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PI3K oncogenic mutations mediate resistance to afatinib in HER2/neu overexpressing gynecological cancers

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HIGHLIGHTS

- Oncogenic PI3K mutations may represent a novel and major mechanism of resistance to single agent afatinib.
- Combinations of c-erb with PIK3CA, AKT or mTOR inhibitors may be necessary to block the PIK3CA pathway in gynecologic tumors.
- PIK3CA/PIK3R1oncogenic mutations may represent a biomarker able to identify patients resistant to single agent afatinib.

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ABSTRACT

Objective. Aberrant expression of HER2/neu and PIK3CA gene products secondary to amplification/mutations are common in high-grade-serous-endometrial (USC) and ovarian-cancers (HGSOC). Because scant information is currently available in the literature on the potential negative effect of PIK3CA mutations on the activity of afatinib, in this study we evaluate for the first time the role of oncogenic PIK3CA mutations as a potential mechanism of resistance to afatinib in HGSOC and USC overexpressing HER2/neu.

Methods. We used six whole-exome-sequenced primary HGSOC/USC cell-lines and three xenografts overexpressing HER2/neu and harboring mutated or wild-type PIK3CA/PIK3R1 genes to evaluate the role of PI3K-mutations as potential mechanism of resistance to afatinib, an FDA-approved pan-c-erb-inhibitor in clinical trials in USC. Primary-USC harboring wild-type-PIK3CA gene was transfected with plasmids encoding oncogenic PIK3CA-mutations (H1047R/E545K). The effect of afatinib on HER2/PI3K/AKT/mTOR pathway was evaluated by immunoblotting.

Results. We found PI3K wild-type cell-lines to be significantly more sensitive (lower IC_{50}) than PI3K-mutated cell-lines $p = 0.004$). *In vivo*, xenografts of primary cell-line USC-ARK2, transfected with the PIK3CA-H1047R or E545K hotspot-mutations, exhibited significantly more rapid tumor growth when treated with afatinib, compared to mice harboring ARK2-tumors transfected with wild-type-PIK3CA ($p = 0.041$ and 0.001, respectively). By western-blot, afatinib effectively reduced total and phospho-HER2 proteins in all cell-lines. However, H1047R/E545K-PIK3CA-transfected-ARK2-cells demonstrated a greater compensatory increase in phosphorylated-AKT proteins after afatinib exposure when compared to controls ARK2.

Conclusions. Oncogenic PI3K mutations may represent a major mechanism of resistance to afatinib. Combinations of c-erb with PIK3CA, AKT or mTOR inhibitors may be necessary to more efficiently block the PIK3CA/AKT/mTOR pathway.

1. Background

Endometrial cancer is the most common gynecologic malignancy in the United States with 63.230 estimated new cases and 11.350

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estimated deaths in 2018 [1]. Ovarian cancer represents the fifth cause of death in women among all tumor types, with 22.240 estimated new cases and 14.070 estimated deaths in 2018 [1]. Despite recent advances in the treatment of gynecologic tumors, prognosis remains poor for a large number of ovarian and endometrial cancer patients diagnosed with advanced stage disease and/or biologically aggressive endometrial cancers (*i.e.*, Type II).

Studies from our group and the TCGA network have recently revealed the genomic landscape of uterine serous carcinoma (USC) and carcinosarcoma (*i.e.*, the most aggressive variants of endometrial cancers) and high grade serous ovarian cancer (HGSOC) (*i.e.*, the most common epithelial ovarian tumor), providing the opportunity to target aberrant pathways crucial for cell growth, survival and proliferation with targeted agents [2–6]. In this regard, the c-erbB2 gene is a member of the erbB receptor tyrosine kinase family, which consists of four transmembrane glycoproteins: erbB1, erbB2, erbB3, and erbB4. The c-erbB2 gene encodes for HER2/neu receptor whose overexpression induces an increased phosphorylation of intracellular tyrosine kinase residues and ultimately leads to cell proliferation, differentiation, migration, and survival [7,8]. Both USC and HGSOC demonstrated aberrant activation of the HER2 gene pathways in a significant percentage of patients with up to 66% of ovarian carcinoma and up to 80% of USC reported to harbor c-erbB2 gene amplification and/or HER2/neu over-expression [3,4,9–14]. These data identify HER2/neu as a potential therapeutic target for both USC and HGSOC.

The PIK3CA/AKT/mTOR pathway is critical for driving diverse cellular responses, including cell proliferation, survival, metabolism, and control of malignant cell growth and is located downstream to HER2/neu. Importantly, comprehensive studies have reported PIK3CA and PIK3R1 among the top five most frequently mutated genes in gynecologic and breast cancers (32% and 11%, respectively) [15], with the PIK3CA E545K and H1047R "hotspot-mutations" identified as the most common "driver mutations" affecting USC and HG-SOC patients [2–6,16]. Similarly to HER2/neu, as previously reported by our own and multiple other research groups, PIK3CA is often constitutively activated secondary to gene amplification or activating mutations, [3,5,13], while PIK3R1 loss of function/mutation is known to increase the binding of p110α–p85 signaling complexes, activating the receptors and augmenting PI3K pathway output [17].

Our research group has recently reported the first clinical evidence that trastuzumab (Herceptin, Genentech/Roche) is highly active when combined to standard carboplatin/paclitaxel chemotherapy in advanced/recurrent USC overexpressing HER2/neu [18]. On the basis of these results, National Comprehensive Cancer Network (NCCN) guidelines, which are widely recognized and used as the standard for clinical policy in oncology, have recently been revised adding carboplatin/paclitaxel/trastuzumab (2A category recommendation) as the preferred regimen for women with HER2+, advanced or recurrent USC (https://www.nccn.org/professionals/physician_gls/default.aspx). In our Institution, USC patients overexpressing HER2/neu progressing on trastuzumab are currently enrolled/treated within a multicenter Phase II clinical trial (NCT02491099) with afatinib (BIBW-2992), an irreversible tyrosine kinase inhibitor (TKI) approved by the FDA as first-line treatment for ErbB1-mutated non-small-cell lung cancer [19]. Afatinib inhibits EGFR, HER2, and HER4 by covalently binding to intracellular phosphorylation sites as well as inhibiting transphosphorylation of ErbB3 [20]. While afatinib may efficiently interrupt the HER2/neu/PIK3CA/AKT/mTOR pathway signaling cascade and decrease proliferation and tumor growth, strong experimental evidence has recently suggested that PIK3CA mutations may represent an important mechanism of resistance to trastuzumab in HER2 overexpressing USC cell lines and xenografts [21]. Because scant information is currently available in the literature on the potential negative effect of PIK3CA mutations on the activity of afatinib, in this study we have evaluated for the first time the role of oncogenic PIK3CA mutations as a potential mechanism of resistance to afatinib in HGSOC and USC overexpressing HER2/neu.

2. Materials and methods

2.1. Primary cell lines

Prior to surgical staging, patients were consented for tumor banking in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board at Yale University. At the time of frozen section, a small portion of the tumor was obtained to establish primary cell lines, as previously described [22]. Briefly, tumors were processed by mechanical disruption in an enzymatic solution of 0.14% collagenase type I (Sigma) and 0.01% DNase (Sigma) in RPMI 1640 (Gibco, Life Technologies, Grand Island, NY), then incubated while stirring for 45 min at room temperature. The samples were washed twice with RPMI 1640 10% FBS and plated in cell culture dishes as a monolayer in RPMI 1640 supplemented with 10% FBS (Gemini, Woodland, CA), 1% Fungizone (Gibco, Life Technologies, Grand Island, NY) and 1% penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2. Primary cell lines were authenticated by whole exome sequencing (WES) at the Yale Center for Genome Analysis and PIK3CA and PIK3R1 gene mutations identified [3,6]. Each primary tumor was also tested using fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) techniques in the pathology department at Yale University to determine HER2/neu expression/amplification, as previously described by our group [22]. Six primary cell lines with limited passages, harboring HER2/neu gene amplification, were selected to use in the experiments listed below (characteristics and tissue source are described in Table 1). ARK2, OM(M)98 and KRCH31 harbored a wild-type PIK3CA gene while ARK20, OVA10, and OVA13 harbored oncogenic PIK3CA or PIK3R1 mutations.

2.2. ARK2 transduction

The pDONR223_PIK3CA WT, p.E545K and H1047R were purchased from ADDGENE (# 81736, 82881 and 82824, respectively). The Gateway LR clonase II kit (Invitrogen) was used to clone these constructs in the pLX302 lentiviral destination vectors. Viruses were generated in 293T cells as previously described [23]. Briefly, 293T cells were transfected by the calcium phosphate method using 8 μg of plasmid DNA of interest, 3 μg of VSVg envelope vector (pMD.G), 3 μg of RSV-Rev, and 3 μg of packaging vector pCMVDR8.2 per 10 cm dishes. Forty hours after transfection, the lentivirus-containing supernatant was collected and used to infect ARK2 cells in the presence of polybrene (6 μg/ml). Transduced cells were selected with puromycin for a week before experiments were performed. The transduction was validated by qRT-PCR using the TaqMan SNP Genotyping Assay to detect the PIK3CA H1047R mutation and the PIK3CA E545K mutation (respectively, Custom Assay ID: ANTZ9XW, and Assay ID: C_150852487_10, Thermo Fisher Scientific).

2.3. Drug

Afatinib (BIBW-2992) free base was obtained from Selleckchem (Houston, TX, USA). The compound was dissolved in DMSO as a 10 mM stock solution and diluted in culture medium immediately before use.

2.4. Chemo-response assay

To determine dose-response, cells were aliquoted into 6-well microtiter plates at 40,000 cells per well. After 24 h the cells were treated with scalar amounts of afatinib ranging from 0.1 nM to 1 μM. Seventy-two hours after treatment the contents of each well were harvested in their entirety and stained with propidium iodide (Sigma Life Sciences, St. Louis, MO) (2 μl of 500 μg/ml stock solution in PBS), to be counted by flow cytometry. The number of viable cells in each well was normalized to the number of viable cells in the control well. The IC_{50} of each cell

Table 1
Cell lines characteristics and tissue source.

Cell line	Age	Stage	Histology	HER2/neu IHC	HER2/neu FISH	PIK3CA Mutations	PIK3R1 Mutations
ARK2	63	IV	USC	3+	AMPLIFIED	NOT DETECTED	NOT DETECTED
ARK20	42	II	USC	3+	AMPLIFIED	H1047R Exon 20	NOT DETECTED
OVA10	51	IIC	HGSOC	3+	AMPLIFIED	NOT DETECTED	Q457fs K459fs
OVA13	42	IIIC	HGSOC	3+	AMPLIFIED	G118D Exon 3	NOT DETECTED
KRCH31	69	IV	HGSOC	3+	AMPLIFIED	NOT DETECTED	NOT DETECTED
OM(M)98	66	IIIC	MMMT	3+	AMPLIFIED	NOT DETECTED	NOT DETECTED

Abbreviation: IHC = immunohistochemistry; FISH = fluorescence *in situ* hybridization; MMMT = malignant mixed müllerian tumor.

line was then determined by comparing the log base 10 of drug concentration in each well to the percentage of viable cells using a non-parametric 3 parameter regression. All IC_{50} data were calculated using Prism 7 software (GraphPad Prism Software Inc., San Diego, CA) and presented as mean ± standard error of the mean. A minimum of 3 independent experiments per cell line was performed.

2.5. Immunoblotting

ARK2 cells transfected with the plasmids encoding for the PIK3CA wild-type, the oncogenic PI3KCA mutation H1047R and E545K were seeded in tissue culture plates (100,000 cells) and left to adhere overnight. Cells were then treated with 20 nM of afatinib or DMSO. After 24 hour incubation, cells were washed three times with ice-cold PBS, mechanically scraped, and lysed for 30 min on ice with 400 μL of radioimmunoprecipitation assay buffer (RIPA) (50 mmol/L Tris–HCl, pH 8, 150 mmol/L NaCl, Triton X-100 1%, Na deoxycholate 0.5%, SDS 0.1%, MgCl 5 mmol/L in H_2O) supplemented with Protease and Phosphatase Inhibitor (cat#78430, Thermo Fisher Scientific). Protein levels were quantified with the Micro BCA Protein Assay Kit (#23225, Thermo Scientific), and equal amounts (30 μg) were resolved using SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were washed with TBS (140 mmol/L NaCl, 50 mmol/L Tris-HCl; pH 7.2) containing 0.1%Tween20 (TBST) and 5% skimmed milk to block nonspecific protein binding. Membranes were incubated with an antibody against HER2 (1:500, 06562S, Millipore), pHER2 (1:1000, 2247S, Cell Signaling), Phospho-AKT (Thr308) (1:500, 4056S, Cell Signaling), Phospho-AKT (Ser473) (1:2000, 4060S, Cell Signaling), Phospho-S6 (1:1000, 5364S, Cell Signaling), or GAPDH (1:1000, 2118S, Cell Signaling) in 5% BSA in TBST overnight at 4 °C, washed three times with TBST, and then incubated with an HRP-linked secondary antibody (1:2000, 7074S, Cell Signaling) in 5% nonfat dry milk in TBST for 1 h at room temperature, before washing three times in TBST. Signals were detected with western blotting detection reagents (Thermo Scientific). Bands were then visualized and the blots developed using a Q10 enhanced chemiluminescent system (GEL Logic 1500).

2.6. In vivo experiments

To determine the *in vivo* activity of afatinib, ARK2 cells transfected with the plasmids encoding for the PIK3CA wild-type or the oncogenic PI3KCA mutation H1047R and E545K were injected subcutaneously into the abdominal region of a total of fifty 5-week-old female CB17/lcrHsd-Prkd/SCID mice (7×10^6 cells in 0.3 ml of PBS with 50% Matrigel® (BD Biosciences)). All the xenografted mice (ARK2/wild-type and ARK2/H1047R and ARK2/E545K) were triaged into treatment groups (7–8 mice each group) when mean tumor burden was 0.15–0.25 cm^3. Dosing (vehicle PO or afatinib 10 mg/kg QD, PO) begun upon reaching target size and was delivered to the ARK2 xenografts for 3 weeks (5 days/week). Tumor measurements and mouse weights were recorded at least two times weekly and reported for each mouse (individually identified). Tumor volumes were calculated using the formula $0.5 \times (\text{width}^2 \times \text{height})$. At the end of the study, the animals were humanely euthanized. All mice were housed and treated in accordance with the policies set forth by the Institutional Animal Care and Use Committee (IACUC) at Yale University.

2.7. Statistical analysis

Statistical analysis was conducted using GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA). Comparison of afatinib efficacy was carried out between PI3K mutated and non-mutated cell lines. The IC_{50} values of the six cell lines were compared using a one-way analysis of variance. Grouped mean IC_{50} values were compared using two-tailed Student's *t*-test. Differences in all comparisons were considered significant at p values <0.05.

3. Results

3.1. HER2/neu amplified PI3K wild-type primary cell lines are more sensitive to afatinib than HER2/neu amplified PI3K mutated primary cell lines in vitro

We exposed six primary cell lines, 3 harboring PIK3CA/PIK3R1 mutations *vs* 3 harboring wild types PIK3CA/PIK3R1 genes, to increasing concentration of afatinib, as described in the Materials and methods section. As shown in Fig. 1A and B, PI3K wild-type cell lines (*i.e.*, ARK2, KRCH31 and OM(M)98, Table 1) demonstrated a statistically significant lower IC_{50} than PI3K mutated cell lines when exposed to afatinib (IC_{50} mean ± SEM, 5.41 ± 1.14 nM *vs* 14.22 ± 1.90 nM respectively, $p = 0.004$). ARK2 cell line was the most sensitive (2.98 nM) to the pan c-erb inhibitor while OVA10 cell line was found to be the most resistant (16.73 nM) (Supplementary Fig. 1).

3.2. ARK2 cell line transfected with H1047R or E545K PIK3CA mutation demonstrated significantly lower sensitivity to afatinib in vitro than ARK2 cell line transfected with PIK3CA wild-type

To evaluate whether driver mutations in the PIK3CA gene may be the cause of the increased resistance of the cell lines to afatinib, the HER2-amplified ARK2 cell line which harbors a wild-type PIK3CA gene, was transfected with lentivirus encoding the E545K or the H1047R PIK3CA activating mutations as well as with a vector encoding the wild-type PIK3CA gene. The efficiency of the transfection was validated by qRT-PCR in all samples before exposure to afatinib (data not shown). Stably transfected cells were then exposed to serial afatinib concentrations *in vitro*. As shown in Fig. 1B and C, we found the ARK2

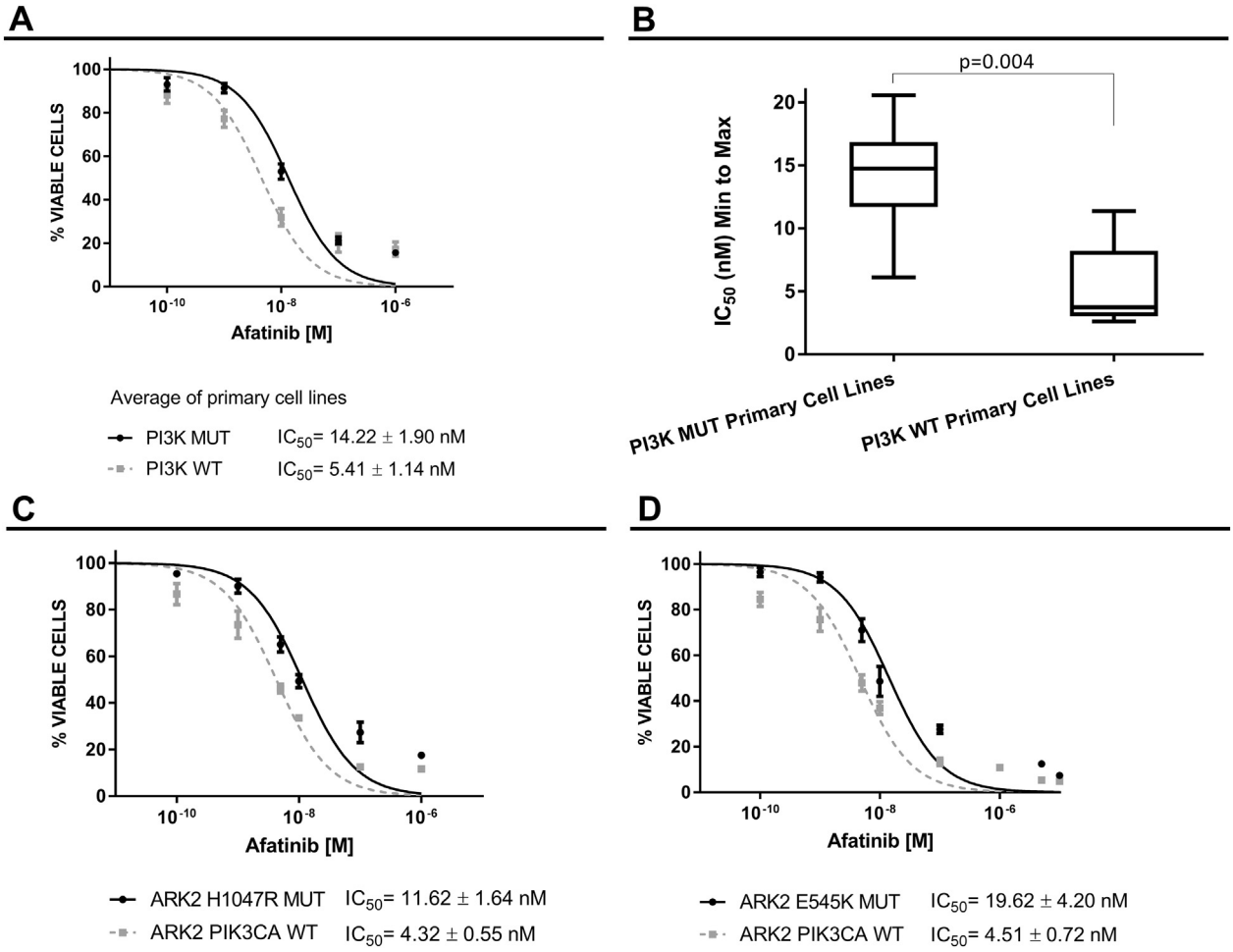

Fig. 1. *In vitro* cell proliferation of A) HER2/neu amplified PI3K wild-type primary cell lines *versus* HER2/neu amplified PI3K mutated primary cell lines C) ARK2 transfected cell line with H1047R PIK3CA mutation *versus* ARK2 transfected cell line with PIK3CA wild-type D) ARK2 transfected cell line with E545K PIK3CA mutation *versus* ARK2 transfected cell line with PIK3CA wild-type, following 72 hour incubation with afatinib at increasing concentrations. B) Afatinib IC_{50} values representation of HER2/neu amplified PI3K wild-type primary cell lines *versus* HER2/neu amplified PI3K mutated primary cell lines.

PIK3CA wild-type cell line to be significantly more sensitive to afatinib, when compared with ARK2 transfected cell lines with the activating PIK3CA mutations (*i.e.*, H1047R or the E545K). Indeed, ARK2 transfected cell line harboring the H1047R PIK3CA mutation demonstrated an IC_{50} 2.7 fold higher when compared to ARK2 wild-type (IC_{50} mean ± SEM: 11.62 ± 1.64 nM *vs* 4.32 ± 0.55 nM, respectively) ($p = 0.009$). Similarly, ARK2 transfected cell line harboring the E545K PIK3CA mutation showed an IC_{50} 4.3 fold higher when compared to ARK2 wild-type (IC_{50} mean ± SEM: 19.62 ± 4.20 nM *vs* 4.51 ± 0.72 nM, respectively) ($p = 0.035$).

3.3. Western blot analysis of USC ARK2 cells transfected with the H1047R and E545K PIK3CA mutations after exposure to afatinib

To determine whether PIK3CA mutations are responsible for the resistance to Afatinib in HER2/neu amplified tumors, the activation of the HER2/PI3K/mTOR pathways in USC-ARK2 cells transfected with the H1047R and E545K PIK3CA mutations and the wild-type PIK3CA was evaluated in western blotting experiments. As shown in Fig. 2, all cell lines expressed high levels of the HER2 and phospho HER2 proteins by western blot. Afatinib was very effective in reducing both total and phospho HER2 proteins at 24 h after treatment in all cell lines. Importantly, a greater increase of phosphorylated AKT protein levels (pAKT Ser473 and pAKT Thr308) was detectable in the mutated cell lines when compared with the wild-type after exposure to afatinib (Fig. 2). In contrast, no significant change was demonstrated after afatinib treatment on the expression of the phosphorylated S6 protein.

3.4. ARK2 tumor transfected with H1047R and E545K PIK3CA gene mutations are more resistant to afatinib when compared to ARK2 tumor transfected with wild-type PIK3CA genes in vivo

To evaluate the impact of afatinib *in vivo*, we generated 3 xenograft models by injecting subcutaneously ARK2 cells transfected with the H1047R and E545K PIK3CA mutations and wild-type PIK3CA into female CB17/lcrHsd-Prkd/SCID mice. The daily oral dose of afatinib 10 mg/kg was well tolerated with no clear impact on body weight compared with vehicle control (Fig. 3F). As shown in Fig. 3A and B, mice harboring ARK2 tumor with wild-type PIK3CA and ARK2 with the H1047R PIK3CA mutation exhibited a significantly slower rate of tumor growth, compared to vehicle control. This difference reached statistical significance starting on day 9 (p-value = 0.000001 and 0.0017 respectively). In contrast, Afatinib was unable to significantly inhibit tumor growth in mice harboring ARK2 tumor transfected with the E545K PIK3CA mutation, compared to vehicle control (Fig. 3C, p-value > 0.07). Importantly, while the 3 group of xenografted animals treated with vehicle demonstrated a similar tumor growth rate (Fig. 3D), only mice harboring ARK2 tumors with the H1047R and E545K PIK3CA mutations demonstrated resistance to afatinib, as demonstrated by the more rapid tumor growth, compared to mice harboring ARK2 tumor with wild-type PIK3CA (p-value = 0.041 and 0.001 respectively) (Fig. 3E).

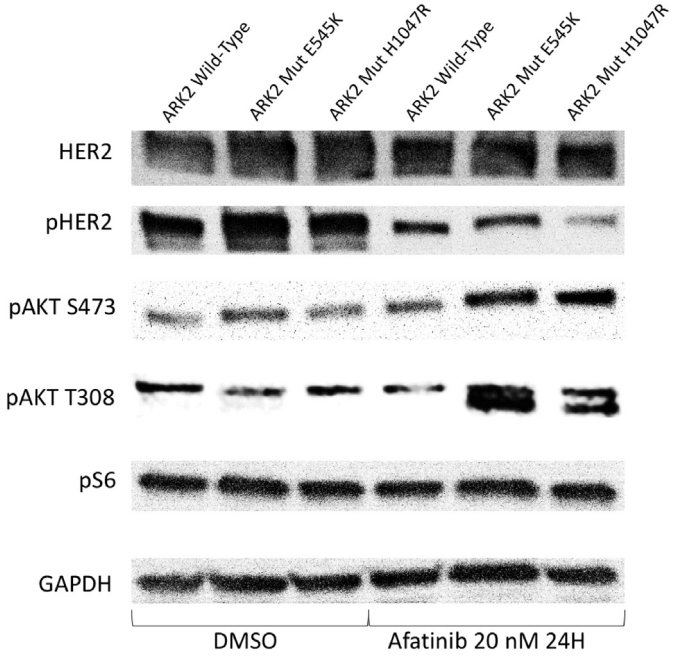

Fig. 2. HER2, pHER2, pAKT Ser473, pAKT Thr308, pS6, and GAPDH protein levels expression in ARK2 cells transfected with the H1047R and E545K PIK3CA mutations and the wild-type PIK3CA, untreated and after exposure to afatinib (20 nM) for 24 h.

4. Discussion

Patients diagnosed with advanced or recurrent biologically aggressive (*i.e.*, Type II) endometrial cancers, such as USC or recurrent HGSOC have an extremely poor prognosis [14,24]. The development of novel, effective therapies for patients with recurrent, chemotherapy-resistant disease remains an unmet medical need. In this study, we investigated the preclinical activity of afatinib, an FDA-approved pan c-erb TK inhibitor currently in Phase II trial (NCT02491099) in HER2/neu amplified endometrial cancer patients, using primary USC and HGSOC cell lines overexpressing HER2/neu with and without PIK3CA mutations.

Overexpression of HER2/neu is associated with aggressive disease and poor prognosis in multiple human tumors including USC and HGSOC [25]. In the attempt to identify alterated gene/pathways that may represent novel therapeutic targets, our group and others have recently performed comprehensive genetic landscape studies of multiple biologically aggressive gynecological cancers [2–6]. We found a large number of USC and HGSOC patients to harbor alterations in the HER2/neu and/or the PIK3CA genes, suggesting the HER2/PI3K/AKT/mTOR pathway may represent a highly attractive therapeutic target option against these aggressive tumors [2–6,26]. Accordingly, we recently initiated and completed a Phase II prospective randomized trial adding trastuzumab, a humanized monoclonal antibody targeting HER2/neu, to carboplatin-paclitaxel in HER2/neu-positive USC patients with advanced/recurrent disease. The trial resulted in a 56% decrease in risk of progression relative to carboplatin-paclitaxel alone and an increase in progression-free survival (PFS) of 4.6 months [18]. Importantly, an overall survival increase was already present at the time of the initial study report in USC patients with stages IIIC–IV disease [18]. The positive results of this prospective randomized study combining chemotherapy with a highly targeted agent such as trastuzumab, which represents a milestone in the treatment of HER2-amplified breast cancers, contrast with the negative results obtained by Fleming et al. in the GOG 181B trial evaluating the efficacy of single-agent trastuzumab in heavily pretreated endometrial cancer patients with HER2 overexpression [27]. While the discrepancy in results is likely related to the different criteria used for the selection of patients, with the majority of patients enrolled in GOG 181B (*i.e.*, 67%) harboring a different histological type of endometrial carcinoma (*i.e.*, Type I endometrioid tumors instead of Type II serous tumors), and about half (45.5%) harboring no HER2/neu amplification [28], these data do suggest that endometrial cancer patients may be primarily resistant and/or rapidly develop multiple strategies including compensatory HER2 downstream effector mechanisms leading to resistance to anti-HER2 therapy [21]. Consistent with this view, in breast cancer cell lines, multiple studies have previously identified aberrant phosphatidylinositol 3-kinase (PI3K) expression, low PTEN expression, AKT phosphorylation, and S6K phosphorylation as potential mediators of primary resistance to trastuzumab [29]. Furthermore, relevant to this discussion, Black et al. have recently demonstrated PIK3CA oncogenic mutations to represent a major mechanism of resistance to single-agent trastuzumab in HER2/neu overexpressing USC cell lines and xenografts [21].

USC patients overexpressing HER2/neu and progressing on trastuzumab are currently enrolled/treated within a multicenter Phase II clinical trial (NCT02491099) with afatinib, an irreversible tyrosine kinase inhibitor (TKI), that inhibits EGFR, HER2, and HER4 by covalently binding to intracellular phosphorylation sites as well as inhibiting transphosphorylation of ErbB3 [20]. Part of the rationale supporting the sequential use of afatinib in these trastuzumab-resistant patients is related to the fact that the truncated form of the HER2/neu receptor, (*i.e.*, p95HER2/neu), that arises through the proteolytic shedding of the extracellular domain (ECD) of the full-length HER2/neu, has been recently identified as one of the potential mechanisms of USC trastuzumab resistance *in vivo* [30]. Indeed, the remaining membrane-bound portion (p95) of HER2/neu, which is no more bound/affected by trastuzumab, has a constitutively activated kinase domain. While this mechanism of resistance has been correlated with poor prognosis and decreased responsiveness to treatments in other human tumors [31,32], dual and pan c-erb inhibitors such as lapatinib, afatinib, and neratinib, respectively, may continue to remain clinically effective in this setting [33,34].

Unfortunately, minimal information is currently available on the potential negative effect of PIK3CA mutations on the activity of afatinib. To fill this gap in knowledge, in this study we have evaluated for the first time the role of oncogenic PIK3CA mutations in afatinib resistance. We found PIK3CA wild-type primary cell lines to be consistently more sensitive to afatinib *in vitro*, with statistically significant lower IC_{50} than PIK3CA mutated primary cell lines. *In vivo*, xenografts of USC-ARK2, a cell line transfected with the H1047R and E545K PIK3CA hotspot mutations, exhibited a significantly higher rate of tumor growth when treated with afatinib, compared to mice harboring ARK2 tumor with wild-type PIK3CA (p-value = 0.041 and 0.001 respectively). Of interest, ARK2 cell xenografts transfected with the E545K PIK3CA hotspot mutation were found more resistant to afatinib exposure when compared to those transfected with the H1047R PIK3CA mutation. While the reason of these results is not completely understood, previous reports have demonstrated the capability of different oncogenic PIK3CA mutations to induce various levels of phosphorylation in the HER2/neu downstream pathway (*i.e.*, phosphorylated AKT) [16]. Consistent with this explanation, in our study, western blot experiments with transfected ARK2 cells demonstrated the highest levels of AKT phosphorylation in the E545K transfectants. Importantly, both E545K and H1047R transfected cells were found to have levels of phosphorylated AKT significantly increased after afatinib treatment, when compared with ARK2 cells transfected with wild-type PIK3CA. Taken together, these results support the hypothesis that a compensatory downstream activation of the PIK3CA pathway may represent the main mechanism of resistance to Afatinib. This interpretation is further supported by previously published data in neratinib-resistant USC cell lines, demonstrating a progressive increase in phosphorylated AKT after pan c-erb inhibitor exposure, specifically in USC cell lines harboring PIK3CA hotspot mutations [21,26,29].

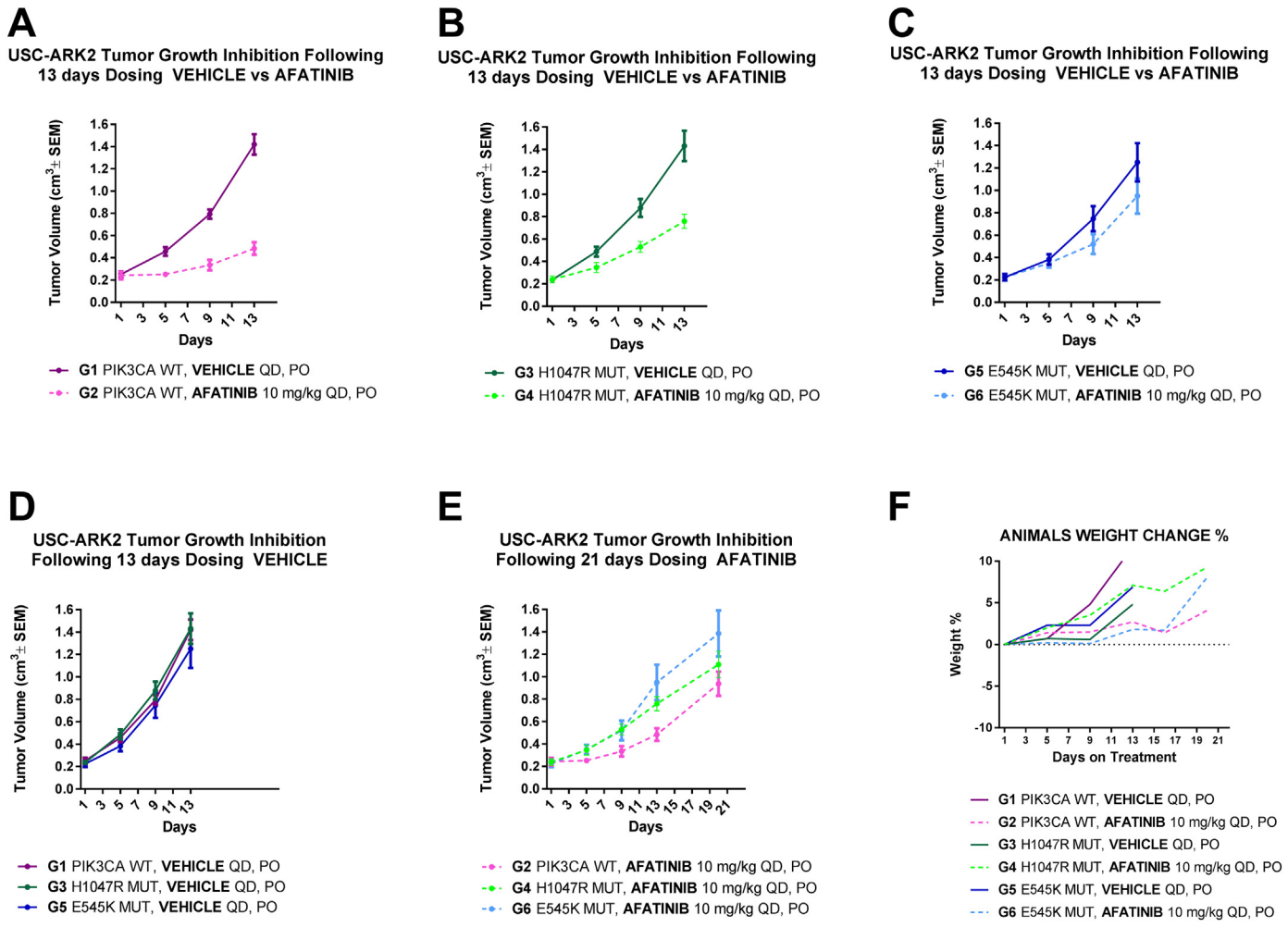

Fig. 3. *In vivo* tumor growth inhibition following 13 days dosing afatinib or vehicle of A) ARK2 tumor transfected with PIK3CA wild-type B) ARK2 tumor transfected with H1047R PIK3CA mutation C) ARK2 tumor transfected with E545K PIK3CA mutation D) merged of A, B and C groups dosing vehicle E) merged of A, B and C groups dosing afatinib for 21 days F) percentage representation of weight change in all the groups of animals.

A large number of patients treated with highly targeted agents such as dual/pan-EGFR inhibitors, to mention just a few, after an initial response to treatment rapidly experience secondary progression [35,36]. Our data revealing PIK3CA mutations as one of the main mechanisms of resistance to afatinib combined with the high percentage of USC patients known to potentially harbor such mutations in their tumors [21], may potentially explain the common resistance of these tumors to anti-HER2/neu treatment when used as single agent. Importantly, the encouraging experimental results obtained with afatinib or neratinib (*i.e.*, another pan c-erb inhibitor) when used in combinations with PIK3CA inhibitors [37] allow the speculation that targeting of the HER2/PI3K/AKT/mTOR pathway with afatinib in combination with PIK3CA, AKT or mTOR inhibitors, may represent a more effective and more durable treatment modality in patients harboring HER2-amplified and PIK3CA mutated tumors [38–40].

In conclusion, the preclinical data presented in this study, suggest for the first time that PIK3CA oncogenic mutation and/or PIK3R1 alterations may represent a major mechanism of resistance not only to trastuzumab but also to afatinib in HER2/neu overexpressing high-grade serous carcinomas of the ovary and uterus. In light of these results, we suggest PIK3CA/PIK3R1oncogenic mutations may represent a biomarker able to identify patients at least partially resistant to single agent afatinib treatment *in vivo* [17,21]. Importantly, the pending sequencing of HER2/overexpressing USC specimens prospectively collected in our ongoing clinical trial with afatinib will help to verify this hypothesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.01.002.

Conflict of interest

The authors declare no conflicts of interest.

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Authorship

A.D.S., E.B., and E.C. designed research; E.B., E.C., S.L., S.B., L.Z., A.B., A.M., G.Y., P.M., E.P., K.H., M.E., K.D., G.M., G. A., C.H., B.Z., performed research; E.B., E.C., S.L., S.B., L.Z., A.B., A.M., G.Y., P.M., E.P., D-A.S., E.R., M.A., B.L., G.H., P.S., contributed new reagents/analytic tools; C. H., A.D.S., and

S.L., analyzed data; and E.B., S.L., and A.D.S. wrote the paper. All authors have revised and approved the manuscript.

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