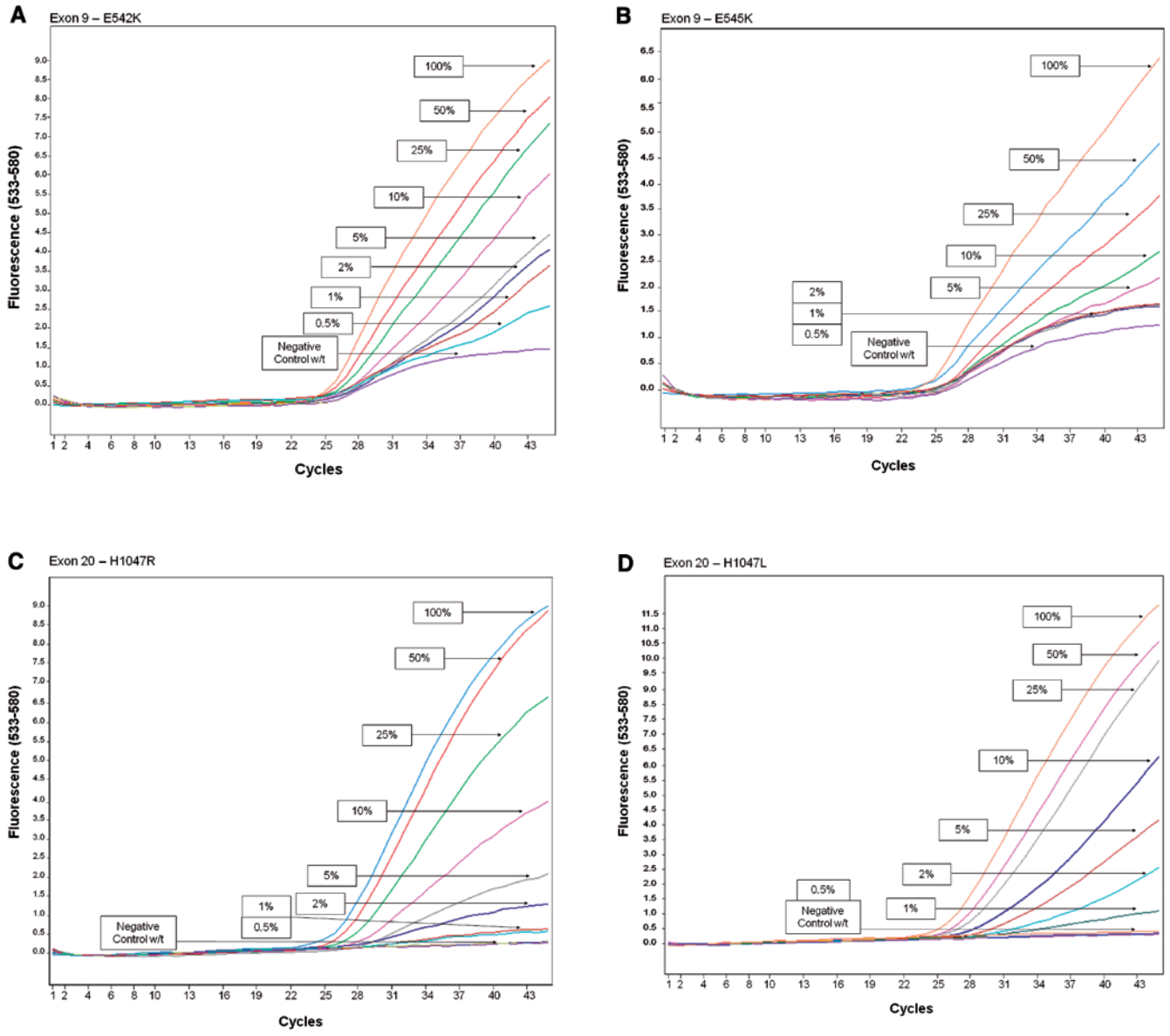

Figure 1. Sensitivity results for (A) E542K, (B) E545K, (C) H1047R and (D) H1047L mutations using PCR-ARMS.

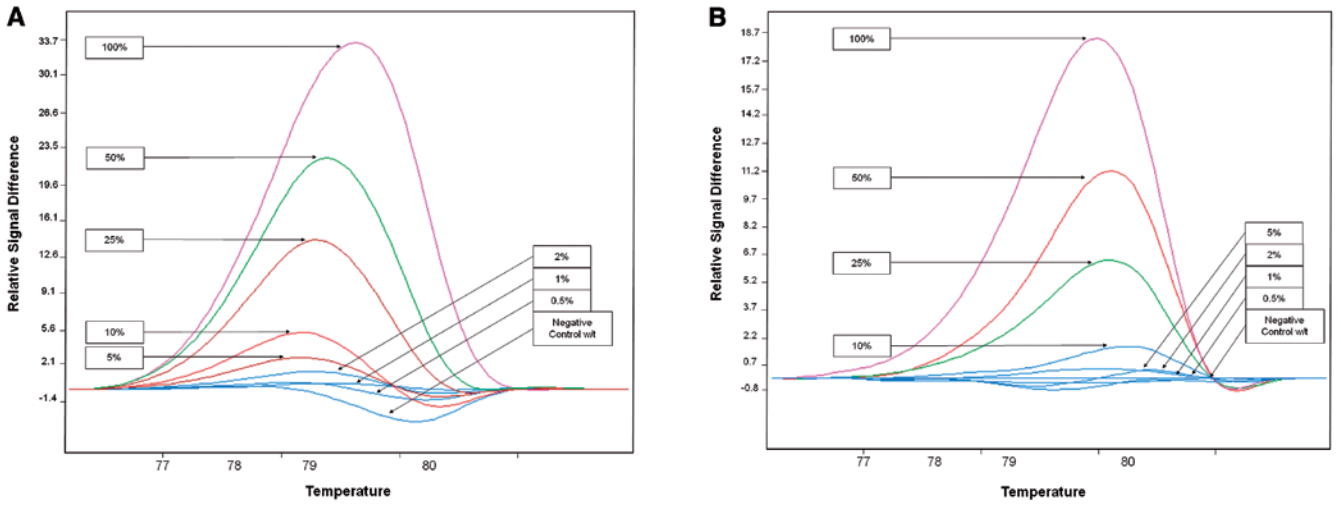

Figure 2. Sensitivity results for (A) exon 9 and (B) exon 20 mutations using PCR-HRM.

reported a decrease in survival in *PIK3CA* exon 20-mutated and HER2-positive patients. This study was retrospective and was based on a small number of cases (only 6 patients in the exon 20-mutated *PIK3CA* and HER2-positive group) and was never confirmed prospectively. Finally, in a larger series of patients, Cizkova *et al* (28) did not find any difference between exon 9- and exon 20-mutated tumors regarding metastasis-free survival. Using immunohistochemistry, Aleskandarany *et al* (29) reported that PIK3CA protein expression in invasive breast cancer was associated with poor prognosis.

The frequencies of *PIK3CA* mutations reported here, with PCR-HRM and PCR-ARMS, respectively, are fully consistent with COSMIC database values and previously published results reporting 18-40% of *PIK3CA* mutations in breast tumors (7,26,30-33).

In the present series, with a relatively limited number of cases, no significant correlation was observed between *PIK3CA* mutations and several clinical and histopathological criteria. Concerning the age at diagnosis, a higher *PIK3CA* mutation rate was noted in older patients (34) that was not observed in other series (25,26,28). The same is true for the histological type of breast cancer, when comparing ductal and lobular types. Some studies reported *PIK3CA* mutations to be more frequently observed in ductal than in lobular carcinomas (25,28,34) while others did not (35).

Although it has been reported that *PIK3CA* mutations are related to hormone receptor and HER2 status (28,34), this issue still remains controversial since no correlation between *PIK3CA* mutations and hormonal status has been observed (25,36,37) similarly to the results of the present study, or limited to estrogen receptor status, but not to progesterone receptor status (35). Similar findings have been found concerning the correlation between *PIK3CA* mutations and HER2 status (28,34) but again are controversial in other series (25,35) or observed when HER2 is determined using immunohistochemistry but not FISH (37). A higher frequency of *PIK3CA* mutation has also been noted in low SBR grade tumors similarly to the observation in the present series and consistently with other studies (28,34) but contrary with others (35).

All these discrepancies among the different studies could probably be explained by the relatively limited number of cases that were analyzed and the different techniques that were used for *PIK3CA* mutation analysis with different sensitivity limits. This also probably explains why a large range of mutation rates were reported. This point clearly justifies that consideration should be given to the validation and especially the endpoints and sensitivity limits of the assays that are routinely used.

Combining PCR-HRM and PCR-ARMS assays, several discrepancies were observed between the two techniques in 6 (5.9%) samples. All were identified as *PIK3CA* wild-type using PCR-ARMS and mutated using PCR-HRM; 5 samples with mutations on exon 9 and 1 sample with mutation on exon 20. Because our PCR-ARMS assay was designed to specifically, but only, identify the four main *PIK3CA* mutations (c.1624G→A, c.1633G→A, c.3140A→G and c.3140A→T), it is obvious that the 6 discordant cases should bear other mutations on exon 9 or 20, that were only detected using PCR-HRM which is an exon-specific method, able to detect all the somatic mutations on the entire exon 9 and 20. Exon 9 and 20 mutations, not located in the four main hotspots, were reported to represent approximately 10% of all exon 9 and 20 mutations in breast cancers (28). Our data were consistent with these values. In the present series, this rate of *PIK3CA* mutations, differing from the four main mutations identified by PCR-ARMS was found to be consistent with data recently reported in breast cancer (28).

Direct Sanger sequencing of these samples could confirm the hypothesis that they may carry another mutation but the lack of sensitivity of Sanger sequencing (approximately 20% mutant DNA) could be not discriminatory between a false-positive with HRM or a false-negative with sequencing (38).

The PCR-HRM and PCR-ARMS assays used here are highly sensitive, able to detect as low as 5% of mutated DNA for exon 9 (2.0 ng) and 10% for exon 20 (4.0 ng) and 0.5% (0.8 ng) of mutated DNA. The PCR-ARMS assay is more sensitive than pyrosequencing-based assay as previously described (38) and which was able to detect 5% of mutated DNA and even higher than another PCR-ARMS assay as previously reported (5 ng) (23). This high sensitivity could be explained by the high specificity of the primers used for this technique.

High sensitivity assays are required for routine analysis of mutations in clinical specimens. Recent results (39) achieved in colon cancer using *KRAS* mutation analysis, reported that sequencing was not sensitive enough to provide clinically relevant results yielding two many false-negative results. The authors recommended the use of more sensitive techniques with a detection limit approximating 1-2% to overcome this problem.

In conclusion, the present study highlighted the potential of PCR-HRM- and PCR-ARMS-based assays for the evaluation of the *PIK3CA* mutation status in breast cancers. No correlation was observed with patient age at diagnosis, histological type, hormone receptor and HER2 status. *PIK3CA* exon 9 and 20 mutations were found to be related to Scarff-Bloom-Richardson (SBR) grade with a lower rate of mutations and a higher frequency of exon 9 mutations in SBRI and exon 20 mutations in SBRII/III tumors. No difference was observed in the frequency of the two different mutations screened for each exon in any subcategory. Thus, we propose to use a combination of both assays, with a screening of full exon 9 and 20 using PCR-HRM and further identification of the four main mutations using PCR-ARMS. Following analysis of our data, using such a procedure would have led to only one false-negative result (<1%) which is quite satisfactory. Combining these two assays should represent a cost-effective rapid procedure that provides highly reliable results that are fully consistent with the attempts of practitioners in view of personalizing therapy for invasive breast cancers.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Engelman JA, Luo J and Cantley LC: The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet 7: 606-619, 2006.

3. Tokunaga E, Oki E, Egashira A, *et al*: Deregulation of the Akt pathway in human cancer. Curr Cancer Drug Targets 8: 27-36, 2008.
4. McAuliffe PF, Meric-Bernstam F, Mills GB and Gonzalez-Angulo AM: Deciphering the role of PI3K/Akt/mTOR pathway in breast cancer biology and pathogenesis. Clin Breast Cancer 10: S59-S65, 2010.
5. Wang L, Zhang Q, Zhang J, *et al*: PI3K pathway activation results in low efficacy of both trastuzumab and lapatinib. BMC Cancer 11: 248, 2011.
6. Jiang BH and Liu LZ: PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv Cancer Res 102: 19-65, 2009.
7. Bachman KE, Argani P, Samuels Y, *et al*: The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther 3: 772-775, 2004.
8. Karakas B, Bachman KE and Park BH: Mutation of the PIK3CA oncogene in human cancers. Br J Cancer 94: 455-459, 2006.
9. Dunlap J, Le C, Shukla A, *et al*: Phosphatidylinositol-3-kinase and AKT1 mutations occur early in breast carcinoma. Breast Cancer Res Treat 120: 409-418, 2009.
10. Orlando L, Schiavone P, Fedele P, *et al*: Molecularly targeted endocrine therapies for breast cancer. Cancer Treat Rev 36: S67-S71, 2010.
11. Miller TW, Balko JM and Arteaga CL: Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol 29: 4452-4461, 2011.
12. Abramson V and Arteaga CL: New strategies in HER2-overexpressing breast cancer: many combinations of targeted drugs available. Clin Cancer Res 17: 952-958, 2011.
13. Ma CX, Crowder RJ and Ellis MJ: Importance of PI3-kinase pathway in response/resistance to aromatase inhibitors. Steroids 76: 750-752, 2011.
14. Razis E, Bobos M, Kotoula V, *et al*: Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. Breast 128: 447-456, 2011.
15. Dave B, Migliaccio I, Gutierrez MC, *et al*: Loss of phosphatase and tensin homolog or phosphoinositol-3 kinase activation and response to trastuzumab or lapatinib in human epidermal growth factor receptor 2-overexpressing locally advanced breast cancers. J Clin Oncol 29: 166-173, 2011.
16. Esteva FJ, Guo H, Zhang S, *et al*: PTEN, PIK3CA, p-AKT, and p-p70S6K status: association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. Am J Pathol 177: 1647-1656, 2010.
17. Baselga J: Treatment of HER2-overexpressing breast cancer. Ann Oncol 21: vii36-40, 2011.
18. Garrett JT and Arteaga CL: Resistance to HER2-directed antibodies and tyrosine kinase inhibitors: mechanisms and clinical implications. Cancer Biol Ther 11: 793-800, 2011.
19. Tanaka H, Yoshida M, Tanimura H, *et al*: The selective class I PI3K inhibitor CH5132799 targets human cancers harboring oncogenic PIK3CA mutations. Clin Cancer Res 17: 3272-3281, 2011.
20. Dan S, Okamura M, Mukai Y, *et al*: ZSTK474, a specific phosphatidylinositol 3-kinase inhibitor, induces G1 arrest of the cell cycle in vivo. Eur J Cancer 48: 936-943, 2012.
21. Bibeau F, Frugier H, Denouel A, Sabourin JC and Boissiere-Michot F: Technical considerations for KRAS testing in colorectal cancer. The pathologist's point of view. Bull Cancer 96: S15-S22, 2009 (In French).
22. Vorkas PA, Poumpouridou N, Agelaki S, Kroupis C, Georgoulias V and Lianidou ES: PIK3CA hotspot mutation scanning by a novel and highly sensitive high-resolution small amplicon melting analysis method. J Mol Diagn 12: 697-704, 2010.
23. Board RE, Thelwell NJ, Ravetto PF, *et al*: Multiplexed assays for detection of mutations in PIK3CA. Clin Chem 54: 757-760, 2008.
24. Dumont AG, Dumont SN and Trent JC: The favorable impact of PIK3CA mutations on survival: an analysis of 2587 patients with breast cancer. Chin J Cancer 31: 327-334, 2012.
25. Barbareschi M, Buttitta F, Felicioni L, *et al*: Different prognostic roles of mutations in the helical and kinase domains of the PIK3CA gene in breast carcinomas. Clin Cancer Res 13: 6064-6069, 2007.
26. Lai YL, Mau BL, Cheng WH, Chen HM, Chiu HH and Tzen CY: PIK3CA exon 20 mutation is independently associated with a poor prognosis in breast cancer patients. Ann Surg Oncol 15: 1064-1069, 2008.
27. Lerma E, Catasus L, Gallardo A, *et al*: Exon 20 PIK3CA mutations decrease survival in aggressive (HER-2 positive) breast carcinomas. Virchows Arch 453: 133-139, 2008.
28. Cizkova M, Susini A, Vacher S, *et al*: PIK3CA mutation impact on survival in breast cancer patients and in ERalpha, PR and ERBB2-based subgroups. Breast Cancer Res 14: R28, 2012.
29. Aleskandarany MA, Rakha EA, Ahmed MA, *et al*: PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis. Breast Cancer Res Treat 122: 45-53, 2009.
30. Campbell IG, Russell SE, Choong DY, *et al*: Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res 64: 7678-7681, 2004.
31. Levine DA, Bogomolniy F, Yee CJ, *et al*: Frequent mutation of the PIK3CA gene in ovarian and breast cancers. Clin Cancer Res 11: 2875-2878, 2005.
32. Miron A, Varadi M, Carrasco D, *et al*: PIK3CA mutations in in situ and invasive breast carcinomas. Cancer Res 70: 5674-5678, 2010.
33. Wu G, Xing M, Mambo E, *et al*: Somatic mutation and gain of copy number of PIK3CA in human breast cancer. Breast Cancer Res 7: R609-R616, 2005.
34. Kalinsky K, Jacks LM, Heguy A, *et al*: PIK3CA mutation associates with improved outcome in breast cancer. Clin Cancer Res 15: 5049-5059, 2009.
35. Maruyama N, Miyoshi Y, Taguchi T, Tamaki Y, Monden M and Noguchi S: Clinicopathologic analysis of breast cancers with PIK3CA mutations in Japanese women. Clin Cancer Res 13: 408-414, 2007.
36. Ellis MJ, Lin L, Crowder R, *et al*: Phosphatidyl-inositol-3-kinase alpha catalytic subunit mutation and response to neoadjuvant endocrine therapy for estrogen receptor positive breast cancer. Breast Cancer Res Treat 119: 379-390, 2010.
37. Perez-Tenorio G, Alkhori L, Olsson B, *et al*: PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. Clin Cancer Res 13: 3577-3584, 2007.
38. Baker CL, Vaughn CP and Samowitz WS: A PIK3CA pyrosequencing-based assay that excludes pseudogene interference. J Mol Diagn 14: 56-60, 2012.
39. Tougeron D, Lecomte T, Pages JC, *et al*: Effect of low-frequency KRAS mutations on the response to anti-EGFR therapy in metastatic colorectal cancer. J Clin Oncol 30: 3520, 2012.