PIK3CA Mutation in the ShortHER Randomized Adjuvant Trial for Patients with Early HER2$^+$ Breast Cancer: Association with Prognosis and Integration with PAM50 Subtype

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ABSTRACT

Purpose: We explored the prognostic effect of *PIK3CA* mutation in HER2$^+$ patients enrolled in the ShortHER trial.

Patients and Methods: The ShortHER trial randomized 1,253 patients with HER2$^+$ breast cancer to 9 weeks or 1 year of adjuvant trastuzumab combined with chemotherapy. *PIK3CA* hotspot mutations in exon 9 and 20 were analyzed by pyrosequencing. Expression of 60 genes, including PAM50 genes was measured using the nCounter platform.

Results: A mutation of the *PIK3CA* gene was detected in 21.7% of the 803 genotyped tumors. At a median follow-up of 7.7 years, 5-year disease-free survival (DFS) rates were 90.6% for *PIK3CA* mutated and 86.2% for *PIK3CA* wild-type tumors [HR, 0.84; 95% confidence interval (CI), 0.56–1.27; $P = 0.417$]. *PIK3CA* mutation showed a favorable prognostic impact in the PAM50 HER2-enriched subtype ($n = 232$): 5-year DFS 91.8% versus 76.1% (log-rank $P = 0.049$; HR, 0.46; 95% CI, 0.21–1.02). HER2-enriched/*PIK3CA* mutated versus wild-type tumors showed numerically higher tumor-infiltrating lymphocytes (TIL) and significant upregulation of immune-related genes (including *CD8A, CD274, PDCD1,* and *MYBL2*, a proliferation gene involved in immune processes). High TILs as well as the upregulation of *PDCD1* and *MYBL2* were associated with a significant DFS improvement within the HER2-enriched subtype (HR, 0.82; 95% CI, 0.68–0.99; $P = 0.039$ for 10% TILs increment; HR, 0.81; 95% CI, 0.65–0.99; $P = 0.049$ for *PDCD1* expression; HR, 0.72; 95% CI, 0.53–0.99; $P = 0.042$ for *MYBL2* expression).

Conclusions: *PIK3CA* mutation showed no prognostic impact in the ShortHER trial. Within the HER2-enriched molecular subtype, patients with *PIK3CA* mutated tumors showed better DFS versus *PIK3CA* wild-type, which may be partly explained by upregulation of immune-related genes.

Introduction

HER2$^+$ breast cancer is a heterogeneous entity with regards to gene expression and gene mutation profile. Such heterogeneity may affect both prognosis and treatment efficacy.

The PI3K pathway is frequently aberrantly activated in breast cancer through activating mutations in the helical (exon 9) or kinase (exon 20) domain of the *PIK3CA* gene. The rate of tumors harboring a *PIK3CA* gene mutation varies according to tumor subtype and has been reported in the range of 20%–25% in early HER2$^+$ breast cancer (1–3). *In vitro* studies have shown that the activation of the PI3K pathway, being downstream to HER2 and hub of multiple intracellular signaling, may drive escape from upstream inhibition of HER2 (4). In the metastatic setting, patients with HER2$^+$ and *PIK3CA* mutated breast cancer treated with chemotherapy and anti-HER2 therapy experience

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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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Translational Relevance

In early HER2$^+$ breast cancer, *PIK3CA* mutation is associated with reduced rate of pathologic complete response after neoadjuvant chemotherapy and anti-HER2 agents without any impact on disease-free or overall survival. This study evaluated the prognostic role of *PIK3CA* mutation in the largest cohort from a randomized trial of HER2$^+$ patients treated with adjuvant chemotherapy and trastuzumab. Beyond showing no prognostic effect in the whole study cohort, our study provides unique data describing for the first time a potential prognostic effect of *PIK3CA* mutation in the HER2-enriched subtype. We also provide data supporting the association of *PIK3CA* mutation and immune activation in the HER2-enriched cohort, which might at least in part explain the favorable prognostic effect. These data warrant further validation and may contribute to the generation of an integrated multiple biomarker score for HER2$^+$ early breast cancer.

poorer prognosis as compared with *PIK3CA* wild-type patients (5, 6). In early breast cancer, a large pooled analysis of individual patients data from prospective trials have demonstrated that *PIK3CA* mutation is associated with reduced rate of pathologic complete response after chemotherapy and anti-HER2 agents (3). However, investigation of *PIK3CA* mutation in HER2$^+$ patients enrolled in adjuvant trials have not demonstrated any prognostic effect of this molecular alteration, nor a predictive effect for trastuzumab added to adjuvant chemotherapy (7–9).

However, because HER2$^+$ disease is heterogeneous, one could hypothesize that the effect of *PIK3CA* mutation may depend on the tumor subtype. For example, in their pooled analysis of neoadjuvant trials for HER2$^+$ breast cancer, Loibl and colleagues showed that *PIK3CA* mutation was associated with an improved long-term outcome in hormone receptor–negative patients and to a worse outcome in hormone receptor–positive patients (3). It is now clear that the simple dichotomization of HER2$^+$ breast cancer in two subtypes according to hormone receptor status is oversimplistic. PAM50 molecular intrinsic subtypes more accurately recapitulate the complexity of HER2$^+$ breast cancer biology (10). All relevant intrinsic subtypes are represented within HER2$^+$ disease, with a distribution that varies according to hormone receptor coexpression (10–12).

Currently, there are no data on the impact of *PIK3CA* mutation within molecular intrinsic subtypes of HER2$^+$ breast cancer.

In this study, we investigated the prognostic role of *PIK3CA* mutation in HER2$^+$ patients with breast cancer enrolled in the prospective randomized ShortHER trial of adjuvant chemotherapy and trastuzumab. Our aim was to evaluate the prognostic effect of *PIK3CA* mutation in the overall population and according to molecular intrinsic subtype.

Patients and Methods

Patients

The ShortHER trial is a phase III multicentric trial of adjuvant therapy that randomized (1:1) 1,253 patients with HER2$^+$ early breast cancer to:

Arm A (long): AC (adriamycin 60 mg/sqm plus cyclophosphamide 600 mg/sqm) or EC (epirubicin 90 mg/sqm plus cyclophosphamide 600 mg/sqm) every 3 weeks for four courses followed by paclitaxel 175 mg/sqm or docetaxel 100 mg/sqm every 3 weeks for four courses combined with trastuzumab every 3 weeks for 1 year starting concomitant with taxane (8 mg/kg loading dose followed by 6 mg/kg thereafter) or Arm B (short): docetaxel 100 mg/sqm every 3 weeks for three courses with concomitant trastuzumab every week for 9 weeks (4 mg/kg loading dose followed by 2 mg/kg thereafter) followed by FEC (5-Fluorouracil 600 mg/sqm, epirubicin 60 mg/sqm, and cyclophosphamide 600 mg/sqm) every 3 weeks for four courses.

The aim was to demonstrate the noninferiority of short (Arm B) versus long (Arm A) treatment in terms of disease-free survival (DFS). Further details and results of the primary study endpoint are reported elsewhere (13). Briefly, the study failed to demonstrate the noninferiority of 9 weeks of the short treatment: 5-years DFS rates were 88% in the long and 85% in the short arm [HR, 1.13; 90% confidence interval (CI), 0.89–1.42, with a predefined noninferiority margin of 1.29]. This analysis was approved by the competent Ethical Committee in November 2014, patients provided signed informed consent for tumor sample centralization and use for research purpose. The study was conducted in accordance with ethical guidelines (1964 Helsinki declaration and its later amendments or comparable ethical standards).

The consort diagram for the analyses described in this work is shown in Supplementary Fig. S1.

PIK3CA gene status

Formalin-fixed paraffin-embedded (FFPE) tumor blocks were reviewed for quality and tumor content. Macro dissection from 5-μmol/L FFPE sections of primary tumor lesions containing at least 20% of tumor cells were carried out to obtain tumor DNA. DNA was extracted with the Maxwell 16 FFPE Tissue LEV DNA Purification Kit (Promega) in the Maxwell 16 Instrument (Promega), according to the manufacturer's instructions. *PIK3CA* status was analyzed by pyrosequencing using anti-EGFR MoAb response (*PIK3CA*status) Kit (Diatech Pharmacogenetics), according to the manufacturer's instructions. Reactions were run on a PyroMark Q96 ID (Qiagen). This kit allows identification of the most important mutations in exon 9 (E542K, E545K, E545A, E545G, Q546E, and Q546K) and exon 20 (M1043I, H1047Y, H1047R, H1047L, G1049R, and G1049S) of the *PIK3CA* gene.

Tumor-infiltrating lymphocytes

Methods for tumor-infiltrating lymphocytes (TIL) assessment and TILs data have been described previously (14). TILs data included in this article derive from previous publication (14).

Gene expression

First, a section of centralized FFPE surgical tumor sample was examined with hematoxylin and eosin staining to confirm diagnosis and determine tumor surface area. If needed, microdissection was then performed to avoid normal breast contamination. RNA was then extracted from FFPE material using the High Pure FFPET RNA Isolation Kit (Roche) following manufacturer's protocol and quantified using the NanoDrop spectrophotometer (Thermo Fisher Scientific).

A minimum of approximately 100 ng of total RNA was used to measure the expression of 55 BC-related genes, including the PAM50 genes, androgen receptor and some immune-related genes (e.g., *CD8A*, *CD4*, *PD1*, and *PD-L1*), and five housekeeping genes (*ACTB*, *MRPL19*, *PSMC4*, *RPLP0*, and *SF3A1*) using the nCounter platform (Nanostring Technologies; ref. 15). Data were log

base 2–transformed and normalized using the housekeeping genes. The complete list of genes can be found in Supplementary Table S1. Intrinsic molecular subtyping at baseline was determined using the previously reported PAM50 subtype predictor (16).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Version 25 and R project software 3.4.4 (17).

Association between variables was evaluated by the Pearson χ^2 test or the Mann–Whitney test, according to the nature of the variables.

DFS was defined as the time between randomization and any of the following events, whichever first: local, regional, and distant recurrence; contralateral breast cancer, excluding *in situ* carcinoma; other second invasive primary cancer; death before recurrence or second primary cancer. Patients without event were censored at the date of last follow-up. The Kaplan–Meier method was used to estimate survival curves, the log-rank test was used to compare between groups. Cox proportional regression models were used to calculate HRs and 95% CIs. The likelihood ratio test was used to evaluate the amount of prognostic information provided by single variables when added to integrated prognostic models within the HER2-enriched subtype. Level of significance was $P < 0.05$.

To identify genes whose expression was significantly different according to *PIK3CA* mutational status, we used a two-class unpaired significance of microarrays (SAM) analysis with a FDR <10%.

No formal sample size calculation was performed, because the sample population was based on the number of cases with centralized tumor sample that was suitable for the present analysis.

The analyses described in this work were not prespecified in the study protocol.

Results

Patients' characteristics

Centralized tumor samples were available for 913 of the 1,253 randomized patients. A total of 803 cases (64% of all randomized patients) had tumor samples suitable for *PIK3CA* assessment and were included in this analysis (Supplementary Fig. S1). The characteristics of the 803 patients analysed for *PIK3CA* and the comparison with the 450 patients from the ShortHER trial not included in this analysis are shown in Supplementary Table S2. As compared with patients not assessed for *PIK3CA*, those included in the *PIK3CA* cohort were older [median age 56 (Q1–Q3: 48–64) vs. 54 (Q1–Q3: 46–62), $P = 0.012$], presented more often hormone receptor positive tumors (71% vs. 64%, $P = 0.010$), and showed lower TILs levels [median 5 (Q1–Q3: 1–15) vs. 5 (Q1–Q3: 1–30), $P = 0.040$].

PIK3CA

A mutation of the *PIK3CA* gene was detected in 21.7% of the 803 genotyped patients ($n = 174$ mutated; $n = 629$ wild-type). Mutations in exon 9 and 20 occurred in 78 (9.7%) and 95 (11.8%) cases, respectively (Supplementary Fig. S2 shows the pattern of mutations).

Association between *PIK3CA* and clinicopathologic characteristics is shown in **Table 1**. *PIK3CA* mutation occurred more frequently in hormone receptor–positive versus hormone receptor–negative cases (23.5% vs. 17.4%, $P = 0.057$) and in postmenopausal vs. premenopausal patients (23.8% vs. 17.7%, $P = 0.050$). No association with stage, age, grade, TILs, and treatment arm was observed. Although the distribution of *PIK3CA* mutation according to molecular subtype was not significant, the rate of *PIK3CA* mutation was numerically higher in Luminal A (22/86, 26%), followed by HER2-enriched (49/232, 21%), Luminal B (8/42, 19%), and Basal-like (2/27, 7%).

PIK3CA and DFS

At a median follow-up of 7.7 years (95% CI, 7.5–7.9), 146 DFS events occurred: 28 in patients with *PIK3CA* mutated tumor (28/174, 16%) and 118 in patients with *PIK3CA* wild-type tumor (118/629, 19%; $P = 0.419$). **Figure 1** shows the Kaplan–Meier DFS curves according to *PIK3CA* gene status. DFS rates at 5 years were 90.6% for *PIK3CA* mutated and 86.2% for *PIK3CA* wild-type groups (HR, 0.84; 95% CI, 0.56–1.27; $P = 0.417$). Similar results were obtained in patients randomized to receive 1 year of trastuzumab (HR, 0.86; 95% CI, 0.48–1.55) and in patients randomized to 9 weeks of trastuzumab (HR, 0.84; 95% CI, 0.47–1.50).

We explored the prognostic effect of *PIK3CA* gene status according to hormone receptor expression and intrinsic molecular subtype. Because of limited sample size and number of events within individual intrinsic molecular sutbypes, we analyzed the impact of *PIK3CA* mutations in two groups: HER2-enriched and non-HER2-enriched (including Luminal A, Luminal B, Basal, and Normal-like). Kaplan–Meier curves are shown in **Fig. 2**. *PIK3CA* mutation had no significant effect on DFS in hormone receptor–positive and hormone receptor–negative subgroups. According to intrinsic subtype, patients with *PIK3CA* tumor experienced better DFS as compared with *PIK3CA* wild-type in the HER2-enriched subgroup: 5-year DFS rate was 91.8% for *PIK3CA* mutated versus 76.1% for *PIK3CA* wild-type groups, log-rank $P = 0.049$ (HR, 0.46; 95% CI, 0.21–1.02; $P = 0.055$). No difference in DFS was observed in non-HER2-enriched patients according to *PIK3CA* gene status. The test for interaction was not significant ($P = 0.269$). Within the HER2-enriched group, the positive prognostic impact of *PIK3CA* mutation was particularly evident in patients with hormone receptor–negative tumor (n tot $= 88$; *PIK3CA* mutated $n = 14$, 0 events; *PIK3CA* wild-type $n = 74$, 21 events): 5-year DFS rate was 100% versus 75.6%, log-rank $P = 0.029$. In HER2-enriched/hormone receptor–positive patients (n tot $= 144$, *PIK3CA* mutated $n = 35$, 7 events; *PIK3CA* wild-type $n = 109$, 30 events), 5-year DFS rate in the *PIK3CA* mutated and wild-type groups was 88.6% and 76.5%, respectively (log-rank $P = 0.329$).

Clinicopathologic characteristics of *PIK3CA* mutated, HER2-enriched tumors

To explore the potential reasons for favorable prognostic effect of *PIK3CA* mutation within the HER2-enriched molecular subtype, we first analyzed the association between *PIK3CA* gene status and classic clinicopathologic factors in this patients' subgroup (Supplementary Table S3). *PIK3CA* gene mutation was numerically associated with hormone receptor–positive status (71% vs. 60%, $P = 0.128$) and higher TILs [median 8 (Q1–Q3:2–30) vs. median 5 (Q1–Q3:1–20), $P = 0.164$]. **Figure 3** shows box plot for TILs levels according to *PIK3CA* mutation in the entire HER2-enriched cohort as well as in HER2-enriched/hormone receptor–positive and HER2-enriched/hormone receptor–negative patients. In the HER2-enriched/hormone receptor–negative subgroup, TILs were significantly higher in PIK3CA mutated tumors [median 24% (Q1–Q3:10–50) for *PIK3CA* mutated vs. median 7 (Q1–Q3:2–20) in *PIK3CA* wild-type, $P = 0.005$]. We have previously demonstrated the independent prognostic role of TILs in the ShortHER trial (14). TILs maintained a significant association with DFS in the subgroup of HER2-enriched patients analyzed for *PIK3CA* in this study: (HR, 0.82; 95% CI, 0.68–0.99; $P = 0.039$ for each 10% TILs increment].

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Table 1. Patients' characteristics in the *PIK3CA* cohort according to *PIK3CA* gene status.

Characteristics		*PIK3CA* mutated *N* (%)	*PIK3CA* wild-type *N* (%)	TOT (*PIK3CA* cohort) *N* (%)	*P*
Total		174 (22%)	629 (78%)	803 (100%)	—
Age (years)					
	<60	104 (60)	393 (62)	497 (62)	
	≥60	70 (40)	236 (38)	306 (38)	0.515
	Median (Q1–Q3)	57 (50–64)	56 (48–64)	56 (48–64)	0.169
Menopausal status					
	Premenopausal	49 (28)	227 (36)	276 (34)	
	Postmenopausal	125 (72)	401 (64)	526 (66)	0.050
AJCC Stage					
	I	70 (40)	259 (41)	329 (41)	
	II	77 (44)	277 (44)	354 (44)	
	III	27 (16)	91 (15)	118 (15)	0.936
N stage					
	N0	85 (49)	343 (55)	428 (53)	
	N1-N2	61 (35)	191 (30)	252 (31)	
	N3	28 (16)	95 (15)	123 (15)	0.393
Hormone receptors					
	Negative	41 (24)	195 (31)	236 (29)	
	Positive	133 (76)	434 (69)	567 (71)	0.057
Histologic grade					
	Grade 1–2	54 (31)	172 (28)	226 (28)	
	Grade 3	118 (69)	448 (72)	566 (72)	0.348
TILs	Median (Q1–Q3)	5 (1–15)	5 (1–15)	5 (1–15)	0.637
PAM50 Intrinsic subtype					
	LumA	22 (24)	64 (19)	86 (20)	
	LumB	8 (9)	34 (10)	42 (10)	
	HER2-enriched	49 (53)	183 (54)	232 (54)	
	Basal-like	2 (2)	25 (7)	27 (6)	
	Normal-like	11 (12)	35 (10)	46 (11)	0.358
Treatment arm					
	A long	93 (53)	312 (50)	405 (50)	
	B short	81 (47)	317 (50)	398 (50)	0.369

Abbreviations: AJCC, American Joint Committee on Cancer; LumA, luminal A; LumB, luminal B; TILs, tumor-infiltrating lymphocytes.

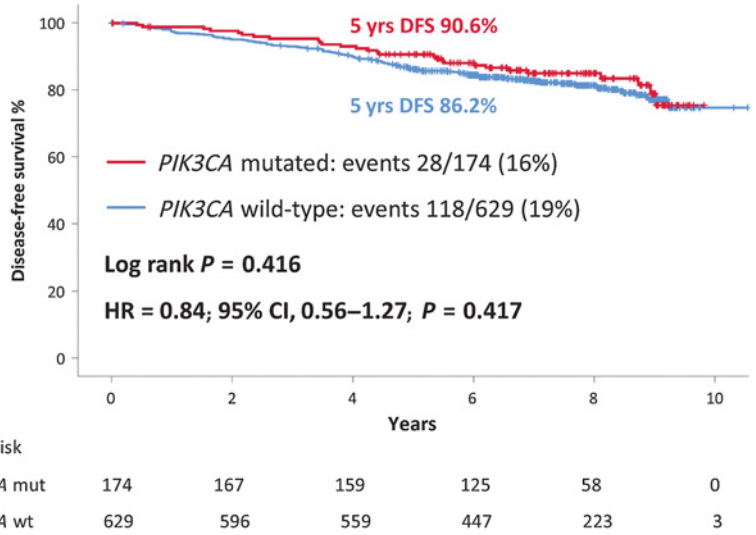

Figure 1.
Kaplan–Meier curves for DFS according to *PIK3CA* gene mutation ($n = 803$ patients from the ShortHER trial).

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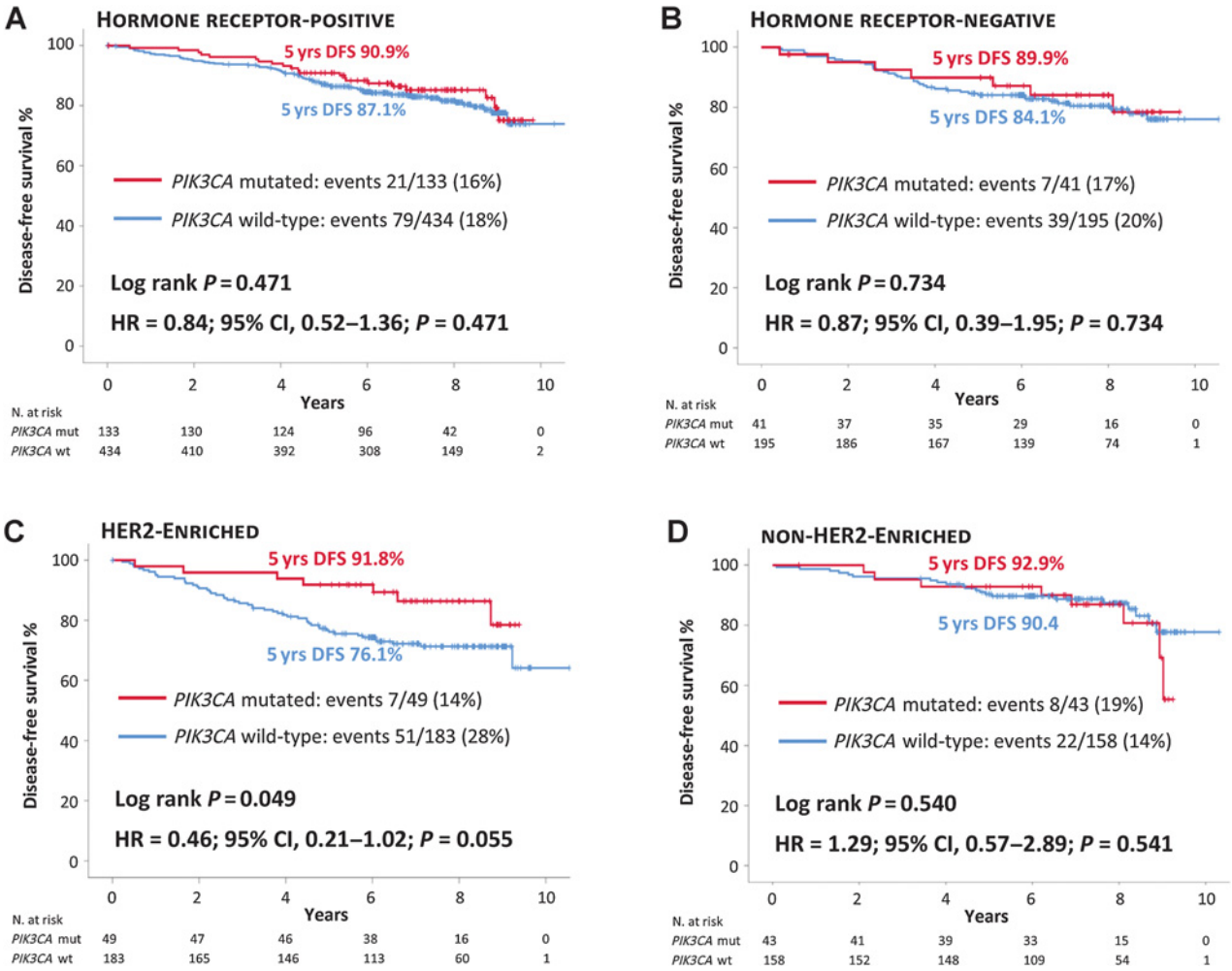

Figure 2.

Kaplan–Meier curves for DFS according to *PIK3CA* gene mutation in subgroups: hormone receptor–positive (**A**), hormone receptor–negative (**B**), HER2-enriched (**C**), and non–HER2-enriched (**D**).

Gene expression profile of *PIK3CA* mutated, HER2-enriched tumors

We analyzed differences in gene expression among HER2-enriched tumors according to *PIK3CA* gene status (**Fig. 4**). As reported in Supplementary Table S4, the following genes were overexpressed in *PIK3CA* mutated tumors (two-class unpaired SAM analysis, FDR < 0.10): genes tracking luminal properties (*ESR1* and *PGR*), genes tracking proliferation and cell-cycle processes (*MYC, MKI67, CEP55, MYBL2*), immune-related genes (*CD8A, CD274* encoding for PD-L1, *PDCD1* encoding for PD-1), and a gene encoding for the microtubule-associated protein tau (*MAPT*). Genes that resulted downregulated in *PIK3CA* mutated tumors were: *ERBB2, GRB7* (which is one of the 105 protein-encoding genes located in the same amplicon as *ERBB2*) and *TMEM45B* [encoding for a member of the transmembrane (TMEM) family, which includes proteins that span biological membranes]. The same analysis was conducted stratified by hormone receptor status (Supplementary Table S4): the only differentially expressed genes were *ERBB2* and *GRB7* that resulted downregulated in *PIK3CA* mutated tumors in the HER2-enriched/hormone receptor–positive subgroup.

We then explored the association between differentially expressed genes in *PIK3CA* mutated versus *PIK3CA* wild-type tumors and DFS, in the cohort of HER2-enriched patients. Univariate cox regression analysis is shown in **Table 2**. Two genes found to be upregulated in HER2-enriched/*PIK3CA* mutated had a significant association with improved DFS: *MYBL2* (HR, 0.72; 95% CI, 0.53–0.00; $P = 0.042$) and *PDCD1* (HR, 0.81; 95% CI, 0.65–0.99; $P = 0.049$). Interestingly, as further described in the Discussion, *MYBL2* may be implicated in processes linked to tumor immune activation. Among the genes found to be downregulated in HER2-enriched/*PIK3CA* mutated tumors, *TMEM45B* was associated with a worse DFS (HR, 1.31; 95% CI, 1.02–1.69; $P = 0.037$).

Integrated prognostic models within the HER2-enriched subtype

We assessed the amount of prognostic information provided by *PIK3CA* mutation, TILs, *PDCD1* expression, and *MYBL2* expression when added to integrated prognostic models for the HER2-enriched subtype. Among classic clinicopathologic factors, stage was significantly associated with DFS (stage II vs. stage III:

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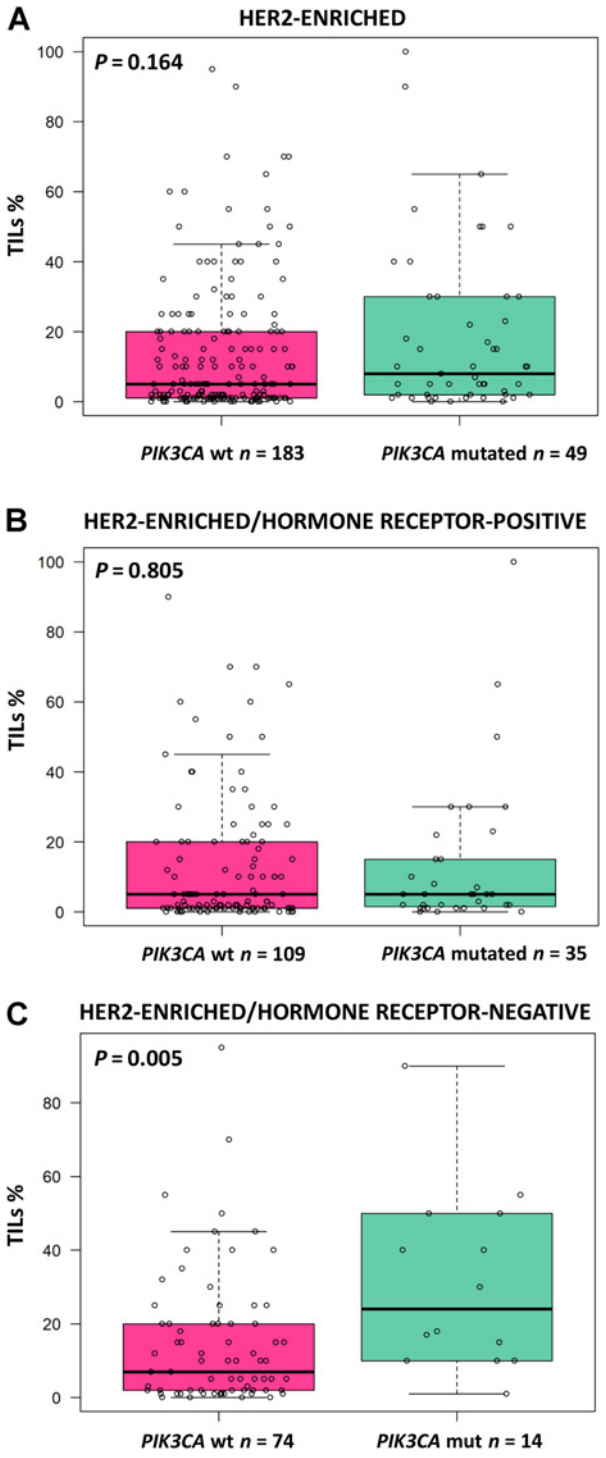

Figure 3.
Box plot showing TILs levels according to *PIK3CA* mutation in HER2-enriched patients: all patients (**A**), hormone receptor–positive (**B**), and hormone receptor–negative (**C**).

HR, 0.48; 95% CI, 0.26–0.88; $P = 0.019$; stage I vs. stage III: HR, 0.37; 95% CI, 0.18–0.75; $P = 0.006$). Age (HR, 1.02; 95% CI, 0.99–1.04; $P = 0.264$), histologic grade (grade 1–2 vs. 3: HR, 1.03; 95% CI, 0.56–1.91; $P = 0.920$), and hormone receptor status (positive vs. negative: HR, 1.11; 95% CI, 0.65–1.90; $P = 0.703$) were not associated with DFS. When TILs, *PIK3CA* mutation, *PDCD1* expression, or *MYBL2* expression were added as single variables to a model containing stage, all provided a significant amount of prognostic information (Supplementary Table S5). The inclusion of either TILs or *PDCD1* expression added significant prognostic information when included to a model containing stage and *PIK3CA* status (Supplementary Table S5). To the opposite, the inclusion of *PIK3CA* mutation to a model containing stage and TILs, stage and *PDCD1* expression, or stage and *MYBL2* expression was not significantly prognostic (Supplementary Table S5).

Discussion

This study represents the largest cohort of patients with HER2$^+$ early breast cancer homogeneously treated with adjuvant chemotherapy and trastuzumab in the context of a clinical trial that were evaluated for *PIK3CA* mutation ($n = 803$) and the first study to date to investigate the prognostic role of *PIK3CA* mutation within molecular intrinsic subtypes of HER2$^+$ breast cancer (7–9).

We did not find any prognostic impact of *PIK3CA* gene mutation in the overall study cohort. This result is concordant with data from other adjuvant trials showing no association between *PIK3CA* mutation and long-term outcome (7–9). Moreover, in the NSABP B-31 and FinHER studies, *PIK3CA* mutation was not predictive of reduced benefit from adjuvant trastuzumab (7, 8). More recently, *PIK3CA* mutation did not result associated with differential benefit from adjuvant neratinib in the ExteNET trial (9). Two pooled analyses also confirmed no robust impact of *PIK3CA* mutation on long-term outcome. Zardavas and colleagues conducted a pooled analysis of more than 10,000 patients with early breast cancer (18). In the HER2$^+$ subgroup, they described a numerically worse overall survival for patients with *PIK3CA* mutation (HR, 1.17; 95% CI, 0.94–1.46). However, there was no impact of *PIK3CA* mutation on invasive DFS (HR, 0.98) or distant DFS (HR, 0.93). Moreover, not all the patients in this cohort received adjuvant trastuzumab. In the pooled analysis of neoadjuvant trials in HER2$^+$ disease, although pathologic complete response rates after chemotherapy and anti-HER2 treatment were lower in patients with *PIK3CA* mutated tumor, this did not translate into a worse DFS (3). Some hypotheses may explain this discrepancy in the effect of *PIK3CA* mutation on pathologic complete response and long-term outcome. First, *PIK3CA* mutation might confer a more indolent biology that may result in lower rates of pathologic complete response without affecting long-term outcome, similarly to what is observed in case of HER2$^+$/hormone receptor–positive breast cancer (19). In our cohort, patients with *PIK3CA* mutation were more frequently postmenopausal and showed more frequently HR-positive disease. However, the association with hormone receptor status is not consistent across studies (3, 8, 9). According to intrinsic subtype, in our study, Luminal A tumors showed the highest frequency of *PIK3CA* mutation, although the distribution of *PIK3CA* mutation across intrinsic subtypes was not statistically significant. In the CALGB 40601 neoadjuvant study, the highest rate of *PIK3CA* mutation was detected in Luminal B/HER2$^+$ tumors (20). A second potential explanation for discrepant data may be linked to the observation that *PIK3CA* mutation not necessarily determines downstream PI3K pathway activation (21), a factor that may act as a confounder in studies looking at *PIK3CA* mutation. Finally, one could also hypothesize a different role of *PIK3CA* mutation in macroscopic versus microscopic disease. It has been previously suggested that antibody-dependent cell-mediated cytotoxicity

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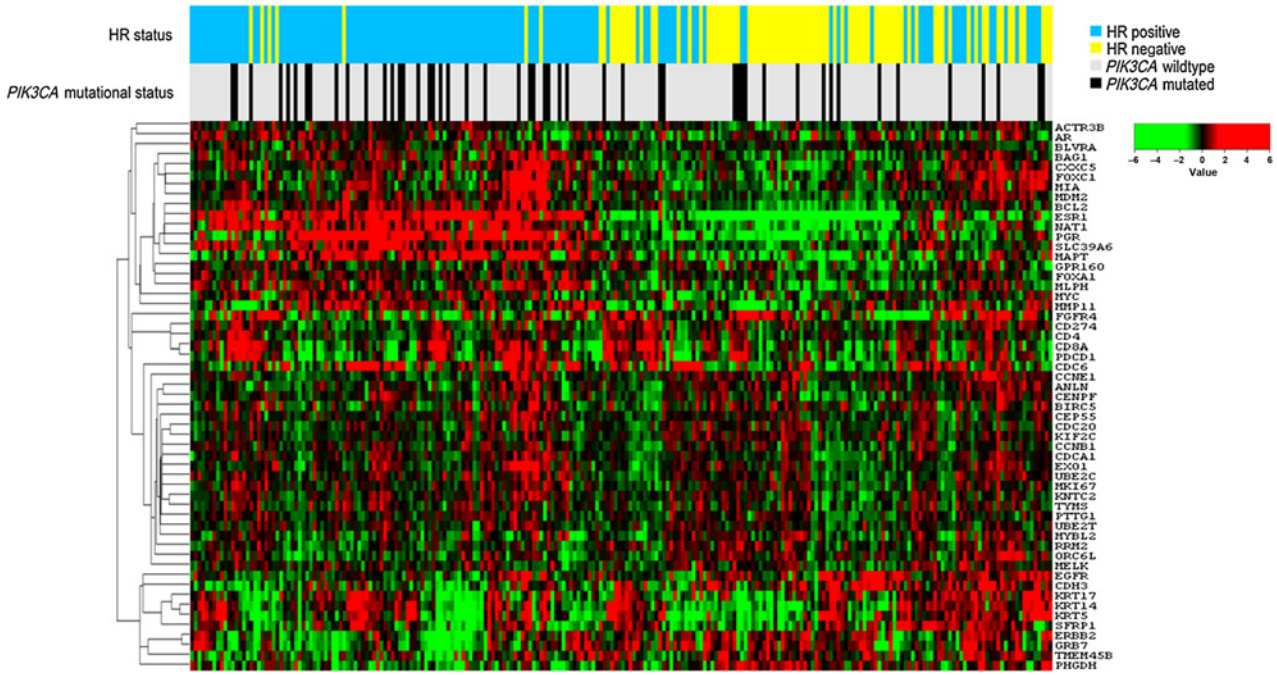

Figure 4.

Unsupervised clustering of 55 genes across HER2-enriched tumors from the ShortHER trial ($N = 232$). Hormone receptor status (positive - blue; negative - yellow) and *PIK3CA* mutational status (mutated - black; gray – wild-type) of each sample is also presented.

(ADCC), which is known as the main mechanisms of action of trastuzumab, may be more effective, in the presence of *PIK3CA* mutation, in case of microscopic disease, whereas in case of macroscopic disease it may be more difficult for ADCC to overcome resistance due to downstream pathway activation (22, 23).

However, another hypothetic explanation may be that the role of *PIK3CA* mutation depends on tumor subtype. A first hint was suggested by the pooled analysis by Loibl and colleagues. In this study, there was a significant interaction between *PIK3CA* mutation and hormone receptor status for both pathologic complete response and DFS: the reduction in pathologic complete response rate was significant in hormone receptor–positive disease and not in hormone receptor–negative disease ($P_{interaction} = 0.036$), *PIK3CA* was associated with significantly worse DFS in hormone receptor–positive patients and with a trend for a better DFS in hormone receptor–negative patients ($P_{interaction} = 0.021$; ref. 3).

Table 2. HER2-enriched patients: univariate DFS Cox regression analysis of genes found to be differentially expressed according to *PIK3CA* gene status.

	All patients	
Gene	**HR (95% CI)**	***P***
Upregulated in *PIK3CA* mut		
ESR1	1.06 (0.95–1.19)	0.300
PGR	1.04 (0.96–0.93)	0.490
MYC	0.84 (0.64–1.09)	0.193
MKI67	0.75 (0.49–1.17)	0.203
CEP55	0.64 (0.39–1.04)	0.073
MYBL2	0.72 (0.53–0.99)	0.042
CD8A	0.84 (0.68–1.05)	0.118
CD274	0.80 (0.57–1.12)	0.197
PDCD1	0.81 (0.65–0.99)	0.049
MAPT	0.98 (0.88–1.19)	0.801
Downregulated in *PIK3CA* mut		
ERBB2	0.98 (0.81–1.20)	0.873
GRB7	0.97 (0.80–1.18)	0.773
TMEM45B	1.31 (1.02–1.69)	0.037

Abbreviations: CI, confidence interval; HR, hazard ratio.

In our study, we did not observe any difference in the impact of *PIK3CA* mutation on outcome according to hormone receptor status. However, we found an association between the presence of *PIK3CA* mutation and improved outcome in HER2-enriched patients (5-year DFS rate 91.8% vs. 76.1%, log-rank $P = 0.049$; HR, 0.46; 95% CI, 0.21–1.02; $P = 0.055$; test for interaction between molecular subtype and *PIK3CA* mutation not significant). In this subgroup, *PIK3CA* mutation was significantly prognostic beyond stage. On the basis of this result, we investigated potential biological differences between *PIK3CA* mutated and wild-type tumors within the HER2-enriched subtype. We observed that HER2-enriched/*PIK3CA* mutated tumors were numerically more frequently hormone receptor–positive, showed increased expression of *ESR1* and *PGR* and a decreased expression of *ERBB2* as compared with HER2-enriched/*PIK3CA* wild-type tumors. These results suggest that *PIK3CA* mutation may be associated, even within the HER2-enriched subgroup, to a more luminal-like and less HER2-addicted gene expression profile as compared with *PIK3CA* wild-type tumors that might account for a more indolent behavior. We also found that HER2-enriched/*PIK3CA*-mutated tumors showed numerically higher TILs levels (statistically significant in hormone receptor–negative patients), and upregulation in genes involved in proliferation and immune response. In particular, when exploring, within the HER2-enriched subtype, the association with DFS of the features and genes found to be differentially expressed between *PIK3CA* mutated and *PIK3CA* wild-type tumors, the

biological processes that seemed to affect prognosis to a larger extent were those attributable to immune pathways. Indeed, increase TILs levels and increased expression of *PDCD1* and *MYBL2* were all significantly associated to improved prognosis. In multivariable models, TILs and *PDCD1* added significant prognostic information beyond stage and *PIK3CA* mutation, whereas *PIK3CA* mutation did not add a significant amount of prognostic information beyond models containing stage and TILs or stage and *PDCD1* expression. This observation corroborates the hypothesis that immune features associated with *PIK3CA* mutation, rather than *PIK3CA* mutation itself, may be the main biological driver of the observed prognostic effect on DFS of *PIK3CA* in univariate analysis. TILs are known to be a strong prognostic factor in HER2$^+$ breast cancer and reflect a general state of immune activation. The significant prognostic effect of TILs in patients with early HER2$^+$ breast cancer treated with adjuvant therapy has been previously demonstrated by our group and others (14, 24). *PDCD1* expression is dynamic and tends to increase to counterbalance an antitumor immune activation; the prognostic role of PD-L1 gene expression in HER2-enriched breast cancer has not been clearly established thus far (25). *MYBL2* is a transcription factor involved in cell cycle; however, there is evidence that it may also be implicated in immune activation or in immune response–promoting processes. Indeed, *MYBL2* may mediate increase mutational load by inducing APOBEC expression (26), may promote *XCL1* expression in breast cancer which is associated with a type 1 dendritic cell signature and improved survival (27) and may be implicated in chromosomal instability (28). In our article, *MYBL2* provided significant prognostic information beyond stage but not to a model containing stage and *PIK3CA* mutation.

Interestingly, other works have linked *PIK3CA* mutation to immune infiltration. A recent study by Sobral-Leite and colleagues focused on hormone receptor–positive patients (also including HER2$^+$ cases) found that tumors with *PIK3CA* mutation tend to have more CD8 cells and tumors enriched for FOXP3-positive cells show downstream activation of the PI3K pathway (29). More functional data are needed to better understand the interactions between the PI3K pathway and the immune microenvironment.

The strengths of our study are: the large prospective cohort of patients from a randomized trial, the large number of samples suitable for molecular analyses, and the study design. Our study has limitations, including: the fact that these analyses were not prespecified in the protocol and the lack of statistical power that precluded the possibility to evaluate the association of *PIK3CA* with DFS within each single molecular intrinsic subtype separately. Another limitation is the lack of correction for multiple comparisons, due to the exploratory nature of the study, raising the possibility that some of the findings might be due to chance. Therefore, our results should not be regarded as conclusive but rather as hypothesis generating. However, they add novel findings to the knowledge in the field that deserve validation.

In conclusion, our study provides unique data exploring the role of *PIK3CA* mutation in HER2$^+$ breast cancer by integrating the classification into molecular intrinsic subtype; we describe for the first time a potential prognostic effect of *PIK3CA* mutation in the HER2-enriched subtype; we provide data supporting the association of *PIK3CA* mutation and immune activation in the HER2-enriched cohort, which might at least in part explain the favorable prognostic effect. These data warrant further validation and may contribute to the generation of an integrated multiple biomarker score for HER2$^+$ early breast cancer. Indeed, beyond classic clinicopathologic factors, it is now clear that intrinsic subtypes and immune features represent independent prognostic factors that could be combined into a prognostic score for patients' stratification. How and if *PIK3CA* mutation should be integrated in prognostic models for HER2-enriched tumors needs to be further evaluated.

Disclosure of Potential Conflicts of Interest

V. Guarneri reports personal fees from Eli Lilly (advisory board, speaker's bureau), Novartis (advisory board, speaker's bureau), and grants from Roche (institutional research grant) outside the submitted work. M.V. Dieci reports personal fees from Eli Lilly, Genomic Health, Novartis, and Celgene outside the submitted work, and is listed as a coinventor on a patent regarding a prognostic index for HER2$^+$ patients with early breast cancer that will be licensed to the University of Barcelona and University of Padova. A. Frassoldati reports personal fees from Roche (nonfinancial support), Novartis (nonfinancial support), Pfizer (nonfinancial support), and Lilly (nonfinancial support) outside the submitted work. A. Musolino reports grants and personal fees from Roche and Eisai, as well as personal fees from Macrogenics, Lilly, Novartis, and Merck-MSK outside the submitted work. O. Garrone reports personal fees from Eisai, Amgen, Eli Lilly, Novartis, and Pfizer outside the submitted work. G. Griguolo reports nonfinancial support from Pfizer (travel support). N. Chic reports other from Novartis (travel expenses) and Eisai (travel expenses) outside the submitted work. A. Prat reports personal fees from Nanostring Technologies during the conduct of the study; grants and personal fees from Roche and Novartis; and personal fees from Daiichi Sankyo, AstraZeneca, Oncolytics Biotech, Pfizer, and MSD outside the submitted work, and is listed as a coinventor of a patent regarding predicting prognosis in early HER2$^+$ disease, owned by IDIBAPS and the University of Padova. P. Conte reports grants from AIFA (institutional research grant) during the conduct of the study; grants from Merck KGA (institutional research grant); grants and personal fees from Bristol-Myers Squibb (institutional research grant; advisory board), Novartis (institutional research grant; advisory board), and Roche (institutional research grant; advisory board); and personal fees from Eli Lilly (advisory board), Tesaro (advisory board), and AstraZeneca (advisory board) outside the submitted work, and is listed as a coinventor on a patent regarding a prognostic index for HER2$^+$ patients with early breast cancer that will be licensed to the University of Barcelona and University of Padova. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

V. Guarneri: Conceptualization, supervision, funding acquisition, writing-review and editing. **M.V. Dieci:** Conceptualization, data curation, formal analysis, writing-original draft. **G. Bisagni:** Data curation, writing-review and editing. **A.A. Brandes:** Data curation, writing-review and editing. **A. Frassoldati:** Data curation, writing-review and editing. **L. Cavanna:** Data curation, writing-review and editing. **A. Musolino:** Data curation, writing-review and editing. **F. Giotta:** Data curation, writing-review and editing. **A. Rimanti:** Data curation, writing-review and editing. **O. Garrone:** Data curation, writing-review and editing. **E. Bertone:** Data curation, writing-review and editing. **K. Cagossi:** Data curation, writing-review and editing. **O. Nanni:** Data curation, writing-review and editing. **F. Piacentini:** Data curation, writing-review and editing. **E. Orvieto:** Data curation, writing-review and editing. **G. Griguolo:** Data curation, formal analysis, writing-review and editing. **M. Curtarello:** Data curation, writing-review and editing. **L. Urso:** Data curation, writing-review and editing. **L. Paré:** Data curation, writing-review and editing. **N. Chic:** Data curation, writing-review and editing. **R. D'Amico:** Data curation, formal analysis, methodology, writing-review and editing. **A. Prat:** Data curation, writing-review and editing. **P. Conte:** Conceptualization, data curation, supervision, funding acquisition, writing-review and editing.

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PIK3CA Mutation in the ShortHER Randomized Adjuvant Trial for Patients with Early HER2 $^{+}$ Breast Cancer: Association with Prognosis and Integration with PAM50 Subtype

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