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Report

A Functional Genetic Approach Identifies the PI3K Pathway as a Major Determinant of Trastuzumab Resistance in Breast Cancer

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SUMMARY

A large-scale RNA interference screen to discover genes involved in trastuzumab resistance in breast cancer identified only *PTEN* as a modulator of drug sensitivity. Oncogenic mutants of *PIK3CA* (activator of the same pathway and frequently mutated in breast cancer) also conferred resistance to trastuzumab in cell culture. In a cohort of 55 breast cancer patients, activation of the PI3K pathway, as judged by the presence of oncogenic *PIK3CA* mutations or low *PTEN* expression, was associated with poor prognosis after trastuzumab therapy, and the combined analysis of PTEN and PIK3CA identified twice as many patients at increased risk for progression compared to PTEN alone. Thus, assessment of PI3K pathway activation may provide a biomarker to identify patients unlikely to respond to trastuzumab-based therapy.

INTRODUCTION

The mechanism by which trastuzumab exerts its antitumor activity is not fully understood. Trastuzumab has been suggested to induce antibody-dependent cellular cytotoxicity (ADCC) (Clynes et al., 2000), inhibit HER2 extracellular domain cleavage (Molina et al., 2001), or inhibit PI3K/AKT survival signaling, either by downregulating HER2 signaling (Yakes et al., 2002) or by increasing PTEN membrane localization and phosphatase activity, leading to a decline in PI3K/AKT pathway activation and inhibition of proliferation (Nagata et al., 2004). In addition, activation of HER-related receptors, such as HER3, or non-HER receptors, such as insulin-like growth factor I receptor, have been suggested in preclinical studies to increase PI3K/AKT signaling thereby limiting trastuzumab efficacy (Lu et al., 2001; Sergina et al., 2007). In spite of this, it is still largely unclear why almost half of the breast cancer patients that overexpress HER2 are initially nonresponsive to trastuzumab-based therapy even when combined with chemotherapy or eventually become resistant to trastuzumab during treatment (Cobleigh et al., 1999; Slamon et al., 2001; Vogel et al., 2002). An understanding of the resistance mechanisms would stimulate the development of rational drug combinations to circumvent resistance and allow better selection of patients likely to respond.

SIGNIFICANCE

Availability of biomarkers that predict responses to cancer therapy is instrumental to the rational use of cancer drugs in the clinic. Elucidating the molecular mechanism of primary or acquired drug resistance can be critical to identify patients that fail to respond to therapy and may help design more efficient treatment protocols. We present here a method to identify biomarkers associated with nonresponsiveness to cancer drugs in cell culture and demonstrate that these biomarkers have predictive value in a patient cohort treated with this drug, thus validating the approach.

RESULTS

As an unbiased approach to identify genes involved in trastuzumab resistance, we used a large-scale RNA interference genetic screen in the *HER2*-overexpressing breast cancer cell line BT-474. We have previously described the generation of a library of 24,000 shRNA retroviral vectors targeting some 8000 human genes for suppression by RNA interference as well as a technology to rapidly screen such libraries, named siRNA barcode screening (Berns et al., 2004; Brummelkamp et al., 2006). In short, this technology allows one to identify shRNAs that are enriched in a population based on the relative abundance of a "barcode" identifier (a unique 19-mer DNA sequence) in the vector, which is measured on a DNA microarray that carries the 24,000 different barcode sequences. BT-474 cells respond to trastuzumab predominantly by a reduction in proliferation rate rather than apoptosis or complete proliferation arrest (Figure 1A). To identify genes whose suppression by shRNA cause resistance to trastuzumab, BT-474 cells were infected with the shRNA library and selected for the presence of the shRNA vectors with puromycin. After selection, cells were split into two populations and plated at low density. One population was left untreated and was used as a reference while the other was exposed to 10 μg/ml trastuzumab. After 4 weeks, cells were harvested, genomic DNA isolated, and shRNA cassettes were recovered by PCR amplification and hybridized to DNA microarrays as described (Berns et al., 2004) (Figure 1B). We combined the data from five independent trastuzumab barcode screens and analyzed the relative abundance of the recovered shRNAs. Figure 1C shows the relative abundance of the shRNA vectors in the trastuzumab-treated population as compared to the untreated population. We selected the top 5 shRNA vectors with the highest enrichment by trastuzumab selection (*IDE*, *NBS1*, *PTEN*, *NRP1*, and *ZAP70*), of which the shRNA targeting the *PTEN* tumor suppressor gene was most prominently enriched (marked with an arrow). When tested in second round selection, only the vector targeting *PTEN* conferred resistance to trastuzumab. Importantly, a second, independent shRNA knocking down *PTEN* expression (Kortlever et al., 2006) also conferred resistance to trastuzumab, effectively ruling out the possibility that "off target" effects of the shRNA vectors caused the resistance phenotype (Figure 2A).

Our finding that knockdown of PTEN in BT-474 cells decreases sensitivity to trastuzumab is consistent with earlier findings which demonstrated that PTEN loss is associated with resistance to trastuzumab-based therapy (Nagata et al., 2004). Importantly, our observation that of the 8000 genes tested, only knockdown of PTEN conferred resistance to trastuzumab suggests that the PTEN pathway plays a dominant role in trastuzumab resistance. However, we cannot eliminate the possibility that other factors were missed in our screen due to false negative results of the shRNA vectors. Since loss of PTEN is only observed in a fraction of breast cancers, it is unlikely that loss of PTEN alone explains the frequent primary and acquired nonresponsiveness to trastuzumab observed in the clinic. Loss-of-function mutations in *PTEN* or decreased *PTEN* expression result in hyperactivation of the PI3K pathway. Significantly, activating mutations in the gene encoding the p110α catalytic subunit of PI3K (*PIK3CA*) have been identified in some 25% of primary breast cancers potentially mimicking the effects of *PTEN* loss (Saal et al., 2005). The majority of these mutations reside in two hotspots in exon 9 and 20 and it has been demonstrated that the two most common mutations (E545K and H1047R) result in increased PI3K pathway signaling (Isakoff et al., 2005).

To test whether activation of the PI3K pathway upstream of PTEN results in trastuzumab resistance, we retrovirally transduced BT-474 cells with a constitutively active mutant of *PIK3CA*, caPIK3CA (p110αCaaX). Figure 2A shows that expression of this mutant rendered BT-474 almost completely insensitive toward trastuzumab. Furthermore, expression of *PIK3CA*(WT) and the breast cancer-derived mutant *PIK3CA*(H1047R) also conferred resistance to trastuzumab in SK-BR3 cells (Figure 2B) and in BT-474 cells (data not shown). As expected, the oncogenic mutant displays higher PI3K activity than wild-type PIK3CA as measured by AKT S473 phosphorylation status (Figure 2C). However, a small increase in PI3K signaling through overexpression of PIK3CA(WT) is already sufficient to completely counteract the growth inhibitory effects of trastuzumab in cell culture as was further shown by analysis of the proliferation rates of BT474 cell lines expressing PIK3CA(WT) (Figure 2D). These findings are consistent with a major role of the PI3K pathway in the development of resistance to trastuzumab.

The high frequency of activating mutations in *PIK3CA* (around 25%; Saal et al., 2005) in breast cancer, together with our observation that activated PI3K signaling induces strong trastuzumab resistance in cell culture, led us to investigate whether PI3K pathway activity, as assessed by cancer-associated mutations of *PIK3CA* or altered levels of PTEN, was able to predict trastuzumab resistance in the clinic. For this, we made use of samples from two series of *HER2*-overexpressing patients with metastatic breast cancer treated at the Antoni van Leeuwenhoek Hospital (n = 34) and the M.D. Anderson Cancer Center (n = 21). These 55 patients received trastuzumab monotherapy (n = 6), or trastuzumab in combination with a chemotherapy regimen (n = 49). Both the PTEN expression levels (using immunohistochemical analysis) and the *PIK3CA* mutation status (by direct sequencing or SNP-based analysis) were evaluated in this study and correlated to the therapeutic response to trastuzumab-based therapy (see Experimental Procedures and Figures S1 and S2 in the Supplemental Data available with this article online).

We observed reduced PTEN expression in 22% (11 out of 51; four samples could not be scored) of the tumors examined (Table S1). Kaplan-Meier survival curves were generated based on clinical follow-up data on time to progression after initiation of the trastuzumab-based treatment. Figure 3A shows that patients with PTEN low tumors

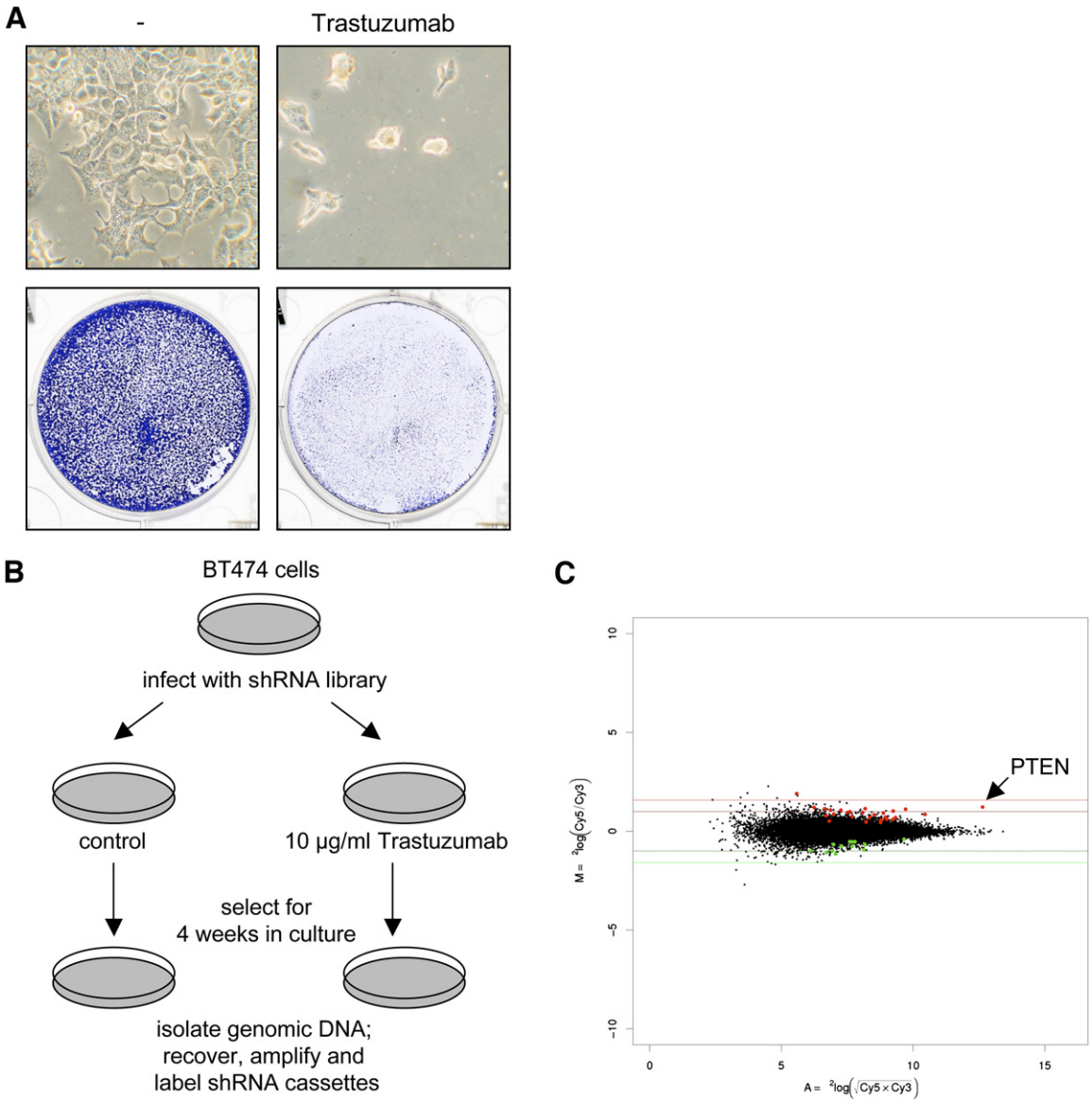

Figure 1. shRNA Barcode Screen Identifies PTEN as a Modulator of Trastuzumab Efficacy

(A) BT-474 cells undergo a stable proliferation arrest upon trastuzumab treatment. One hundred thousand BT-474 cells were seeded and cultured for 10 d in absence or presence of 2 μg/ml trastuzumab, after which cells were photographed, fixed, and stained.

(B) Schematic outline of the trastuzumab resistance barcode screen performed in BT-474 cells. High titer shRNA library polyclonal virus was produced and used to infect BT-474 cells. Infected cells were left untreated (control) or were treated with 10 μg/ml trastuzumab. After 4 weeks of selection, shRNA cassettes were recovered from both control and treated populations and labeled as described (Berns et al., 2004).

(C) Analysis of the relative abundance of the recovered shRNA cassettes from trastuzumab barcode experiment. Data were normalized and 2log transformed. Depicted is the average of five independent experiments. All hybridizations were performed with reverse color swap.

have a somewhat worse progression-free survival, although this is not statistically significant ($p = 0.127$). Subsequent *PIK3CA* sequence analysis of the 55 tumor samples identified 10 mutations in exon 20 (H1047R) and 4 in exon 9 (E542K and E545K) corresponding to a *PIK3CA* mutation frequency of 25%, in agreement with the published frequency of *PIK3CA* mutations in breast cancer (Saal et al., 2005; Table S1). Interestingly, 10 of 14 of the *PIK3CA* mutations were identified in PTEN high tumors (for three PIK3CA mutations PTEN data were missing), which is in agreement with the finding that PTEN loss and *PIK3CA* mutation are rarely present in the same tumor in breast cancer (Saal et al., 2005). Apparently, abrogation of either PTEN expression or oncogenic *PIK3CA* mutation relieves the selective pressure to target the other. We then determined whether activation of the PI3K pathway by oncogenic *PIK3CA* mutation would predict trastuzumab-based treatment outcome. The Kaplan-Meier survival

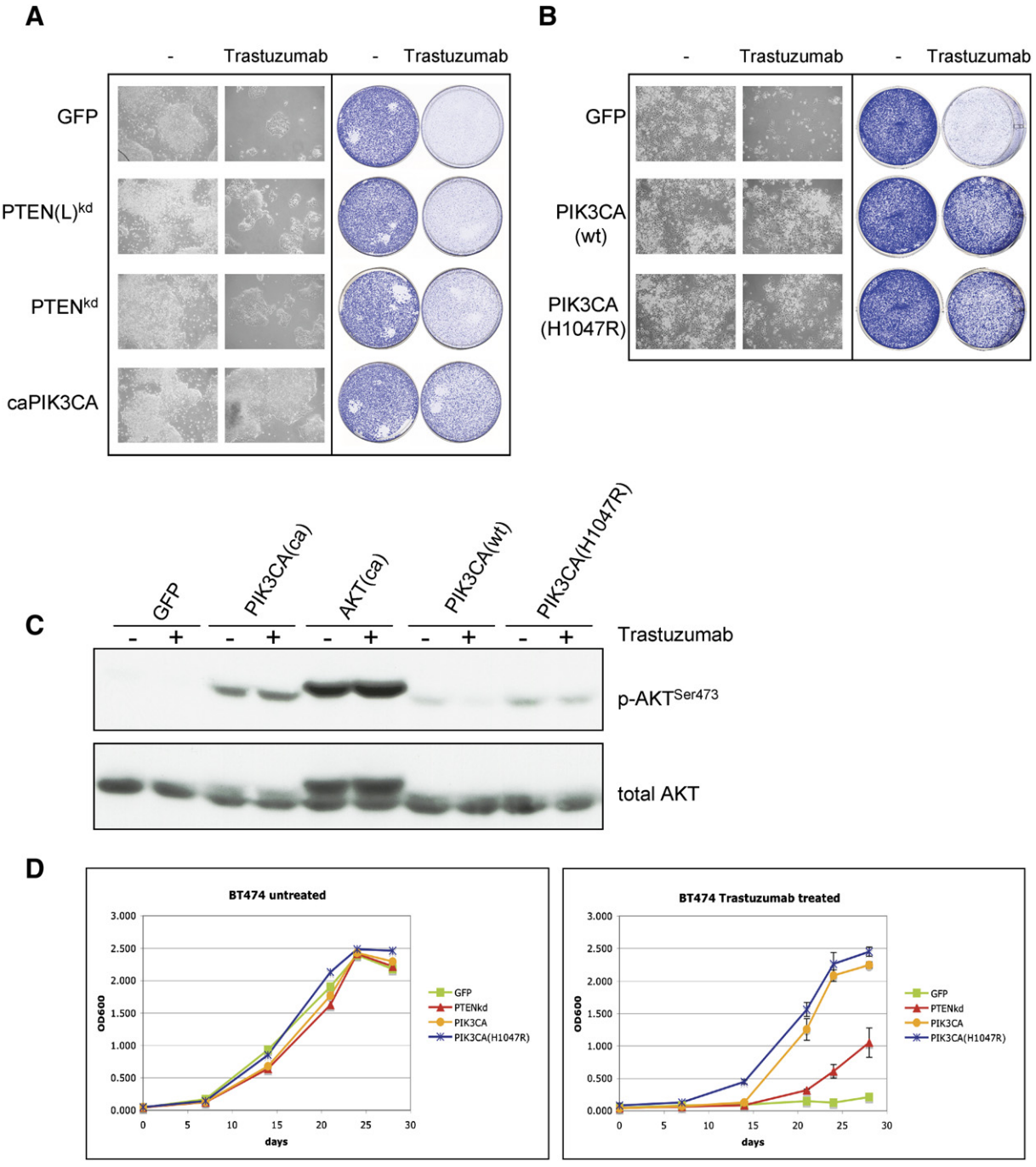

Figure 2. PTEN Downregulation and Active PI3K Signaling Confer Trastuzumab Resistance in Cell Culture

(A) Knockdown of PTEN confers resistance to trastuzumab arrest in BT-474 cells. The NKi library vector targeting PTEN (L), a second independent shRNA vector targeting PTEN, and a constitutive active PIK3CA mutant were introduced into BT-474 cells. Infected cells were left untreated (−) or incubated with trastuzumab (10 μg/ml). After 3 weeks cells were photographed, fixed, and stained.

(B) SK-BR3 cells were infected with PIK3CA(WT) and PIK3CA(H1047R) retroviral vectors and cultured in the absence (−) or presence of 10 μg/ml trastuzumab for 3 weeks, after which cells were photographed, fixed, and stained.

(C) Phospho AKT western analysis of SK-BR3 cell lines stably infected with indicated retroviral vectors. Lysates were generated from cells cultured in the absence (−) or presence (+) of 10 μg/ml trastuzumab overnight.

(D) Growth curves of stably infected BT474 cells that were cultured for 4 weeks in the absence and presence of 2 μg/ml trastuzumab. Cell numbers were quantified as described in the Experimental Procedures. Growth curves were performed multiple times in triplicate with error bars depicting the mean ± SD.

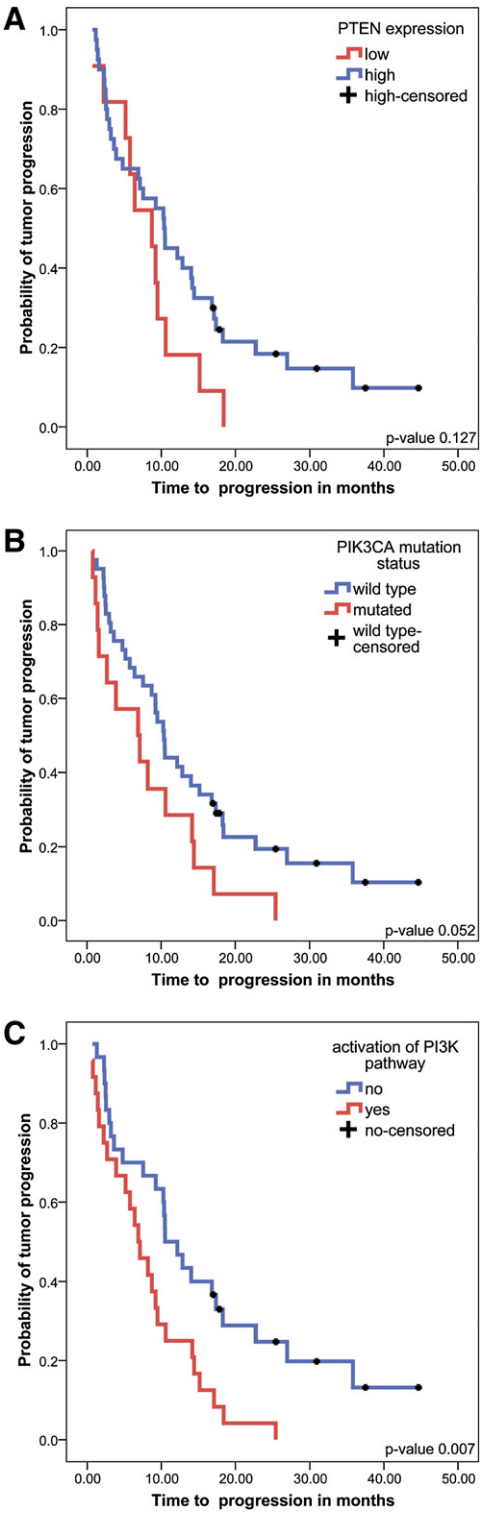

Figure 3. Kaplan-Meier Survival Curves for Trastuzumab-Treated HER2-Positive Patients

(A) Patients from both cohorts were divided into PTEN low (IRS 0–3) and PTEN high (IRS 4–12) based on IHC and semiquantitation of PTEN levels using immunoreactive scores (IRS; see the Experimental Procedures). Time to progression was calculated from start date of trastuzumab-based treatment until time of first progression of disease.

(B) Progression-free survival for patients with a hotspot PIK3CA mutation and for patients with no mutation in exon 9 or 20.

(C) Based on the PTEN scores and PIK3CA mutation data, patient groups were divided in activated PI3K pathway (PTEN low or oncogenic mutation in PIK3CA) and not-activated pathway (PTEN high + wild-type PIK3CA).

curve in Figure 3B indeed demonstrates shorter progression-free survival among patients with mutation-positive tumors, which has a borderline statistical significance ($p = 0.052$). However, the *PIK3CA* wild-type tumors are contaminated with 25% (10 out of 40) PTEN low tumors, which appear associated with shorter time to disease progression (Figure 3A). Since PTEN loss and *PIK3CA* mutation both contribute to PI3K pathway activation, we classified the patients in two groups having either "activated" PI3K pathway (PTEN low or PIK3CA mutants; $n = 24$) and "not-activated" PI3K pathway (PTEN high + PIK3CA WT; $n = 29$) and generated Kaplan-Meier survival plots. The curves shown in Figure 3C demonstrate that patients with an activated PI3K pathway due to either PTEN loss or *PIK3CA* mutation have a significantly shorter progression-free survival following trastuzumab-based treatment than patients without PTEN loss or *PIK3CA* mutation. Importantly, the progression-free survival difference between the two groups only reached statistical significance ($p = 0.007$) when both events (PTEN loss or *PIK3CA* mutation) were considered together. Hazard ratios based on multivariate Cox regression analysis with age as time scale, stratified for center and adjusted for ER status, indicate that PI3K pathway status is an independent significant risk factor for disease progression ($HR = 1.9$, $p = 0.048$; Table 1). Hazard ratios for the separate risk factors PTEN loss or *PIK3CA* mutation were not significant (Table 1). These data suggest that combined PTEN expression and PIK3CA hotspot mutation analysis may serve as an important predictor for risk for progression after trastuzumab-based therapy. There was no evidence of heterogeneity of the effects of PTEN and PIK3CA between the two centers.

DISCUSSION

We identified the tumor suppressor *PTEN* in an unbiased genome-wide RNAi screen for genes that modulate resistance to trastuzumab and demonstrate the importance of PI3K pathway activation as a biomarker in a cohort of trastuzumab-treated breast cancer patients. This study illustrates the power of in vitro RNAi screens combined with confirmation on patient samples to identify biomarkers useful for predicting treatment response in the clinic.

Significant loss of PTEN expression is seen in some 20%–25% of HER2 positive breast cancers (Fujita et al., 2006; Nagata et al., 2004; Saal et al., 2005; and our data). Activating mutations in *PIK3CA* have also been found in approximately 25% of primary breast cancers and these occur almost exclusively in the PTEN positive samples (Saal et al., 2005; and our data). Since both

Table 1. Multivariate Cox Regression Analysis: Individual and Joint Effects of PTEN Expression and PIK3CA Mutation on Time to Progression

	n (Patients)	n (Events)	HR[a]	95% CI	p
PTEN high	39	34	1.0		
PTEN low	11	11	1.5	0.7–3.3	0.300
PIK3CA WT	40	34	1.0		
PIK3CA mutant	14	14	1.6	0.8–3.3	0.210
Not-activated PI3K pathway	29	24	1.0		
Activated PI3K pathway	24	24	1.9	1.0–3.6	0.048

HR, hazard ratio; CI, confidence interval; WT, wild-type; Not-activated PI3K pathway, PTEN high + PIK3CA wild-type; Activated PI3K pathway, PTEN low or PIK3CA mutant.
[a] Based on Cox regression with age as time scale, stratified for center, and adjusted for ER status.

loss of PTEN and oncogenic mutation in *PIK3CA* lead to activation of PI3K/AKT signaling (Isakoff et al., 2005) one would expect that both genes can contribute to prediction of response to trastuzumab. Indeed, our present data provide evidence that *PIK3CA* mutations contribute to increased risk for progression (Figure 3B). In our analyses PTEN status or *PIK3CA* mutation status alone had only limited ability to predict prognosis after trastuzumab treatment (Figure 3A). However, combined analysis of PTEN status and *PIK3CA* status not only identified twice as many patients at increased risk for disease progression, but the combined analysis also reached statistical significance as a biomarker for prognosis after trastuzumab therapy (Figure 3C). Our data therefore indicate that assessment of both *PIK3CA* mutation status and PTEN expression level likely reflecting pathway activation status is required for optimal prediction of disease progression after trastuzumab therapy in *HER2* amplified breast tumors. A recent study has identified the presence of activating mutations in AKT1 in breast cancer (Carpten et al., 2007). However, none of the 55 breast cancer tumor samples studied here carried this mutation (unpublished data), suggesting that *AKT1* mutation was not a major player in modulating trastuzumab responsiveness in this patient cohort. It should be pointed out that most patients analyzed here received a combination of trastuzumab and chemotherapy, which may confound the results of this study.

The present study highlights the central importance of PI3K signaling in risk for progression after trastuzumab-based therapy, which in turn suggests combination therapeutic strategies to treat trastuzumab unresponsive breast cancer or to prevent emergence of resistance. Our data merit the design of larger scale prospective clinical trials to validate the use of PI3K pathway activation as a biomarker for trastuzumab resistance in the clinic.

EXPERIMENTAL PROCEDURES

shRNA Barcode Screen

BT-474 cells were infected with retroviruses representing the complete NKi RNAi library, selected with puromycin (2.0 μg/ml) and plated into two populations at low density. One population was left untreated, while the other population was cultured in 10 μg/ml trastuzumab. After 4 weeks in culture the treated and untreated populations were collected. Genomic DNA was isolated with the use of DNAzol (Life Technologies). The shRNA inserts were amplified from genomic DNA by PCR. Purified PCR products were used for linear RNA amplification, and purified RNA probes were labeled with cyanine-3 (Cy3) or cyanine-5 (Cy5) fluorescent groups (Kreatech). Labeled RNA probes from untreated and trastuzumab-treated cells were combined and hybridized to oligonucleotide arrays as described (Berns et al., 2004). Quantification of the resulting fluorescent images was performed with Imagene 5.6 (BioDiscovery), local background was subtracted, and the data were normalized and 2log transformed. Additional information on barcode screens can be found at http://www.screeninc.nki.nl/.

Phospho AKT Immunoblotting

Cell lysates were separated by 10% SDS-PAGE, transferred, and blots were first probed with anti-pAKT (Ser473 #9271 Cell Signaling, Beverly, MA) and subsequently reprobed with total AKT1/2 (H136 Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting was performed according to the antibody manufacturer's recommendations using enhanced chemiluminescence.

Growth Curves

BT-474 cells were infected with the indicated retroviruses and polyclonal cell lines were seeded in 12-well plates (2.10^4 cells/well) and cultured both in the absence and presence of 2 μg/ml trastuzumab. Cell numbers were quantified at the indicated time points by fixing the cells with 4% formaldehyde, staining the cells with crystal violet (0.1% Sigma), extracting dye with 10% acetic acid, and determining the optical density at 600 nm. Growth curves were performed multiple times in triplicate.

Patients

For the NKI/AVL cohort, patients were eligible based on HER2 overexpression by immunohistochemistry (IHC 3+) and/or *HER2* gene amplification by chromogenic in situ hybridization (CISH). Primary material from 34 patients with *HER2*-overexpressing primary breast carcinomas who subsequently developed metastatic breast cancer and received either trastuzumab monotherapy (n = 3), trastuzumab plus taxane (n = 8), vinorelbine (n = 16), vinorelbine and lonafarnib (n = 5), paclitaxel and carboplatin (n = 1), or adjuvant trastuzumab with paclitaxel and carboplatin for a locoregional recurrence (n = 1), was collected from the NKI/AVL hospital and surrounding hospitals. This study was approved by the Institutional Review Board of the Netherlands Cancer Institute. Twenty-six of the tumors had high nuclear grade, and ER status by IHC was positive in 14 and negative in 19 tumors

(for one tumor it was not possible to retrieve the ER status). For the M.D. Anderson cohort, primary tumor material from 21 patients with breast cancer that was confirmed as *HER2* amplified (by FISH and/or 3+ positivity on IHC) was obtained from the frozen breast tissue tumor bank at M.D. Anderson Cancer Center under the auspices of an IRB-approved protocol. Seventeen of the tumors had high nuclear grade and ER status by IHC was positive in 12 and negative in 9 tumors. Patients were treated with trastuzumab monotherapy (n = 3) or trastuzumab plus taxane (n = 8), vinorelbine (n = 6), or other chemotherapy (n = 4). Data on time to progression were available for all patients.

Average age at diagnosis was 46.6 years and ranged from 25 to 74.1 (NKI: 47.8, 28.9–74.1, M.D. Anderson: 44.5, 25–65). Average time to progression was 11.8 and ranged from 0.7 to 44.7 months (NKI: 11.3, 0.7–37.5, M.D. Anderson: 12.6, 0.8–44.7). During follow-up, 48 events occurred, and seven patients were censored (NKI: 29 and 5, M.D. Anderson: 19 and 2). The prevalence of PIK3CA mutation was 25% (NKI: 21%, M.D. Anderson: 33%). Low PTEN expression was observed in 22% of the patients (NKI: 24%, M.D. Anderson: 17%).

PIK3CA PCR, Sequencing, and Mutational Analysis

PCR reactions were performed on 10–100 ng of genomic DNA using a standard protocol. PCR products were purified over a QIAquick spin column (QIAGEN) and were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 3730 automated capillary sequencer. For all PCR products with sequence variants, both forward and reverse sequence reactions were repeated for confirmation.

PIK3CA SNP Analysis

DNA around the known potential PIK3CA mutation hotspot sites 111, 542, 545, and 1047 was amplified and a primer extension reaction was run to determine a potential SNP base using the Sequenom (San Diego, CA) MALDI TOF MassArray system. The PCR reactions were done and cleaned according to manufacturer's instructions. The primer extension reactions were done using Sequenom's IPLEX chemistry, and were then desalted using Sequenom's Clean Resin and spotted onto Spectrochip matrix chips using a Samsung Nanodispenser. The chips were then run on the Sequenom MassArray. Sequenom Typer Software was used to interpret the mass spectra that were generated and to report the SNPs based on expected masses. All spectra generated were run in duplicate and were visually inspected.

Immunohistochemistry

Serial sections of 3 μm from the paraffin blocks were deparaffinized in xylene, and hydrated in a graded series of alcohol. Staining was performed using the Lab Vision Immunohistochemical Autostainer (Lab Vision Corporation, Fremont, CA) with primary antibodies toward PTEN (DAKO, 1:200), HER2 (clone 3B5, 1:3000) (van de Vijver et al., 1988), and ER (estrogen receptor-α; 1D5+6F11, dilution 1:50, Neomarkers, Lab Vision Corporation, Fremont, CA). Detection was performed with antigen retrieval method (citrate pH 6.0).

Scoring

The PTEN expression level was scored semiquantitatively based on staining intensity and distribution using the immunoreactive score (IRS) as described elsewhere (Chui et al., 1996; Friedrichs et al., 1993) (see Supplemental Experimental Procedures). Samples were scored as ER positive by IHC, when at least more than 10% of the tumor cells showed staining of the ER in the nuclei. A sample was considered to be HER2 positive when either a strong membrane staining (3+) could be observed by IHC or CISH revealed amplification of HER2 in samples with weak (1+ or 2+) membrane staining at IHC (Hannemann et al., 2006).

Statistical Analysis

We evaluated the association between time to progression and two candidate risk factors, PIK3CA mutation and PTEN expression, using multivariate Cox regression with age as the time scale. Follow-up started at the age of diagnosis and ended at the age of progression, the age at death, or the age at censoring, whichever came first. For categorization of the continuous PTEN expression values into low and high, we chose a commonly used cut point for the measurements (range 0–12, cut point < 3 versus > 3), which resulted in 22% of patients being low. PTEN expression was missing for four subjects. All analyses were stratified by center and adjusted for age by using this variable as the time scale. Confounding was further evaluated for grade and ER status. Kaplan-Meier plots were produced in order to graphically illustrate the event history by the two candidate risk factors. Log-rank tests were performed to evaluate the homogeneity of group-specific survival curves.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, two supplemental figures, and one supplemental table and can be found at http://www.cancercell.org/cgi/content/full/12/4/395/DC1/.

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