**ARTICLE**

# Somatic Mutation Profiling and Associations With Prognosis and Trastuzumab Benefit in Early Breast Cancer

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**Background**    Certain somatic alterations in breast cancer can define prognosis and response to therapy. This study investigated the frequencies, prognostic effects, and predictive effects of known cancer somatic mutations using a randomized, adjuvant, phase III clinical trial dataset.

**Methods**    The FinHER trial was a phase III, randomized adjuvant breast cancer trial involving 1010 women. Patients with human epidermal growth factor receptor 2 (HER2)–positive breast cancer were further randomized to 9 weeks of trastuzumab or no trastuzumab. Seven hundred five of 1010 tumors had sufficient DNA for genotyping of 70 somatic hotspot mutations in 20 genes using mass spectrometry. Distant disease-free survival (DDFS), overall survival (OS), and interactions with trastuzumab were explored with Kaplan-Meier and Cox regression analyses. All statistical tests were two-sided.

**Results**    Median follow-up was 62 months. Of 705 tumors, 687 were successfully genotyped. *PIK3CA* mutations (exons 1, 2, 4, 9, 13, 18, and 20) were present in 25.3% (174 of 687) and *TP53* mutations in 10.2% (70 of 687). Few other mutations were found: three *ERBB2* and single cases of *KRAS, ALK, STK11/LKB1*, and *AKT2*. *PIK3CA* mutations were associated with estrogen receptor positivity ($P < .001$) and the luminal-A phenotype ($P = .04$) but were not statistically significantly associated with prognosis (DDFS: hazard ratio [HR] = 0.88, 95% confidence [CI] = 0.58 to 1.34, $P = .56$; OS: HR = 0.603, 95% CI = .32 to 1.13, $P = .11$), although a statistically significant nonproportional prognostic effect was observed for DDFS ($P = .002$). *PIK3CA* mutations were not statistically significantly associated with trastuzumab benefit ($P_{interaction}$: DDFS $P = .14$; OS $P = .24$).

**Conclusions**    In this dataset, targeted genotyping revealed only two alterations at a frequency greater than 10%, with other mutations observed infrequently. *PIK3CA* mutations were associated with a better outcome, however this effect disappeared after 3 years. There were no statistically significant associations with trastuzumab benefit.

Gene expression profiling divides breast cancer into distinct molecular portraits according to the presence of the estrogen receptor (ER) and amplification/overexpression of the *ERBB2*/HER2/*neu* oncogene (1). Notably, HER2 amplification/overexpression (HER2-positive) predicts response to anti-HER2 therapy, suggesting that somatic alterations in breast cancer are associated with prognosis and potentially amenable to targeted therapy (2). This has inspired efforts to better understand the spectrum of somatic "driver" mutations and, in particular, targetable mutated kinases.

An abundance of data suggests that genetic aberrations and activation of the phosphatidylinositol 3-kinase (PI3K) pathway are important in determining breast cancer prognosis and the efficacy of standard chemo- and endocrine therapies (3). Furthermore, mutations in the *PIK3CA* gene, which encodes the p110α catalytic subunit of the class IA PI3K, are frequent in breast cancer (4–7).

These mutations have been shown to be oncogenic in mammary epithelial cells by driving constitutive, growth factor–independent PI3K pathway activation (8,9).

Despite being the focus of intense research interest, a clear association between *PIK3CA* mutations and a poorer prognosis has not been shown. To the contrary, *PIK3CA* mutations have been associated with statistically significantly better survival when compared with *PIK3CA* wild-type breast cancers in larger series obtained from single institutions (4,7–10). An association with resistance to endocrine therapy has also not been demonstrated (6,11,12). *PIK3CA* mutations have also been shown to be associated with trastuzumab resistance in preclinical models overexpressing HER2 (13–15). Clinical validation of this association could have important clinical utility given the emergence of a broadening array of anti-HER2 agents and the concept of dual anti-HER2 therapy

(16–18). Hence, given their frequency, oncogenic capabilities, and the potential to induce resistance to commonly prescribed breast cancer treatments, the clinical relevance of *PIK3CA* mutations deserves further clarification.

High levels of evidence on the clinical utility of prognostic and predictive biomarkers can be achieved from the use of archived tumor specimens from appropriate randomized clinical trial datasets (19). Therefore, the main purpose of this study was to clarify in a well-characterized, randomized clinical trial dataset the predictive relevance of *PIK3CA* mutations to trastuzumab efficacy and its prognostic abilities in both HER2-positive and HER2-negative disease. Given that *PIK3CA* genotyping can be performed with other somatic hotspot mutations, we also set out to determine prevalence and prognostic associations of other known cancer driver mutations. Our objective was to identify other potentially targetable genetic alterations that contribute to resistance to standard therapy in breast cancer.

## Methods

The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria were followed in this study (20).

### Patients in the FinHER Study
This study is based on formalin-fixed, paraffin-embedded (FFPE) primary breast tumor tissue samples of Finnish women who were aged <66 years and diagnosed with either node-positive or node-negative breast cancer and who participated in the FinHER trial (N = 1010) from 2000 to 2003, a multicenter adjuvant trial sponsored by the Finnish Breast Cancer Group (21,22). All women were randomly assigned to receive three cycles of docetaxel or vinorelbine, followed by (in both groups) three cycles of fluorouracil, epirubicin, and cyclophosphamide. The 232 women whose tumors had an amplified HER2/*neu* gene were further randomly assigned to receive or to not receive nine weekly trastuzumab infusions. The primary end point of the FinHER study, distant disease-free survival (DDFS), has been previously reported to be superior for the docetaxel- and trastuzumab-containing arms. The final median follow-up was 62 months (21).

The determination of steroid hormone receptor status and HER2 expression by immunohistochemistry (IHC) was required locally and was performed according to the guidelines of each institution during the time of the study. Samples were considered hormone receptor positive if their level of ER and/or progesterone receptor (PR) was ≥10%. All patients with ER- or PR-positive tumors received five years of endocrine therapy, which was tamoxifen when the study commenced, but aromatase inhibitors were permitted midway through the study. Ki67 IHC was assessed in locally by IHC using the Mib-1 monoclonal antibody (Dako, Glostrup, Denmark). When HER2 expression was scored as 2+ or 3+ (on a scale of 0, 1+, 2+, or 3+), the number of copies of the HER2/*neu* gene was centrally determined by means of chromogenic in situ hybridization in one of two reference laboratories.

### Mutation Testing
Study participants provided written informed consent to allow further research analyses to be carried out on their tumor tissue.

The ethical committee of the Helsinki University Central Hospital also approved the current study. Of the 1010 samples, 935 (92.5%) could be retrieved. All samples were reevaluated by one reference pathologist to ensure tumor was present in the tissue sample. Of these samples, 705 (75.4%) were able to have adequate DNA extracted. DNA was extracted from FFPE tumor tissue using a salt precipitation method (23).

Because few data were available about the mutational landscape of breast cancer at the time of genotyping, we queried the COSMIC (Catalogue of Somatic Mutations in Cancer) database to identify a broad range of genes for hotspot somatic mutation profiling (24). We ultimately covered 94% of reported *PIK3CA* mutations (exons 1, 2, 4, 9, 13, 18, 20) reported in COSMIC to be occurring in breast cancer in this study, 12% of *TP53* mutations (all cancer types), selected *ERBB2*, *PTEN*, *AKT1/2* mutations, and known *EGFR*, *BRAF*, *KRAS*, *MAP3K1*, and *CDK4* mutations, as well as a scattering of other "druggable" mutations. In total, 70 mutations in 20 genes were evaluated (Supplementary Table 1, available online).

The samples were genotyped centrally using the Sequenom MassARRAY Assay Design 3.1 with the default parameters. Multiplex polymerase chain reaction was done in 5-μL volume containing 5–10 ng of DNA. Samples were considered to be of sufficient quality when genotyping could be performed for >75% of the mutations. A total of 687 samples (68.1% of the original FinHER cohort) were successfully genotyped (2.5% [18 of 705] samples were discarded for this reason). Sixteen samples were genotyped in duplicate and were found to have 100% concordance. Details about the assay and independent validation have been previously published: the Sequenom can detect low-frequency mutant alleles to maximize mutation detection in impure samples (≥5% for the PIK3CA hotspot mutations) with sensitivity and specificity of 90% and 99%, respectively, in FFPE-derived DNA, and 100% concordance with other technologies (25,26). In this study, we further confirmed one sample of each positive *PIK3CA* mutation, as well as a wild-type sample, using Sanger sequencing (except for the rarer G241A, G3019C, and C473T); another 9 samples (both positive and wild type) were confirmed with the Qiagen Rotor-gene Kit. All of these were found to be 100% concordant with the Sequenom results. *ERBB2* mutations were also confirmed using Sanger sequencing (primers TCCTGGAAGGCAGGTAGGATCCAG and AGTCTAGGTTTGCGGGAGTCATATCTC).

### Statistical Analysis
In this study, a sample was considered to be wild type for a given gene if no mutation was found. Associations between mutations and clinicopathologic characteristics were investigated with $\chi^2$ tests for categorical variables. For the survival analyses, the primary end point was DDFS, which was defined as the time period from the date of random treatment assignment to the date of first cancer recurrence outside the ipsilateral locoregional region or to death, whenever death occurred before distant recurrence (21). Relapse-free survival (RFS) was defined as the time from the date of random assignment to the date of the local, distant, or contralateral invasive cancer recurrence or death. Overall survival (OS) was defined as the time period from the date of random assignment to the date of death, whenever death occurred before distant recurrence.

Cox proportional hazards regression models were used to test the prognostic value of *PIK3CA* mutation status (hazard ratios [HRs] and 95% confidence intervals [CIs]) and its possible interaction with trastuzumab treatment (after adding a trastuzumab main effect and a product interaction term) using the Wald test. The Cox models used a separate baseline hazard for chemotherapy type (docetaxel or vinorelbine). Departures from the proportional hazards assumption were assessed based on the Schoenfeld residuals (27).

All *P* values were two-sided and a *P* value of less than .05 was considered statistically significant. The Kaplan-Meier survival curves were calculated (with group differences assessed using the log-rank test). Interaction effects were also displayed using forest plots. No adjustment was planned for multiple testing of the prespecified hypotheses. For this study, breast cancer subtypes were classified using IHC as previously published (28): luminal (ER-positive and/or progesteron receptor [PgR]–positive, HER2-negative), HER2-positive/overexpressing by (chromogenic in situ hybridization), and triple negative: ER-negative/PgR-negative/HER2-negative. Luminal A and B phenotypes were defined using IHC staining of Ki67-positive cells using a cutoff of less than 14% and greater than 14%, respectively (28).

## Results

### Patient Characteristics

The patient characteristics of the genotyped series (n = 687) are compared with the original series and those who were not genotyped in Supplementary Table 2 (available online). There were more tumors that were ER-negative, of larger size and higher grade, and from younger patients genotyped compared with those not genotyped. There were no statistically significant differences in survival between groups (DDFS log-rank *P* = .19, RFS *P* = .34, OS *P* = .64).

### Frequency and Associations Between Mutations

Despite genotyping this cohort for 70 known cancer somatic "driver" mutations in 20 genes, only *PIK3CA* and *TP53* somatic mutations occurred at frequencies >10%.

*PIK3CA* mutations were successfully genotyped in 100% of samples that passed the quality control criteria. 176 *PIK3CA* mutations were found (Supplementary Table 3, available online). The vast majority of these were located on the hotspots on the helical and kinase domains (exons 9 and 20, respectively—161 of 176 [91.5%]), with two samples having a double *PIK3CA* mutation present (A3140G + C473T; T1035A + G1633A). The overall frequency of tumor samples with a PIK3CA mutation was 25.3% (174 of 687). *TP53* mutations, with coverage of approximately 12% of known mutations, were present in 10.2% (70 of 687) of samples. Three *ERBB2* kinase domain mutations (two *T2264C, C2313T) were present in 0.5% of samples genotyped (3 of 659 [28 of 687 samples could not be assigned]). Mutations that occurred only once were *KRAS* (G35A), *AKT2* (G49A), *ALK* (G3824A), and *STK11/LKB1* (C1062G) (Figure 1).

### Association With Clinicopathological Features and Breast Cancer Subtypes

*PIK3CA* mutations were statistically significantly associated with smaller tumor size (T1, *P* = .03), histological grade 1 (*P* < .001), positive expression of the ER (*P* < .001), and the luminal-A phenotype (*P* = .04; Table 1). As expected, *TP53* mutations were associated with ER negativity (*P* = .005), histological grade 3 (*P* = .007), larger tumor size (*P* = .009), and four or more positive lymph nodes (*P* = .003). All three *ERBB2* mutant samples were ER-positive and HER2-negative (luminal). In the three main breast cancer subtypes defined using IHC, as expected, *PIK3CA* mutations were highly frequent in luminal and HER2-positive subtypes (*P* < .001) and TP53 mutations in the triple-negative group (*P* = .003; Table 2).

### Associations With Prognosis

In the whole cohort that was genotyped, *PIK3CA* mutations were not statistically significantly associated with prognosis (DDFS: HR = 0.88 [95% CI = 0.58 to 1.34], *P* = .56; OS: HR = 0.603 [95% CI = 0.32 to 1.13], *P* = .11; Figure 2). However, we noted that there was a statistically significant nonproportional prognostic effect over time for DDFS (*P* = .002) and RFS (*P* = .007) but not for OS
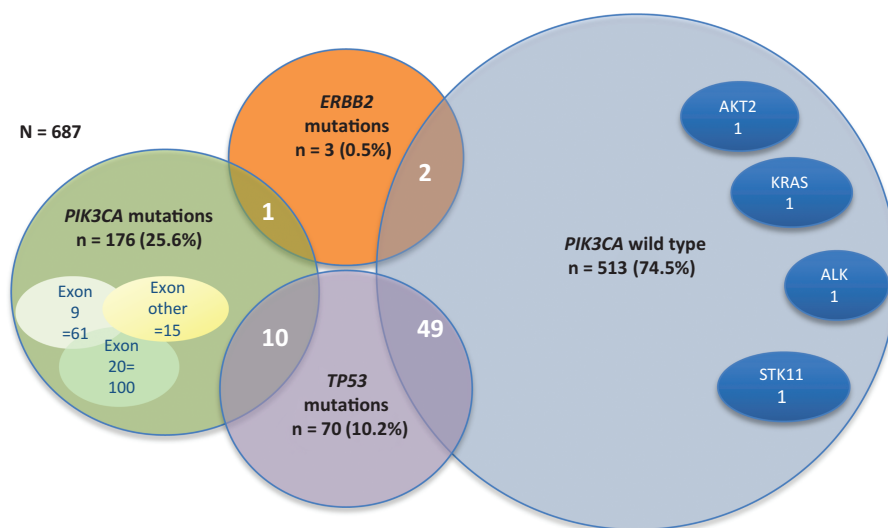


**Figure 1.** Frequency and associations between mutations. Absolute numbers are shown of *PIK3CA* mutant, *PIK3CA* wild type, *ERBB2* mutant, and *TP53* mutant, as well as those tumors with coexisting mutations. *PIK3CA* exon 9 and 20 mutations (and other locations) are also shown.

**Table 1.** Patient and tumor characteristics by PIK3CA genotype*

| Characteristic | Whole cohort (N = 687) | PIK3CA genotype | | | TP53 genotype | | |
|---|---|---|---|---|---|---|---|
| | | WT (n = 511) | Any mt PIK3CA (n = 176) | P | WT (n = 617) | Any mt TP53 (n = 70) | P |
| Age category | | | | | | | |
| ≤50 y | 364 (53%) | 274 (53.6%) | 90 (51.1%) | .57 | 330 (53.5%) | 34 (48.6%) | .44 |
| >50 y | 323 (47%) | 237 (46.4%) | 86 (48.9%) | | 287 (46.5%) | 36 (51.4%) | |
| Tumor stage | | | | | | | |
| T1 | 275 (40%) | 192 (37.8%) | 83 (47.2%) | .003 | 258 (42%) | 17 (24.3%) | .009 |
| T2 | 364 (53%) | 274 (53.9%) | 90 (51.1%) | | 319 (52%) | 45 (64.3%) | |
| T3 | 45 (6.6%) | 42 (8.3%) | 3 (1.7%) | | 37 (6%) | 8 (11.4%) | |
| Missing | 3 (0.4%) | | | | | | |
| Nodal status | | | | | | | |
| Negative | 81 (11.8%) | 64 (12.5%) | 17 (9.7%) | .33 | 64 (10.4%) | 17 (24.3%) | .003 |
| 1–3 | 410 (59.7%) | 297 (58.1%) | 113 (64.2%) | | 373 (60.5%) | 37 (52.9%) | |
| >3 | 196 (28.5%) | 150 (29.4%) | 46 (26.1%) | | 180 (29.2%) | 16 (22.9%) | |
| Histological grade | | | | | | | |
| I | 80 (11.6%) | 46 (9.3%) | 34 (20.2%) | <.001 | 73 (12.2%) | 7 (8.8%) | .007 |
| II | 270 (39.3%) | 187 (37.8%) | 83 (49.4%) | | 254 (42.5%) | 16 (24.2%) | |
| III | 313 (96.5%) | 262 (52.9%) | 51 (30.4%) | | 270 (45.2%) | 43 (54.2%) | |
| Missing | 23 (3.5%) | | | | | | |
| ER IHC | | | | | | | |
| Positive | 475 (69.1%) | 335 (69.7%) | 140 (79.5%) | <.001 | 437 (70.8%) | 32 (45.7%) | .005 |
| Negative | 212 (30.9%) | 176 (26.3%) | 36 (20.5%) | | 180 (29.2%) | 38 (45.7%) | |
| HER2 amplification | | | | | | | |
| Positive | 157 (22.9%) | 123 (24.1%) | 34 (19.3%) | .20 | 138 (22.4%) | 19 (27.1%) | .37 |
| Negative | 530 (77.1%) | 388 (68.8%) | 142 (28.8%) | | 479 (77.6%) | 51 (72.9%) | |
| Histology | | | | | | | |
| Ductal | 558 (81.2%) | 422 (83.6%) | 136 (78.6%) | .14 | 501 (82.3%) | 57 (82.6%) | .94 |
| Lobular | 120 (17.5%) | 83 (16.4%) | 37 (21.4%) | | 108 (17.7%) | 12 (17.4%) | |
| Other | 9 (1.3%) | | | | | | |
| Breast cancer subtype (defined by IHC) | | | | | | | |
| Luminal (ER-positive/ HER2-negative) | 410 (59.7%) | 284 (55.6%) | 126 (71.6%) | <.001 | 380 (61.6%) | 30 (42.9%) | .003 |
| HER2-amplified | 157 (22.9%) | 123 (24.1%) | 4 (19.3%) | | 138 (22.4%) | 19 (27.1%) | |
| Triple negative (ER-negative/ PgR-negative/HER2-negative) | 120 (17.5%) | 104 (20.4%) | 16 (9.1%) | | 99 (16%) | 21 (30%) | |
| Luminal A/B | | | | | | | |
| Ki67 IHC <14% | 127 (30%) | 80 (31.7%) | 47 (42.7%) | .04 | 121 (36.2%) | 6 (21.4%) | .12 |
| Ki67 IHC ≥14% | 235 (57.3%) | 172 (68.3%) | 63 (57.3%) | | 213 (63.8%) | 22 (78.6%) | |
| NA | 48 (11.7%) | | | | | | |

* P values were calculated using a two-sided $\chi^2$ test. ER = estrogen receptor; IHC = immunohistochemistry; mt = mutation; NA = not applicable; WT = wild type.

**Table 2.** Frequency of mutations by breast cancer subtype*

| Subtype | PIK3CA mutations, No. | TP53 mutations, No. |
|---|---|---|
| Luminal (ER-positive/HER2-negative) | 126/410 (30.7%) P < .001 | 30/409 (7.3%) |
| HER2-positive | 34/157 (21.7%) | 19/157 (12.1%) |
| ER-negative/HER2-negative | 16/120 (13.3%) | 21/120 (17.5%) P < .001 |

* P values were calculated using a two-sided $\chi^2$ test. ER = estrogen receptor.

(P = .12). An exploratory subdivision of the time axis at three years shows a favorable prognostic effect before three years (DDFS: HR = 0.57 [95% CI = 0.31 to 1.03], P = .06; RFS: HR = 0.55 [95% CI = 0.31 to 0.98], P = .04, respectively, and statistically nonsignificant effect after 3 years: DDFS: HR = 1.69 [95% CI = 0.90 to 3.16], P = .10; RFS: HR = 1.58 [95% CI = 0.86 to 2.88], P = .14).

No statistically significant differences in patient outcome were observed when *PIK3CA* mutations were evaluated separately according to their location (Figure 3). Patients whose tumors contained a *PIK3CA* mutation were also not found to have a statistically significantly different survival than those with wild type in any of the breast cancer subtypes (Supplementary Figure 1, available online).

*TP53* mutations were not statistically significantly associated with prognosis in the whole genotyped cohort (DDFS log-rank P = .36; RFS P = .34; OS P = .11). Of the three *ERBB2* mutated tumors, one patient had a distant relapse and died of her disease.
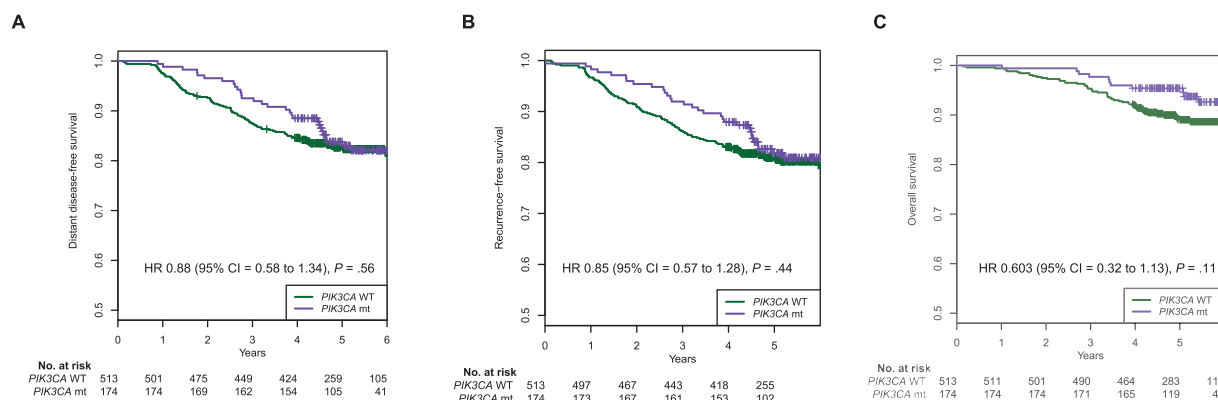
**Figure 2.** Prognostic associations between patients who had a *PIK3CA* mutation (mt) vs wild type (WT) and clinical outcome. **A–C)** Kaplan-Meier plots of the cumulative proportion of patients surviving with the time in years. Various clinical end points are shown: distant disease-free survival (A), recurrence-free survival (B), and overall survival (C). Cox regression hazard ratios (HRs) and 95% confidence intervals (CIs) are shown, stratified by chemotherapy type given. All statistical tests are two-sided. The number of patients at risk in each group is given below the graphs.
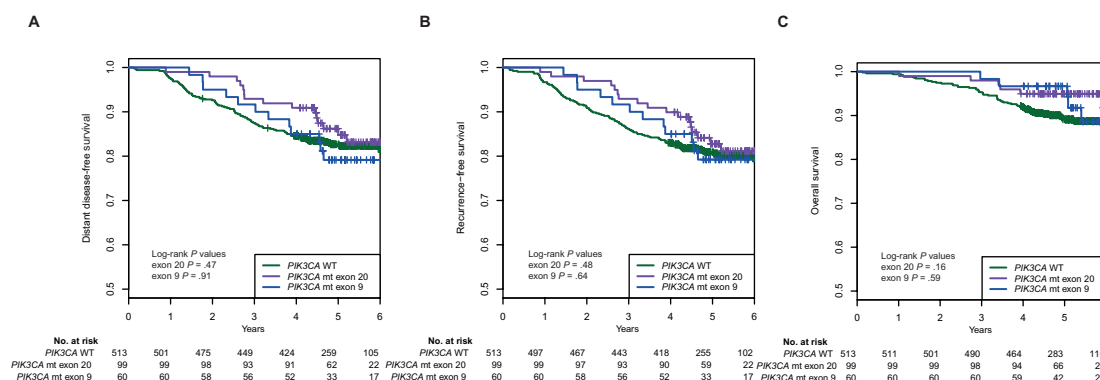


**Figure 3.** Prognostic associations between *PIK3CA* genotype and clinical outcome according to mutation location on the gene (helical/exon 9 vs kinase/exon 20 domain). The number of patients at risk in each group is given below the graphs. **A–C)** Kaplan-Meier plots of the cumulative proportion of patients surviving with the time in years. Various clinical end points are shown: distant disease-free survival (A), recurrence-free survival (B), and overall survival (C). The two patients with dual mutations were excluded and all treatment arms were pooled. *P* values correspond to log-rank tests; mt = mutant; WT = wild type. All statistical tests are two-sided.

## Association Between *PIK3CA* Mutations and Trastuzumab Efficacy in HER2-Positive Breast Cancer

We subsequently evaluated the association between *PIK3CA* genotype and trastuzumab efficacy in the HER2-positive population, considering preclinical data suggesting that *PIK3CA* mutations contribute to trastuzumab resistance (13,15). We found that in our dataset, the magnitude of trastuzumab benefit (with cytotoxic chemotherapy) did not differ statistically significantly according to *PIK3CA* genotype (Figure 4; $P_{interaction}$: DDFS $P = .14$; RFS $P = .17$; OS $P = .24$). For the primary endpoint of DDFS and trastuzumab benefit, patients who were *PIK3CA* mutant had an HR of 0.19 (95% CI = 0.04 to 1.04; $P = .06$) vs patients who were *PIK3CA* wild type (HR = 0.98 [95% CI = 0.47 to 2.8], $P = .97$).

## Discussion

The primary objective of this study was to investigate the clinical relevance of *PIK3CA* mutations with regard to prognosis and benefit from adjuvant trastuzumab. While confirming the dominance of *PIK3CA* and *TP53* mutations in breast cancer with few other

known mutations being present in breast cancer at a high rate, we showed that *PIK3CA* mutations, regardless of location, were not statistically significantly associated with prognosis in breast cancer over the entire follow-up period, although, interestingly, there was a statistically significant nonproportional prognostic effect for DDFS and RFS over time. Initially, a better outcome for the mutant genotype compared with wild type was seen, consistent with the mutant genotype's association with favorable clinicopathological features; however, this effect disappeared after three years. Perhaps this pattern can be explained by the high-risk population studied or reflect a biological tendency for long-term relapse, as endocrine therapy resistance could conceivably develop through enhanced PI3K signaling. In general, however, the prognostic direction in the first three years supports the results from many of the larger cohort series reported in the literature, even though the prognostic association has yielded conflicting reports overall (4–7,29).

The unique advantage and strength of our study was that we could evaluate interactions between *PIK3CA* mutations and trastuzumab benefit in the context of a randomized clinical trial in which patients with HER2-positive breast cancer received treatment with or without trastuzumab. To our knowledge, this study
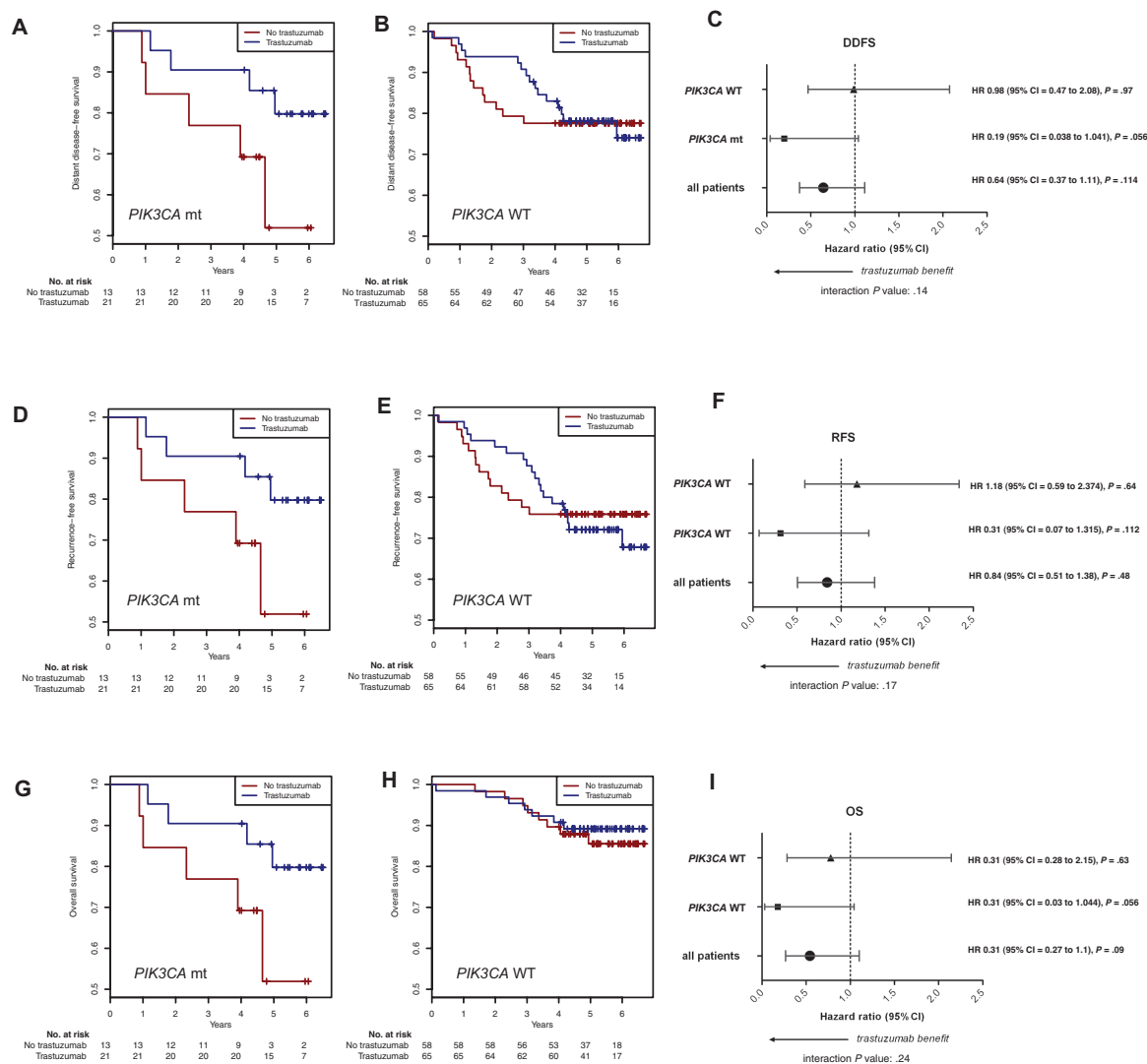
**Figure 4.** Interaction between *PIK3CA* genotype and trastuzumab efficacy. **A)** Kaplan-Meier plots comparing trastuzumab vs no trastuzumab treatment arms for *PIK3CA* mutated (mt), HER2-positive cohorts. Cumulative proportions of patients surviving distant disease free are shown. **B)** Kaplan-Meier plots comparing trastuzumab vs no trastuzumab for *PIK3CA* wild-type (WT), HER2-positive cohorts. Cumulative proportions of patients surviving distant disease free are shown. **C)** Interaction forest plots indicate Cox regression hazard ratios (HRs) and 95% confidence intervals (CIs) stratified by chemotherapy type given for trastuzumab benefit for distant disease-free survival (DDFS). according to *PIK3CA* genotype and by overall series. **D)** Kaplan-Meier plots comparing trastuzumab vs no trastuzumab treatment arms for *PIK3CA* mt, HER2-positive cohorts. Cumulative proportions of patients surviving relapse free are shown. **E)** Kaplan-Meier plots comparing trastuzumab vs no trastuzumab treatment arms for *PIK3CA* WT, HER2-positive cohorts. Cumulative proportions of patients surviving relapse free are shown. **F)** Interaction forest plots indicate Cox regression HRs and 95% CIs stratified by chemotherapy type given for trastuzumab benefit for recurrence-free survival (RFS) according to *PIK3CA* genotype and by overall series. **G)** Kaplan-Meier plots comparing trastuzumab vs no trastuzumab treatment arms for *PIK3CA* mt, HER2-positive cohorts. Cumulative proportions of patients alive are shown. **H)** Kaplan-Meier plots comparing trastuzumab vs no trastuzumab treatment arms for *PIK3CA* WT, HER2-positive cohorts. Cumulative proportions of patients alive are shown. **I)** Interaction forest plots indicate Cox regression HRs and 95% CIs stratified by chemotherapy type given for trastuzumab benefit for overall survival. according to *PIK3CA* genotype and by overall series. All statistical tests are two-sided. The number of patients at risk in each group is given below the graphs.

also represents the largest breast cancer cohort with clinical outcome data to be genotyped for *PIK3CA* and multiple other known cancer somatic mutations. Furthermore, we covered greater than 94% of known *PIK3CA* mutations, rather than limiting to hotspot areas. Preclinical data suggest that *PIK3CA* mutations could identify a subgroup of patients with HER2-positive disease resistant to trastuzumab, but our data do not support this. In fact, the *PIK3CA* mutant compared with wild-type, HER2-positive tumors seemed to derive more benefit from adjuvant trastuzumab, suggesting increased dependency on p110α, although the interaction

test is not statistically significant (30). All the patients in this study also received chemotherapy with trastuzumab, which is standard practice, so we cannot discount the possibility that mutations could cause resistance to trastuzumab as a single agent. It has been proposed that scheduling of chemotherapy either before or after administration of trastuzumab could affect clinical outcomes, particularly through immune mechanisms (31). As the generation of antitumor immunity has been proposed as a dominant mechanism of action for the efficacy of trastuzumab, it is plausible that *PIK3CA* mutations could alter the immune microenvironment to be either

or antitumor or protumor (31,33). PI3K signaling per se is known to affect immune signaling, although no data currently exist with regard to specific mutation-related events (34). Therefore, despite *PIK3CA* mutations being oncogenic activators of the PI3K pathway, overall our data support the notion that *PIK3CA* mutant tumors when compared with the *PIK3CA* wild-type tumors are not resistant to standard adjuvant chemotherapy, trastuzumab, and endocrine therapy regimens.

A biological mechanism for these observations is currently unknown. We have speculated previously that *PIK3CA* mutations are not effective at completely activating the pathway and negative feedback mechanisms may serve to weaken the oncogenic signal (6). Full AKT activation has not been associated with the mutation, and AKT-independent signaling has been proposed through PDK1-SGK3, with SGK3 also implicated with estrogen signaling (7,35–37). Estrogen has also been shown predominantly to repress transcription of many genes, which may also reduce the final signaling output (38,39). High levels of pathway activation could be detrimental for tumor growth (ie, result in senescence), analogous to PTEN deficiency (40). Regardless, it seems that high levels of pathway activation are not associated with *PIK3CA* mutations per se. We hypothesize that *PIK3CA* mutations may be more important in breast cancer initiation and malignant transformation whereas other mutations may be required to drive the acquisition of aggressive biological features: it is notable that *PIK3CA* mutations often coexist with other lesions in the same pathway (30,41–43). It remains to be seen if primary and/or metastatic breast cancer patients with *PIK3CA* mutations will derive increased benefit from PI3K pathway–targeted drugs, which has been observed in vitro (44–46). Many clinical trials evaluating potential benefit from specific PI3K targeted drugs are currently ongoing.

This study, as well as others using massively parallel sequencing, have confirmed that breast cancers contain a large number of known cancer driver mutations that occur infrequently (42,43,47,48). In this cohort we have identified three *ERBB2* as well as single *KRAS*, *ALK*, *STK11/LKB1*, and *AKT2* mutations. These are known "driver" mutations, yet it is unknown how these influence outcomes or are amenable to targeted therapies in breast cancer. *ERRB2* kinase domain mutations have recently been shown to be important in breast cancer; hence, this mutation could represent a new target for non-HER2-amplified/overexpressing breast cancer (49–52).

To our knowledge, this is the only study thus far to address the relevance of *PIK3CA* genotype and trastuzumab benefit. We acknowledge several limitations of our study, specifically the low number of events in the HER2-positive subgroup, which does not exclude the possibility that an effect might be seen in a larger series; less than 100% coverage of all reported *PIK3CA* mutations in breast cancer; and sequencing from one tumor section, given emerging data on intratumoral heterogeneity (53). Next-generation sequencing technologies may give us a more complete picture of the clonal composition and molecular landscape of these tumors. However, it is becoming clear that elucidating the relationship between infrequent but known driver genetic aberrations, prognosis, and drug response will require the genotyping of tumors from many thousands of breast cancer patients. This may also prove challenging for drug development. Nevertheless, our study provides important information from a large randomized clinical trial dataset about the prevalence and relationship between targetable and known somatic driver mutations, trastuzumab efficacy, and prognosis.

## References

1. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406(6797):747–752.
2. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235(4785):177–182.
3. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9(8):550–562.
4. 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*. 2012;14(1):R28.
5. Kalinsky K, Jacks LM, Heguy A, et al. PIK3CA mutation associates with improved outcome in breast cancer. *Clin Cancer Res*. 2009;15(16):5049–5059.
6. Loi S, Haibe-Kains B, Majjaj S, et al. PIK3CA mutations associated with gene signature of low mTORC1 signaling and better outcomes in estrogen receptor-positive breast cancer. *Proc Natl Acad Sci U S A*. 2010;107(22):10208–10213.
7. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res*. 2008;68(15):6084–6091.
8. Isakoff SJ, Engelman JA, Irie HY, et al. Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. *Cancer Res*. 2005;65(23):10992–11000.
9. Zhao JJ, Liu Z, Wang L, et al. The oncogenic properties of mutant p110alpha and p110beta phosphatidylinositol 3-kinases in human mammary epithelial cells. *Proc Natl Acad Sci U S A*. 2005;102(51):18443–18448.
10. 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*. 2007;13(12):3577–3584.
11. Baselga J, Semiglazov V, van Dam P, et al. Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. *J Clin Oncol*. 2009;27(16):2630–2637.
12. 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*. 2010;119(2):379–390.
13. Berns K, Horlings HM, Hennessy BT, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell*. 2007;12(4):395–402.
14. Junttila TT, Akita RW, Parsons K, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer. Cell* 2009;15(5):429–440.
15. Serra V, Markman B, Scaltriti M, et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer Res*. 2008;68(19):8022–8030.
16. Baselga J, Bradbury I, Eidtmann H, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet*. 2012;379(9816):633–640.
17. Baselga J, Cortes J, Kim SB, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med*. 2011;366(2):109–119.
18. Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2011;13(1):25–32.
19. Simon RM, Paik S, Hayes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. *J Natl Cancer Inst*. 2009;101(21):1446–1452.
20. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst*. 2005;97(16):1180–1184.
21. Joensuu H, Bono P, Kataja V, et al. Fluorouracil, epirubicin, and cyclophosphamide with either docetaxel or vinorelbine, with or without trastuzumab,

as adjuvant treatments of breast cancer: final results of the FinHer Trial. *J Clin Oncol*. 2009;27(34):5685–5692.

22. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med*. 2006;354(8):809–820.

23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;16(3):1215.

24. Welcome Trust Sanger Institute. *Catalogue of somatic mutations in cancer*. http://cancer.sanger.ac.uk/cancergenome/projects/cosmic Accessed August 2011.

25. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol*. 2010;11(8):753–762.

26. MacConaill LE, Campbell CD, Kehoe SM, et al. Profiling critical cancer gene mutations in clinical tumor samples. *PLoS One*. 2009;4(11):e7887.

27. Grambsch PM, Therneau TM. Proportional hazards tests and diagnostics based weighted residuals. Biometrika. 1994;81(3):515–526.

28. Cheang MC, Chia SK, Voduc D, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J Natl Cancer Inst*. 2009;101(10):736–750.

29. Jensen JD, Knoop A, Laenkholm AV, et al. PIK3CA mutations, PTEN, and pHER2 expression and impact on outcome in HER2-positive early-stage breast cancer patients treated with adjuvant chemotherapy and trastuzumab. *Ann Oncol*. 2012;23(8):2034–2042.

30. Oda K, Okada J, Timmerman L, et al. PIK3CA cooperates with other phosphatidylinositol 3'-kinase pathway mutations to effect oncogenic transformation. *Cancer Res*. 2008;68(19):8127–8136.

31. Park S, Jiang Z, Mortenson ED, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell*. 2010;18(2):160–170.

32. Clynes RA, Towers TL, Presta LG, et al. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat Med*. 2000;6(4):443–446.

33. Stagg J, Loi S, Divisekera U, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A*. 2011;108(17):7142–7147.

34. Dituri F, Mazzocca A, Giannelli G, et al. PI3K functions in cancer progression, anticancer immunity and immune evasion by tumors. *Clin Dev Immunol*. 2011;2011:947858.

35. Wang Y, Zhou D, Phung S, et al. SGK3 Is an estrogen-inducible kinase promoting estrogen-mediated survival of breast cancer cells. *Mol Endocrinol*. 2011;25(1):72–82.

36. Vasudevan KM, Barbie DA, Davies MA, et al. AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. *Cancer Cell*. 2009;16(1):21–32.

37. TCGA. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490(7418):61–70.

38. Frasor J, Danes JM, Komm B, et al. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology*. 2003;144(10):4562–4574.

39. Yarden RI, Wilson MA, Chrysogelos SA. Estrogen suppression of EGFR expression in breast cancer cells: a possible mechanism to modulate growth. *J Cell Biochem*. 2001;81(S36):232–246.

40. Carracedo A, Alimonti A, Pandolfi PP. PTEN level in tumor suppression: how much is too little? *Cancer Res*. 2011;71(3):629–633.

41. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene*. 2008;27(41):5497–5510.

42. Ellis MJ, Ding L, Shen D, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature*. 2012;486(7403):353–360.

43. Stephens PJ, Tarpey P, Davies HR, et al. The landscape of cancer genes and mutational processes in breast cancer. *Nature*. 2012;486(7403):400–404.

44. Brachmann SM, Hofmann I, Schnell C, et al. Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. *Proc Natl Acad Sci U S A*. 2009;106(52):22299–22304.

45. O'Brien C, Wallin JJ, Sampath D, et al. Predictive biomarkers of sensitivity to the phosphatidylinositol 3' kinase inhibitor GDC-0941 in breast cancer preclinical models. *Clin Cancer Res*. 2010;16(14):3670–3683.

46. Weigelt B, Warne PH, Downward J. PIK3CA mutation, but not PTEN loss of function, determines the sensitivity of breast cancer cells to mTOR inhibitory drugs. *Oncogene*. 2010;30(29):3222–3233.

47. Banerji S, Cibulskis K, Rangel-Escareno C, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature*. 2012;486(7403):405–409.

48. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature*. 2012;486(7403):395–399.

49. Lee JW, Soung YH, Seo SH, et al. Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas. *Clin Cancer Res*. 2006;12(1):57–61.

50. Kancha RK, von Bubnoff N, Bartosch N, et al. Differential sensitivity of ERBB2 kinase domain mutations towards lapatinib. *PLoS One*. 2011;6(10):e26760.

51. Trowe T, Boukouvala S, Calkins K, et al. EXEL-7647 inhibits mutant forms of ErbB2 associated with lapatinib resistance and neoplastic transformation. *Clin Cancer Res*. 2008;14(8):2465–2475.

52. Bose R, Kavuri SM, Searleman AC, et al. Activating HER2 mutations in HER2 gene amplification negative breast cancer. *Cancer Discov*. 2013;3(2):224–237.

53. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366(10):883–892.

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