Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: A review

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Deregulation of the cyclin D-CDK4/6-INK4-RB pathway leading to uncontrolled cell proliferation, is frequently observed in breast cancer. Currently, three selective CDK4/6 inhibitors have been FDA approved: palbociclib, ribociclib and abemaciclib. Despite promising clinical outcomes, intrinsic or acquired resistance to CDK4/6 inhibitors has limited the success of these treatments; therefore, the development of various strategies to overcome this resistance is of great importance. We highlight the various mechanisms that are directly or indirectly responsible for resistance to CDK4/6 inhibitors, categorizing them into two broad groups; cell cycle-specific mechanisms and cell cycle-nonspecific mechanisms. Elucidation of the diverse mechanisms through which resistance to CDK4/6 inhibitors occurs, may aid in the design of novel therapeutic strategies to improve patient outcomes. This review summarizes the currently available knowledge regarding mechanisms of resistance to CDK4/6 inhibitors, and possible therapeutic strategies that may overcome this resistance as well.

Introduction
Breast cancer is the most common malignancy in women, accounting for approximately 25% of all malignancies worldwide.1 Breast cancer is categorized into three subtypes according to estrogen receptor (ER), progesterone receptor (PR) and HER2 status: hormone receptor (HR)-positive, HER2-positive and triple negative subtypes.2 Of these, HR-positive breast cancers constitute approximately 60%–70%.3 Endocrine therapy is considered to be the mainstay therapy for HR-positive breast cancer.4 Despite having remarkable improvement in treatment of advanced disease, a large proportion of patients eventually acquire resistance to endocrine therapy.5 Previous studies have suggested multiple mechanisms responsible for endocrine resistance, including ER loss or mutation, alteration of the ER pathway, deregulation of cell cycle signaling molecules, and activation of various escape pathways.6,7 These limitations regarding endocrine resistance have enabled researchers to identify novel therapeutic targets, such as cyclin-dependent kinase (CDK) 4/6 inhibitors.

CDK4/6 kinases associate with cyclin D proteins during transition from G1 to S phase of the cell cycle. The cyclin D-CDK4/6 complex phosphorylates retinoblastoma proteins (RB) and dissociates them from the E2F transcription factors, which are ultimately responsible for cell cycle progression.8 Various factors including the overexpression of cyclin D, mutation or amplification of CDK4/6 and loss of cyclin D-CDK4/6 negative regulators, elicit the activity of cyclin D-CDK4/6, which hyperphosphorylates RB, ultimately leading to uncontrolled cell proliferation.9 Thus, specific targeting of CDK4/6 has garnered special interest as an anticancer therapy.

Currently, three CDK 4/6 inhibitors are in clinical development: palbociclib, ribociclib and abemaciclib.10 These three CDK4/6 inhibitors have demonstrated greater efficacy in combination with endocrine therapies, leading to FDA approval.11

Key words: CDK4/6, estrogen receptor-positive breast cancer, drug resistance

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When used to treat HR-positive metastatic breast cancer in postmenopausal women as a first-line therapy, palbociclib, ribociclib and abemaciclib significantly prolonged progression-free survival (from 12 to 14 months to ≥25 months) in combination with aromatase inhibitors (PALOMA-2, MONALEESA-2 and MONARCH-3 trials).\textsuperscript{12-14} Ribociclib demonstrated similar efficacy in combination with ovarian suppressor and tamoxifen or aromatase inhibitor as a first-line therapy in premenopausal women (MONALEESA-7 trial)\textsuperscript{15} as well. Furthermore, combining fulvestrant with palbociclib, ribociclib, or abemaciclib doubled progression-free survival compared to fulvestrant alone (PALOMA-3, MONALEESA-3 and MONARCH-2 trials)\textsuperscript{16-18} as a second-line therapy after progression occurred with aromatase inhibitors.

Despite improved disease control that CDK4/6 inhibitors offer to patients with HR-positive breast cancer, not all patients respond to these drugs and most patients whose tumors respond to CDK4/6 inhibitors eventually develop acquired resistance.\textsuperscript{19} The early and late adaptation mediated by persistent G1–S-phase cyclin expression and other bypass signaling limits the effectiveness of CDK4/6 inhibitors.\textsuperscript{20} In addition, various other mechanisms also exist, which are responsible for intrinsic or acquired resistance to CDK4/6 inhibitors.

This review explores various preclinical biomarkers, which may contribute to intrinsic or acquired resistance to CDK4/6 inhibitors. The main cancer type we will discuss resistance mechanisms in is breast cancer based on current clinical approval status of CDK4/6 inhibitors, despite resistance mechanisms having been studied in several other cancers, too. We will discuss various possible strategies to overcome resistance as well.

**Mechanisms of Resistance to CDK4/6 Inhibitors**

**Cell cycle-specific mechanisms**

The cyclin D-CDK4/6–INK4–RB pathway is the key regulator of the G1–S transition of the cell cycle.\textsuperscript{21} Both CDK4 and CDK6 may associate with all three types of cyclin D (cyclin D1, cyclin D2 and cyclin D3), with cyclin D1 being the best characterized.\textsuperscript{21} Various mitogenic signals activate cyclin D1, which then forms a complex with CDK4/6, thereby phosphorylating RB, and promoting the dissociation of the RB-E2F complex. Once E2F is released from the complex, it activates genes required for DNA replication, and the cell progresses into the S phase.\textsuperscript{8,21} As cyclin D-CDK4/6 is the key initiator of the G1–S transition, selectively targeting the ATP binding site of CDK4/6 blocks cellular transition from G1 to S phase of the cycle.

The p16INK4A protein, a member of the INK4 family of cell cycle inhibitors, is a tumor suppressor which inhibits cyclin D-CDK4/6 activity and contributes to G1 arrest by directly binding to CDK4 and inhibiting its catalytic activity.\textsuperscript{9} In addition, Cip/Kip member proteins, p21 and p27 in particular, which inhibit a broader spectrum of cyclin-CDK complexes, also influence CDK4/6.\textsuperscript{9,22,23} However, specific mutations in the CDKN2A locus, (which encodes p16INK4A) are common in various malignancies, suggesting that the proteins, primarily responsible for inhibiting CDK4/6-driven signaling, are absent, resulting in aberrant activation of the pathway. This supports the selective inhibition of CDK4/6 as an attractive therapeutic strategy. However, intrinsic or acquired resistance to CDK4/6 inhibitors is an emerging issue, which limits its therapeutic efficacy. Various cell cycle-specific mechanisms responsible for resistance to CDK4/6 inhibitors are summarized below (Fig. 1).

**Loss of RB.** The tumor suppressor RB is the aforementioned key checkpoint in the cell cycle. As the primary target of CDK4/6 inhibitors, RB was considered to be one of the most important biomarkers of sensitivity to therapy.\textsuperscript{24-26} In this scenario, loss of RB is the evident cause of resistance to CDK4/6 inhibitors,\textsuperscript{24} and various preclinical studies have supported this hypothesis.\textsuperscript{20,27,28} In addition, some preclinical and clinical studies have also reported that mutations in RB are responsible for the resistance.\textsuperscript{29,30} A study using glioblastoma xenograft cells, a missense mutation in exon 2 of RB(A193T) resulted in resistance to CDK4/6 inhibitors.\textsuperscript{29} Despite the loss of RB, constitutive progression of the cell cycle continues via the activation of other cell cycle machinery, including such as E2F and the cyclinE-CDK2 axis, demonstrating the loss of dependence on CDK4/6 for progression from G1 to S phase.\textsuperscript{10,11} Therefore, when RB has been lost, inhibition of the cyclin E-CDK2 axis in combination with CDK4/6 inhibitors may be effective in overcoming resistance to CDK4/6 inhibitors.

**p16 amplification.** The p16, a member of INK4 family and a natural inhibitor of CDK4, is an important tumor suppressor involved in the regulation of cell cycle.\textsuperscript{31} Naturally, p16 works as a tumor suppressor in the presence of functional RB because CDK4/6 (a target of p16) requires RB for its own kinase activity.\textsuperscript{32} Overexpression of p16 occurs during oncogenic stress, with or without the loss of RB.\textsuperscript{33,34} Loss of RB with concurrent p16 overexpression resulted in failure to respond to CDK4/6 inhibitors because of the absence of RB function.\textsuperscript{35} Alternatively, p16 overexpression in the presence of functional RB, also confers resistance to CDK4/6 inhibitors as a result of diminished CDK4, indicating depletion of a target of CDK4/6 inhibitors. Although the loss of RB and p16 overexpression seems to occur consequently together, further studies revealing the mechanistic association of RB loss and p16 overexpression might be beneficial in designing the strategies to overcome acquired resistance to CDK4/6 inhibitors.

**CDK6 amplification.** In addition to CDK4, CDK6 also plays an important role in the progression of cell cycle from G1 to S phase, as mentioned above. Function of CDK6 is mainly kinase-dependent. However, CDK6 functions partly kinase-independently, too.\textsuperscript{36} CDK6 has a role in transcriptional regulation independently of its protein kinase activity,\textsuperscript{37} that is, CDK6 has been reported to upregulate the transcription of p16 in the presence of STAT3 and cyclin D. In other way,
CDK6 upregulates VEGF-A in concert with c-Jun, inducing tortuous angiogenesis, which generally promotes cancer progression and drug resistance. In some studies, CDK6 overexpression was reported to promote resistance to CDK4/6 inhibitors in preclinical models. Possible mechanisms how CDK6 amplification confers resistance to CDK4/6 inhibitors might be due to kinase-independent function of CDK6, which involves VEGF-A or p16.

CCNE1/2, CDK2 amplification. Cyclin E-CDK2 is also known to play a key role in the progression of cell cycle from G1 to S phase. Cyclin E-CDK2 phosphorylates RB, allowing the release of E2F and promoting entry into the S phase. Overexpression of CCNE1, which encodes cyclin E, is a well-accepted mechanism for resistance to CDK4/6 inhibitors, as demonstrated by previous studies. Some of these studies have emphasized that CDK4/6 inhibitor-resistant cells have lost their dependence on cyclin D1-CDK4/6 signaling, instead using the bypass signaling pathway. Several bypass mechanisms are described elsewhere in this review. For instance, previous studies have mentioned that, cells may follow the MAPK-AKT signaling cascade as a bypass to compensate for CDK4/6 inhibition. This bypass mechanism is described in detail here, in the section entitled “PI3K/AKT/mTOR pathway.” Activation of the cyclin E-CDK2 pathway is also an important bypass mechanism; in one study, CDK2 inhibitors effectively decreased the growth of cells overexpressing cyclin E1. Taken together, inhibiting cyclin E-CDK2 as well as upstream targets such as PI3K/AKT/mTOR in combination with inhibiting CDK4/6 may also be a successful strategy to overcome resistance.

E2F amplification. E2F is a downstream transcription factor of RB. The RB-E2F complex plays an important role in the regulation of cell cycle progression from G1 to S phase. Phosphorylation of RB by cyclin D-CDK4/6 releases E2F, leading to the transcription of proteins, including cyclin E, required for cell cycle progression. The cyclin E-CDK2 complex also phosphorylates RB, releasing E2F and promoting entry into S phase, as mentioned earlier. Loss of RB is correlated with the increased expression of E2F, resulting in the constitutive activation of its downstream target proteins.

Figure 1. Cell cycle-specific mechanisms for the resistance to CDK4/6 inhibitors. Multiple factors involved in the regulation of cell cycle are associated with resistance to CDK4/6 inhibitors. Loss of drug target genes, such as RB and FZR1, as well as overexpression of various genes which are directly or indirectly involved in the progression of cell cycle, as shown in Figure 1, are responsible for resistance to CDK4/6 inhibitors. Abbreviations: CDK, Cyclin dependent kinase; RB, Retinoblastoma protein; CHK1, Checkpoint kinase 1; STAT3, Signal transducer and activator of transcription 3; VEGF-A, Vascular endothelial growth factor A; HDAC, Histone deacetylases; MDM2, Mouse double minute 2 homolog. [Color figure can be viewed at wileyonlinelibrary.com]
Moreover, E2F has also been shown to upregulate AKT signaling via Gab2. Above all, the overexpression of E2F causes the cell to circumvent CDK4/6 inhibition and rely upon signaling pathways other than the cyclin D-CDK4/6 axis for cell cycle progression. Further studies are required to explore the detailed mechanism of this escape pathway. Moreover, inhibition of proteins downstream of E2F, in concert with CDK4/6 inhibition, may increase the efficacy of CDK4/6 inhibitors, overcoming resistance.

**CDK4 amplification.** CDK4 is one component of the cyclin D-CDK4/6-RB pathway. Various mechanisms, such as gene amplification, mutations and epigenetic alterations, serve to activate the cyclin D-CDK4/6-RB pathway. Overexpression of CDK4, which has been described in several cancers, may limit the efficacy of CDK4/6 inhibitors. A study in alveolar rhabdomyosarcoma cells Rh 28 and Rh 41 demonstrated the decreased activity of CDK4/6 inhibitors in cells overexpressing CDK4. In addition, glioma cells overexpressing CDK4 were completely resistant to CDK4/6 inhibitors. Further investigations are needed to confirm whether such a pattern of CDK4 expression associated with resistance to CDK4/6 inhibitors is confined to specific cancer types or is similar in other cancer types as well.

**CDK7 overexpression.** CDK7 is a cell cycle regulator. In addition, it also acts as a transcription factor, after complexation with cyclin H and MAT1. Increased expression of CDK7 is reported to confer resistance to CDK4/6 inhibitors. It acts as a CDK-activating kinase (CAK) and is involved in the G2/M phase by maintaining CDK1 and CDK2 activity. Reportedly, CDK7 has CAK activity toward CDK4 and CDK6, which may play a role in mitogen signaling during G1 phase progression. Though CDK7 appears to be involved in the G1 phase of the cell cycle, it remains unclear whether it induces resistance to CDK4/6 inhibitors. Further studies are warranted to reveal the detailed underlying mechanism of CDK7’s role in G1 phase progression, and to determine whether, the combined inhibition of CDK7 and CDK4/6 could overcome resistance to CDK4/6 inhibitors.

**WEE1 overexpression.** WEE1 plays an important role in the G2/M checkpoint. It inhibits the entry of DNA-damaged cells into mitosis in coordination with CDK1. Though the involvement of WEE1 in inducing resistance to CDK4/6 inhibitors is unknown, inhibition of WEE1 has been shown to increase sensitivity to CDK4/6 inhibitors in resistant cells. As WEE1 is associated with a resistant phenotype in preclinical models, targeting the G2/M phase via the inhibition of WEE1 in combination with CDK4/6 inhibition could be a therapeutic option in overcoming resistance.

**MDM2 overexpression.** Mouse double minute 2 homolog (MDM2) is a protein that negatively regulates p53 activity, in destabilizing and inhibiting cellular senescence. Approximately 20%–30% of breast cancer patients show overexpression of MDM2, and this overexpression contributes particularly to the progression of HR-positive breast cancer. It is reported that CDK4/6 inhibitor-resistant cells have disrupted senescence pathways and insensitivity to the induction of senescence. Therefore, interruption of the senescence pathway by MDM2 in a p53-dependent manner may cause resistance to CDK4/6 inhibitors. MDM2 inhibitors activate p53 by disrupting the MDM2-p53 complex. A combination of palbociclib with an MDM2 inhibitor (RG7388) produced a synergistic anticancer effect in human liposarcoma. In addition, another MDM2 inhibitor (CGM097) has shown synergistic effects in combination with CDK4/6 inhibitors or fulvestrant, abrogating cells that are resistant to CDK4/6 inhibitors, as well as those resistant to endocrine therapy in vitro and in vivo. These studies highlight the importance of MDM2 in overcoming resistance to CDK4/6 inhibitors.

**HDAC activation.** Histone deacetylases (HDACs) remove the acetyl group from the ε-N-acetyl lysins of histones and play an important role in gene regulation. HDACs suppress the natural CDK inhibitor, p21, which interacts with cyclin D throughout the cell cycle, and they associate with cyclin A or cyclin B in the later part of the cell cycle at the G2/M phase. Although the involvement of HDAC in resistance to CDK4/6 inhibitors is currently unknown, inhibition of HDAC may increase the efficacy of CDK4/6 inhibitors in CDK4/6 inhibitor-resistant cells by activating p21, resulting in cell cycle arrest at the G1 and G2/M phases, as demonstrated in CDK4/6 inhibitor-sensitive cells. In other way, HDAC inhibition was reported to induce proapototic proteins such as Noxa and Bim resulting in apoptosis, that is independent of p21, and providing a synergistic effect with CDK4/6 inhibitors in ER-positive breast cancer cells. This supports the hypothesis that HDAC inhibition may enhance the activity of CDK4/6 inhibitors through a non-overlapping mechanism, suggesting combining HDAC inhibitors with CDK4/6 inhibitors might be beneficial in resistant cases of CDK4/6 inhibitors.

**Loss of FZR1.** The ubiquitin (Ub) ligase APC/C, which is activated via the co-activator FZR1, interacts with RB during the G1 phase of cell cycle. More notably, APC/C[FZR1] complex degrades S-phase kinase associated protein 2 (SKP2), which inhibits p27, natural CDK inhibitors, resulting in decreased CDK2, CDK4 and CDK6. Accordingly, the loss of FZR1 results in uncontrolled cell cycle progression from G1 to S phase. In addition, it is noted that FZR1 serves as a substrate of cyclin D-CDK4/6, similar to RB and phosphorylated FZR1 loses its function to activate APC/C. Furthermore, simultaneous knockdown of both genes was shown to bypass the requirement of cyclin D-CDK4/6 for the progression of cell cycle. Therefore, in addition to RB, FZR1 status may also be correlated with resistance to CDK4/6 inhibitors. Though the detailed mechanism remains unknown, the loss of
FZR1 may correspond with the loss of RB to confer resistance to CDK4/6 inhibitors. Further studies are required to explore the mechanism and to overcome resistance to CDK4/6 inhibitors associated with the loss of FZR1.

**Cell cycle-nonspecific mechanisms**

Various cell cycle-nonspecific mechanisms which may confer resistance to CDK4/6 inhibitors are depicted in Figure 2a and b.

*Activation of the FGFR pathway.* The fibroblast growth factor receptor (FGFR) signaling pathway is involved in key biological processes such as proliferation, differentiation and cell survival.65 The FGFR pathway is frequently activated in several types of cancer, including breast cancer.66,67 Of the five FGFRs, FGFR 1–4 have been reported to play an important role in cancer progression.68 Furthermore, FGFR1 and FGFR2 also appear to be associated with resistance to CDK4/6 inhibitors, as well as with endocrine resistance.68,69 Mechanistic investigation showed that FGFR1 amplification activated the PI3K/AKT and RAS/MEK/ERK signaling pathways in endocrine-resistant breast cancer cells.70 Preclinically forced overexpression of FGFR1 induced resistance to a combination of fulvestrant and palbociclib, as well as fulvestrant alone.71 The FGFR pathway is primarily activated via FGF2 amplification, rather than FGF3 and FGF4,65 and FGFR1 signaling via FGF2 promotes endocrine resistance.70 Accordingly, silencing FGFR1 prevented FGF2-mediated endocrine resistance in preclinical studies.70 In addition, a recent study reported that an FGFR2-activating mutation may also contribute to the development of resistance to CDK4/6 inhibitors, as well as selective estrogen receptor degraders.69 Therefore, the combined inhibition of CDK4/6 and FGFR pathway may be a viable option to overcome resistance to CDK4/6 inhibitors.

*Activation of the PI3K/AKT/mTOR pathway.* The PI3K/AKT/mTOR signaling pathway is activated in approximately 30%–40% of breast cancers, particularly in the HR-positive subtype.20,72 Aberration of this pathway is known to be a crucial factor in resistance to endocrine therapy. Furthermore correlation of the PI3K3/AKT/mTOR pathway with resistance to CDK4/6 inhibitors has also been reported recently.20,42,73,74 For instance, CDK4/6 inhibitor-resistant breast cancer cells were more dependent on PI3K/AKT/mTOR signaling than ER signaling.73 CDK4/6 inhibitors activated the PI3K/AKT pathway via the phosphorylation of S477/T479 of AKT by PDK1 in ribociclib-resistant breast cancer cells.42 Notably, herein, S477/T479 of p-AKT is specifically a CDK2-dependent phosphorylation site. Reactivation of phospho-RB and E2F, which was noted in CDK4/6 inhibitor-resistant cell lines, may occur via pathways other than the CDK pathway, such as the mTOR pathway. This may be inferred from preclinical results, wherein mTORC1/2 inhibition suppressed phospho-RB and E2F in CDK4/6 inhibitor-resistant cells, and consequently restored sensitivity to CDK4/6 inhibitors.75 Another recent study suggests that PI3K inhibitors may decrease the cyclin D1 expression and prevent early adaptations to CDK4/6 inhibition.20 Likewise, in CDK4/6 inhibitor-sensitive preclinical model, complete tumor regression was also observed after combined inhibition of CDK4/6 and PI3K compared to single-agent treatment.20 These results suggest that the inhibition of the PI3K/AKT/mTOR pathway in combination with CDK4/6 inhibitors may have potential therapeutic benefits in overcoming resistance to CDK4/6 inhibitors as well as in augmenting anticancer activity in CDK4/6 inhibitor-sensitive setting.

*Loss of ER or PR expression.* A major driver of cyclin D-CDK4/6 activity in breast cancer cells is hormone-mediated activation of the ER.76 Loss of ER/PR expression has been observed in an abemaciclib-resistant preclinical model.39 Furthermore, in a small number of patient series in which paired biopsies were performed during pre-CDK4/6 inhibitor treatment and post-progression,39 three out of seven patients had lost expression of ER or PR. These data suggested that a subset of patients who develop resistance to CDK4/6 inhibitors may be associated with tumoral changes in ER/PR levels. That is, loss of ER dependence may drive cells to escape CDK4/6 inhibition. Therapeutic approaches similar to those that have been successful in HR-negative subtypes may be required for the treatment of CDK4/6 inhibitor-resistant patients who have lost ER or PR expression.

*Higher transcriptional activity of AP-1.* AP-1 is a transcription factor that regulates a wide variety of genes, including cyclin D1.77 AP-1 family is composed of homodimers and heterodimers of the Jun, Fos, activation transcription factor (ATF) and transcription factor MAF sub-families.77,78 Approximately 20%–40% of human breast tumors have high levels of activated c-Jun,79 which is known to interact with the ER and inhibit its activity.80 There are some reports that overexpression of AP-1 proteins, including c-Jun and c-Fos, may account for resistance to CDK4/6 inhibitors, as well as to endocrine therapy. For example, overexpression of c-Jun in the MCF7 breast cancer cell line was linked with resistance to antiestrogen therapy.80 In addition, higher transcriptional activity of AP-1 and increased c-Fos levels were observed in MCF7 cells having acquired resistance to palbociclib and tamoxifen.81 Mechanistically, it is not understood why the overexpression of AP-1 is correlated with resistance to CDK4/6 inhibitors; however, a plausible explanation is that the suppression of ER by c-Jun80 may drive cells to escape CDK4/6 inhibition due to the loss of ER dependence. In addition, cyclin D1 overexpression transcribed by c-Jun may also explain the mechanism.77 Blockade of AP-1 through the downregulation of c-Jun via genetic modification synergistically inhibited breast cancer cell growth when applied in combination with palbociclib.81 Further, blockade of AP-1 in combination with palbociclib and fulvestrant was more
effective in inhibiting cell growth than dual- or mono-treatment. Various natural products, bioactive phytochemicals, and small molecules targeting AP-1 are currently under development. However, only one selective c-Fos/AP-1 inhibitor (T-5224), has progressed to phase II clinical trials. Combining such AP-1 specific inhibitors with CDK4/6 inhibitors may have a synergistic effect in overcoming the acquired resistance to CDK4/6 inhibitors.

**EMT pathway.** The transdifferentiation of cells from epithelial to mesenchymal is known as epithelial–mesenchymal transition (EMT). EMT is an essential phenomenon for tissue morphogenesis in multicellular organisms; however, it promotes invasion and metastasis of cancer cells. In addition, the involvement of EMT in anticancer drug resistance has been suggested by many previous studies. Furthermore, there are some evidences that EMT may be correlated with resistance to CDK4/6 inhibitors. It was reported that the inhibition of CDK4/6, induced EMT via the activation of TGF-β, which then form a complex with Smad4, leading to EMT via the activation of EMT transcription factors. Additionally, TGF-β also induces EMT via PI3K/AKT/mTOR signaling, which is independent of the

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**Figure 2.** Cell cycle-non specific mechanisms for the resistance to CDK4/6 inhibitors. (a) Overexpression of various factors which are upstream of the cell cycle, such as FGFR, PI3K/AKT/mTOR, and AP-1, act as bypass pathways for the progression of the cell cycle, resulting in decreased efficacy of CDK4/6 inhibitors. Loss of ER dependence also drives cells to escape CDK4/6 inhibition. (b) TGF-β induces the expression of several transcription factors involved in EMT via Smad and the PI3K/AKT/mTOR pathway. The cyclin-CDK complex phosphorylates and suppresses Smad 3, recovering cell cycle arrest. In addition, inhibition of cyclin D activates autophagy, leading to the reversal of cell cycle arrest mediated by CDK4/6 inhibitors. CDK4/6 inhibitors may activate various immune-related genes, which may also play a role in the development of resistance. Abbreviations: FGFR2, fibroblast growth factor 2; FGFR1, fibroblast growth factor receptor 1; ER, estrogen receptor; TGF-β, transforming growth factor β; MEK, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; PI3K, phosphatidylinositide-3-kinases; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent protein kinase-1; AP-1, Activator protein 1; EMT, epithelial-mesenchymal transition; NFAT, Nuclear factor of activated T-cells; IL-2, Interleukin-2; DNMT, DNA methyltransferase. [Color figure can be viewed at wileyonlinelibrary.com]
Smad pathway. Therefore, the inhibition of TGF-β or EMT in combination with CDK4/6 inhibitors may overcome the resistance to CDK4/6 inhibitors.

**Smad 3 suppression.** Smad3 is a component of the TGF-β signaling pathway, having antiproliferative effects that contribute to G1 cell cycle arrest. From this perspective, it was demonstrated that the suppression of Smad3 was involved in mechanisms responsible for resistance to certain anticancer drugs, such as trastuzumab. Furthermore, some evidences suggested that Smad3 may be correlated with resistance to CDK4/6 inhibitors. Mechanistically, Smad3 was reported to be suppressed through phosphorylation by the cyclin E-CDK2 or cyclin D1-CDK4/6 complexes. This suppression of Smad3 released the RB-E2F blockade induced by of Smad3, and finally recovered cell cycle arrest in breast cancer cells. The link between the cyclin E-CDK2 complex and Smad3 was demonstrated in a trastuzumab-resistant preclinical model; CDK2 inhibition or transfection of trastuzumab-resistant breast cancer cells with a Smad3 construct containing inhibitory mutations in CDK2 phosphorylation sites led to decreased phosphorylation of Smad3, resulting in decreased proliferation of trastuzumab-resistant cells. Accordingly, based on the activation of the cyclin E-CDK2 axis observed in CDK4/6 inhibitor-resistant models, resistance to CDK4/6 inhibitors may result from suppression of Smad3 by the activated cyclin E-CDK2 axis. Therefore, combined inhibition of CDK2 and CDK4/6 may be a promising strategy in this setting. However, augmenting Smad3 should not be overlooked as a means of overcoming resistance to CDK4/6 inhibitors.

**Autophagy activation.** Autophagy is thought to be a mechanism of stress tolerance and survival in cancer cells. During cell cycle arrest, stress response activates autophagy, which can then degrade ROS and mediate the reversal of G1 arrest and senescence. In preclinical studies, autophagy inhibition has been shown to augment the efficacy of many anticancer drugs. There are also some evidences that suggest autophagy accounts, partly, for imparting resistance to CDK4/6 inhibitors. In addition, CDK4/6 inhibition activated autophagy, maybe through the inhibition of cyclin D1, which was reported to suppress autophagy in mammary epithelial cells, or as a stress response. Accordingly, cell cycle arrest mediated by CDK4/6 inhibitors was reversed by autophagy. This phenomenon may support the hypothesis that the activation of autophagy is involved in resistance to CDK4/6 inhibitors. Preclinically, combined inhibition of CDK4/6 and autophagy synergistically increased senescence and sustained growth inhibition. The inhibition of autophagy enhanced the efficacy of CDK4/6 inhibitors, and may be help overcome resistance to CDK4/6 inhibitors.

**Immune mechanisms.** Immune checkpoint blockades in cancer have demonstrated promising efficacy in recent years. Various targeted therapies, such as gefitinib, erlotinib, cetuximab and axitinib, are also known to modulate immune responses. On the other hand, immune-related pathways are correlated with the emergence of resistance to various anticancer drugs. Moreover, immune-related pathways, such as those of IFN-α and IFN-β, were reported to be enriched in CDK4/6 inhibitor-resistant breast cancer cells. In one preclinical study, CDK4/6 inhibitors were reported to promote anti-tumor immunity, similar to other targeted therapies. Mechanistically, CDK4/6 inhibitors reduced the activity of DNA methyltransferase, an E2F target protein which promotes cytotoxic T cell-mediated tumor inhibition. In addition, CDK6 phosphorylated nuclear factor of activated T cell 4 (NFAT4) and suppressed its activity, resulting in reduced IL2 levels, whereas CDK4/6 inhibitors increased IL2 levels by dephosphorylating NFAT4 and enhancing its activity. Taken together, CDK4/6 inhibition potentiates anti-tumor immunity and enhances the response to PD-1 blockade, providing a rationale for new anti-cancer therapeutic strategies combining CDK4/6 inhibitors with immunotherapies. Therefore, although it is yet to be preclinically verified, we could hypothesize that combinatorial strategies might have some role in overcoming resistance to CDK4/6 inhibitors.

**Potential Biomarkers Tested in Clinical Studies**

Based on preclinical observations, potential biomarker analyses were performed using biosamples collected from patients enrolled in palbociclib clinical trials. Five hundred sixty-six tumor samples from 666 participants enrolled in the PALOMA-2 trial, in which palbociclib plus letrozole were compared to letrozole alone as a first-line therapy, were immunohistochemically evaluated for ER, RB, p16, cyclin D1 and Ki-67. The ER-negative subgroup was not expected to benefit from palbociclib but, unexpectedly, this subgroup (n = 62) showed similar benefits from palbociclib as the ER-positive subgroup (n = 499) by central confirmation of ER status. Although it was thought that ER-negative subgroup herein derived benefits from palbociclib because of their PR-positive status, it was not proved. Although the loss of RB is considered the evident resistant mechanism of CDK4/6 inhibitors, as mentioned earlier, this could not be substantiated because only a small number of patients were RB negative (n = 51). None of the other markers were correlated with sensitivity or resistance to palbociclib. In other biomarker studies using liquid biopsies from the PALOMA-3 trial, in which palbociclib plus fulvestrant was compared to fulvestrant alone as a second-line therapy, palbociclib was similarly beneficial, irrespective of mutations in PIK3CA or ESR1 mutation.

**Conclusions**

CDK4/6 inhibitors are currently the prevailing standard therapy in combination with endocrine therapy in HR-positive metastatic breast cancer. However, issues resulting from
resistance to CDK4/6 inhibitors are emerging. Evidence collected from preclinical studies has suggested that various mechanisms may contribute to intrinsic or acquired resistance to CDK4/6 inhibitors. However, none of the potential resistance mechanisms which were preclinically demonstrated could be confirmed in clinical studies. Therefore, further investigations are warranted for both mechanistic and clinical validation in order to define more precise mechanisms of resistance to CDK4/6 inhibitors, and to develop successful therapeutic strategies to overcome resistance.

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