Mechanisms of Resistance to CDK4/6 Blockade in Advanced Hormone Receptor–positive, HER2-negative Breast Cancer and Emerging Therapeutic Opportunities

Maxwell R. Lloyd[1,2], Laura M. Spring[2,3], Aditya Bardia[2,3], and Seth A. Wander[2,3]

ABSTRACT

The cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) have become the standard of care, in combination with antiestrogen therapy, for patients with hormone receptor–positive (HR^+)/$HER2^-$ advanced breast cancer. Various preclinical and translational research efforts have begun to shed light on the genomic and molecular landscape of resistance to these agents. Drivers of resistance to CDK4/6i therapy can be broadly subdivided into alterations impacting cell-cycle mediators and activation of oncogenic signal transduction pathways. The resistance drivers with the best translational evidence supporting their putative role have been identified via next-generation sequencing of resistant tumor biopsies in the clinic and validated in laboratory models of HR^+ breast cancer. Despite the diverse landscape of resistance, several common, therapeutically actionable resistance nodes have been identified, including the mitotic spindle regulator Aurora Kinase A, as well as the AKT and MAPK signaling pathways. Based upon these insights, precision-guided therapeutic strategies are under active clinical development. This review will highlight the emerging evidence, in the clinic and in the laboratory, implicating this diverse spectrum of molecular resistance drivers.

Introduction

Three cyclin-dependent kinase 4 and 6 inhibitors (CDK4/6i)—palbociclib, ribociclib, and abemaciclib—have emerged as therapeutic options for hormone receptor–positive, HER2-negative advanced breast cancer (HR^+/$HER2^-$ ABC). These agents have been approved in the first-line setting, typically in combination with an aromatase inhibitor (AI), or in later line settings, in combination with fulvestrant (1). Their approval and integration into clinical practice was based upon the landmark PALOMA, MONALEESA, and MONARCH series of prospective randomized clinical trials (2–8). Subsequent analyses have also demonstrated an overall survival advantage for patients treated with these agents (9–14). Among these drugs, abemaciclib has distinct pharmacokinetic and pharmacodynamic properties and remains the only CDK4/6i approved as monotherapy (15).

In the metastatic setting, patients often develop disease progression in the setting of drug resistance. Recent clinical, translational, and laboratory-based research efforts have shed light upon the heterogeneous genomic landscape of CDK4/6i resistance, and a multitude of potential resistance mediators have been identified. In this review article, we will summarize the growing body of translational evidence implicating these various resistance mechanisms and the potential therapeutic implications.

[1]Beth Israel Deaconess Medical Center, Boston, Massachusetts. [2]Harvard Medical School, Boston, Massachusetts. [3]Massachusetts General Hospital Cancer Center, Boston, Massachusetts.

Corresponding Author: Seth A. Wander, Medical Oncology, Massachusetts General Hospital Cancer Center, 55 Fruit Street, Boston, MA 02114. Phone: 617-726-6500; E-mail: swander@partners.org

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Many CDK4/6i resistance drivers can be broadly subdivided into two categories including (i) alterations in cell-cycle mediators and (ii) activation of oncogenic signal transduction pathways, which are reviewed in detail below.

Alterations in cell-cycle mediators

The G_1-to-S cell-cycle checkpoint is regulated by the tumor suppressor protein retinoblastoma (RB1), which is active and bound to the transcription factor E2F in its unphosphorylated state (16). D-type and E-type cyclins, whose activation can be mediated by various cellular growth signals, form complexes with CDK4/CDK6 and cyclin-dependent kinase 2 (CDK2), respectively, to facilitate the phosphorylation of RB1, release of E2F, and downstream G1–to–S phase transition (16). The CDK4/6 inhibitors effectively disrupt cellular proliferation by blocking this pathway. Aberrations in a specific subset of cell-cycle regulatory proteins detailed below have a demonstrable role in intrinsic and acquired resistance to CDK4/6 blockade (**Fig. 1**).

RB1 loss

Given its central role downstream of CDK4/6 signaling, initial expectations were that *RB1* alterations would be a common mechanism of CKD4/6i resistance, however multiple studies now suggest that biallelic loss of *RB1* is rare (<10%; refs. 17–19). Despite this, a growing body of clinical and preclinical evidence supports biallelic *RB1* loss as a mediator of acquired and intrinsic resistance to CDK4/6 blockade. Serial circulating tumor DNA (ctDNA) sequencing from 195 patients in the PALOMA-3 trial demonstrated uniquely enriched *RB1* alterations at disease progression in the palbociclib arm (6/127 vs. 0/68; ref. 18), and targeted sequencing of 348 CDK4/6i-naïve tumors in another study identified a significant association between loss-of-function mutations in *RB1* ($n = 9$) and decreased progression-free survival (PFS; ref. 19). Our own effort leveraged whole-exome

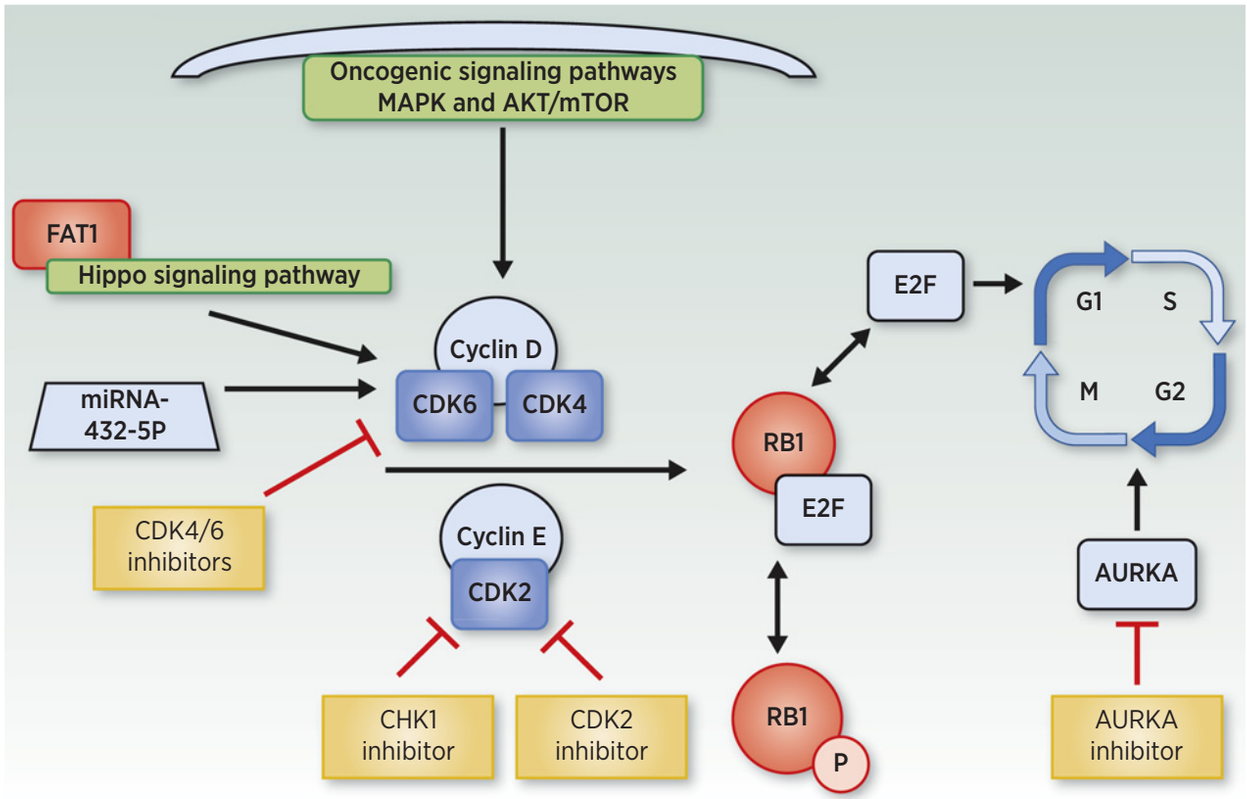

Figure 1.
Cell-cycle mediators that are implicated in driving intrinsic and acquired resistance to CDK4/6 blockade. Blue, Factors that promote cell-cycle progression; Red, Factors that inhibit the cell cycle; Yellow, Targeted therapies under investigation. P, phosphate.

sequencing (WES) from 59 HR^+ ABC tumors pre- and post-CDK4/6i exposure, revealing biallelic *RB1* alterations in 4 patients with intrinsic and acquired resistance to CDK4/6 blockade (17). In this analysis, a variety of RB1 alterations were appreciated including point mutations, frameshift alterations, and splice-site mutations, highlighting the diverse nature of potential "second hits" in patients with biallelic disruption (17).

Preclinical data has supported these observations. HR^+ breast cancer cells with RB1 loss demonstrated *de novo* CDK4/6i resistance (20). Subsequent work in laboratory-based models also demonstrated that RB1 loss confers resistance to CDK4/6 blockade, and multiple breast cancer cell lines demonstrated decreased expression of RB1 when cultured to resistance in the presence of CDK4/6i (17). Notably, given that RB1 expression can decrease in response to CDK4/6 inhibition, low RB1 expression in resistant tumors may be a passenger finding rather than a resistance modulating event and must be interpreted within this context. Other preclinical work demonstrated that RB1 mRNA levels are associated with sensitivity to CDK4/6 blockade (21, 22), but further work is required to validate the impact of these findings clinically, alone and in the context of other mutational events that can occur in *RB1*.

Aurora Kinase A upregulation

Aurora Kinase A (AURKA) promotes mitotic spindle assembly and regulates the G_2–M transition by phosphorylation and activation of Cyclin B1-CDK1 (23). WES from 59 biopsy specimens in patients with CDK4/6i exposure implicated *AURKA* amplification as a mediator of resistance: *AURKA* was the only gene with a statistically meaningful copy-number difference between CDK4/6i-sensitive and CDK4/6i-resistant tumor samples in this analysis (17). This observation was supported by preclinical correlative efforts, where AURKA overexpression conveyed resistance to CDK4/6 blockade *in vitro*, and HR^+ breast cancer cells cultured to resistance in the presence of CDK4/6i demonstrated high levels of AURKA expression (17).

CDK6 upregulation

Acquired *CDK6* amplification in breast cancer cells diminished response to CDK4/6 inhibition, while *CDK6* knockdown restored drug sensitivity (24). Clinically, *CDK6* amplifications and mutations have not yet been demonstrated in resistant tumor samples, but other mechanisms that appear to converge on CDK6 upregulation have been identified. Targeted sequencing of 348 hormone-sensitive breast tumors prior to CDK4/6i treatment identified that *FAT1* loss-of-function events, while rare (6/348), were associated with resistance to CDK4/6 blockade (19). Complementary efforts in the laboratory demonstrated that loss of the tumor suppressor *FAT1* in HR^+ breast cancer cells led to increased CDK6 expression, via Hippo pathway signaling, and provoked resistance to CDK4/6 blockade (19).

Increased expression of miR-432-5P may play a role in mediating CDK4/6i resistance (25). miR-432-5P was shown to upregulate CDK6 expression via suppression of the TGF-b pathway, provoking

resistance to palbociclib *in vitro* (25). Interestingly, it was shown that extracellular miRNA signaling can also confer CDK4/6 resistance to neighboring cell populations (25).

Cyclin E1/E2 amplification

Cyclin E1/E2 (CCNE1/2) promotes cell-cycle progression via association with CDK2 (26). Clinically, gene expression analysis from the PALOMA-3 study suggested that palbociclib efficacy was diminished in patients with high CCNE1 mRNA expression (27). The median PFS in the high expression group was 7.6 months, compared with 14.1 months in patients with low CCNE1 (27). CCNE2 amplification was also identified as a potential mediator of resistance via WES of resistant tumor biopsies (17).

Preclinical work demonstrated that overexpression of CCNE1 and CCNE2 in HR^+ breast cancer cells reduced sensitivity to palbociclib secondary to activation of CDK2, effectively creating a bypass route for cellular growth signaling (28). Both *CCNE1* (20) and *CCNE2* overexpression (17) are sufficient to provoke resistance to CDK4/6 blockade *in vitro*. Furthermore, cells cultured to resistance in the presence of a CDK4/6i demonstrated increased CCNE2 protein expression (17).

Preclinical therapeutic insights related to cell-cycle mediators of resistance

The work outlined above has implicated a variety of cell-cycle regulatory proteins in modulating CDK4/6i resistance while also highlighting several emerging therapeutic opportunities.

RB1 loss and AURKA upregulation

In breast cancer cell lines with acquired resistance to CDK4/6i and *RB1* loss or *AURKA* amplification, enhanced sensitivity to the novel AURKA inhibitor LY3295668 was seen, demonstrating that Aurora Kinase may serve as a viable therapeutic target in RB1-deficient and AURKA-amplified tumors (17).

CDK6 upregulation

With regard to CDK6, FAT1-deficient cells were resensitized to abemaciclib following *CDK6* knockdown (19); a drug holiday, defined by discontinuation of therapy for 28 days, was sufficient to reset CDK4/6i susceptibility in miRNA-432-5P–overexpressing breast cancer models (25). These studies also suggested that Hippo pathway signaling (19) and TGF-b (25) play important roles in facilitating CDK6 overexpression, however a therapeutic modality targeting these pathways has not yet been characterized in models of CDK4/6i resistance.

CCNE1/2 amplification

Cyclin E upregulation may provoke unique tumor cell susceptibility to checkpoint kinase 1 (CHK1) inhibition. Preclinical efforts have demonstrated that DNA damage and replication stress activate CHK1 to halt cell-cycle progression until DNA damage can be repaired (29, 30), and CHK1 inhibitors are progressing through clinical development (29). Evidence suggests that cells do not tolerate CDK2 activity in S-phase, with CHK1 regulating CDK2 inactivity, and cellular replication stress makes cells especially sensitive to CHK1 inhibition (29). Based upon this rationale, *CCNE2*-amplified breast cancer cells were sensitive to the CHK1 inhibitor prexasertib (17). A downstream therapeutic target has also shown efficacy, with research demonstrating that the CDK2 inhibitor roscovitine abrogates resistance to CDK4/6 blockade in *CCNE1*-amplified models (20).

Oncogenic signaling pathway activation—upstream tyrosine kinase receptors, MAPK and AKT/mTOR signaling

Oncogenic transduction pathways mediate the intracellular response to growth factor activation of membrane receptor tyrosine kinases (31). The MAPK and PI3K pathways play well characterized roles in modulating a myriad of cellular processes including growth, proliferation, invasion, and apoptosis (31, 32). Activation of the oncogenic signaling pathways detailed below have been shown to provoke resistance to CDK4/6 blockade (**Fig. 2**).

PTEN loss and AKT1 activation

AKT1 expression leads to a downstream increase in CCNE1/2 and CDK2 activation via reduced p27 activity (33–36). Multiple diverse AKT1 activating events were implicated in driving resistance to CDK4/6 blockade, including point mutations and amplification events in matched pre- and posttreatment tumor samples (17). Elevated phospho-AKT levels correlated with shorter PFS in patients treated with a combination of CDK4/6i and endocrine-directed therapy (ET; ref. 37). Overexpression of AKT1 in HR^+ breast cancer cells provoked resistance to CDK4/6 inhibition, as well as antiestrogen therapy (17, 38).

Loss of the PTEN tumor suppressor can also upregulate AKT/mTOR signaling, leading to increased CDK2 and CDK4 expression (33). A study examining serial tumor biopsies from patients receiving ribociclib and letrozole demonstrated loss of PTEN function at disease progression (33). Despite its pivotal role in regulating the PI3K/AKT/mTOR signaling pathway, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) aberrations have not been associated with CDK4/6i resistance (17, 18) and are not likely to be a clinically relevant predictor of CDK4/6 resistance.

RAS/MAPK activation

The *RAS* family of oncogenes promote cellular growth signaling secondary to downstream activation of the MAPK and AKT/mTOR pathways (39, 40). Activating mutations in *RAS* family oncogenes *(KRAS*, *HRAS*, and *NRAS*) were identified in tumor biopsy specimens of patients with CDK4/6i resistance (17). Introduction of mutant *KRAS* via lentiviral overexpression provoked resistance *in vitro*, and *KRAS* mutations emerged spontaneously when breast cancer cells were cultured to resistance in the presence of a CDK4/6 inhibitor (17). Increased ERK activation was demonstrated in these resistant models, implicating the downstream role of the MAPK pathway in facilitating resistance to CDK4/6 blockade (17).

FGFR1/2 upregulation

Similar to RAS, the FGFR pathway promotes tumor growth and has been implicated in driving tumor progression in multiple cancers (41, 42). Previous work suggests that dysregulation of this pathway drives the development of tumor resistance to antiestrogen therapy (41, 42). Several studies have implicated activating mutations and amplification events in *FGFR1* and *FGFR2* in mediating resistance to CDK4/6 blockade in breast cancer. From the PALOMA-3 trial, ctDNA analyses demonstrated acquired *FGFR2* mutation or amplification events at disease progression in a subset of patients (4/195; ref. 18) and associated worse PFS with *FGFR1* amplification (43). Sequencing of 212 samples from the MONALEESA-2 study identified inferior PFS in patients with *FGFR1* amplification events (10.61 months vs. 24.84 months; $P = 0.075$), and higher levels of FGFR1 mRNA correlated with a significantly shorter PFS (44).

Preclinical efforts have also demonstrated that activation events across multiple *FGFR* family members provoke resistance to CDK4/6 blockade in HR^+ breast cancer cells (42, 44, 45). FGFR1/2 upregulation

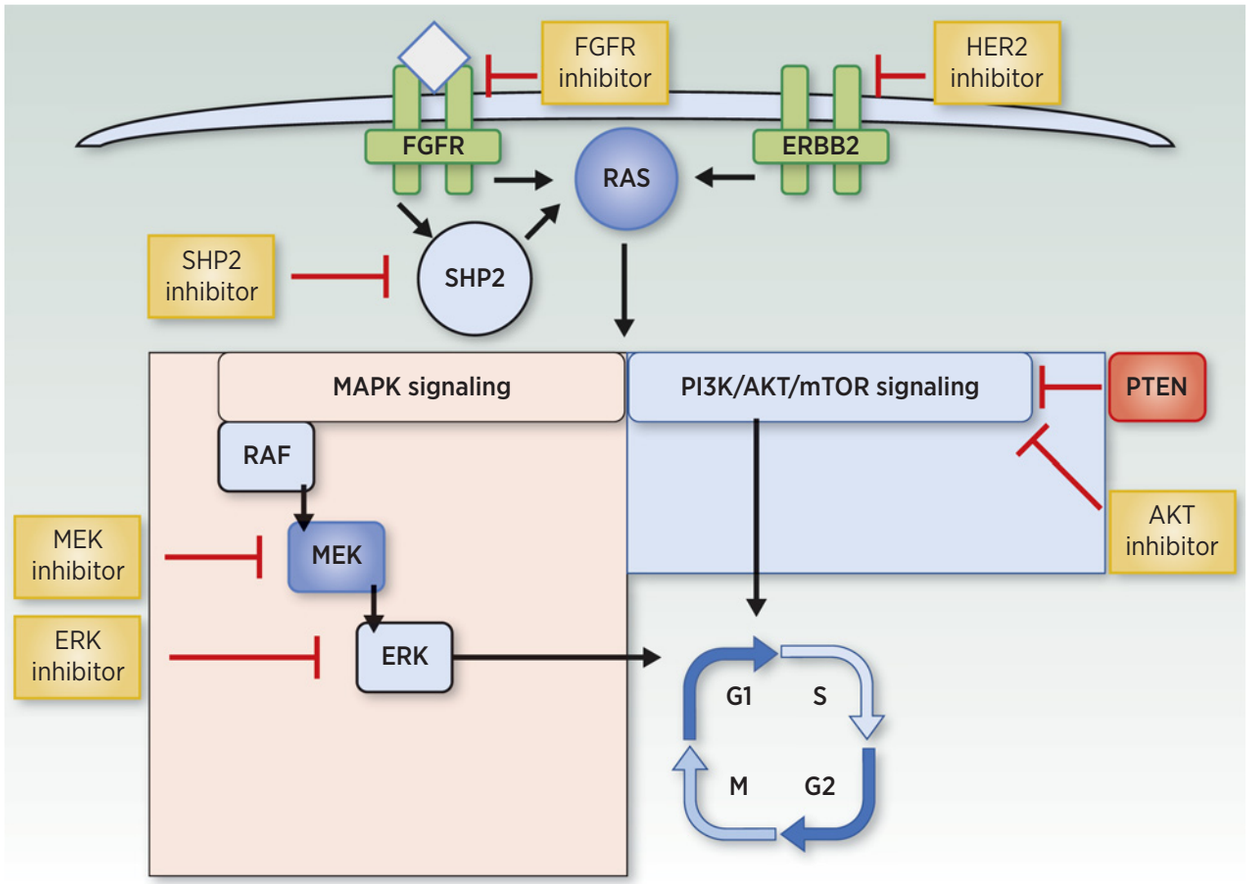

Figure 2.

Oncogenic signaling pathways and upstream tyrosine kinase receptors that mediate CDK4/6i resistance. Green, Tyrosine kinase receptors; Blue, Factors that transduce downstream oncogenic signals; Red, Factors that inhibit the cell cycle; Yellow, Targeted therapies under investigation.

led to ERK overexpression in these cells, again implicating convergent downstream activation of the MAPK pathway as a common node of CDK4/6i resistance (42). AKT/mTOR pathway activation and cyclin D1 (CCND1) overexpression were also demonstrated in *FGFR1/2* amplified cells (42).

ERBB2 activating mutation

ERBB2 activation has been shown to stimulate oncogenic signaling pathways and has been implicated in mediating resistance to estrogen-directed therapy (46). *ERBB2* mutations were identified via WES in 5 of 41 CDK4/6i resistant biopsy samples (17). Preclinical work from multiple studies revealed that *ERBB2* activating mutations in breast cancer cells conferred resistance to CDK4/6 blockade (46, 47). *ERBB2* mutant cell lines had activation of downstream effectors such as MAPK and AKT/mTOR, again highlighting the convergence of multiple CDK4/6i resistance mechanisms on these common nodes of signal transduction (46).

Preclinical therapeutic insights related to oncogenic signal transduction pathways

A variety of therapeutic agents targeting the oncogenic pathways outlined above are in clinical development and may emerge as important tools to circumvent CDK4/6i resistance.

PTEN loss and AKT1 activation

Laboratory-based efforts revealed that cells with activation of AKT1, either via *AKT1* amplification (48) or PTEN loss (33), could be resensitized to CDK4/6i by treatment with the AKT inhibitor MK2206 (33, 48). The triple combination of an AKT inhibitor with a CDK4/6i and ET inhibits the growth of breast cancer cells and patient-derived xenografts resistant to combined CDK4/6i and ET (37). Interestingly, preclinical models overexpressing FGFR1/2 demonstrated that CDK4/6i resistance was only partially overcome by mTOR inhibition, suggesting that AKT/mTOR signaling may have a diminished role in mediating resistance compared with MAPK in that context (42).

RAS/MAPK activation

The convergence of these CDK4/6i resistance mechanisms on the MAPK signaling node suggests that this may serve as a common therapeutic target in resistant patients (17). In breast cancer models with activating mutations in *KRAS*, treatment with an ERK inhibitor abrogated resistance to CDK4/6 blockade (17). *FGFR1/2*-amplified cells were sensitive to treatment with meiotic chromosome-axis–associated kinase (MEK) inhibitors and SH2 containing protein tyrosine phosphatase-2 (SHP2) inhibitors (42). Thus, treatments targeting downstream MAPK pathway proteins could potentially

overcome or prevent resistance via multiple upstream mechanisms. While MEK and ERK inhibitors have not been tested in *ERBB2* mutant breast cancer cells, this is another area for future investigation

FGFR/ERBB2 activation

FGFR1/2-amplified breast cancer cells can be resensitized to CDK4/6 blockade by treatment with the FGFR inhibitors lucitanib and erdaftinib (42, 44). Cells with *ERBB2* activating mutations were sensitive to treatment with the HER2 kinase inhibitor neratinib (47), alone or in combination with endocrine therapy (46). These preclinical findings have served to inform the development of new therapeutic strategies in the clinic, which are discussed below.

Comparing the genomic resistance landscape between research studies

As discussed in the sections above, clinical data from CDK4/6i resistant tumors has primarily come from DNA sequencing studies. Two research efforts by O'Leary and colleagues in 2018 (18) and Wander and colleagues in 2020 (17) helped elucidate much of the genomic resistance landscape highlighted in this review. These two studies had several key differences in their design, execution, and results (**Table 1**). One possibility to account for these differences is to examine the next-generation sequencing (NGS) techniques used between the two studies: the first utilized ctDNA to perform targeted sequencing of a gene panel and hotspot mutations, while the other collected metastatic tissue biopsies for WES. The genomic insights yielded by these approaches may account for some of the dissimilarities in driver alterations found in the latter study that were absent in the former. For example, ctDNA has the potential to miss mutations due to low levels of tumor DNA that are misclassified as sequencing noise, and targeted sequencing may not identify genomic events appreciated on WES. In addition, alterations in AURKA were only appreciated via copy-number analysis of WES, and may have been missed via targeted sequencing of ctDNA. Another important difference includes the granularity of clinical detail provided at the patient level. In O'Leary and colleagues, there were rare examples of potential driver alterations identified (such as AKT1 and ERBB2) though details relating the clinical outcomes for each patient were not available. It is possible, based on insights from the latter study, that these alterations arose at low frequencies at baseline in patients with intrinsic resistance or at progression after clinical benefit in patients with acquired resistance. Finally, another key difference is that O'Leary and colleagues had matched pre- and post-CDK4/6i treatment samples collected on all participants, and resistant tumor samples were archived precisely at end of treatment (18). In the study by Wander and colleagues, only 12% of participants had matched pre- and posttreatment tissue collected and, in some instances, there were intervening lines of therapy between progression on a CDK4/6i and obtaining the resistant biopsy specimen (17). Specimen collection methodology could influence the frequency of mutations observed and may help explain differences in the cohorts.

Many studies have investigated intrinsic and acquired drivers of CDK4/6i resistance. Intrinsically resistant tumors do not demonstrate any durable clinical benefit, and typically progress within 3 months after treatment initiation, while acquired resistance is seen in patients who demonstrate clear radiographic response or durable clinical benefit (typically exceeding 6 months, although definitions may vary between studies). Observations made in distinguishing intrinsic versus acquired resistance mechanisms can be impacted by timing of tumor sample collection in relation to CDK4/6i treatment. Posttreatment samples, with or without a pretreatment match, can be interrogated for intrinsic or acquired driver events based upon the patient's response to therapy. The gold standard is to collect the posttreatment specimen at time of progression, as was done in the PALOMA-3 trial (18), to minimize emergence of confounding genomic alterations on interval lines of therapy.

On the other hand, studies sequencing exclusively pretreatment samples may more readily identify intrinsic resistance mechanisms. One such study by Li and colleagues identified *FAT1* loss-of-function events in mediating intrinsic CDK4/6i resistance (19), but this mechanism has not, as of yet, been demonstrated in acquired drug resistance. Many of the driver events implicated above have been demonstrated in both intrinsic and acquired resistant tumors, such as alterations in *RB1, AURKA, AKT1, RAS, FGFR2*, and *ERRB2* (17, 18, 42, 46). Notably, events in *CCNE2* have been predominantly shown in intrinsic resistance (17), and patients with alterations in *PTEN* were described as exhibiting acquired resistance (33). These differences could be attributable to sampling, and it may be that *FAT1*, *PTEN*, and *CCNE2* alterations could be identified in both intrinsic and acquired resistance if a large enough population was studied. Alternatively, there may be unappreciated cellular or micro-environmental factors that favor tumor acquisition of certain mechanisms of resistance over others, but there is no data currently to support this.

Table 1. Comparing study characteristics between two genomic landscape research efforts in the field of CDK4/6i resistance.

Study characteristic	O'Leary and colleagues; Genetic landscape of palbociclib-resistant breast cancer, 2018 (18)	Wander and colleagues; Genomic mechanisms of CDK4/6i resistance in breast cancer, 2020 (17)
Cohort analyzed	Randomized clinical trial	Retrospective study
Number of participants	521 participants enrolled in PALOMA-3, and 195 participants analyzed in this study	58 participants
Tumor samples collected	ctDNA	Metastatic tissue biopsy
DNA sequencing technology used	Paired exome sequencing ($n = 14$), paired targeted sequencing ($n = 184$); 195 total participants	WES on all samples (59 samples from 58 participants)
Implicated CDK4/6i resistance drivers	RB1, possible signal for FGFR2	RB1, AURKA, CCNE2, AKT1, RAS, FGFR2, ERBB2
Prior lines of endocrine therapy	All patients received 1 or more prior lines of endocrine therapy in any setting	91% of patients received 1 or more prior lines of endocrine therapy in any setting
First-line CDK4/6i	22% of patients enrolled in PALOMA-3 received a CDK4/6i first-line in the metastatic setting	45% of patients received a CDK4/6i first-line in the metastatic setting
CDK4/6i received	Palbociclib	Palbociclib, ribociclib, and abemaciclib

Several of the large, randomized clinical trials, such as PALOMA and MONALEESA, have performed robust translational analyses designed to establish baseline biomarkers for response or resistance to CDK4/6i (18, 27, 43, 44, 49, 50), however reliable predictors of patient outcome (beyond ER positivity) were not established by these efforts. The lack of positive data from these translational studies may be due to a variety of factors. Many of the efforts utilized IHC analysis, which may be impacted by tissue preservation and issues with sampling. RB1 protein staining, for example, may be falsely read as negative due to issues with antibody fixation or tissue quality (and a low occult level of RB1 may be sufficient to provoke CDK4/6i efficacy). Alternatively, RB1 protein staining may be intact, however the protein may harbor an inactivating mutation impacting its tumor suppressor function. In addition to these issues, many of the genomic alterations which drive response or resistance to CDK4/6i occur rarely, and will not emerge with statistical significance unless exceedingly large patient populations are studied. Without granular insight into the clinical experience of individual patients, it is likely that the impact of alterations in these pathways may have been underestimated or missed in the translational studies outlined above.

Future Directions and Emerging Therapeutic Opportunities in the Clinic

Modeling resistance to CDK4/6 blockade in combination with antiendocrine therapy

CDK4/6i are used in combination with ET for the treatment of HR^+/HER^- ABC. One of the advantages of the PALOMA-3 analysis was that it had an ET monotherapy control arm, and results suggested that several genomic mechanisms of resistance might be common to ET and CDK4/6i plus ET (18). Efforts to interrogate resistance in the laboratory often rely upon cellular response to single drug treatments and modeling exposure to multiple drugs simultaneously presents unique challenges (**Table 2**). Several studies have demonstrated that preclinical models harboring putative mediators of CDK4/6i resistance have variable sensitivity to ET (17, 28, 42, 46). For example, research suggests that upregulation in AKT1 (17), CCNE1/2 (28), FGFR1/2 (42), ERBB2 (46), and AURKA (51) can provoke concurrent resistance to CDK4/6 blockade and ET. On the other hand, RB1 loss may require a second, cooperating event to confer resistance to endocrine-directed treatment (17). Furthermore, alterations in ESR1 (17, 52) and PIK3CA (17, 18) may convey resistance to ET while retaining sensitivity to CDK4/6i. These data suggest that, in some instances, a single driver mutation may provoke resistance to both CDK4/6 blockade and ET, while in other circumstances, multiple cooperating events may be necessary. Establishing better models in the laboratory to interrogate these contributions represents an important priority.

In addition, specific mechanisms of resistance emerge with some degree of cell line specificity in the laboratory (17). For example, MDA-MB-361 cells cultured in the presence of a CDK4/6i acquired resistance by overexpressing AURKA and activating RAS (17). On the other hand, these alterations did not emerge in MCF7 cells in similar conditions, and exogenous upregulation of AURKA in MCF7 also failed to provoke resistance to CDK4/6 blockade (17). These results suggest there may be other idiosyncratic features that cooperate to influence the phenotype of response or resistance to a given drug exposure in a specific cell line or tumor.

Beyond cellular context, there may be important contributions related to cooperativity between concurrent mutations. In our own effort, nearly 30% of CDK4/6i resistant tumor biopsies harbored two (or more) putative driver alterations (17). In patients with more than one driver, it remains unclear if those events are cooperating within the same tumor cells, or may have arisen in contemporary subclones independently. Future efforts utilizing single-cell RNA sequencing (RNA-seq) may be useful to characterize alterations within subclonal populations and will allow us to investigate the degree of cooperativity in provoking resistance. Other emerging sequencing technologies, such as methylation profiling, will also aid in establishing whether nongenomic alterations converge on the common resistance pathways implicated above, or promote the identification of entirely new and unrelated signaling pathways which were missed by conventional genomic sequencing.

Novel therapeutic strategies in the clinic to circumvent CDK4/6i resistance

Emerging insight into the heterogeneous landscape of CDK4/6i resistance has prompted interest in novel therapeutic combinations designed to exploit tumor susceptibilities and common oncogenic signaling nodes. Several regimens combine CDK4/6i with a targeted

Table 2. Practical challenges that are faced in modeling resistance to CDK4/6i and their combination with endocrine-directed therapy.

Challenges in modeling resistance	Potential solutions
Challenge 1: Characterizing resistance to a multi-drug regimen of a CDK4/6i and its ET partner, given that models of resistance often examine cellular response to a single drug.	Establish better laboratory models to interrogate driver alterations of resistance to CDK4/6i and ET, both alone and in combination.
Challenge 2: Determining if a single driver mutation is sufficient or multiple cooperating events are necessary to provoke resistance to a CDK4/6i in combination with ET.	Serial NGS of resistant tumors and cancer cells may shed light on the emergence of a dominant single or multiple cooperative resistance drivers.
Challenge 3: Investigating cellular, context-specific factors that may influence tumor sensitivity to CDK4/6 blockade.	Cell lines that demonstrate variable emergence of CDK4/6i resistance drivers could be probed for cell-specific molecular factors that mediate drug response.
Challenge 4: Examining potential cooperativity between concurrent resistance-driving alterations that arise.	Single-cell RNA-seq may be used to investigate the degree of cooperativity between simultaneously arising resistance mechanisms, and whether they emerge within the same tumor cell or within contemporary subclonal populations independently.
Challenge 5: Characterizing nongenomic alterations that mediate resistance.	Emerging sequencing technologies, such as methylation profiling, could aid in the discovery of nongenomic events that play unique roles in CDK4/6i resistance.

Table 3. Selected clinical trials evaluating novel therapeutic regimens targeting pathways implicated in CDK4/6i resistance.

Clinical Trial	Resistance Mechanism/Pathway
NCT03092934: A phase I/II trial of the AURKA inhibitor erbumine in ABC and other cancers.	RB1 loss and AURKA upregulation
NCT03955939: A phase Ib trial evaluating the AURKA inhibitor erbumine, with or without ET, in HR^+ ABC after progression on a CDK4/6i.	RB1 loss and AURKA upregulation
NCT02860000: A phase II trial characterizing the AURKA inhibitor alisertib, in combination with fulvestrant, in endocrine resistant ABC.	RB1 loss and AURKA upregulation
NCT02124148: A phase Ib trial evaluating the CHK1/2i prexasertib, in combination with chemotherapy or the PI3Ki samotolisib, in ABC and other cancers	CCNE1/2 amplification
NCT04553133: A phase I/II trial investigating the CDK2 inhibitor PF-07104091, without and without palbociclib and letrozole, in HR^+ ABC	CCNE1/2 amplification
NCT02857270: A phase I study assessing the antitumor activity of the ERK1/2 inhibitor LY3214996, with and without abemaciclib, in ABC and other advanced cancers.	RAS/MAPK activation
NCT01160718: A phase II trial of the MEK1/2 inhibitor selumetinib, with and without fulvestrant, in HR^+ ABC after progression on AI therapy.	RAS/MAPK activation
NCT04045496: A phase I study evaluating the SHP2 inhibitor JAB3312 in ABC and other advanced cancers.	RAS/MAPK activation
NCT03959891: A phase I trial evaluating the AKT1 inhibitor ipatasertib with ET, with and without a CDK4/6i, in HR^+ ABC.	AKT/mTOR activation
NCT04191499: A phase III study evaluating the efficacy of the PI3K inhibitor inavolisib plus a CDK4/6i and fulvestrant.	AKT/mTOR activation
NCT03056755: A phase II trial investigating the PI3K inhibitor alpelisib plus ET following progression on a CDK4/6i.	AKT/mTOR activation
NCT03238196: A phase Ib study of the FGFR inhibitor erdafitinib in combination with CDK4/6i and ET in *FGFR* amplified HR^+ ABC.	FGFR1/2 upregulation
NCT04024436: A phase II trial investigating the FGFR inhibitor TAS-120 plus ET in ABC harboring an *FGFR* amplification.	FGFR1/2 upregulation
NCT01670877: A phase II trial assessing the efficacy of the HER2 inhibitor neratinib in combination with ET in $HER2^-$mutant HR^+ ABC.	ERBB2 activation
NCT04460430: A phase II study of the HER2 inhibitor neratinib plus ET in HER2-enriched ABC.	ERBB2 activation
NCT03147287: A phase II study of palbociclib and ET, with or without avelumab, in HR^+ ABC after progression on palbociclib and ET.	Continued CDK4/6i after progression on CDK4/6i
NCT02632045: A phase II trial investigating the efficacy of palbociclib or ribociclib with ET in patients with HR^+ ABC after progression on CDK4/6i.	Continued CDK4/6 after progression on CDK4/6i
NCT03809988: A phase II study of palbociclib and ET in HR^+ ABC after progression on palbociclib and ET.	Continued CDK4/6i after progression on CDK4/6i

therapy, and some have shown efficacy in published phase I/II trials. The efforts outlined below do not represent a comprehensive list, but rather serve to illustrate active research efforts being undertaken to translate therapeutic vulnerabilities into the clinic (**Table 3**).

RB1 loss, AURKA upregulation, and CCNE1/2 amplification

A phase I/II trial of the AURKA inhibitor erbumine will examine its efficacy as monotherapy in ABC (NCT03092934; ref. 53). A notable index case from this study demonstrated 11 months of clinical benefit in a patient with CDK4/6i resistant breast cancer (17), which led to a phase Ib trial evaluating erbumine with or without ET after progression on a CDK4/6i (NCT03955939; ref. 54). The AURKA inhibitor alisertib, in combination with fulvestrant, is also under investigation (NCT02860000; ref. 55). A phase Ib trial is examining the efficacy of the CHK1/2 inhibitor prexasertib in combination with chemotherapy or the PI3K inhibitor samotolisib in ABC (NCT02124148; ref. 56). A phase I/II trial is evaluating the CDK2 inhibitor PF-07104091 without and without palbociclib and letrozole in HR^+/HER^- ABC (NCT04553133; ref. 57).

AKT/mTOR and RAS/MAPK activation

Many of the resistance alterations identified and outlined above converge upon MAPK and AKT, and multiple studies are underway exploring agents that target these pathways. A phase I study is assessing the antitumor activity of the ERK1/2 inhibitor LY3214996 with and without abemaciclib in ABC (NCT02857270; ref. 58). A phase II trial of the MEK1/2 inhibitor selumetinib with and without fulvestrant in $HR^+/HER2^-$ ABC, postprogression on AI, completed accrual in 2019 (NCT01160718; ref. 59). A phase I study evaluating the SHP2 inhibitor JAB3312 is underway in ABC (NCT04045496; ref. 60). Emerging data from several trials examining the anti-tumor activity of the AKT inhibitor capivasertib in $HR^+/HER2^-$ ABC have been promising. These include results from a phase II study of capivasertib in combination with fulvestrant (61), and a phase I study involving patients with tumors harboring an *AKT1 E17K* mutation (62). The active phase Ib/II TAKTIC trial is evaluating the efficacy of the AKT1 inhibitor ipatasertib with ET with and without a CDK4/6i (NCT03959891; ref. 63).

The mTOR inhibitor everolimus combined with exemestane is an approved line of therapy following progression on a CDK4/6i. This treatment is based on the results of the BOLERO-2 trial (64); however, this trial predates the approval of CDK4/6 inhibitors and study participants did not have prior exposure to CDK4/6 blockade. The phase I/II TRINITI-1 trial helped shed light on this sequence of treatments, investigating the mTOR inhibitor everolimus plus exemestane and ribociclib in $HR^+/HER2^-$ ABC after progression on a CDK4/6i (NCT02732119; ref. 65). Published results showed safety and efficacy of this triplet combination, supporting further investigative efforts in the clinic (65). Published data from another phase 1b trial

demonstrated evidence of clinical benefit from this triplet therapy combination in $HR^+/HER2^-$ ABC that was either naïve or refractory to CDK4/6i therapy (NCT01857193; ref. 66).

On the basis of the results of the SOLAR-1 trial, the PI3K inhibitor alpelisib plus fulvestrant is a treatment approved in *PIK3CA*-mutant breast cancer after progression on standard front-line therapy (67). Notably, less than 6% of study participants had received a prior line of CDK4/6i therapy (67). The BYLieve trial is an active phase II study investigating this sequence of therapies by evaluating alpelisib plus ET following progression on a CDK4/6i (NCT03056755; ref. 68), with published results from one cohort of the study demonstrating treatment response and a reasonable safety profile (69). Data presented from a phase I trial of the PI3K inhibitor inavolisib showed promising antitumor activity when combined with ET and a CDK4/6i (70), and led to an ongoing phase III study examining the triplet combination inavolisib plus a CDK4/6i and fulvestrant (NCT04191499; ref. 71).

FGFR and ERBB2 activation

Agents targeting the FGFR and HER2 pathways are also in clinical development. A phase Ib study is evaluating the FGFR inhibitor erdafitinib in combination with CDK4/6i and ET in *FGFR* amplified $HR^+/HER2^-$ ABC (NCT03238196; ref. 72). A phase II trial is investigating the FGFR inhibitor TAS-120 plus ET in ABC harboring an *FGFR* amplification (NCT04024436; ref. 73). The HER2 inhibitor neratinib is being investigated in combination with ET in ongoing phase II trials in HER2-mutant (NCT01670877; ref. 74) and HER2-enriched (NCT04460430; ref. 75) HR^+ ABC.

Continued CDK 4/6i blockade

Despite these ongoing efforts, following progression on a CDK4/6i, the optimal therapeutic approach remains unclear. We previously analyzed a retrospective cohort of 87 patients who received abemaciclib after prior palbociclib progression, and these results suggested that a meaningful subset of patients derive clinical benefit (76). Similar to the landmark MONARCH-1 trial, which demonstrated a median PFS of 6.0 months in CDK4/6i-naïve patients receiving abemaciclib monotherapy (15), our analysis demonstrated that the median PFS was 5.3 months in patients receiving abemaciclib who had progressed on a prior line of CDK4/6 blockade (76). Preliminary assessment of available ctDNA sequencing revealed alterations in putative resistance drivers such as *RB1*, *ERBB2*, and *CCNE1* among patients with rapid progression on abemaciclib (76). A second course of CDK4/6 inhibition, with or without endocrine and other targeted therapies, is being explored in several clinical studies including PACE (NCT03147287; ref. 77), TAKTIC (NCT03959891; ref. 63), MAINTAIN (NCT02632045; ref. 78), and PALMIRA (NCT03809988; ref. 79).

Conclusion

CDK4/6 inhibitors have become the standard of care for $HR^+/HER2^-$ metastatic breast cancer. The emerging evidence highlighted here suggests that the landscape of CDK4/6i resistance is heterogeneous, with many putative drivers already identified and the expectation that more will emerge. The clinical approach for patients with CDK4/6i progression may come to rely on precision-guided therapy selection which can ultimately inform clinicians (i) whether a second round of CDK4/6 blockade is likely to be successful, and (ii) whether a resistance driver is present and which targeted therapeutic option might be optimal. Ongoing efforts in the preclinical, translational, and clinical settings will allow us to elucidate new predictive biomarkers and inform treatment selection strategies in $HR^+/HER2^-$ ABC.

Authors' Disclosures

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