Review Article

A combination of the PI3K pathway inhibitor plus cell cycle pathway inhibitor to combat endocrine resistance in hormone receptor-positive breast cancer: a genomic algorithm-based treatment approach

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Abstract: Cyclin-dependent kinase 4 (CDK4) and CDK6 together with D-type cyclins (D1, D2 and D3) to promote cell cycle entry and progression through G1 by inactivating retinoblastoma protein (RB) by inhibiting an INK4 family of CDK inhibitors (CDKN2A/B). Selective cyclin-dependent kinase inhibitors are game changers in the clinical management of hormone receptor-positive/HER2-negative advanced breast cancers. There are currently three CDK4/6 inhibitors that have been approved by the US Food and Drug administration: palbociclib, ribociclib, and abemaciclib. Although, the bulk of the data supporting the use of selective CDK4/6 inhibitors is currently in breast cancer patients with other tumor types are expected to benefit as well from this class of agents, which can counter proliferative signaling pathways and arrest cell cycle in early G1 phase. Areas of active interest include identifying predictive biomarkers for CDK4/6 inhibitors, deciding whether to continue CDK4/6 inhibitor after disease progression, creating novel treatment combinations and expanding the use of CDK4/6 inhibitors beyond hormone receptor-positive/HER2-negative advanced breast cancer. Right now, *CCND*1 amplification and *CDKN2A/B* loss have not sorted out biomarkers useful for the purpose. One of the most important clinical questions is how to use a CDK4/6 inhibitor with other targeted therapies. Here we provide a rationale that oncologists can use to sequence the CDK4/6 inhibitors along with the PI3K-AKT-mTOR pathway-specific inhibitor(s); future data will better guide this approach. In this review we have tried to (a) describe the specific cellular signals initiated following alterations in the cell cycle pathway genes and the PI3K pathway genes, (b) interrogate how these alterations/co-alterations influence the action of PI3K and cell cycle pathway-targeted drugs in different clinical trials and (c) understand the role of co-alterations towards the development of cell cycle inhibitors induced drug-resistance in ER+ breast cancers.

Keywords: ER+ breast cancer, PI3K/AKT/mTOR pathway, CYCLIN D1, CDK4/6, retinoblastoma protein (RB)

Introduction

Approximately 75% of breast cancer (BC) patients express ER and/PR biomarkers, and these are indicative of steroid hormone dependency [1]. This hormone dependency in hormone receptor-positive (ER+) breast cancer is exploited therapeutically in three major ways, 1) selective ER modulators (SERMs; tamoxifen), 2) selective ER down-regulators (SERDs; fulvestrant) and 3) blocking estrogen biosynthesis (aromatase inhibitors; AIs). Endocrine therapy remains the therapeutic backbone for the treatment of ER+ cancers. However, anti-estrogen therapies are initially frequently effective, 50% of ER+ patients develop resistance to hormonal manipulation within their lifetime, ultimately leading to therapeutic failure. Two emerging mechanisms of endocrine resistance include activation of growth signaling pathways such as the phosphatidylinositol 3-kinase (PI3K) pathway and more recently, the decoupling of cell cycle control from ER-signaling, via deregulation of the CYCLIN D dependent kinase (CDK-4/6-INK4-RB) pathway besides HER2 amplification [2, 3].

Literature references have shown that activation of the PI3K-AKT-mTOR signaling pathway promotes resistance to endocrine-based thera-

pies [4, 5]. The PI3K-AKT-mTOR pathway has been the focus of attention in ER+ breast cancer for the last few years. Everolimus (mTOR inhibitor) in combination with aromatase inhibitor was approved in ER+/HER2-negative advanced breast cancers [6]. Pan-PI3K inhibitor in combination with hormonal therapy (HR) showed a modest response in advanced hormone receptor-positive, HER2-negative breast cancer but was associated with significant toxicities [7]. Most recently, p110α specific PI3K inhibitors with better toxicity profile have been developed and are being studied in clinical trials in patients with HR-positive, HER2-negative advanced breast cancer [8].

Cyclin-dependent kinase 4 (CDK4) and CDK6 together with D-type cyclins (D1, D2 and D3) to promote cell cycle entry and progression through G1 by inactivating retinoblastoma protein (RB) by inhibiting an INK4 family of CDK inhibitors (CDKN2A/B). Recent data demonstrated that CDK4 induces cell cycle progression also plays a key role in hormone-independent cell growth [9]. Aberrant activation of CDK leads to dysregulated cell cycle progression from G1 to S phase and is a common feature of many cancers, including BC. Recently, some cell cycle proteins particularly those of CYCLIN D1-CDK4/6-RB network has been shown to exert oncogenic roles in a variety of cancers including BC, suggesting that their therapeutic exploitation may improve outcome in BC patients [9, 10]. Published data (PALOMA-1) showed that the addition of palbociclib (an oral small molecule inhibitor for CDK4/6) to letrozole significantly improved progression-free survival (PFS) in women with HR-positive, HER2-negative advanced BC who had not received systemic treatment for advanced disease [11]. Based on this trial, FDA granted accelerated approval to palbociclib and letrozole for treatment of postmenopausal women with HR-positive, HER2-negative metastatic BC who had not received prior endocrine-based therapy. Ribociclib was the second CDK4/6 inhibitor which recently got FDA approval as a first-line treatment for HR-positive, HER2-negative advanced BC in combination with an aromatase inhibitor (AI) in postmenopausal women based on MONALESSA-2 trial [12]. Most recently, abemaciclib (another CDK4/6 inhibitor) in combination with AI has been approved as a first-line treatment in postmenopausal women with

ER+/HER2-negative advanced or metastatic breast cancers [13].

Multiple fundamental studies have led to CDK4/6 inhibitors such as palbociclib, ribociclib, and abemaciclib becoming the standard of care for women with metastatic HR-positive/HER2-negative BC. Genetic analyses reveal that these agents are necessary but not sufficient for an effective treatment strategy for some ER+/HER2-negative metastatic breast cancer patients. At the 11th European Breast Cancer Conference in Barcelona, Spain (June 2018), Prof, Nick Turner, a consultant medical oncologist with The Royal Marsden Hospital NHS Trust, explained how HR-positive tumors resist treatment with CDK4/6 inhibitors and how that resistance can be overcome. The PI3K/mTOR pathway is critical in ER-positive breast cancer. In the PALOMA-3 trial of palbociclib, *PIK3CA* mutations were associated with approximately 5 folds increased the risk of development of resistance of this inhibitor within several months. Prof. Turner mentioned that PI3 kinase inhibitors had demonstrated synergy in several studies with CDK4/6 inhibitors. Remarkably, a recent disclosure of the results of the SOLAR 1 study from Novartis at ESMO, October 2018 showed that BYL719 (PI3K alpha-selective inhibitor, alpelisib) is highly effective in patients' tumors with ER+/*PIK3CA* mutations who were already exposed to CDK4/6 inhibitor along with fulvestrant. This article describes a rationale that oncologists can use to sequence the CDK4/6 inhibitors along with the PI3K-AKT-mTOR pathway-specific inhibitor(s); future data will better guide this approach.

Estrogen receptor pathway in breast cancer

Estrogen receptor (ER) is a group of nuclear receptor family. Estrogen receptor (ER) alpha is composed of NH2-terminal domain, two transcriptional activation function domains (AF1, AF2), DNA binding domain, and C-terminal binding domain [14]. The NH2-terminal encodes both ligand-dependent and ligand-independent activation function. AF1 is activated by phosphorylation. AF-2 is integral to ligand binding domain, and its activation requires ligand binding domain to bind to E2. The DNA binding domain contains two zinc finger structures which play an important role in receptor dimerization and the binding of the receptor to spe-

cific DNA sequences [14-16]. The C-terminal domain, upon ligand binding causes receptor dimerization, nuclear translocation, and trans-activation of target gene expression. The membrane-bound ERs cross-talk with other signaling pathways like IGF-1R, EGFR, FGFR and HER2 which are involved in cell proliferation, survival, differentiation, and apoptosis [17]. The membrane-bound ER can cause activation of mitogen-activated protein kinase (MAPK) signaling, activation of cyclic adenosine monophosphate (cAMP) and activation of the PI3K pathway [18]. Another mechanism by which ER modulates gene expression through protein-protein interactions for example activation protein-1 (AP-1) and nuclear factor κ-β (NF-kβ) [19]. Phosphorylation of estrogen receptor can cause ligand-independent ER activation through other signaling pathways including regulation of general phosphorylation state such as protein kinase A (PKA), protein kinase C (PKC), extracellular signals like peptide growth factors, cytokines, cell-cycle regulators [15]. Several cytokines, growth factors, and other pathways phosphorylate ER at a key position in the AF-1 domain and cause ligand-independent activation of ER. For example, ERK 1/2 pathway phosphorylates at serine 104 and serine 106 in the AF-1 domain and causes ligand-independent activation of ER [20]. AKT mediated phosphorylation of ER at serine 167 increases ER α binding to DNA and interaction with co-activator SRC3 in the presence of E2 [20]. Serine 167 is also phosphorylated by p90 ribosomal S 6 kinase (RSK), a kinase downstream of the MEK-ERK pathway [21].

Tamoxifen in ER-positive breast cancer

Tamoxifen is a selective ER modulator which when binds to AF2 causes a conformational change in AF2, releasing the suppression of AF1 and causes the repression of AF2 transcriptional activity [22]. A meta-analysis from EBCTCG showed that adjuvant tamoxifen for 5 years reduced the recurrence of annual BC death by 31% [23]. International breast-cancer-study-group (IBCSG) trial showed that premenopausal women with HR-positive, node-positive BC who received adjuvant tamoxifen for 5 years, after receiving chemotherapy had disease-free survival of 75% as compared to 62% in patients who did not receive adjuvant tamoxifen (HR: 0.59) [24]. ATLAS trial showed that

continuing adjuvant tamoxifen for up to 10 years further decreases the cumulative risk of BC during 5-14 years to 21.4% versus 25.1% for women who stopped adjuvant tamoxifen after five years. There was 2.8% decrease in BC mortality during years 5-14 in women who continued adjuvant tamoxifen for 10 years versus who stopped tamoxifen after five years (breast cancer mortality during years 5-14 was 12.2% for women allocated to continue versus 15.0% for controls) [25]. NSABP trial showed that adjuvant tamoxifen in node-negative, HR-positive BC increases disease-free survival to 83% versus 77% in patients who did not receive adjuvant treatment [26]. At 10 years follow-up, patients who received five years of adjuvant treatment with tamoxifen, disease-free survival was 69% as compared to 57% in women who did not receive adjuvant treatment with tamoxifen [27]. Overall, tamoxifen is an effective treatment in the adjuvant setting to decrease the risk of recurrent BC, especially in premenopausal women. Data from the Arimidex study group showed that in postmenopausal women with advanced BC, tamoxifen was associated with increased risk of thromboembolic events and vaginal bleeding as compared to anastrozole [28]. Therefore, aromatase inhibitors (AI) are usually the treatment of choice in postmenopausal women with HR-positive breast cancer in adjuvant and advanced settings.

Aromatase inhibitor (AI) in ER-positive breast cancer

In postmenopausal women, estrogen is no longer produced by ovarian tissue and is predominantly synthesized from non-glandular sources via the aromatase enzyme. Aromatase can be found in several tissues including subcutaneous fat, liver, and muscle; the enzyme has also been isolated in breast cancer cells [*Bland KI, Copeland EM. The Breast E-Book: Comprehensive Management of Benign and Malignant Diseases. Elsevier; 2009*]. Aromatase inhibitors emerged as an important class of drugs in the early 2000s which significantly improved disease outcome in HR-positive BC, especially in postmenopausal women. AIs block the conversion of adrenal androgens to estrogen in non-ovarian tissue by targeting aromatase enzyme which is the key source of estrogen synthesis in postmenopausal women. Three different AIs are active in clinical use (ste-

roidal and non-steroidal) namely anastrozole (Arimidex), letrozole (Femara) and exemestane (Aromasin).

ATAC trial demonstrated that adjuvant treatment with anastrozole in postmenopausal women with early-stage BC was associated with superior disease-free survival at 4 years (DFS: 86.9% in anastrozole group) as compared with tamoxifen (DFS: 84.5% in tamoxifen group) [29]. A ten-year analysis of ATAC trial continued to show superior DFS, time to recurrence and time to distant metastasis in anastrozole group as compared to tamoxifen [30]. Nabholtz JM et al. showed that anastrozole was associated with improved time to progression (TTP) as compared with tamoxifen when used in the first-line setting in postmenopausal women with HR-positive advanced BC. Median time to progression was 11.1 months in anastrozole group versus 5.6 months in the tamoxifen group [31]. Interestingly, the survival benefits from aromatase inhibitor versus tamoxifen as first-line treatment in postmenopausal women with advanced or metastatic HR-positive, HER2-negative BC is less clear [31, 32]. A Cochrane review showed 10% overall survival benefit with AI compared with other non-AI therapies [HR: 0.90, 95% CI: 0.84-0.74] [33]. *ESR1* mutant clones evolve in ER+ advanced BC during AI treatment, with *ESR*1 mutations resulting in constitutively active ER and its downstream signaling activity [34-36]. *ESR1* mutations have been recently recognized as one of the mechanisms of endocrine resistance and thought to evolve secondary to hormonal treatment. *ESR1* mutations are rarely found in primary BC and have been found in high prevalence in metastatic BC treated with an AI. These mutations occur in the hotspot domain of ligand binding domain (LBD) and cause constitutive activation of the ER pathway and resistance to AI [36]. Jeselsohn R et al. showed that 11 out of 76 patients (11%) with metastatic BC had *ESR1* mutation in ligand binding domain (LBD). Nine of these mutations were substitution mutations affecting Y537 and D538 in LBD. None of the 58 primary tumors had the *ESR1* mutation [35].

Fulvestrant in advanced breast cancer

Fulvestrant binds to ER monomers and inhibits receptor dimerization, activating function 1 (AF1), and AF2 are rendered inactive, transloca-

tion of the receptor to the nucleus is reduced, and degradation of the estrogen receptor is accelerated [37]. Ellis MJ et al. compared fulvestrant 500 mg intramuscularly on day 0, 14, 28 and then every 28 days with anastrozole 1 mg daily in postmenopausal women with HR-positive, HER2-negative metastatic BC. Median overall survival (OS) was 54.1 months in fulvestrant group versus 48.4 months in the anastrozole group [37]. FALCON, a phase 3 randomized, double-blind trial compared fulvestrant 500 mg intramuscularly on day 0, 14, 28 for the first cycle and then every 28 days with anastrozole 1 mg daily in HR-positive, HER2-negative locally advanced breast cancer who had not received prior endocrine therapy. Median PFS was 16.6 month in fulvestrant group versus 13.8 months in anastrozole group [38].

Fribbens C et al. assessed baseline plasma samples of patients (via circulating tumor DNA by digital PCR) enrolled in SOFEA and PALOMA-3 trials for the *ESR1* mutation [36]. The SOFEA study randomized patients with advanced HR-positive BC who had progressed on a non-steroidal aromatase inhibitor (NSI), in the adjuvant or metastatic setting, to fulvestrant plus anastrozole, fulvestrant plus placebo, or exemestane [36]. In SOFEA study, *ESR1* mutations were detected in 39.1% of patients (63/161). Patients with an *ESR1* mutation had improved PFS with fulvestrant containing treatment (N = 45) compared to exemestane alone (N = 18), (HR: 0.52; 95% CI, 0.30 to 0.92; P = .02), whereas patients with wild-type (WT) *ESR1* had similar PFS on either treatment (HR: 1.07; 95% CI, 0.68 to 1.67; P = .77).

In PALOMA 3 (Palbociclib Combined with fulvestrant in HR-positive HER2-negative metastatic BC after endocrine failure) trial, patients who had prior endocrine treatment (tamoxifen or an AI) were randomized to receive fulvestrant plus palbociclib, a CDK4/6 inhibitor, or fulvestrant plus placebo [39]. *ESR1* mutations were found in the 25.3% of patients (91 of 360), of whom 28.6% (26 of 91) were polyclonal, with mutations associated with acquired resistance to prior AI. Fulvestrant plus palbociclib improved PFS compared with fulvestrant plus placebo in both *ESR1* mutant (9.4 months vs. 3.6 months, HR: 0.43; 95% CI, 0.25 to 0.74; P = .002) and *ESR1* wild-type patients (9.5 vs. 5.4 months HR: 0.49; 95% CI, 0.35 to 0.70; P < .001). Although median PFS seemed to be slightly

worse in the *ESR1* mutated patients treated with fulvestrant alone (3.6 months 95% CI, 2.0-5.5) compared to *ESR1* wildtype (5.4 months 95% CI, 3.5-7.4), this was not statistically significant. One interesting finding was that *ESR1* mutations were almost exclusively found in patients with prior AI exposure with or without tamoxifen and were rare in patients with prior tamoxifen exposure only (28.9% [90 of 311] vs. 2.0% [one of 49], respectively; P = .001) [33, 40, 41]. Hamilton et al. studied AZD9496, a new oral selective estrogen receptor degrader in ER+, HER2-negative advanced BC in a phase I trial. Total of 45 patients was enrolled; one patient showed partial response and 10 patients (22.2%) showed stable disease at 6 months or longer. The main adverse effects were diarrhea, nausea, upper abdominal pain [42].

In a recent San Antonio breast cancer meeting (2017) Dickler M et al. presented their phase I data with GDC-0927. GDC-0927, an oral selective estrogen receptor degrader (SERD) showed promising activity in postmenopausal women with ER+, HER2-negative metastatic breast cancer who had progressed on prior endocrine treatment and cytotoxic chemotherapy. Nine out of 12 patients who had baseline FES-PET [^{18}F-fluoroestradiol] avid disease showed complete or near complete (> 90%) suppression of FES uptake to background levels, including patients with *ESR1* mutations. Evidence of reduced ER levels and Ki67 staining was observed in on-treatment biopsies. Clinical benefit rate (number of patients who remain on study for 24 weeks or greater) was seen in 5 of 12 (CBR = 41.6%). One patient with *ESR1* mutation (D538G) remained on study for over 490 days. GDC-0927 had a favorable toxicity profile as almost all adverse effects were grade 1-2. The most common treatment-related adverse effects were nausea (54%, n = 7), diarrhea (46%, n = 6), elevated aspartate aminotransferase (39%, n = 5) and anemia, constipation, (each 31%, n = 4) [43].

Resistance to hormonal therapy in hormone receptor-positive breast cancer

Endocrine treatment in HR-positive metastatic BC is associated with only 30% objective regression, and about another 20% of patients experience prolonged stable disease [18]. There are several mechanisms that have been

identified as the cause of endocrine resistance including aberrations at the level of ER, over-expression of ER co-activators, down-regulation of co-repressors, hyperactivation of the PI3K-AKT-mTOR pathway, upregulation of positive regulators of cell cycle causing cell proliferation, downregulation of negative regulators of cell cycle which block the antiproliferative effects of endocrine therapy [18, 44, 45]. Here, we discuss few mechanisms of endocrine resistance which have been studied extensively and have therapeutic agents already in clinical use or in clinical trials for the treatment of ER-positive BC.

Role of PI3K/AKT/mTOR pathway in endocrine resistance and targeting PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is frequently upregulated in estrogen-deprived BC cells. The downstream effectors of the PI3K pathway, AKT-mTOR axis impacts on tumor cell growth, survival, motility, and metabolism (**Figure 2A**). AKT is an important downstream target of the PI3K pathway that regulates cell survival, proliferation, growth, apoptosis, and glycogen metabolism [46, 47]. Increased AKT kinase activity is detected in 20% to 55% of cancers [47]. mTOR is a downstream effector of AKT. mTOR comprises two different structurally similar but functionally different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 is the target of rapamycin and rapamycin analogs (rapalogues), such as everolimus. Rapalogues exert their effect mainly on mTORC1, and the incomplete inhibition can lead to feedback loops causing paradoxical activation of AKT to orchestrate proliferative effects via downstream targets. mTOR's downstream substrate S6 kinase can phosphorylate and activate functional domain of ER leading to the ligand-independent receptor activation [48-52]. The enzymatic activity of PI3K is antagonized by the phosphatase and tensin homolog (PTEN), a protein that catalyzes the dephosphorylation of PIP3, and by inositol polyphosphate-4-phosphatase type II (INPP4B), a protein that catalyzes the dephosphorylation of PIP2 [53].

The study that led to the FDA approval of everolimus was the BOLERO-2-trial. BOLERO-2 compared everolimus (10 mg/day) plus exemestane (25 mg/day) to exemestane alone in

724 postmenopausal women with advanced HR-positive, HER2-negative breast cancer, whose cancer had either progressed or recurred while receiving non-steroidal aromatase inhibitor [6]. The primary endpoint after a median follow-up of 18 months indicated that a co-inhibition of the compensatory pathway via everolimus led to a doubling of PFS (local assessment: 7.8 vs. 3.2 months, HR 0.45, 95% CI 0.38-0.54, log-rank $p < 0.0001$; central assessment: 11.0 vs. 4.1 months, HR 0.38, 95% CI 0.31 to 0.48, log-rank $p < 0.0001$) [54]. The biomarker studies from the BOLERO-2 trial showed a greater benefit from everolimus in patients with minimal genetic alterations in *PIK3CA/ PTEN/CCND1* or *FGFR1/2* genes combined, which represented 76% of the next-generation sequencing population. Patients with a single alteration in one of these pathways had a median PFS of 214 days with everolimus, compared to 77 days with placebo (HR: 0.26) [55]. Components of the PI3K pathway are frequently altered in human cancers [56]. Mutations in the gene that constitute the phosphatidylinositol 3-kinase (PI3K) pathway occur in > 70% of breast cancers [45]. The somatic mutations in the components of PI3K pathway include mutations and/or amplification of the genes encoding PI3K catalytic subunits p110α (*PI3KCA*), p110β (*PIK3CB*), *PIK3R1* which encodes the PI3K regulatory subunit p85α and other genes encoding the PI3K associated modulators (namely *PTEN*, *AKT*, *MTOR*, *RICTOR*, *INPP4B* and *STK11*) [45]. *PIK3CA* mutations are the most common genetic alterations of this pathway and are seen in 36% of ER+ breast cancers [45, 56], Cancer Genome Atlas (TCGA) and the Catalogue of Somatic Mutations in Cancer (COSMIC) databases. These mutations frequently occur in the helical domain (hotspots E545K and E542K) or in the kinase domain (hotspot H1047R) of the *PIK3CA*-encoded p110α [49, 56]. The *PIK3R1*, the p85α regulatory subunit which inhibits the catalytic activity of p110α (**Figure 1B**). The p85α also binds to PTEN, preventing PTEN ubiquitination and increasing its protein stability [57]. The increase in PTEN protein expression and stability induced by WT p85α was also inhibited by co-expression of E160 *PIK3R1* mutant [58]. Functional mutants in *PIK3R1* cannot bind p110α and PTEN, leading to upregulation of PI3K signaling [58]. Very recently, it has been reported by others that a high proportion of

tumors harbors multiple mutations, especially *PIK3CA* plus *PIK3R1* mutations [59]. Several preclinical studies showed that PI3K mutant cancers are sensitive to PI3K inhibitors and show synergy with endocrine therapies to decrease tumor progression (**Figure 1A**) [4, 5]. This concept was first studied in the clinical setting by Dr. Baselga et al. in the BELLE-2 trial. BELLE-2 was a phase III double-blind, placebo-controlled randomized trial which included patients with advanced HR-positive, HER2-negative BC whose cancer had progressed on or after AI. The patients were randomly assigned to receive buparlisib (BKM120, a pan PI3K class inhibitor) combined with fulvestrant versus placebo and fulvestrant. Overall median PFS was modestly improved in the buparlisib group versus the placebo group (6.9 months [95% CI 6.8-7.8] vs. 5.0 months [4.0-5.2]; HR: 0.78 [95% CI 0.67-0.89], P = 0.00021). However, buparlisib was poorly tolerated with side-effects including hyperglycemia, diarrhea, stomatitis, and rash, increase in alanine transaminase and aspartate transaminase and frequent mood disorders, including three cases of suicidal ideation in the buparlisib group [7, 60]. Exploratory analysis of BELLE-2 trial showed that patients with *PIK3CA* mutation detected in circulating tumor DNA derived benefit from buparlisib (median PFS 7.0 months vs. 3.2 months in the placebo group, stratified HR: 0.58) whereas patients without *PIK3CA* mutations did not drive any benefit from buparlisib (median 6.8 months vs. 6.8 months for the placebo group, stratified HR: 1.02). This exploratory analysis strongly suggests that *PIK3CA* is a potential target, although more tolerable PI3K inhibitors are needed to exploit this potential. PI3K has several catalytic subunits, and the toxicity from buparlisib is partly a consequence of non-selective inhibition of all four isoforms of class 1 PI3Ks (α, β, γ and d). More selective inhibition of the mutant *PIK3CA* α-subunit has the potential to open the therapeutic window and improve efficacy through more potent inhibition of PI3K in tumor cells [61].

Recently, alpelisib (BYL719, a PI3K alpha-specific Inhibitor) in combination with letrozole was evaluated in phase Ib study in patients with HR-positive, HER2-negative metastatic BC whose tumor has progressed on endocrine therapies. The combination of alpelisib and letrozole showed clinical activity with a much better safety profile compared with pan P13K

A. PI3K and Cell Cycle Pathways: Functional Interaction
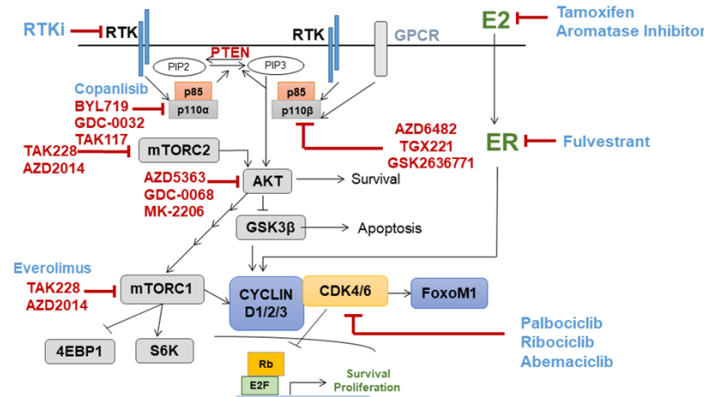
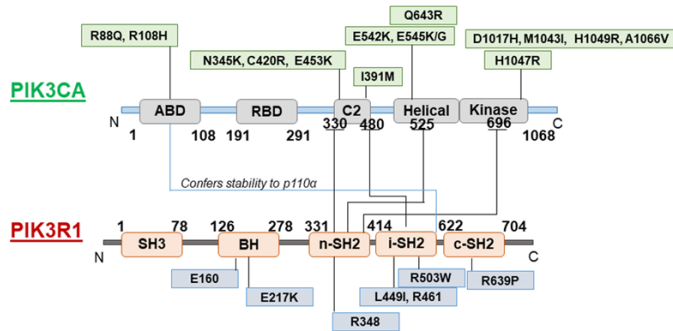## B. Intramolecular Interactions between PIK3CA and PIK3R1

Figure 1. A. The Mechanistic relationship between PI3K pathway activation and cell-cycle in tumor cells: PI3K signaling engages AKT, which drives mTORC1 CAP-dependent mRNA translation encoding CYCLIN D1/D2/D3. CYCLIN D1/D2/D3 bind CDK4/6 to promote RB phosphorylation, which depresses E2F transcription factor to drive expression of genes that promote proliferation and suppress apoptosis. CDK6 can phosphorylate FOXOM1 to enhance FOXOM1 stability, which suppresses senescence. Blue: FDA approved drugs and RED: Active in a clinical trial. B. Intramolecular interactions between PIK3CA and PIK3R1: Domain-specific regulation of PIK3CA (a p110α catalytic subunit of PI3K) by PIK3R1 (p85α regulatory subunit of PI3K) as scaffolds for regulated complex formation. Distribution of some important non-synonymous mutations of PIK3CA and PIK3R1 were depicted.

0221). During the recent ASCO 2018 meeting, Prof. Baselga revealed the SANDPIPER trial (dated 06-02-18) results. The study enrolled 516 postmenopausal women with locally advanced or metastatic HR-positive, HER2-negative breast cancer that worsened or recurred despite initial hormone treatment with AIs. Women were randomly assigned to receive fulvestrant and placebo (176 women) or fulvestrant and taselisib (GDC-0032) (340 women). Women who received taselisib and fulvestrant had a 30% lower chance of cancer worsening than those who received fulvestrant and a placebo, and taselisib extended the time until cancer worsened by a median of 2 months (7.4 months with taselisib/fulvestrant vs. 5.4 months with fulvestrant/placebo). The response rate to treatment was more than doubled when taselisib was added (28% vs. 11.9%). Overall survival data is not yet available. The most common severe side effects for patients who received taselisib were diarrhea, hyperglycemia, and colitis. Due to side effects, 17% of women who received taselisib stopped treatment early, compared to only 2% of those who did not receive the targeted therapy. Most interestingly, when investigators looked at outcomes by geographic area, they noted that taselisib provided more benefit to study participants who received treatment in North America and Europe, where cancer worsening was delayed by a median of 3.5 months (7.9 with taselisib/fulvestrant vs. 4.5 months with fulvestrant/placebo). In other countries including Eastern Europe and Latin America, taselisib appeared to provide very little or no added benefit. More research is needed to understand the reasons for this discrepancy [62].

inhibitors. The clinical benefit rate (lack of progression [greater than or equal to 6 months] was 35% (44% in patients with PIK3CA mutated and 20% in PIK3CA wild-type tumors; 95% CI: 17%-56%) including five objective responses. Of eight patients remaining on treatment (greater than or equal to) for 12 months, six had tumors with PIK3CA mutation. Those with FGFR1/2 amplification, KRAS, and TP53 mutations did not derive clinical benefit [8]. Results from two phase III trials of more α-selective PI3K inhibitors are awaited: SOLAR-1 (alpelisib and fulvestrant; NCT 02437318) and SANDPIPER (taselisib and fulvestrant; NCT 0234-

Role of cell cycle pathway in endocrine resistance and targeting CYCLIN D-CDK4/6-RB pathway

The mammalian cell cycle is a series of highly regulated processes that cause replication and cell division. The cell cycle is divided into four phases G1, S, G2 and M phase [63, 64]. The transition from G1 to S phase is regulated by cyclin-dependent kinases (CDKs). In the presence of mitogenic signals, cyclin-dependent kinases (CDK4 and CDK6) interact with CYCLIN D and form CYCLIN D-CDK4/6 complexes which phosphorylate retinoblastoma protein (RB) and lift repression of E2F transcription genes and causes progression from G1 to S phase (**Figure 1A**) [65, 66]. Activities of CDKs are affected by naturally occurring cyclin-dependent kinase inhibitors (CKI). Depending on target CDK, CKI belongs to two families. INK4 protein family includes p16 INK4a, p18INK4c, p19 INK4d, p15 INK4b. CYCLIN D, E, and A-dependent kinases are inhibited by Cip/kip family of CDKI [67].

Several oncogenic pathways including the RAS-MAPK and PI3K pathways can stimulate CYCLIN D-CDK4/6 complexes which phosphorylate RB (inactivation of RB) and cause cell proliferation. Several mechanisms including amplification of genes encoding *CYCLIN D1* (*CCND1*), *CDK4* or *CDK6* mutation, loss of tumor suppressor genes (*CDKN2A*) which encodes p16 INK4a activate CYCLIN D-CDK4/6-INK4-RB pathway. *CCND1* amplification is seen in 35% of HR-positive BCs [56, 68]. *CDK4* amplification is seen in 16% of BCs [56, 69]. Loss of p16 INK4a is seen in 49% of BCs [56, 69].

Pre-clinical data showed that inhibition of CYCLIN D dependent kinase activity causes tumor shrinkage, reversal of oncogenicity, and in some cases, transformed cell death [10, 70]. Selective CDK4/6 inhibitors "turn off" these kinases and dephosphorylated RB (active), resulting in a block of cell cycle progression in mid-G1 phase (**Figure 1A**). This causes cell cycle arrest and prevents the proliferation of tumor cells [10]. Tumor cells must express wild-type *RB* for the selective CDK4/6 inhibitor to be effective. Currently, three CDK4/6 inhibitors (Palbociclib, Abemaciclib, Ribociclib) have been approved for the treatment of HR-positive, HER2-negative BC in an advanced and metastatic setting in combination with endocrine

therapy. ER+ breast cancer seems to be especially dependent upon CDK4 for proliferation. The tight linkage of the ER signaling pathway to CYCLIN D1-CDK4 may be one reason why BC cells are highly sensitive to the anti-proliferative effects of drugs when they are combined with endocrine therapy. With the combination of a CDK4/6 inhibitor and anti-estrogen therapy, there is an enhancement of G1 and cell-cycle arrest and probably enhanced entry into a senescent state. To date, ER+BC is the malignancy for which this class of drugs has proven most effective and for which we have the most mature data from randomized trials comparing endocrine therapy alone or with endocrine therapy plus CDK4/6 inhibitors.

PALOMA-1 was a double-blind study which included 165 postmenopausal women with HR-positive, HER2-negative BC who had not received systemic treatment for advanced disease. The patients were randomized to receive palbociclib and letrozole or placebo plus letrozole. The median PFS in palbociclib and letrozole group was 20.2 months versus 10.2 months in letrozole group (HR: 0.488, 95% confidence interval (CI) 0.319-0.748, *P* = 0.0004) [11]. Based on results from the PALOMA-1 trial, FDA granted accelerated approval to palbociclib in combination with letrozole for the treatment of postmenopausal women with ER+/HER2-negative advanced BC [71]. PALOMA-3 (*NCT01942135*) compared palbociclib and fulvestrant versus placebo and fulvestrant in (pre and post-menopausal) women with ER+/HER2-negative BC who had relapsed or progressed during or after endocrine therapy. Pre and peri-menopausal women also received goserelin (Zoladex). Palbociclib and fulvestrant showed improved efficacy versus fulvestrant alone (median PFS was 9.5 months versus 4.6 months, HR: 0.46, 95% confidence interval 0.36-0.59, P ≤ 0.001) [72]. FDA approved palbociclib in combination with fulvestrant for the treatment of women with HR+, HER2-negative advanced or metastatic BC with disease progression following endocrine therapy in February 2016 [73].

MONARCH-3 (*NCT02246621*) compared abemaciclib and nonsteroidal aromatase inhibitor versus placebo and a nonsteroidal aromatase inhibitor in HR-positive, HER2-negative advanced BC who had no prior systemic therapy in the advanced setting. The combination of
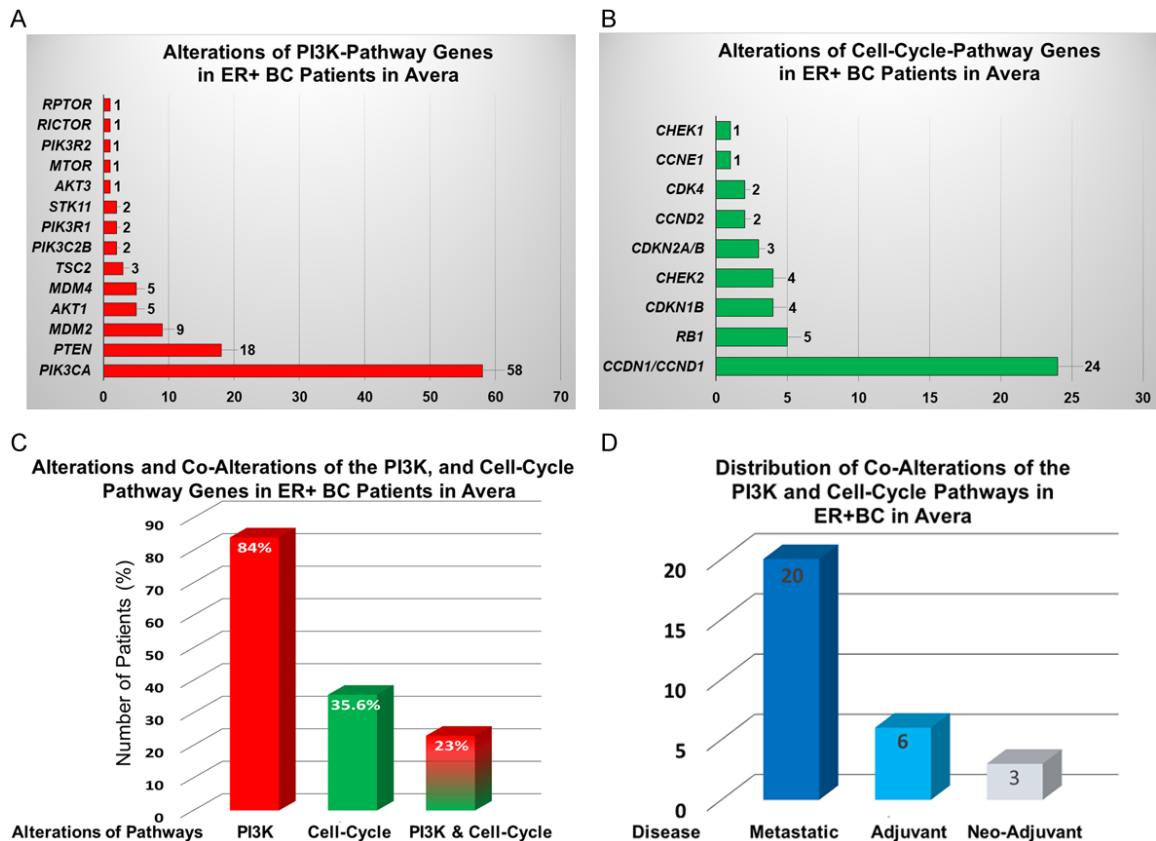
Figure 2. Number of alterations in 14 PI3K-AKT-mTOR signaling genes and 9 cell-cycle pathway genes in patients with ER+ breast cancer at Avera: Alterations in genes in patients with breast cancers enrolled at Avera Cancer Institute with ER+ disease were evaluated for the study. We identified alterations in 14 PI3K-AKT-mTOR pathway genes and 9 cell-cycle pathway genes in 129 patients with ER+ breast cancer at Avera. Alterations of genes were determined from the reports of Foundation Medicine (for DNA sequencing). Genes of the PI3K pathway is altered in 109 patients out of 129 ER+/PR+BC patients (84%; n = 129). A number of alterations of the PI3K pathway showed that the highest alterations were observed in the *PIK3CA* gene (A). Genes of the cell-cycle pathway are altered in 46 patients out of 129 ER+/PR+BC patients (35.6%; n = 129). A number of alterations of the cell-cycle pathway showed that the highest alterations were observed in the *CCND1* gene (B). Percentage of alterations and co-alterations of the PI3K, and cell-cycle pathway genes in ER+BC patients showed 23% of co-alterations (C). Distribution of co-alterations of the PI3K and cell-cycle pathways in ER+BC in Avera showed that co-alterations are predominantly present in patients with metastatic diseases (D).

abemaciclib and AI was statistically better compared to placebo and AI alone (HR: 0.54; 95% CI, 0.41 to 0.72; P = .000021; median PFS: not reached in the abemaciclib arm, 14.7 months in the placebo arm) [13].

MONALEESA-2 (*NCT01958021*), a phase III study compared combination of ribociclib with letrozole versus placebo and letrozole as first-line treatment in postmenopausal women with ER+, HER2-negative advanced BC. The duration of PFS was significantly longer in Ribociclib plus letrozole group than the placebo group (HR: 0.56; 95% CI, 0.43-0.72; P = 3.29 × 10⁻⁶ for superiority) [12]. Results of the second interim analysis of MONALEESA-2 were recently

published. The median duration of follow-up was 26.4 months. Ribociclib and letrozole was associated with improved median PFS vs. placebo and letrozole alone [median PFS was 25.3 months in ribociclib and letrozole group (95% CI, 23.0-30.3) and 16.0 months (95% CI, 13.4-18.2) in placebo and letrozole group (HR: 0.568; 95% CI, 0.457-0.704; log-rank P = 9.63 × 10⁻⁸)]. The objective response rate (ORR) was 42.5% versus 28.7% for all patients treated with ribociclib plus letrozole versus placebo plus letrozole, respectively, and 54.5% versus 38.8%, respectively, for patients with measurable disease. Overall survival data remain immature, with 116 deaths observed; 50 in the ribociclib arm and 66 in the placebo arm (HR:

0.746; 95% CI, 0.517-1.078). The benefit with ribociclib was maintained irrespective of *PIK3CA* or *TP53* mutation status, total RB, Ki67, or p16 protein expression, and *CDKN2A*, *CCND1*, or *ESR1* mRNA levels. Ribociclib benefit was more pronounced in patients with wild-type receptor tyrosine kinase (RTK) genes as compared to altered RTK genes [74]. Interestingly, biomarker analysis from PALOMA-2 did not show any correlation between expression of RB, Ki67, CYCLIN D1, and p16 in response to palbociclib plus letrozole [75].

Several mechanisms of resistance to CDK4/CDK6 inhibitors have been proposed including *CYCLIN E* amplification or overexpression, *E2F3* amplification, loss of p27 kip1 or p21 cip1, activation of *CDK2*, acquired *CDK6* amplification [76-78]. Herrera-Abreu TM et al. showed that chronic inhibition by CDK4/6 inhibitor causes an increase in AKT phosphorylation which correlates with sustained expression of E2F induced G1-S phase regulators such as CYCLIN E2 or CDK2 which cause failure to complete inhibition of RB phosphorylation [79]. They also showed that combining PI3K and CDK4/6 inhibition resulted in loss of RB phosphorylation with concomitant reduction of CYCLIN E2 and CDK2 expression, resulting in cancer cell apoptosis and tumor regression *in vivo* and in patient-derived tumor xenograft models, providing rationale for combination of PI3K and CDK4/6 combination treatment for synergistic response and to prevent resistance to CDK4/6 inhibitors when used alone. Yang C et al. found that in cell line models several clones emerged after prolonged exposure to CDK4/6 inhibitor (abemaciclib) that were found to harbor amplification of the CDK6 kinase. Amplification of *CDK6* resulted in a marked increase in CDK6 expression and reduced response to CDK4/6 inhibitors. CDK6 overexpression also led to reduced expression of the ER and progesterone receptor (PR), and diminished responsiveness to ER antagonism. Alternative mechanisms of resistance to CDK4/6 inhibitors such as loss of pRB and CYCLIN E1 overexpression also exhibited decreased hormone responsiveness [80]. Recently, Condorelli R et al. analyzed genotyping samples of three patients with metastatic BC via circulating tumor DNA before initiation of treatment with CDK4/6 inhibitor and after disease progression while on CDK4/6 inhibitor. All three patients showed acquired polyclonal *RB1* mutation after exposure to the CDK4/6 inhibitor. None of these patients had *RB1* mutation before exposure to CDK4/6 inhibitor [81].

Combined inhibition of CDK4/6 and PI3K/AKT/mTOR pathway signaling

Combined CDK4/6 inhibitors and PI3K inhibitors have been investigated pre-clinically primarily in models of BC, where co-activation of the PI3K and cell cycle pathways frequently occur, with the most promising results emerging with PI3K α-isoform-selective drug, since co-alteration of *PIK3CA* and *CYCLIN D1* are often found in ER+BC cohort [82] (See **Figure 2**). Vora SR et al. showed that combination of CDK4/6 inhibitors and PI3K inhibitor have a synergistic effect of decreasing cell proliferation in *PI3KCA* mutant BC cell lines which were resistant to PI3K inhibitor [83]. The addition of a PI3K inhibitor to combined CDK4/6 inhibition and hormonal therapy is particularly attractive for *PIK3CA* mutant ER+BC patients. A recent preclinical data showed that the triplet combination (letrozole/fulvestrant plus ribociclib plus PI3K pathway inhibitor) increased the tumor growth inhibition (TGI) even further, inducing tumor regressions in all xenograft models. The response was independent of *PIK3CA* or *PTEN* mutation status. Complete tumor regressions were observed for a subset of mice within each of the triple combination arms. Tumor regressions were maintained for up to four weeks post-interruption of treatment [84]. Interestingly, it has been reported by others that the combined inhibition of CDK4/6 and PI3K alpha isoform is synergistic and immunogenic (synergistically increased tumor infiltrating lymphocytes activation, cytotoxicity and decreased the frequency of myeloid-derived suppressor cells) in triple negative BC models. It simply suggests that this combination is not restricted to ER+/HER2-negative breast cancer [85].

Alterations of PI3K and cell-cycle pathway genes in patients with ER+ breast cancers at Avera

We have included 129 breast cancer patients evaluated at Avera with ER+ disease for the study. A close to 130 genes of different pathways have been identified to be altered in this set of patients. We identified alterations in 14 PI3K-AKT-mTOR signaling genes and 9 cell-cycle pathway genes in 129 patients with ER+

Table 1. Specific co-alterations in the PI3K and the cell-cycle pathway genes in ER+ BC patients in Avera

Metastatic	Adjuvant	NeoAdjuvant
AKT1 (E17K) & *CCND1* (Amp)	*PIK3CA* (E545K) & *CCND1* (Amp)	*PIK3CA* (E545K) & *CCND1* (Amp)
PIK3CA (E542K) & *CCND1* (Amp)	*PIK3CA* (H1047R) & *CDKN2A* (Loss)	*PIK3CA* (E970K, H1047R)/*PTEN* (R130Q) & *RB1* (E31*)
CDK4 (Amp) & *MDM2* (Amp)	*PIK3CA* (K111 del)/*TSC2* (R1753fs*58+) & *CCNE1* (Amp)	*PIK3CA* (E542K) & *CCND1* (Amp)
PIK3CA (Amp, H1047R)/*MDM4* (Amp) & *CCND1* (Amp)	*PIK3CA* (C420R) & *CCND1* (Amp)	
PIK3CA (Amp, H1047R)/*MDM4* (Amp) & *CCND1* (Amp)	*TSC2* (A607T) & *CHK1* (R160H)	
PIK3CA (E724K, H1047L) & *CCND1* (Amp)/*CHK2* (T533fs*12+)	*PIK3CA* (E545K) & *CHK2* (Splice site 444+1G>A)	
PIK3CA (E545K)/*MDM2* (Amp)/*RICTOR* (Amp) & *CCND1* (Amp)/*CDK4* (Amp)		
PIK3CA (E545K)/*PTEN* (Loss) & *RB1* (E580*)		
MTOR (I1973F-subclonal)/*MDM2* (Amp) & *CCND1* (Amp)		
AKT1 (E17K) & *CDKN1 B* (Q65*)		
AKT3 (Amp)/*PIK3CA* (Amp) & *CCND1* (Amp)		
PIK3CA (E726K, Q546R) & *RB1* (R556*)		
PIK3CA (E545K) & *CCND1* (Amp)		
PIK3CA (H1047L)/*PTEN* (R233*) & *CHK2* (Loss exons 9-10)		
STK11 (Loss)/*RPTOR* (Amp) & *CDKN2A* (Loss)		
PIK3CA (C420R) & *CDKN1B* (F33fs*9)		
PIK3CA (E545K)/*PIK3R1* (D464H) & *CCND1* (Amp)		
PIK3R2 (Rearrangement intron 13) & *CDKN2A/B* (Loss)		
PIK3CA (E545K) & *CCND1* (Amp)		
PIK3CA (E545K)/*PTEN* (C134F) & *CCND1* (Amp)		

Specific types of alterations in genes of the PI3K and the cell-cycle pathways co-altering in ER+ BC patients with metastatic, adjuvant and neo-adjuvant diseases are presented in separate columns with different colors.

breast cancer at Avera. Alterations of genes were determined from the reports of Foundation Medicine (for DNA sequencing). The genes of the PI3K pathway is altered in 109 patients out of 129 ER+/PR+BC patients (84%; n = 129). The identified genes of the PI3K pathway are *PIK3CA, PTEN, MDM2, AKT1, MDM4, TSC2, PIK3C2B, PIK3R1, STK11, AKT3, MTOR, PIK3R2, RICTOR and RPTOR* in their descending order of magnitude. The highest number of alterations were observed in the *PIK3CA* gene (45%; n = 129) which was followed by alterations in the *PTEN* gene (14%; n = 129) (**Figure 2A**). Prominent alterations in the PI3K pathway included mutations [e.g. *PIK3CA (E542K), AKT1* (E17K)], amplifications (*MDM2*), frame shifts [*TSC2 (R1753fs*58+)*] and loss [*PTEN*] (**Table 1**). The genes of the cell-cycle pathway are altered in 46 patients out of 129 ER+/PR+BC patients (35.6%; n = 129). The identified genes of the cell-cycle pathway are *CCND1, RB1, CDKN1B, CHEK2, CDKN2A/B, CCND2, CDK4, CCNE1* and *CHEK1* in their descending order of magnitude. The highest number of alterations were observed in the *CCND1* gene (18.6%; n = 129) which was followed by alterations in the *RB1* gene (3.87%; n = 129) (**Figure 2B**). Prominent alterations in the cell-cycle pathway included mutations [e.g. *CHEK1 (R160H)*], amplifications (*CCND1*), frame shifts [*CHEK2 (T533fs*12+)*] and loss [*CDKN2A*] (**Table 1**). We also evaluated the co-alteration of the PI3K and the cell-cycle pathway genes in these 129 patients with ER+ breast cancers. The co-alteration was observed in 29 patients (23%; n = 129) (**Figure 2C**). Interestingly, the majority of the co-alterations were observed in patients with metastatic disease (20) followed by adjuvant (6) disease. The least number of co-alterations were observed in patients with neo-adjuvant disease (3) (**Figure 2D**). Remarkably, a recent disclosure of the results of the SOLAR 1 study from Novartis at ESMO, October 2018 showed that BYL719 (PI3K alpha-selective inhibitor) is highly effective in patients' tumors with ER+/*PIK3CA* mutations who were already exposed to CDK4/6 inhibitor along with fulvestrant. We tested the anti-tumor efficacy of the combination of isoform-specific PI3K inhibitors (e.g., BYL719 and AZD6284) and ribociclib using cell line (*PIK3CA* mutated T47D, MCF7, and PTEN-null MDA-MB415 cells) based model. Data showed a synergistic effect of PI3K inhibitor and CDK4/6 inhibitor as determined from

the changes in proliferative and apoptotic signals as well as cell cycle arrests. Results of our study showed that such a combinatorial strategy with PI3K inhibitor and CDK4/6 inhibitor might be beneficial in RB wt ER+ breast cancer with concurrent genetic alterations of these pathways [82]. Several clinical trials are underway to evaluate the synergistic effect of the PI3K inhibitor with CDK4/6 inhibitor. Phase Ib/II study of combination of PI3K inhibitor, taselisib (GDC-0032), or pictilisib (GDC-0941) with palbociclib with subsequent addition of fulvestrant (*NCT 02389842*) or a combination buparlisib (BKM120)/alpelisib (BYL719) plus ribociclib with fulvestrant in patients with *PIK3CA* mutant breast cancer is ongoing (*NCT 02088684*). Michaloglou C et al. showed that mTORC1/2 inhibitor (AZD2014) causes modulation of E2F mediated transcription and cooperate with CDK4/6 inhibitor (palbociclib) in inhibition of E2 function in HR-positive BC [86]. mTOR inhibitor in combination with CDK4/6 inhibitor and exemestane is being studied in a clinical trial (*NCT 01857193*). In recently disclosed results of PALOMA-3 trial data, *PIK3CA* alterations levels (from ctDNA) above the median cut off were associated with approximately a fivefold increased risk for development of resistance to this CDK4/6 inhibitor within several months [87]. This recent data clearly indicate that patients with advanced ER+; high *PIK3CA* mutant burden BC could benefit from the introduction of alpha-specific PI3K inhibitor along with CDK4/6 inhibitor and fulvestrant.

Conclusion

Hormone receptor-positive breast cancer is a very dynamic field and clinical advances in the last 20 years have significantly improved the outcome of this disease. Progression-free survival and overall survival has significantly improved in patients with HR-positive BC. There has been significant research to understand the mechanism of hormone resistance and develop strategies to overcome hormone resistance in BC. Most recently, CDK4/CDK6 inhibitors were added in the armamentarium for the treatment of HR-positive, HER2-negative advanced BC and are being studied in a clinical setting. PI3K alpha specific inhibitors are also being evaluated in clinical trials. Clinical trials are exploring the efficacy of the triplet combination of hormonal therapy, CDK4/CDK6

inhibitor, and inhibitor of the PI3K pathway in advanced HR-positive, HER2-negative breast cancers. The role of immunotherapy is being evaluated in several clinical trials in advanced BC and have shown a more objective response in triple negative and HER2-positive BCs and less in HR-positive breast cancer [88]. The role of immunotherapy, fibroblast growth factor inhibitors in the treatment of HR-positive BC are also being explored, and several clinical trials are underway to evaluate the efficacy of FGFR inhibitors and immunotherapy. Recent studies in the mouse model of breast cancer demonstrated that CDK4/6 inhibitors not only induces tumor cell arrest but also promote anti-tumor immunity in two different ways: 1) CDK4/6 inhibitors activate tumor cell expression of endogenous retroviral elements, increasing intracellular double-stranded RNA, which stimulates the production of interferon III, which then enhance tumor antigen presentation and 2) CDK4/6 inhibitors suppress proliferation of regulatory T cells (Tregs) [89]. Eventually, these events activate cytotoxic T cell-mediated tumor cell clearance.

Next step would be to identify the optimal sequence of combination treatments and to identify predictive biomarkers which would help clinicians to tailor the treatment for the individual patient. The question that which patients with HR-positive BC could be treated by hormonal therapy alone at the time of initial diagnosis and which patients would benefit from combination treatment at the beginning of treatment has yet to be answered. The results from the ongoing clinical trials would help us to better answer these questions and to personalize treatment for each patient with HR-positive BC.

Disclosure of conflict of interest

None.

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