SHORT COMMUNICATION

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Correlation between *PIK3CA* mutations in cell-free DNA and everolimus efficacy in HR$^+$, HER2$^-$ advanced breast cancer: results from BOLERO-2

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Background: The current analysis was performed to evaluate the impact of *PIK3CA* hotspot mutations on everolimus efficacy in BOLERO-2 participants, using cell-free DNA (cfDNA) from plasma samples collected at the time of patient randomisation.

Methods: *PIK3CA H1047R*, *E545K*, and *E542K* mutations in plasma-derived cfDNA were analysed by droplet digital PCR (ddPCR). Median PFS was estimated for patient subgroups defined by *PIK3CA* mutations in each treatment arm.

Results: Among 550 patients included in cfDNA analysis, median PFS in everolimus *vs* placebo arms was similar in patients with tumours that had wild-type or mutant *PIK3CA* (hazard ratio (HR), 0.43 and 0.37, respectively). Everolimus also prolonged median PFS in patients with *PIK3CA H1047R* (HR, 0.37) and *E545K/E542K* mutations (HR = 0.30) with a similar magnitude.

Conclusions: Mutation analysis of plasma-derived cfDNA by ddPCR suggests that PFS benefit of everolimus was maintained irrespective of *PIK3CA* genotypes, consistent with the previous analysis of archival tumour DNA by next-generation sequencing.

Aberrant signalling through the PI3K/AKT/mTOR pathway has been implicated in tumorigenesis, progression, and therapeutic resistance in various cancers (Rodon *et al*, 2013; Thorpe *et al*, 2015). *PIK3CA* mutations result in constitutive activation of p110α, which in turn activates AKT1 and its downstream target mTOR. Somatic *PIK3CA* mutations are frequent in human breast cancer, occurring in up to 45% of luminal A tumours (Cancer Genome Atlas Network, 2012); the majority are missense mutations clustering in the helical (*E524K* and *E545K*) and catalytic (*H1047R*) domains (Kalinsky *et al*, 2009).

In the phase 3 BOLERO-2 trial, everolimus plus exemestane prolonged the progression-free survival (PFS) *vs* placebo plus exemestane (median PFS, 7.8 *vs* 3.2 months; hazard ratio (HR), 0.45; $P < 0.0001$) in patients with hormone receptor positive (HR$^+$), human epidermal growth factor receptor-2 negative (HER2$^-$) metastatic breast cancer (MBC) progressing after a non-steroidal aromatase inhibitor (NSAI) (Yardley *et al*, 2013). An exploratory analysis of predominantly primary tumour samples from roughly 40% of the BOLERO-2 trial population suggested that the PFS benefit from everolimus was largely maintained irrespective of *PIK3CA*

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genotype (Hortobagyi *et al*, 2016). However, given that differences between primary and MBC may arise, we performed an analysis of *PIK3CA* hotspot (HS) mutations by droplet digital PCR (ddPCR) using cell-free DNA (cfDNA) from plasma samples collected at the time of patient randomisation and evaluated their impact on everolimus efficacy in 76% of the BOLERO-2 participants.

MATERIALS AND METHODS

Patients and cfDNA analysis. BOLERO-2 study design has been described previously (Baselga *et al*, 2012). Briefly, patients with HR^+, $HER2^-$ MBC recurring/progressing on/after NSAI were randomised 2 : 1 to everolimus plus exemestane or placebo plus exemestane. Plasma collection, cfDNA extraction, and quantification and analysis by ddPCR for detection were performed as described previously (Chandarlapaty *et al*, 2016). *PIK3CA* mutations in cfDNA were detected by ddPCR in singleplex assays using inventoried assays for *PIK3CA H1047R* (dHsaCP2000077), *E545K* (dHsaCP2000075), and *E542K* (dHsaCP2000073) (BioRad, Hercules, CA, USA) on a BioRad QX200 Droplet Digital PCR System. Each assay run included mutation positive template, wild-type template, and no template controls. The fractional abundance for the *PIK3CA* mutation was calculated from the number of FAM-positive events (mutation positive) over total positive events (both FAM and HEX positive for mutation and wild type, respectively) using QuantaSoft Version 1.7.4.0917 (BioRad). Mutation analyses were performed on anonymised samples.

Written informed consent was obtained from all patients included in the study. The study was undertaken in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. The study protocol was approved by an independent ethics committee or institutional review board at each site.

Statistical analysis. Median PFS was estimated by Kaplan–Meier method in patient subgroups defined by *PIK3CA* mutations in each treatment arm. Hazard ratios with 95% confidence intervals (CIs) were calculated using Cox proportional hazards model for treatment *vs* placebo arm in the wild-type and mutant subgroups. Multivariate analysis was performed to adjust for the potential effects of prior hormonal therapy, visceral disease, and Eastern Cooperative Oncology Group status.

RESULTS

***PIK3CA* mutation frequency.** Of the 724 patients in BOLERO-2, 550 patients (76%) underwent *PIK3CA* cfDNA analysis. The baseline characteristics and clinical outcomes were similar between the cfDNA and overall population (Supplementary Table 1). *PIK3CA* HS mutations were identified in 238 patients (43.3%); the most prevalent was *H1047R* (25.1%), followed by *E545K* (11.1%) and *E542K* (7.1%). Four patients had multiple HS mutations; three, of which, were dual helical mutations. Prevalence of total and domain-specific *PIK3CA* mutations was higher in the everolimus arm (47.3%) than the placebo arm (35.8%; Table 1).

Concordance between mutations detected in cfDNA and archival tumour samples. Plasma-derived cfDNA samples were available in 247 of the 302 patients who underwent mutation analysis on archival tumour samples (198 primary; 49 metastatic) by next-generation sequencing (NGS) (Hortobagyi *et al*, 2016). The overall concordance in *PIK3CA* mutation status between archival tumour and cfDNA sample pairs was 70.4%, with a higher concordance (81.6%) for metastatic lesions. For concordant samples, the cfDNA mutant fractional abundance was generally higher than in the discordant samples where mutations were detected only by ddPCR in cfDNA (Supplementary Figure 1).

Correlation between *PIK3CA* genotypes and everolimus efficacy. Clinical benefit with everolimus as measured by PFS was similar in patients with tumours that had wild-type or mutant *PIK3CA* (HR, 0.43 (95% CI, 0.34–0.56) and 0.37 (95% CI, 0.27–0.51), respectively) (Table 2; Figure 1A). A multivariate analysis yielded similar results (HRs for PFS: *PIK3CA* wild-type HR, 0.39 (95% CI, 0.30–0.51); *PIK3CA*-mutant HR, 0.35 (95% CI, 0.25–0.49)) (Supplementary Table 3).

To examine the role of domain-specific mutations in *PIK3CA* on everolimus efficacy, patients were categorised by mutation site, *H1047R* in the catalytic domain, and *E545K* and *E542K* in the helical domain. Everolimus prolonged median PFS *vs* placebo in patients with *PIK3CA H1047R* mutation (HR, 0.37; 95% CI, 0.24–0.56) and *E545K/E542K* mutations (HR, 0.30; 95% CI, 0.18–0.51) with a similar magnitude (Table 2; Figure 1B). These data suggest that PFS benefit of everolimus was maintained irrespective of *PIK3CA* genotypes.

In the placebo arm, patients with *E545K/E542K* mutation had shorter PFS and overall survival than those with wild-type *PIK3CA* (Supplementary Table 2), suggesting that *PIK3CA* mutations in the helical domain might play a role in resistance to hormone therapy. However, this analysis by mutation site involved small numbers and was highly exploratory.

DISCUSSION

The advent of cfDNA analyses has facilitated the analysis of large clinical trial populations through minimally invasive technologies. We recently determined the feasibility for detection of *ESR1*

Table 1. *PIK3CA* mutation prevalence – overall and by treatment arm

	Overall		PBO + EXE		EVE + EXE	
***PIK3CA* genotype**	***N***	**% Genotype**	***N***	**% Genotype**	***N***	**% Genotype**
WT	312	56.7%	124	64.2%	188	52.7%
MT	238	43.3%	69	35.8%	169	47.3%
H1047R	138	25.1%	43	22.3%	95	26.6%
E545K	61	11.1%	26	13.5%	74	20.7%
E542K	39	7.1%				
Multiple	(4)[a]	0.7%				

Abbreviations: EVE = everolimus; EXE = exemestane; MT = mutant; PBO = placebo; WT = wild type.
[a]Samples with multiple mutations were categorised by allele with highest mutant fraction.

Table 2. PFS benefit in patient subgroups defined by overall and domain-specific mutation status of *PIK3CA*

***PIK3CA* status**	**Treatment**	***n***	**Events**	**Median PFS, months (95% CI)**	**Hazard ratio (95% CI)**
WT	EVE + EXE	188	132	7.36 (6.77–9.69)	0.43 (0.34–0.56)
	PBO + EXE	124	111	2.96 (2.76–4.17)	
MT	EVE + EXE	169	123	6.90 (5.55–8.31)	0.37 (0.27–0.51)
	PBO + EXE	69	60	2.69 (1.51–4.11)	
H1047R	EVE + EXE	95	65	7.59 (5.59–9.76)	0.37 (0.24–0.56)
	PBO + EXE	43	38	4.04 (1.51–4.70)	
E545K/ E542K	EVE + EXE	74	58	5.59 (4.14–7.82)	0.30 (0.18–0.51)
	PBO + EXE	26	22	2.22 (1.38–2.76)	

Abbreviations: CI = confidence interval; EVE = everolimus; EXE = exemestane; MT = mutant; PBO = placebo; PFS = progression-free survival; WT = wild type.

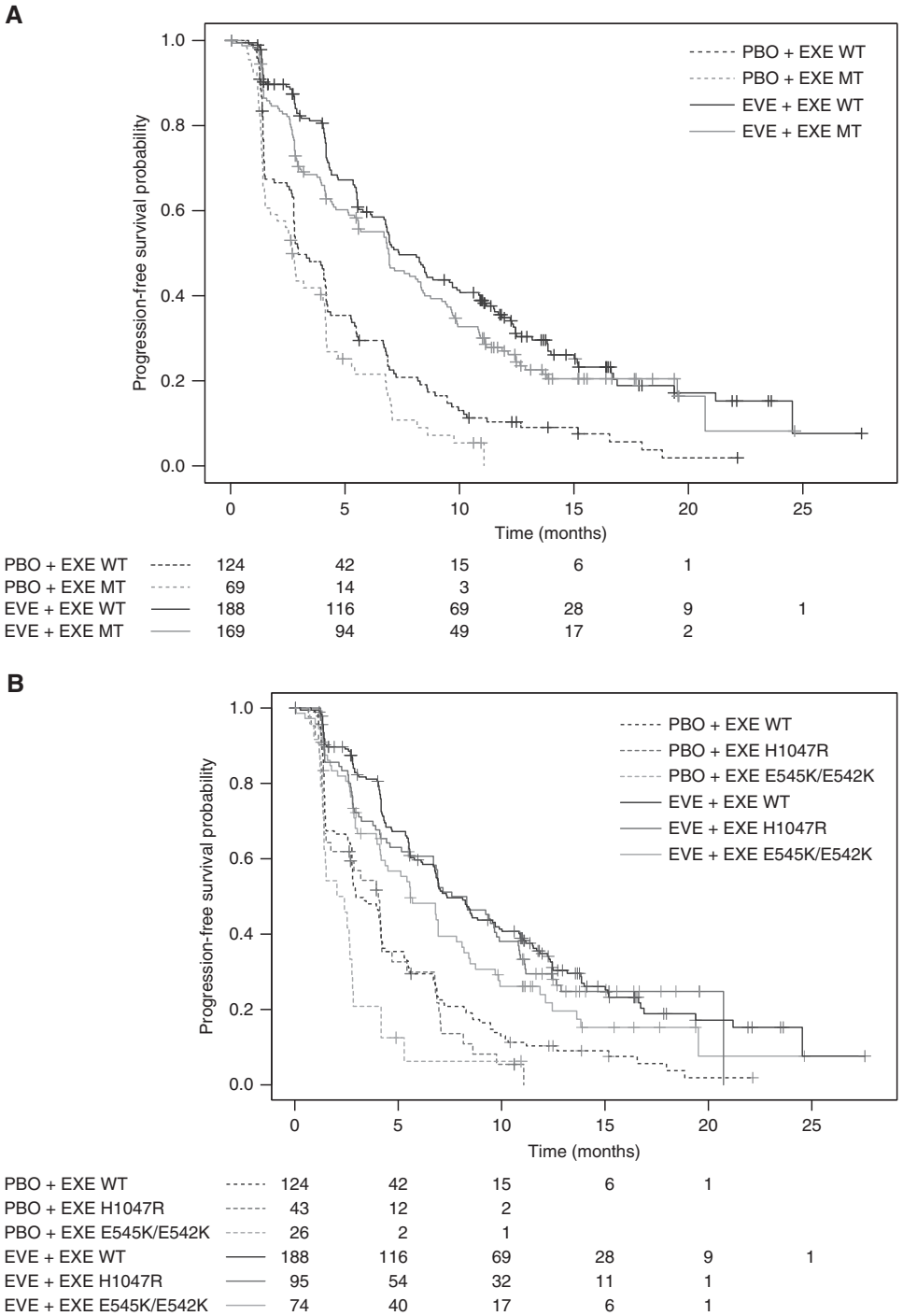

Figure 1. Kaplan-Meier Plot of Progression-free Survival by PIK3CA mutation status. (**A**) *PIK3CA* wild-type *vs* hotspot mutations. (**B**) *PIK3CA* wild-type *vs* domain-specific hotspot mutations.

mutations in cfDNA by ddPCR on stored plasma samples from the BOLERO-2 clinical trial (Chandarlapaty *et al*, 2016). Notably, collection of these plasma samples began in mid-2009 at trial enrolment in the absence of a plan for subsequent cfDNA analysis. Thus, data from large, mature clinical trials can be extended through the use of this emerging technology. In this report, we assessed the impact of three most prevalent *PIK3CA*-activating mutations on everolimus efficacy in >75% of the BOLERO-2 trial population, thus enabling a robust mutation analysis that demonstrates everolimus efficacy regardless of *PIK3CA* genotypes.

In the BOLERO-2 analysis, patients with tumours harbouring HS mutations in both helical and catalytic domains of *PIK3CA* benefit from the addition of everolimus. Interestingly, in the placebo arm, there is a suggestion of inferior clinical outcome in the small group harbouring *E545K/E542K* mutations. This is in contrast to a favourable prognosis associated with these mutations in early-stage cancer (Kalinsky *et al*, 2009; Sabine *et al*, 2014), no effect on prognosis in patients with MBC undergoing first-line hormonal therapy with tamoxifen and an improved outcome in patients with MBC with first-line aromatase inhibitor therapy (Ramirez-Ardila *et al*, 2013). From a biologic perspective, mutation site-specific differences are noted for interacting proteins (Zhao and Vogt, 2008; Hao *et al*, 2013), oncogenic potency and behaviour (Bader *et al*, 2006; Pang *et al*, 2009), and the phosphoproteome (Wu *et al*, 2014; Zahari

et al, 2015) that may be clinically important and deserve further analysis in clinical studies and preclinical modelling.

Approximately, 70% concordance for *PIK3CA* genotype by *PIK3CA* analysis of archival tumour DNA by NGS and contemporaneous plasma-derived cfDNA by ddPCR is similar to that reported for BEAMING of cfDNA and sequencing of archival tumour (Higgins *et al*, 2012). There are several potential biologic and technical reasons for this degree of discordance. ddPCR of plasma cfDNA is more sensitive for mutation detection than tumour sequencing (Guttery *et al*, 2015; Schiavon *et al*, 2015; Chu *et al*, 2016) with mutant allele fraction (mAF) <1–3% not detected by NGS (Guttery *et al*, 2015). When both concurrent biopsy and plasma collection are performed and the material is analysed by the same highly sensitive methodology, concordance is quite high (Higgins *et al*, 2012).

In breast cancer, *PIK3CA* mutation is thought to be truncal, identified in DCIS, primary breast cancer, and selected for in metastatic tumour (Kalinsky *et al*, 2011). However, compelling evidence from tumour genome sequencing highlights that reliance on a single analysis of the primary tumour may not be suitable for guiding therapy in patients with metastatic disease. Genomic heterogeneity in multifocal primary tumours identifies *PIK3CA* mutations occurring in $\sim$44% of patients, but in only half is the mutation identified in all tumours (Desmedt *et al*, 2015). Even in metastatic disease, emergence of a *PIK3CA E545K* mutation during progression has been reported by serial cfDNA analysis (Guttery *et al*, 2015). Conversely, absence of an *H1047R* mutation in hepatic metastasis and cfDNA is reported, when present in the primary tumour at a high mAF of 40% (Butler *et al*, 2015). In larger data sets of paired samples, both the loss and gain of *PIK3CA* mutation in metastatic progression is observed (Dupont Jensen *et al*, 2011; Arthur *et al*, 2014; Markou *et al*, 2014). Thus, although selection for a *PIK3CA*-activating mutation is expected, more dominant subclonal populations may give rise to the metastatic tumour.

CONCLUSIONS

Mutational analysis of plasma-derived cfDNA by ddPCR suggests that PFS benefit of everolimus was maintained irrespective of *PIK3CA* genotypes, consistent with the previous analysis of archival tumour DNA by NGS. These results suggest that *PIK3CA* genotype is not a predictive determinant for everolimus benefit.

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CONFLICT OF INTEREST

MEM: Grant and personal fees from Novartis; patent related to biomarkers for response to PI3K inhibitors pending. GNH: personal fees from Merck, Eli Lilly, Peregrine Pharmaceuticals, Novartis, and Celgene as consultant; personal fees from Novartis, Bayer, Metastat, Pfizer, Antigen Express, and Galena Biopharma as scientific/advisory committee member. SC: Grant from Novartis and Eli Lilly; personal fees from Foresite Capital, Chugai Pharmaceuticals, Oncotheryeon, and Astra Zeneca. DC, WH, PP, and FR are Novartis employees. The remaining authors declare no conflict of interest.

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