Biology of Human Tumors

Clinical Cancer Research

PIK3CA Mutational Status in Circulating Tumor Cells Can Change During Disease Recurrence or Progression in Patients with Breast Cancer

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Abstract

Purpose: Molecular characterization of circulating tumor cells (CTC) is crucial for the investigation of molecular-targeted therapies while *PIK3CA* somatic mutations play a crucial role in therapy response. We investigated the presence of *PIK3CA* mutations in CTC and whether this is associated with clinical outcome.

Experimental Design: We developed and validated an ultrasensitive methodology for the detection of *PIK3CA* mutations that is based on a combination of allele-specific, asymmetric rapid PCR and melting analysis. We analyzed *PIK3CA* hotspot mutations in: (i) a training group consisting of EpCAM-positive CTC fraction from 37 patients with clinically confirmed metastasis, and 26 healthy female volunteers and 15 primary breast tumor tissues and (ii) an independent group consisting of EpCAM-positive CTC fraction from 57 metastatic and 118 operable breast cancer patients and 76 corresponding primary tumors.

Results: The assay could detect 0.05% of mutated dsDNA in the presence of 99.95% wtDNA for both exons (9 and 20) and was highly specific (0/26 healthy donors). *PIK3CA* mutations were identified in EpCAM-positive CTC in 20 of 57(35.1%) and in 23 of 118 (19.5%) patients with metastatic and operable breast cancer, and in 45 of 76(59.2%) corresponding FFPEs. Our data indicate that *PIK3CA* mutational status in CTCs can change during disease progression and is associated with worse survival ($P = 0.047$).

Conclusions: *PIK3CA* hotspot mutations are present at a relatively high frequency in CTCs and their presence is associated with worse survival in patients with breast cancer with metastasis. Evaluation of *PIK3CA* mutational status in CTCs is a strategy with potential clinical application. *Clin Cancer Res; 20(22); 5823–34.* ©*2014 AACR.*

Introduction

Circulating tumor cells (CTC) detection and enumeration can serve as a "liquid biopsy" and an early marker of response to systemic therapy, while their molecular characterization has a strong potential to be translated to individualized targeted treatments and spare breast cancer patients unnecessary and ineffective therapies (1–3).

In early breast cancer, our group had shown the prognostic value of CTCs many years ago (4–7). Another recent prospective clinical study has shown that detection of one or more CTCs in 7.5 mL of blood before neoadjuvant chemotherapy can accurately predict overall survival (OS; ref. 8). Persistent detection of CTCs during the first 5 years of follow-up was associated with an increased risk of late disease relapse and death and indicates the presence of chemo- and hormone therapy–resistant residual disease (9), whereas a recent prospective clinical study confirmed that the presence of one or more CTCs predicted for early recurrence and decreased OS (10).

In metastatic breast cancer (MBC), CTCs represent an independent prognostic factor for progression-free survival (PFS) and OS, and the CTC enumeration assay (CellSearch, Veridex) was cleared by FDA for metastatic breast, prostate, and colorectal cancer and many groups have verified these findings (11–13). Increased numbers of CTC before the second cycle of therapy was an early predictive marker of poor PFS and OS, and could be used to monitor treatment benefit, whereas CTC decrease under treatment was stronger with targeted therapy (14). The detection of CTCs in patients with MBC before front-line therapy could define a subgroup of patients with dismal clinical outcome (15). A recent meta-analysis clearly indicated that the detection of CTCs is a reliable prognostic factor in patients with early-stage and MBC (16), whereas numerous ongoing trials

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-14-0149

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Translational Relevance

PIK3CA somatic mutations play a crucial role in response to molecular-targeted therapies and their presence in circulating tumor cells (CTC) could have important clinical applications. The detection of mutations in CTCs is challenging as CTCs are heterogeneous and cells carrying these mutations are a minority in the CTC population. In this study, we report for the first time that *PIK3CA* hotspot mutations are present at a relatively high frequency in CTCs both in metastatic and operable breast cancer and their detection in CTCs is associated with worse survival in patients with clinically confirmed metastasis, whereas *PIK3CA* mutational status in CTCs can change during disease recurrence or progression. The evaluation of *PIK3CA* mutational status on CTCs is a strategy with potential clinical applications and could have an important impact on therapeutic interventions as the presence of *PIK3CA* mutations is associated with response to molecular-targeted therapies.

evaluate CTCs as markers for early prediction of treatment efficacy (17).

Molecular characterization of CTCs can provide important information for the identification of therapeutic targets and resistance mechanisms in these cells as well as for the stratification of patients and real-time monitoring of systemic therapies (2, 18). Molecular characterization of CTCs has been explored so far at the gene expression (19–22) and DNA methylation (23, 24) level. Mutations in known driver genes, e.g., *BRAF*, *KRAS*, *PIK3CA*, or *EGFR* found in the primary tumor and metastasis were also detected in corresponding CTCs (25–29) and in plasma DNA (30, 31).

PIK3CA (PI3K catalytic subunit) somatic mutations are very frequent in many human cancers and play a crucial role in response to molecular-targeted therapies (32). Aberrant activation of the PI3K pathway correlates with a diminished response to HER2-directed therapies, as the outcome of HER2-positive patients treated with trastuzumab is significantly worse in patients with *PIK3CA*-mutated compared with wild-type tumors (33). The presence of *PIK3CA* mutations accelerates HER2-mediated breast epithelial transformation and metastatic progression, alters the intrinsic phenotype of HER2-overexpressing cancers, and generates resistance to approved combinations of anti-HER2 therapies (34). The importance of individual *PIK3CA* mutations as predictors of sensitivity and resistance to targeted therapies is leading to the use of novel PI3K/mTOR/AKT inhibitors as "personalized" treatment (35, 36).

The presence of *PIK3CA* mutations has been investigated in CTCs in only three studies so far (27–29), where a relatively low percentage of *PIK3CA* mutations was reported in CTCs and only in patients with advanced disease. However, it is very important to note that a very high heterogeneity of CTCs even among the same individuals exist (37), so even if a pure population of CTC is isolated, highly sensitive methods are needed to detect mutations that are present in a minority of this CTC population.

In this study, we investigated the presence of *PIK3CA* mutations in CTC isolated from peripheral blood of patients with breast cancer and evaluated whether *PIK3CA* mutations are associated with clinical outcome. We developed and validated an ultrasensitive and highly specific methodology for the detection of *PIK3CA* hotspot mutations (exons 9 and 20) in CTCs, based on the combination of allele-specific priming, competitive probe blocking of wild-type amplification, asymmetric PCR, and probe melting analysis (38).

We report that *PIK3CA* hotspot mutations are present at a relatively high frequency in the EpCAM-positive CTC fraction both in patients with metastatic and early breast cancer as well as in their corresponding primary tumors. Our data also suggest that *PIK3CA* mutational status can change during disease recurrence or progression in patients with breast cancer and that the presence of *PIK3CA* mutations in CTC is associated with worse survival in patients with clinically confirmed metastasis.

Materials and Methods

Cell lines

We used two gDNA samples isolated from MCF-7 (c.1633G>A: E545K; heterozygous) and T47D (c.3140A>G: H1047R; heterozygous) breast cancer cell lines as *PIK3CA*-mutant controls. MCF-7 cells were obtained by the Laboratory of Tumor Biology, University of Crete (Rethimno, Greece) and were purchased from ATCC. Authentication was done by staining experiments with antibodies for estrogen, progesterone, and HER-2 and EGFR receptors, just before the beginning of the study and spontaneously during the research. The T47D cell line was kindly donated by Dr. M. Alexis in the National Hellenic Research Foundation, (purchased by ATCC) and authentication was done with experiments on estrogen and progesterone receptor expression before the beginning of the study and spontaneously during our experiments. Both cell lines revealed the expected phenotype.

Patients

As a training group, we analyzed a total of 78 samples: (i) 63 peripheral blood samples, 37 from patients with clinically confirmed metastasis, and 26 from healthy female volunteers, used to define the specificity of the assay, and (ii) 15 primary breast tumor tissues (formalin-fixed paraffin embedded [FFPEs]). As an independent group, we obtained a total of 175 peripheral blood samples from 118 patients with operable breast cancer and 57 patients with clinically confirmed metastasis; in addition, for 76 of these patients with breast cancer (32 with metastasis and 44 with operable breast cancer), FFPEs from the primary tumor were also analyzed. For 157 of these samples, information on the expression of *CK-19* in the EpCAM-positive CTC fraction was also available through our previous studies (19, 20). Patient characteristics in respect to the presence of *PIK3CA* mutations are outlined in Supplementary Table S1. In the

independent group of the 118 patients with operable breast cancer, 9 patients relapsed and 6 died due to disease progression (median follow up: 42 months). Supplementary Table S2 indicates the chemotherapy regimens used in the front-line setting in patients with metastatic disease. In addition, patients with $HER2^+$ tumors received trastuzumab for 12 months, whereas patients with HR^+ tumors received endocrine treatment (either LH/RH analogs plus tamoxifen or aromatase inhibitors). Adjuvant radiotherapy was also administered according to the guidelines. All study participants signed an informed consent form to participate in the study, which was approved by the ethics and scientific committees of our institutions.

Positive immunomagnetic selection of CTCs

CTCs were isolated from 20 mL peripheral blood as previously described (19, 20, 23, 24).

DNA extraction from CTCs

gDNA was extracted from CTCs as previously described (23, 24). The DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer. Peripheral blood from 26 healthy female volunteers that was collected for specificity studies was processed by using exactly the same procedure as used for the samples of patients.

Primer and probe designs

All oligonucleotides were *de novo in silico* designed for each of *PIK3CA* exons 9 and 20, by using the PrimerPremier 5 software (Premier Biosoft International), and synthesized by IDT (Intergraded DNA Technologies). For each exon, we designed one allele-specific primer (matched to 1633 G>A mutation for exon 9 and to 3140 A>G mutation for exon 20), one unlabeled competitive blocking probe, and one primer for asymmetric amplification according to the study of Zhou and colleagues (38). For exon 9, primer set S1 was designed to amplify the region (70 bp) that includes the hotspot mutation of exon 9. Reverse primer (allele-specific primer) was designed to amplify the mutant allele by matching the 3′ end to the derived allele. Unlabeled probe and forward primers were designed to be matched with wild-type. Blocking probe competes with the allele-specific primer for increased sensitivity. For exon 20, primer set S2 was designed to amplify the region (104 bp) that includes the hotspot mutation of exon 20. Forward primer (allele-specific primer) was designed to amplify the mutant allele by matching the 3′ end to the derived allele. Unlabeled probe and reverse primers were designed to be matched with wild-type. Hotspot mutations were placed as close to the center of the unlabeled probe as possible. All primers and probes were designed with attention to avoiding amplification of a pseudogene on chromosome 22 that has >95% homology to exon 9 of *PIK3CA*. All primers and probes sequences are given in detail in Supplementary Table S3.

PCR and melting analysis

Real-time PCR and melting curves were obtained using the LightScanner 32 (Idaho Technology) using glass capillary tubes (Roche Applied Science). The LC-Green Plus (Idaho Technology) was used for fluorescence measurements. PCR conditions and melting analysis protocols for each exon are described in detail in Supplementary Table S4. The PCR reaction mix for each exon is described in detail in Supplementary Table S5.

Statistical analysis

Correlations between *PIK3CA* mutational status in CTCs and primary tumors were assessed by using the χ^2 test. We used Cohen kappa coefficient, a statistical measure of inter-rater agreement or inter-annotator agreement for qualitative items, for the evaluation of agreement between *PIK3CA* mutations in CTCs and primary tumors, as well as between *PIK3CA* mutations in CTCs and *CK-19* mRNA expression (39). In the group of patients with verified metastasis, PFS and OS curves were calculated by using the Kaplan–Meier method and comparisons were performed using the log-rank test. P values <0.05 were considered statistically significant. Statistical analysis was performed using the SPSS Windows version 19.0 (SPSS).

Results

The experimental flowchart of the study is outlined in Fig. 1.

Development and validation of an ultrasensitive and highly specific method for *PIK3CA* hotspot mutations

Initially, an ultrasensitive and highly specific methodology for the detection of *PIK3CA* hotspot mutations (exons 9 and 20) in CTCs was developed and validated. This assay is performed in a closed tube format and is based on the combination of allele-specific priming, competitive probe blocking of wild-type amplification, asymmetric PCR, and probe melting analysis (38). In this assay design, we enhanced allele-specific PCR sensitivity and specificity with an unlabeled competitive wild-type–specific blocking probe by asymmetric amplification and probe melting analysis. The melting analysis peak of this unlabeled competitive probe at 60°C was able to indicate the presence of *PIK3CA* mutations in both exons. The peak of the derivative melting curve of the unlabeled blocking probe and the DNA template of the WT *PIK3CA* exon 9, as amplified with the WT allele–specific primer, and the peak of the melting curve of the unlabeled blocking probe and the DNA template of the mutant *PIK3CA* exon 9, as amplified with the mutant allele–specific primer differ around 4°C in both cases (results not shown). In all our experiments, as we are targeting the mutant allele, we are detecting the mutation by the derivative melting of this unlabeled blocking probe and mutant *PIK3CA* sequence, as amplified with the mutant allele–specific primer. So, we detect a mutation only if this peak at this lower temperature 60°C is present. The other peak that is due to the PCR product and can be detected at higher temperatures can be seen for both the

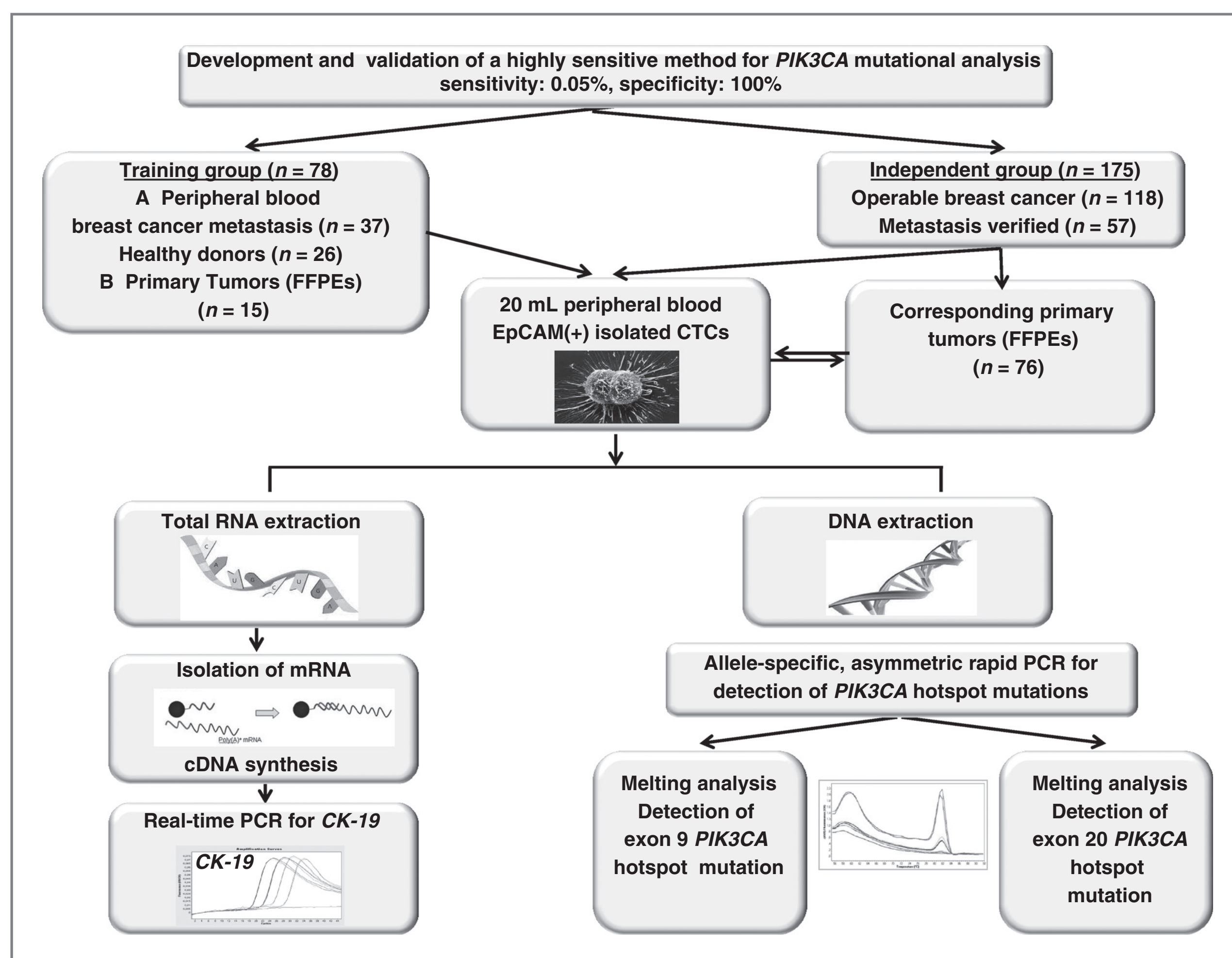

Figure 1. Experimental flowchart of the current study.

mutant and WT, in case that there is a nonspecific amplification of the WT, by using the mutant allele–specific primer.

Protocol optimization. We extensively optimized the *PIK3CA* mutation assay for both exons, using gDNA samples as positive and negative controls from cancer cell lines (MCF-7 and T47D) and wild-type (WT) gDNA isolated from healthy donors, with respect to PCR annealing temperature, Mg^{2+} concentration, primer and unlabeled probe concentration, the number of PCR cycles, duration of each asymmetric PCR step, primer ratio for asymmetric PCR and amount of target DNA, and melting analysis conditions (data not shown).

Specificity study. We evaluated the assay specificity by analyzing gDNA isolated from 26 healthy female volunteers, in exactly the same way that we followed for patients with breast cancer. The developed method is highly specific, as we did not detect these *PIK3CA* mutations in any of these samples (Fig. 2A and B). In exon 9, one of the healthy donor's gDNA (N18) was amplified by the amplification-refractory mutation system (ARMS)-PCR–specific primer and gave a peak at 77.5°C, but not at 60.0°C (Fig. 2A). This could be explained by the fact that even by using the *PIK3CA* hotspot mutation–specific primers, a very low amount of the wild-type sequence that is present at very high concentrations could be nonspecifically amplified. To avoid this, we used the unlabeled probe that plays a key role as a blocker, as it is wild-type–specific and binds at the same sequence as the mutant-specific primer. In the case of N18, this WT sequence was nonspecifically amplified and this is why we detect the melting curve peak at 77.5°C. However, we do not detect any peak at the melting curve for the unlabeled probe at 60.0°C that is specifically indicating the presence of the specific mutation that we are looking for.

Sensitivity study. The assay sensitivity was evaluated by mixing mutated gDNA from cell lines, with WT gDNA at ratios of 50%, 25%, 12.5%, 2.5%, 1.25%, 0.5%, 0.25%, 0.125%, and 0.05%. The WT gDNA samples that were used for dilutions were selected to match mutated gDNA quantity, quality, and quantification cycle (C_q), to minimize PCR bias. Melting curves were generated and the ability to discriminate melting transitions of the cell line dilutions

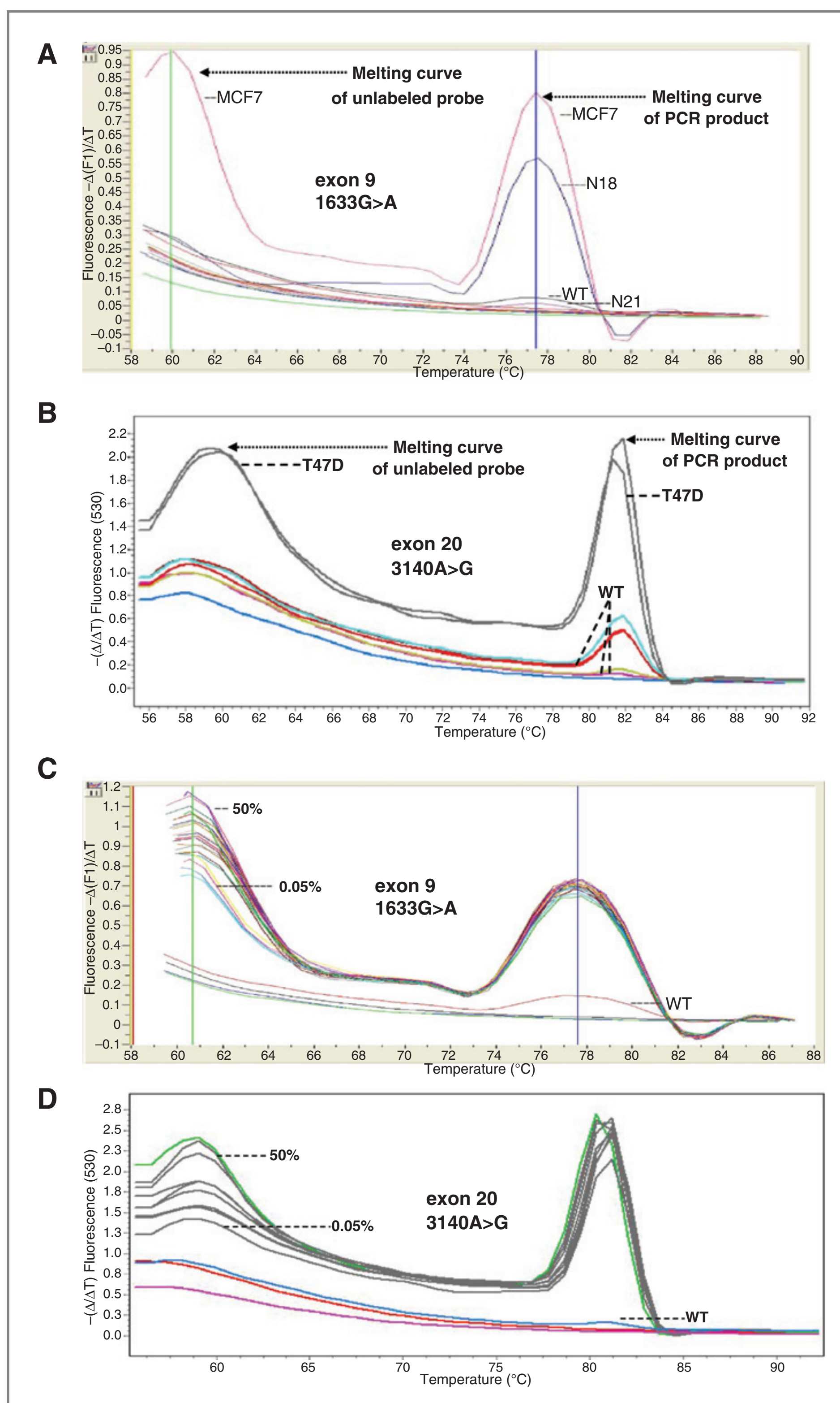

Figure 2. Specificity of the developed *PIK3CA* mutation assay: characteristic derivative melting curves obtained after PCR in the presence of unlabeled blocking probes that detect: exon 9 1633G>A, hotspot mutation (A) and exon 20 3140A>G, hotspot mutation (B). Sensitivity of PIK3CA mutation assay: characteristic derivative melting curves obtained after PCR in the presence of unlabeled blocking probes in synthetic mixtures of different percentages of mutant DNA in wild-type DNA that detect exon 9 1633G>A, hotspot mutation (C) and exon 20 3140A>G, hotspot mutation (D). Red line, PCR negative control. *PIK3CA* mutations are detected by the derivative melting of the unlabeled blocking probe and mutant *PIK3CA* sequence, as amplified with the mutant allele–specific primer. Mutations are detected only if this peak at 60°C is present. The other peak at higher temperatures is due to the PCR product and can be seen for both the mutant and WT, in case that there is a nonspecific amplification of the WT, by using the mutant allele–specific primer.

from that of WT sample was assessed. For exon 9, it was possible to clearly discriminate a dilution corresponding to 0.05% of MCF-7 cell line (Fig. 2C), whereas for exon 20, the assay could also discriminate a ratio of 0.05% of T47D cell line dilution (Fig. 2D). Melting curves were highly reproducible.

Detection of *PIK3CA* mutations in CTCs of breast cancer patients

Training group. As a training group, we analyzed 37 peripheral blood samples from patients with clinically confirmed metastasis and 15 primary breast tumor tissues (FFPEs) for *PIK3CA* mutations. We detected *PIK3CA* mutations in 6 of 37 patients (16.2%) for exon 9 and 4 of 37 patients (10.8%) for exon 20, in the EpCAM-positive CTC fractions; in total, *PIK3CA* mutations were detected in 10 of 37 (27.0%) patients with metastatic breast cancer. In FFPEs, we detected *PIK3CA* mutations in 9 of 15 (60.0%) for exon 9 and 7 of 15 (46.7%) for exon 20. There were six cases where both hotspot mutations were detected in the same FFPE sample; in total, *PIK3CA* mutations were detected in 10 of 15 (66.7%) FFPEs.

Independent group. Subsequently, the assay was evaluated in an independent group of 118 patients with operable breast cancer and 57 patients with clinically confirmed metastasis. For 76 of these patients with breast cancer, FFPEs from the primary tumor were also available.

Detection of PIK3CA mutations in CTCs of early breast cancer patients. The EpCAM-positive CTC fractions from 118 patients with operable breast cancer after the primary cancer had been removed and before adjuvant chemotherapy had been initiated were analyzed. *PIK3CA* mutations were detected in 3 of 118 (2.5%) for exon 9 (Fig. 3A) as well as in 21 of 118 (17.8%) for exon 20 (Fig. 3b). There was one case where both hotspot mutations were detected in the same CTC sample. This patient has a T1 (1.5 cm), N1 (2/18 involved lymph nodes), grade III, ER$^+$/PR$^-$/HER2$^-$ tumor. The patient received adjuvant chemotherapy consisting of 4 cycles with cyclophosphamide + epirubicin and rhG-CSF support every 2 weeks followed by 4 cycles of docetaxel every 2 weeks and rhG-CSF support. In total, *PIK3CA* mutations were detected in 24 of 118 (20.3%) of operable breast cancer patients.

Detection of PIK3CA mutations in CTCs of breast cancer patients with clinically confirmed metastasis. The EpCAM-positive CTC fractions from 57 patients with metastatic breast cancer were analyzed. From these 57 patients, 24 had bone metastasis, 3 in the liver, 2 in the brain, 9 in the lung, 3 both bone and liver, and 6 both in the lung and bone and 2 in more than two different sites. *PIK3CA* mutations were detected in 8 of 57 (14.0%) for exon 9 (Fig. 3C) and in 12 of 57 (21.1%) for exon 20, (Fig. 3D). In total, *PIK3CA* mutations were detected in 20 of 57 (35.1%) in this group of patients. The peaks at 60°C in the melting curves in Fig. 3C and D indicate the presence of exon 9 and exon 20 mutations, respectively. In this context, if no peak is detected at 60°C, the sample is considered as wild-type for the mutation examined.

Detection of *PIK3CA* mutations in corresponding primary tumors

In the independent group, the *PIK3CA* mutational status in CTCs and corresponding primary tumors was compared in 76 patients with breast cancer (32 with clinically confirmed metastasis and 44 with operable breast cancer) as for these patients corresponding FFPEs were also available (Table 1).

Exon 9, 1633G≥A. Concerning all patients, 1633G>A was observed in 38 of 76 (50%) of the primary tumor samples and in 6 of 76 (7.9%) corresponding CTC samples. For 4 patients that were carrying this *PIK3CA* hotspot mutation in their primary tumor, the identical mutation was also detected in the CTCs. In 34 patients, we identified this mutation in the primary tumor, but not in CTCs, whereas 36 patients were found negative for this mutation both in the primary tumor and in the CTCs. However, in two cases, this hotspot mutation was identified in the CTC fraction, but not in the corresponding primary tumor. In patients with operable breast cancer, 1633G>A was observed in 21 of 44 (47.7%) of FFPEs and in 1 of 44 (2.3%) corresponding CTC samples; none of the patients carrying this *PIK3CA* hotspot mutation in her primary tumor had the identical mutation in CTC. In 21 patients, this mutation was identified in the primary tumor, but not in CTCs, whereas 22 patients were found negative for this mutation both in the primary tumor and in CTCs. However, in one case, this hotspot mutation was identified in the CTC fraction, but not in the corresponding primary tumor. In patients with metastasis, 1633G>A was observed in 17 of 32 (53.1%) of FFPEs and in 5 of 32 (15.6%) corresponding CTCs. For 4 patients that were carrying this *PIK3CA* hotspot mutation in their primary tumor, the identical mutation was also detected in the CTCs. In 13 patients, this mutation was identified in the primary tumor, but not in CTCs, whereas 14 patients were found negative for this mutation both in the primary tumor and in the CTCs. However, in one case, this hotspot mutation was identified in the CTC fraction, but not in the corresponding primary tumor.

Exon 20, 3140 A≥G. Concerning all patients, 3140 A>G was observed in 13 of 76 (17.1%) of FFPEs and in 14 of 76 (18.4%) corresponding CTC samples. For 4 of 13 patients that were carrying this *PIK3CA* hotspot mutation in their primary tumor, the identical mutation was also detected in the CTCs. In 9 patients, this mutation was identified in the primary tumor, but not in CTCs, whereas 53 patients were found negative for this mutation both in the primary tumor and in CTCs. It is remarkable that in 10 patients this hotspot mutation was detected only in the CTC fraction, but not in the corresponding primary tumor. In the group of patients with operable breast cancer, 3140 A>G was observed in 5 of 44 (11.4%) of FFPEs and in 10 of 44 (22.7%) corresponding CTCs. For 2 of 5 patients that were carrying this *PIK3CA* hotspot mutation in their primary tumor, the identical mutation was also detected in CTCs. In 3 patients, this mutation was identified in the primary tumor but not in CTCs, whereas 31 patients were found negative for this mutation both in the primary tumor and in CTCs. However, in 8 patients, this hotspot mutation was detected only in CTCs, but not in corresponding FFPEs. In the group of patients with metastasis, 3140 A>G was observed in 8 of 32 (25.0%) of the primary tumor samples and in 4 of 32 (12.5%) corresponding CTCs. For 2 of 8 patients that were carrying this *PIK3CA* hotspot mutation in their primary

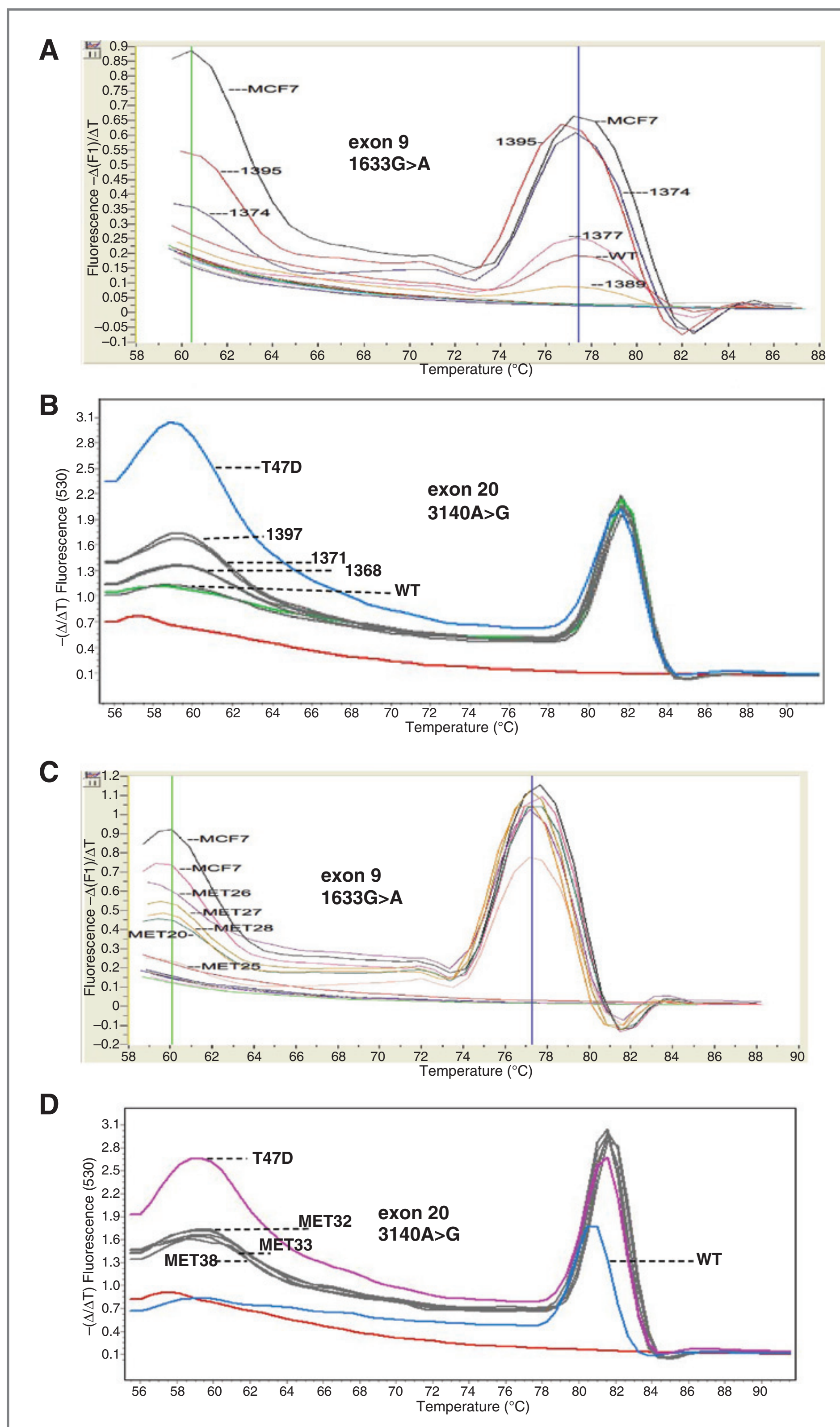

Figure 3. Detection of *PIK3CA* mutations in CTC: characteristic derivative melting curves for exon 9 1633G>A (A), hotspot mutation in patients with operable breast cancer, exon 20 3140A>G, hotspot mutation in operable breast cancer patients (B), exon 9 1633G>A, hotspot mutation in patients with clinically confirmed metastasis (C), exon 20 3140A>G, hotspot mutation in patients with clinically confirmed metastasis (D). Red line, PCR negative control.

Table 1. Independent group: *PIK3CA* mutational status in CTC and corresponding primary tumors (FFPEs)

PIK3CA mutations in primary tumors (FFPEs)	*PIK3CA* mutations in CTC exon 9, 1633G>A		*PIK3CA* mutations in CTC exon 20, 3140A>G	
Early breast cancer, ($n = 44$)				
	CTC positive	CTC negative	CTC positive	CTC negative
FFPEs				
Positive	0	21	2	3
Negative	1	22	8	31
Concordance%	22/44 = 50%		33/44 = 75%	
P	$P = 0.523$		$P = 0.317$	
Cohen kappa coefficient	$\kappa = 0.045$		$\kappa = 0.136$	
Clinically confirmed metastasis, ($n = 32$)				
	CTC positive	CTC negative	CTC positive	CTC negative
FFPEs				
Positive	4	13	2	6
Negative	**1**	14	**2**	22
Concordance%	18/32 = 56.2%		24/32 = 75%	
P	$P = 0.208$		$P = 0.254$	
Cohen kappa coefficient	$\kappa = 0.161$		$\kappa = 0.200$	
All patients, ($n = 76$)				
	CTC positive	CTC negative	CTC positive	CTC negative
FFPEs				
Positive	4	34	4	9
Negative	**2**	36	**10**	53
Concordance%	40/76 = 52.6%		57/76 = 75%	
P	$P = 0.337$		$P = 0.188$	
Cohen kappa coefficient	$\kappa = 0.053$		$\kappa = 0.145$	

tumor, the identical mutation was also detected in CTCs. In 6 patients, this mutation was identified in the primary tumor, but not in CTCs, whereas 22 patients were found negative for this mutation both in the primary tumor and in CTCs. However, in 2 patients, this hotspot mutation was detected only in CTC, but not in the corresponding primary tumor.

There was no concordance between the presence of both these hotspot *PIK3CA* mutations in primary tumors and corresponding CTC (Table 1).

PIK3CA mutation status in CTC in respect to *CK-19* mRNA expression

In the independent group, we further evaluated whether *PIK3CA* mutational status in CTC is correlated with *CK-19 mRNA* expression, for 157 of these patients (57 with clinical metastasis and 100 with early breast cancer; Table 2). In operable breast cancer, only 3 of 100 samples were positive for both *PIK3CA* mutations and *CK-19* mRNA expression, all in exon 20. There were 33 of 100 samples positive for *CK-19*, not carrying mutations in *PIK3CA*, while it is highly remarkable that *PIK3CA* hotspot mutations were identified in CTC of 17 patients who were negative for *CK-19* mRNA expression. In patients with clinically confirmed metastasis, 11 of 57 (19.3%) samples were positive both for *PIK3CA* mutations and *CK-19* expression, 6 in exon 9 and 5 in exon 20. There were 14 of 57 (24.6%) samples positive for *CK-19*, not carrying these hotspot mutations in *PIK3CA*. It is highly remarkable that *PIK3CA* hotspot mutations were identified in CTCs of 9 patients who were negative for *CK-19* mRNA expression.

These 26 samples (17 from patients with operable breast cancer and 9 from patients with clinically confirmed metastasis) that were found positive for *PIK3CA* mutations in CTC, but were negative for *CK-19 mRNA* expression, would have been characterized as CTC-negative if *PIK3CA* mutations were not detected. There was no concordance concerning the presence of both these hotspot *PIK3CA* mutations and *CK-19* mRNA expression in CTCs (Table 2).

Clinical significance of *PIK3CA* mutational status in CTCs in patients with verified metastasis

We further evaluated in the independent group the correlation between *PIK3CA* mutational status in CTCs and the clinical outcome of this relatively small group of patients with clinically confirmed metastasis. Kaplan–Meier survival analysis, performed by using patients' postoperative survival, demonstrated that patients who carried *PIK3CA* hotspot mutations on CTC ($n = 20$) had a significant shorter OS

Table 2. Independent group: comparison between *PIK3CA* mutations in CTC and *CK-19* expression

Patients	PIK3CA mutations in CTC exon 9, 1633G>A		PIK3CA mutations in CTC exon 20, 3140A>G		PIK3CA mutations in CTC At least one mutation	
Early breast cancer, ($n = 100$)						
	Positive	Negative	Positive	Negative	Positive	Negative
CK-19						
Positive	0	36	3	33	3	33
Negative	**2**	62	**15**	49	**17**	47
Concordance (%)	62/100 (62%)		52/100 (52%)		50/100 (50%)	
P	$P = 0.407$		$P = 0.049$		$P = 0.023$	
Cohen kappa coefficient	$\kappa = 0.59$		$\kappa = 0.49$		$\kappa = 0.46$	
Clinically confirmed metastasis verified ($n = 57$)						
	Positive	Negative	Positive	Negative	Positive	Negative
CK-19						
Positive	6	19	5	20	11	14
Negative	**2**	30	**7**	25	**9**	23
Concordance%	36/57 (63.2%)		30/57 (52.6%)		44/57 (77.2%)	
P	$P = 0.073$		$P = 0.564$		$P = 0.167$	
Cohen kappa coefficient	$\kappa = 0.61$		$\kappa = 0.48$		$\kappa = 0.52$	
All patients, ($n = 157$)						
	Positive	Negative	Positive	Negative	Positive	Negative
CK-19						
Positive	6	55	8	53	14	47
Negative	4	92	**22**	74	**26**	70
Concordance%	98/157 (62.4%)		82/157 (52.2%)		84/157 (53.5%)	
P	$P = 0.140$		$P = 0.093$		$P = 0.350$	
Cohen kappa coefficient	$\kappa = 0.61$		$\kappa = 0.48$		$\kappa = 0.48$	

than those without ($n = 37$; $P = 0.047$, log-rank test; Fig. 4). However, the presence of *PIK3CA* hotspot mutations in CTCs was not correlated with PFS.

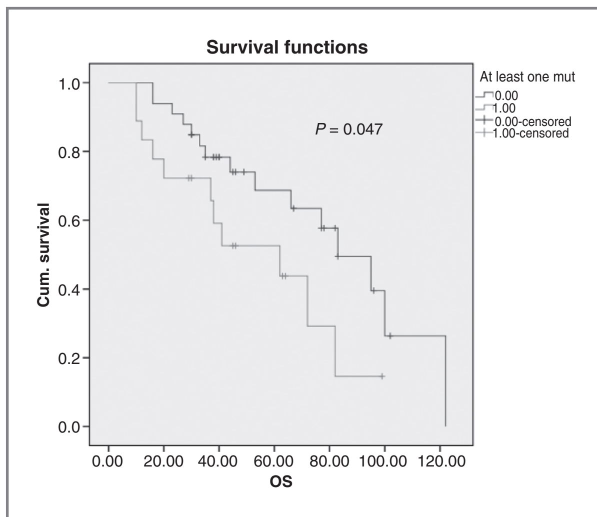

Figure 4. Kaplan–Meier estimates of OS in months for patients with breast cancer with clinically confirmed metastasis, with respect to *PIK3CA* mutational status in CTCs.

Discussion

The clinical relevance of analyzing the *PIK3CA* genotype on CTCs could be very important, as the presence of *PIK3CA* mutations is correlated with drug resistance, especially against HER2-targeted therapy (32–36). Mutations in known driver genes, for example, *BRAF*, *KRAS*, *PIK3CA*, or *EGFR* found in the primary tumor and metastasis were also detected in corresponding CTCs. First, Maheswaran and colleagues have shown that patients with lung cancer who have CTCs harboring the T790M *EGFR* mutation, which is known to cause resistance to tyrosine kinase inhibitors, had faster disease progression compared with patients who have CTCs lacking this specific mutation (25). Moreover, in CTCs of patients with colorectal cancer, genetic alterations were found in *EGFR*, *KRAS*, and *PIK3CA* (26, 27)

However, mutations that are present in very low levels in clinical tumor samples often reside below mutation detection limits, thus leading to false negative that may impact clinical diagnosis and patient management (40). A variety of highly sensitive mutation detection assays have been developed to overcome these limitations. Combination of allele-specific PCR, competitive probe blocking and melting analysis (38), COLD-PCR (40), high-resolution melting analysis (HRMA; ref. 41), locked nucleic acid (LNA)-PCR

sequencing (42), and PCR-HRM and PCR-ARMS (43) can detect low levels of mutant DNA and have superior sensitivity than the standard Sanger sequencing approach. Recently, a simple, sensitive, and fully automated molecular diagnostic system was developed for *KRAS*, *BRAF*, and *PI3KCA* somatic mutations (44), whereas another assay was shown to detect a wide range of mutations in therapeutically relevant genes from very small amounts of sample DNA (45).

Especially, to get reliable information for the molecular characterization of CTCs, sensitivity, specificity, and robustness of the mutation detection systems used is extremely important. We have previously developed a highly sensitive method for *PIK3CA* mutations based on HRMA (41). Despite the fact that this method is much more sensitive (1%) than the traditional Sanger sequencing, when we applied it in our CTC samples, we failed to detect CTC mutations as we initially expected.

We thus developed and validated a novel method for *PIK3CA* hotspot mutations that is characterized by extreme sensitivity (0.05%) and high specificity (100%). We report that by using this method, we could detect *PIK3CA* mutations in CTCs isolated from early breast cancer patients at a very high percentage (20.3%). Moreover, we detected *PIK3CA* mutations in CTCs isolated from patients with clinically confirmed metastasis at a much higher percentage (35.1%) than reported (27–29). Schneck and colleagues recently reported on the development of a novel methodology for analyzing the mutational status of *PIK3CA* in CTCs isolated with the Cellsearch platform from patients with MBC (29). They report that they found *PIK3CA* hotspot mutations in 7 of 44 (15.9%) patients. These differences could be explained by the higher sensitivity of our method with respect to the methods used so far for detecting *PIK3CA* mutations in CTCs (27–29).

The main reason that we decided to develop such a sensitive protocol is that by using this method, *PIK3CA* mutations could be detected even in the presence of an excess of leucocytes, as we know that EpCAM-based isolation methods are also capturing nonspecific leucocytes.

According to our findings, there was one case where both *PIK3CA* hotspot mutations were detected in the same CTC sample. Double mutations in *PIK3CA* are a rare phenomenon (31, 46, 47). Cases with apparent double mutations could present one cell population, uniformly harboring both mutations, or alternatively, could represent heterogeneity within the population in which subpopulations each contain one of the mutations.

Our findings suggest that *PIK3CA* mutational status can change during disease recurrence or progression in patients with breast cancer. When we compared *PIK3CA* mutational status in CTCs and corresponding primary tumors in a subgroup of 76 patients, we observed that the same mutation was present both in the primary tumor and in CTCs in a minority of samples. In most patients, we identified the mutation in the primary tumor but not in CTCs, whereas many patients were negative for this mutation both in primary tumor and in CTCs. However, there were 12 cases where we identified hotspot mutations in CTCs, but not in the corresponding primary tumor; in two patients, we identified exon 9, 1633G>A hotspot mutation in CTCs, but not in corresponding FFPEs, whereas in 10 patients we identified exon 20, 3140 A>G hotspot mutation in CTCs, but not in corresponding primary tumor. Similar findings, reflecting the heterogeneity of CTCs have been reported. By using array-comparative genomic hybridization and next-generation sequencing, Heitzer and colleagues have recently shown that *PIK3CA* mutations found in the primary tumor and metastasis were also detected in corresponding CTCs; however, they also observed mutations exclusively in CTCs (28). Fabbri and colleagues evaluated the presence of *KRAS* mutations in primary tumors and corresponding CTCs, and reported a mutational concordance in 8 of 16 (50.0%) patients. However, they also reported that wild-type CTCs were found in patients with mutations in the primary tumor, that *KRAS*-mutated CTCs were found in one patient harboring a wild-type *KRAS* primary tumor and that different mutations were present in three groups of CTCs recovered from the same patient (48). Finally, Bai and colleagues have already shown that first-line chemotherapy may influence EGFR mutation status in both tissue and peripheral blood samples (49).

Another important observation of our study was that a significant number of patients (17/118 in the operable breast cancer group and 9/57 in the metastasis verified group) that were negative for *CK-19 mRNA* expression were carrying *PIK3CA* hotspot mutations in CTCs. These patients would have been characterized as CTC-negative if *PIK3CA* mutations were not detected. Molecular characterization of CTCs has demonstrated that CTC are highly heterogeneous. This could be, at least part, attributed to epithelial–mesencymal transition and mesencymal–epithelial transition; refs. 1–3, 21). In this context, we did not expect that all CK-19–positive CTCs would be *PIK3CA* mutation–positive, neither that all our samples that are CK-19–negative would not carry *PIK3CA* mutations in CTCs. Our CK-19 real-time PCR assay is very specific and sensitive, and we have already demonstrated in previous studies its clinical significance (4–7, 9). However, there is always a number of patients that do not relapse even if they are CK-19–positive, or do relapse even if they are CK-19–negative, and the same has been shown even when using the FDA-cleared CellSearch system, a clear indication that one marker is not a panacea and not enough to verify the presence of a malignant CTC population in our samples.

A striking finding is also that the presence of *PIK3CA* mutations in CTCs is associated with worse survival in metastatic patients. To the best of our knowledge, this is the first time that the presence of gene mutations in CTCs is correlated with patient survival, in any type of cancer. Matching patients who have cancers with activating mutations in the PI3K signaling pathway to phase I protocols testing PI3K inhibitors have improved response rates and survival (50). In respect to this, our findings that *PIK3CA* mutations can be exclusively present in CTCs, while absent in the primary tumor, can be beneficial for patients, as was

recently shown in a pilot prospective study where administration of herceptin was based on the presence of *CK-19 mRNA*–positive CTCs (51). Mutations in the PI3K/AKT signaling pathway, which is frequently deregulated in tumor cells, have been recently identified in breast cancer stem cells that are thought to have a central role in the initiation, progression, and clinical response of breast cancer (52).

In conclusion, we report for the first time that (i) *PIK3CA* hotspot mutations are present at a relatively high frequency in the *EpCAM*–positive CTC fraction both in metastatic and early breast cancer patients; (ii) a group of patients that were negative for *CK-19 mRNA* expression were carrying *PIK3CA* hotspot mutations in CTC; (iii) *PIK3CA* mutational status can change during disease recurrence or progression in breast cancer; and (iv) most importantly, the presence of *PIK3CA* mutations in CTC is associated with worse survival in patients with clinically confirmed metastasis. In this context, the evaluation of *PIK3CA* mutational status on CTC is a strategy with potential clinical application.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: V. Georgoulias, E. Lianidou
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Markou, V. Georgoulias, E. Lianidou
Writing, review, and/or revision of the manuscript: A. Markou, V. Georgoulias, E. Lianidou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Lianidou
Study supervision: E. Lianidou

Acknowledgments

The authors thank IDAHO technology for kindly donating the LS32 High Resolution Melting Analysis instrument to our laboratory. The authors would also like to thank Dr. G. Kalergi (Laboratory of Tumor Biology, University of Crete, Rethimno, Greece) and Dr. M. Alexis (National Hellenic Research Foundation, Athens, Greece) for kindly donating the MCF-7 and T47D cell lines, respectively.

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Received January 21, 2014; revised August 1, 2014; accepted August 1, 2014; published online November 14, 2014.

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