High CDK6 protects cells from fulvestrant-mediated apoptosis and is a predictor of resistance to fulvestrant in estrogen receptor-positive metastatic breast cancer

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**Translational relevance:** The combination of CDK4/6 inhibitors and endocrine therapy has shown to improve clinical outcome in advanced estrogen receptor-positive (ER+) breast cancer patients. However, not all patients will benefit from such combination therapy and there is currently no accompanying diagnostics available. Herein, we show that CDK6 was highly expressed in fulvestrant-resistant breast cancer cells and that CDK4/6 inhibitor palbociclib was effective in inhibiting growth of ER+ breast cancer cells with high expression of CDK6 that responded poorly to fulvestrant alone. High expression of CDK6 in metastatic ER+ lesions of breast cancer patients treated with fulvestrant was associated with shorter progression-free survival upon fulvestrant treatment. Our findings offer preclinical and clinical evidence for the use of CDK6 as a predictive biomarker of response to fulvestrant treatment in ER+ metastatic breast cancer and for selection of patients who may benefit from combined targeted therapy with CDK4/6 inhibitors and fulvestrant.
Abstract

**Purpose:** Resistance to endocrine therapy in estrogen receptor-positive (ER+) breast cancer remains a major clinical problem. Recently, the CDK4/6 inhibitor palbociclib combined with letrozole or fulvestrant was approved for treatment of ER+ advanced breast cancer. However, the role of CDK4/6 in endocrine resistance and their potential as predictive biomarkers of endocrine treatment response remains undefined.

**Experimental Design:** We investigated the specific role of increased CDK6 expression in fulvestrant-resistant cells by gene knockdown and treatment with palbociclib, and evaluated the effect in cell proliferation, apoptosis and kinase activity. Furthermore, we evaluated CDK6 expression in metastatic samples from breast cancer patients treated or not with fulvestrant.

**Results:** We found increased expression of CDK6 in two fulvestrant-resistant cell models vs. sensitive cells. Reduction of CDK6 expression impaired fulvestrant-resistant cell growth and induced apoptosis. Treatment with palbociclib re-sensitized fulvestrant-resistant cells to fulvestrant through alteration of retinoblastoma protein phosphorylation. High CDK6 levels in metastatic samples from two independent cohorts of breast cancer patients treated with fulvestrant (N=45 and N=46) correlated significantly with shorter progression-free survival (PFS) of fulvestrant treatment (p=0.0006 and p=0.018), while no association was observed in patients receiving other first- or second-/third-line endocrine treatments (N=68, p=0.135 and p=0.511, respectively).

**Conclusions:** Our results indicate that upregulation of CDK6 may be an important mechanism in overcoming fulvestrant-mediated growth inhibition in breast cancer cells. Patients with
advanced ER+ breast cancer exhibiting high CDK6 expression in the metastatic lesions show shorter PFS upon fulvestrant treatment and thus may benefit from the addition of CDK4/6 inhibitors in their therapeutic regimens.
Introduction

Postmenopausal women with estrogen receptor-positive (ER+) breast cancer who experience recurrence are primarily treated with third generation aromatase inhibitors (AI), which have shown better overall response and clinical benefit rates compared with tamoxifen treatment [1-3]. Upon disease progression, patients are switched either to the selective estrogen receptor modulator tamoxifen or to fulvestrant, a selective estrogen receptor downregulator. Unlike tamoxifen, fulvestrant is a pure ER antagonist that prevents ER dimerization and leads to the degradation of the antiestrogen-ER complex, inducing loss of cellular ER and subsequent complete inhibition of estrogen signaling through the ER [4]. Phase III trials in postmenopausal women with metastatic breast cancer have shown that fulvestrant is as effective as anastrozole in patients in whom disease has progressed during tamoxifen treatment [5], and is similar in efficacy to first-line tamoxifen therapy [6]. Fulvestrant has further been demonstrated to be efficacious in postmenopausal women with advanced ER+ breast cancer in whom disease progressed despite previous aromatase inhibitor therapy [7,8].

Although endocrine therapy is a very effective treatment for ER+ breast cancer, a significant number of patients with localized disease, and all patients with metastatic disease, eventually develop resistance to the drugs that antagonize ER activation by estrogen [9]. Clearly, blocking other essential pathways in ER+ breast cancer cells is required, and current studies using cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors have shown promising results [10]. CDK4/6, which associate with D-type cyclins, phosphorylate and inactivate retinoblastoma (Rb), thereby
promoting cell cycle entry from G1 to S phase. Cancer cells may overcome the tumor-suppressive function of Rb by sustained activation of cyclin D/CDK4/6 or loss of Rb [11]. The majority of breast cancers overexpress cyclin D1, and approximately 20% show amplification of the gene (CCND1) [12,13]. The cyclin D/CDK4-6/p16\(^{INK4A}\)/Rb pathway has been shown to be particularly important in luminal breast cancer, which is comprised mainly of ER+ and human epidermal growth factor receptor 2 (HER2) unamplified tumors. Specifically, amplification of CCND1 and CDK4 and loss of p16, the major inhibitor of CDK4/6 encoded by CDKN2A, is enriched in both luminal A and B breast tumors [14]. Further, a preclinical study exploring sensitivity to CDK4/6 inhibitor across a panel of human breast cancer cell lines showed that luminal ER+ cells, including those that were HER2-amplified, were most sensitive to growth inhibition by CDK4/6 inhibitor, while non-luminal and basal subtypes were less sensitive [15].

Palbociclib (Ibrance, Pfizer), an CDK4/6 inhibitor, was recently approved by the FDA for treatment of postmenopausal women with ER+/HER2- metastatic breast cancer in combination with letrozole or fulvestrant [16,17]. Clinical trials in advanced disease with combinations of aromatase inhibitor or fulvestrant and CDK4/6 inhibitors, including palbociclib, ribociclib (LE011, Novartis), and abemaciclib (LY2835219, Lilly) are ongoing, and recent completed trials have shown improved progression-free survival [10,18]. The combination of CDK4/6 inhibitors and endocrine therapy is being used in the clinic and in clinical trials in unselected patient populations, but not all patients will benefit from such combination therapy. Thus, there is an urgent need to identify biomarkers that predict response to the different endocrine treatments.
alone, as well as in combination with new pathway inhibitors in clinical development, as ER remains the only routinely used clinical predictive biomarker [19].

In the present study, we found increased expression of CDK6 in two breast cancer cell line models of fulvestrant resistance by gene array, and showed that reduction of CDK6 expression in fulvestrant-resistant cells with specific small interfering RNAs (siRNAs) impaired growth and induced apoptosis of fulvestrant-resistant cells. Inhibition of CDK6 using palbociclib re-sensitized the resistant cells to fulvestrant by reducing phosphorylation of Rb. Evaluation of the expression of CDK6 in metastatic ER+ lesions of breast cancer patients treated with different endocrine treatments suggests that CDK6 is a potential predictive biomarker of fulvestrant response. In the context of the approval of CDK4/6 inhibitors in combination with endocrine therapy to overcome resistance in ER+ advanced breast cancer, the use of CDK6 as a biomarker may provide useful information for patient selection.
Materials and Methods

Cell lines and culture conditions

The MCF-7 and T47D cell lines were originally obtained from the Breast Cancer Task Force Cell Culture Bank, Mason Research Institute. MCF-7 cells were gradually adapted to grow in low serum concentration [20] and the subline, MCF-7/S0.5, was used to establish two fulvestrant-resistant cell line models, ICI 182,780R (182R, consisting of 182R-1 and 182R-6), and ICI 164,384R (164R, consisting of 164R-1 and 164R-4), by long-term treatment with 10⁻⁷ M of ICI 182,780 or ICI 164,384, as previously described [21]. Tamoxifen-resistant (TamR) cell lines, TamR1, TamR4, TamR7 and TamR8, were established from MCF-7/S0.5 by long-term treatment with 10⁻⁶ M of tamoxifen [22], whereas the formestane-resistant (AIR) cell line was derived from MCF-7/S9 cells grown with 5 % newborn calf serum (NCS), 10⁻⁷ M androstenedione and 10⁻⁷ M of formestane [23]. T47D fulvestrant-resistant cells (T47D-R1 and T47D-R2) were established from T47D cells by long-term treatment with 100 nM fulvestrant, as previously described [24]. Cells were grown in a humidified atmosphere of 5% CO₂ at 37°C. All cell lines underwent DNA authentication using Cell ID™ System (Promega) before the described experiments to ensure consistent cell identity.

Global gene expression analysis

182R, 164R, TamR and AIR cell line models and the corresponding parental cell lines MCF-7/S0.5 and MCF-7/S9 were separately grown to 70-80% confluence and total RNA was purified using RNA kit (Qiagen) and arrayed separately in Affymetrix Gene Chip® Human Genome U133
plus 2.0 arrays (Affymetrix) as described [25]. Genes from the cyclin D/CDK4-6/p16\textsuperscript{INK4A}/Rb pathway showing $\geq$2-fold alteration in expression, false discovery rate (FDR) <0.05 cutoff and $p<0.01$ were considered as significantly altered expressed. Raw data were deposited in the Gene Expression Omnibus (GEO) database (GSE74391).

**siRNA-mediated gene knockdown**

Cells were transfected with single small interfering RNAs (siRNAs) or combinations of two siRNAs using Ingenio Electroporation kit (Mirus Bio) in a Nucleofector\textsuperscript{TM} II device (Amaza, Lonza) according to manufacturer’s instructions. The following siRNAs were used: CDK6-5 (SI00605052) and CDK6-6 (SI00605059) siRNA (Qiagen) and siRNA Mission siRNA Universal Negative Control (SIC001) (Sigma-Aldrich). Transfected cells were seeded in 24-well plates (5 x $10^4$ cells per well) and incubated for 48 hours to evaluate gene knockdown efficiency by RT-qPCR. Cells (6 x $10^5$) were seeded in T25 flasks and incubated for 96 hours to assess protein expression by Western blotting.

**Quantitative real time PCR (RT-qPCR)**

Total RNA was extracted using Isol-Lysis Reagent, TRIzol\textsuperscript{®} (Life technologies) and cDNA synthesis was performed using RevertAid Premium Reverse Transcriptase kit (Fermentas). Relative quantification of gene expression was performed using SYBR\textsuperscript{®} Green PCR Mastermix (Applied Biosystems) according to manufacturer’s instructions. All primers were purchase from
Qiagen and PUM1 was used as reference gene for normalization and relative expression levels were calculated using the comparative threshold method [26].

**Immunocytochemistry and Western blotting**

The generation of cell line arrays and Western blotting were performed as previously described [27]. The following antibodies were used according to the manufacturer’s protocol: anti-cyclin D1 (#sc-718) and anti-CDK4 (#sc-23896) from Santa Cruz; anti-CDK6 (#ab54576), anti-pRb T821 (#ab32015), anti-pRb T826 (#ab133446) and anti-β-actin (#ab6276) as loading control from Abcam; anti-pRb S780 (#3590), anti-pRb S807/811 (#9308), anti-pS6 S235/236 (#2211), anti-pS6 S240/244 (#2215), anti-S6 (#2217), anti-pAkt S473 (#4060), anti-Akt (pan) (#4685), anti-S6K T389 (#9206), anti-S6K T421/S424 (#9204) and anti-S6K (#2708) from Cell Signaling; anti-Rb1 (#554136) from BD Biosciences; Horseradish peroxidase (HRP)-conjugated goat anti-mouse (#P0447) and HRP-conjugated goat anti-rabbit (#P0448) from Dako. For detection of immunoreactive bands, ECL Prime Western Blotting Detection Reagents (GE Healthcare) were used and membranes were visualized on a Fusion-Fx7-7026 WL/26MX instrument (Vilbaer Lourmat).

**Cell growth assays**

For evaluation of the effect of siRNA-mediated gene knockdown on cell growth, 5 x 10^4 cells per well were seeded in 24-well plates and growth was measured using crystal violet-based colorimetric assay [28] 24 and 96 hours after siRNA transfection. The effect of specific CDK6
knockdown on cell growth was confirmed using BrdU cell proliferation kit (Cell Signaling Technology) according to the manufacturer’s instructions.

**Measurement of cell death**

The effect of CDK6 knockdown on apoptotic cell death was assessed using cell death detection ELISA Plus assay (Roche) according to the manufacturer’s instructions. Briefly, cells transfected with CDK6 siRNA were seeded in 24-well plates at a density of $4 \times 10^4$ cells per well. After 96 hours, the supernatant was removed, adherent cells were lysed in 200 μl lysis buffer and the lysates were assayed for levels of nucleosomes using the immunoreagent from the kit. The percentage of apoptotic cells was calculated by determining cell numbers using standard curves plotting the number of viable or apoptotic cells vs. optical density readings for crystal violet colorimetric assay and cell death assay, respectively.

**Flow cytometry analysis of cell cycle**

Changes in cell cycle distribution were evaluated 96 hours after transfection with specific siRNA against CDK6. Cells were harvested and fixed in ice-cold 70% ethanol overnight. After centrifugation, the pellets were resuspended in 50 μl RNase A in PBS (1 mg/ml) (Sigma-Aldrich) and stained with 200 μl propidium iodide (50 μg/ml) (Sigma-Aldrich). Cells were incubated for 30 min at room temperature and DNA content was determined using FACS flow cytometer (BD Biosciences) at 488 nm. For each measurement $1 \times 10^4$ events were collected and the data was analyzed by FlowJo software vX0.7 (Tree Star, Inc.).
Kinomics analysis

The activation status of various kinases by measuring specific peptide phosphorylation by protein kinases was determined by PamGene microarray technology (PamGene). Serine/threonine kinase (STK) activity profiles were determined for lysates of MCF-7/S0.5 and fulvestrant-resistant cells transfected with control siRNA and CDK6-specific siRNA using PamGene’s STK peptide microarrays, comprising 144 peptides that are substrates for human protein phosphorylation. Fluorescence-labeled anti-phospho-antibodies were used to detect phosphorylation according to the manufacturer’s instructions. Kinomics analysis was performed using BioNavigator software v.6.0. Raw signal intensity data per each of the 144 spots was captured over multiple 50 ms exposures sequentially as lysates were pumped through the array, and then over multiple exposure times (10, 20, 50, 100, 200 ms) after lysates were rinsed off. These values were converted to slopes of intensity by exposure time, and slopes were multiplied by 100 and log2-transformed for visualization, labeled “log signal”. Significantly altered peptides (by t-test) were cross-compared for recurrent upstream kinases as listed per residue in Kinexus kinase predictor (www.phosphonet.ca), and scored according to percent occurrence.

Specific chemical inhibitor

Fulvestrant-resistant and -sensitive cell lines were treated with 100 and 200 nM palbociclib (PD0332991, Sigma-Aldrich) dissolved in water and 120 nM pan-Akt inhibitor (GSK2141795,
MedChemexpress) dissolved in DMSO (Sigma-Aldrich). Cell growth measurements were performed 48 or 72 hours later using crystal violet-based colorimetric assay.

Clinical samples

Formalin-fixed, paraffin-embedded (FFPE) metastatic tumor samples from ER+ breast cancer patients treated with fulvestrant were selected by database extraction from the archives of the Department of Pathology at Odense University Hospital (OUH) between 2009 and 2013 (N=84) (initial cohort) and between 2013 and 2016 (N=67) (validation cohort). Generally, these patients had also received other endocrine treatments (tamoxifen/AI) for their advanced disease. Furthermore, FFPE metastatic tumor samples from ER+ breast cancer patients not treated with fulvestrant were also collected from OUH encompassing the period 2004-2016 (N=154). The inclusion criteria for all groups were ER+ breast cancer patients operated/biopsied at OUH for metastatic disease with complete clinical information as well as pathological verification that the metastatic lesion was of breast cancer origin. Exclusion criteria were competing cancer(s), cytological biopsies or insufficient material in the FFPE block. These parameters yielded the following number of metastatic lesions from advanced breast cancer patients treated with fulvestrant: initial cohort (N=45), validation cohort (N=46), and a cohort treated with tamoxifen/aromatase inhibitors (N=68). The metastatic biopsies used for evaluation of CDK6 expression were obtained soon after disease recurrence diagnoses and prior to fulvestrant or tamoxifen/aromatase inhibitor treatment in the advanced setting. ER+ tumors were defined as ≥ 1% positively stained tumor cells. All clinical samples were coded to maintain patient

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confidentiality and studies were approved by the Ethics Committee of the Region of Southern Denmark and Copenhagen and Frederiksberg Counties (approval no S-2008-0115).

Immunohistochemical stainings

Whole FFPE sections of the first metastatic lesions following recurrence were incubated with anti-CDK6 (#ab54576, Abcam) and immunostained using the HRP-conjugated PowerVision+TM system on the autostainer (TechMateTM 500, Dako) as described [27]. A Leica DMLB microscope (100×/numerical aperture 1.25, Leica Microsystems) and LasV3.6 acquisition software were used for tissue microscopy. Evaluation of the stainings was performed by an experienced breast pathologist in a blinded setup. CDK6 expression was observed in cell nucleus and tumors were scored based on the staining intensity score (0-3) multiplied by the percentage of positive cells, on a scale from 0 to 300. The cut-off value for high vs. low CDK6 expression used in all three cohorts (H-score = 200) was determined from the median expression of the initial cohort of fulvestrant-treated patients. The cut-off value was further examined using the web-based tool Cutoff Finder [29] and comparable H-score values were identified using the function 'Significance of correlation with binary variable'.

Clinical endpoints

Progression-free survival (PFS) was defined as the time from the date of starting the respective endocrine treatment, in the advanced setting, and disease progression within a three year
period. Patients without progression were censored at the date of database retrieval from the registry or 3 years after the date of initiating endocrine treatment, whichever came first.

**Statistical Analysis**

One-way ANOVA was used to select genes differentially expressed between fulvestrant-resistant and -sensitive cell lines, based on ≥2-fold change in expression and a FDR of <0.05. A two tailed t-test compared groups for cell growth, apoptosis and cell cycle analysis assays following treatment with siRNAs or chemical inhibitor. Associations between CDK6 expression and other clinicopathological characteristics were analyzed by Fisher’s exact and χ² tests. Univariate analysis was conducted on CDK6 expression levels, age, tumor size, nodal status, tumor grade and site of relapse. For multivariate analysis, the Cox proportional hazards regression model was applied to assess the adjusted hazard ratio (HR) of PFS by expression level of CDK6 and clinicopathological characteristics to assess interactions. Survival curves were generated by Kaplan-Meier estimates and differences between groups were evaluated using the log-rank test. For statistical analysis, STATA v14.0 (STATACorp) and GraphPad Pisma v5.0 (GraphPad Software, Inc.) were used. P values < 0.05 were considered statistically significant.
Results

Gene expression analysis reveals altered CDK6 expression in fulvestrant-resistant breast cancer cell lines

Using gene expression profiling, we evaluated whether regulators of the cyclin D/CDK4-6/p16INK4a/Rb pathway exhibited altered expression in different endocrine-resistant cell models. Analysis of the microarray data showed that a fulvestrant-resistant cell line model consisting of two resistant cell lines, 182R-1 and 182R-6, showed significant upregulation of CDK6 compared to parental fulvestrant-sensitive MCF-7/S0.5 cells (≥2-fold alteration in expression, \( p < 0.01 \)), as shown in Supplementary Table S1. Analysis of a second fulvestrant-resistant cell line model, 164R, including the resistant 164R-1 and 164R-4 cell lines, showed similarly higher CDK6 expression in fulvestrant-resistant vs. parental MCF-7/S0.5 cells. The remaining regulators of the cyclin D/CDK4-6/p16INK4a/Rb pathway were slightly downregulated in both fulvestrant-resistant cell line models vs. sensitive cells. Analysis of the gene array data in other endocrine-resistant cell line models, including four tamoxifen-resistant and one formestane-resistant MCF7-based cell lines, showed no significant alterations in the expression of any of the regulators of cyclin D/CDK4-6/p16INK4a/Rb pathway (Supplementary Table S1), suggesting that CDK6 was selectively deregulated in fulvestrant-resistant breast cancer cells.

The altered expression of CDK6 observed by gene array was verified by RT-qPCR in both fulvestrant-resistant cell line models, 182R and 164R vs. MCF-7/S0.5 cells. The expression of CDK6 was highly upregulated, whereas CCND1 (cyclin D1), CDK4 and Rb were slightly downregulated in fulvestrant-resistant lines compared with the parental MCF-7/S0.5 cell line.
The altered CDK6 expression in fulvestrant-resistant cells observed at the mRNA level was also reflected at the protein level, as confirmed by Western blotting (Figure 1B) and by immunocytochemical staining of FFPE breast cancer cell lines (Figure 1C). We further investigated the expression of CDK6 in another ER+ breast cancer cell line model derived from T47D cells using RT-qPCR and Western blotting (Figure 1D and 1E). Contrary to the MCF-7-based fulvestrant-resistant cell lines, fulvestrant-resistant cells derived from the parental ER+ T47D cell line showed a stable ER- phenotype [24]. Down-regulation of CDK6 was observed in T47D-fulvestrant-resistant vs. parental cell lines, suggesting that the CDK6 pathway may be of less importance in the absence of ER expression.

**Knockdown of CDK6 impairs growth and induces apoptosis in fulvestrant-resistant breast cancer cell lines**

To evaluate the role of high CDK6 expression in fulvestrant responsiveness, CDK6 was significantly reduced in the two MCF-7-based fulvestrant-resistant cell line models by transfection with two CDK6-specific siRNAs individually and in combination. CDK6 knockdown in all fulvestrant-resistant cell lines, as well as in the parental MCF-7/S0.5 cell line, was confirmed by both RT-qPCR (Figure 2A) and Western blotting (Figure 2B). Knockdown of CDK6 resulted in significantly reduced growth of both 182R and 164R cells (p < 0.05) compared with cells transfected with control siRNA, as assessed by both crystal violet-based colorimetric evaluation of overall cell numbers (Figure 2C and 2D) and BrdU incorporation assay (Figure 2E) measuring
newly synthesized DNA. In contrast, no significant growth reduction was observed in parental MCF-7/S0.5 cells transfected with CDK6-specific siRNAs compared with cells transfected with control siRNA. The effect of CDK6 knockdown on the growth of breast cancer cell lines was assessed both in the presence (Figure 2C) and absence of fulvestrant (Figure 2D), and the effect was comparable in both cases, indicating that the importance of increased expression of this gene to cell growth and survival was independent of the ER pathway. Furthermore, the effect of CDK6 knockdown on apoptosis in fulvestrant-resistant cells was measured using an apoptosis assay based on detection of cytoplasmic nucleosomes. The analysis showed that CDK6 knockdown resulted in a significant increase in apoptosis ($p < 0.05$) in fulvestrant-resistant cells when compared to cells transfected with control siRNA, while no significant change in apoptosis was observed following CDK6 knockdown in the parental cell line (Figure 2F).

**CDK6-specific knockdown alters cell cycle regulation in fulvestrant-resistant breast cancer cell lines**

CDK6 is known to be an important regulator of cell cycle [31], and we therefore investigated the effect of CDK6 knockdown on cell cycle phase distribution in 182R and 164R cell line models using flow cytometric analysis of propidium iodide-stained cells. Reduction of CDK6 levels with CDK6-specific siRNA induced measurable changes in the distribution of the cell cycle phase fractions of all fulvestrant-resistant cells. The percentage of cells in the G1 phase was increased and that of cells in S and G2/M phase was decreased compared with cells transfected with control siRNA (Figure 3A and 3B). For the parental MCF7/S0.5 cell line, fewer changes in the
proportion of cells in the different phases of the cell cycle were observed following CDK6-specific knockdown. Furthermore, Rb protein, which is a direct target of CDK6, exhibited decreased phosphorylation at threonine 826 (p-Rb T826), threonine 821 (p-Rb T821) and serine 807/811 (p-Rb S807/811) following CDK6-specific knockdown in some fulvestrant-resistant cells and in the parental MCF-7/S0.5 cell line (Figure 3C). No detectable changes were observed in the levels of Rb phosphorylated at serine 780 (p-Rb S780) in resistant and sensitive cells.

**Activity-based kinase analysis reveals decreased activity of Akt following CDK6 knockdown**

Since CDK6 knockdown showed reproducible changes in Rb phosphorylation levels, but differed between the fulvestrant-resistant cell lines, we investigated whether the effect of CDK6 in inhibiting fulvestrant-induced cell death was independent of Rb-phosphorylation by activity-based kinase analysis. Knockdown of CDK6 in the four MCF-7-based fulvestrant-resistant breast cancer cells decreased overall serine/threonine kinase activity compared with cells transfected with control siRNA using PamGene arrays (Figure 4A, Supplementary Figure S1). Changes in kinase activities following CDK6 knockdown in fulvestrant-resistant cells and parental MCF-7/S0.5 cells predicted involvement of different upstream kinases (Supplementary Figure S1 and S2), indicating differences in CDK6 signaling associated with the two phenotypes. Akt2 was identified as the kinase with the most significant decrease in activity between CDK6 siRNA and control siRNA-treated groups in fulvestrant-resistant cells, followed by p70 ribosomal S6 kinase 2 (S6K2, a downstream target of mTOR) and Akt1 (Supplementary Figure S1). Western blot analysis confirmed that CDK6 knockdown decreased phosphorylation of Akt (p Akt-S473), p70
ribosomal S6 kinase (pS6K-T389) and S6 (pS6-S235/236 and pS6-S240/244), a downstream target of S6K2 that controls protein synthesis and cell proliferation, resulting in the inactivation of these kinases in two fulvestrant-resistant cells (182R-1 and 164R-4) (Figure 4B). Treatment of the four MCF-7-based fulvestrant-resistant cell lines with an Akt inhibitor equally decreased their growth and increased their sensitivity to fulvestrant treatment (Figure 4C). Further, Akt inhibitor alone more effectively inhibited the growth of fulvestrant-resistant cells than fulvestrant-sensitive MCF-7 cell line (Figure 4C), with the latter showing no significant decrease in activity of Akt/mTOR pathway in the PamGene analysis (Supplementary Figure S2). Although Akt and CDK4/6 inhibitors showed comparable effects in decreasing proliferation of fulvestrant-resistant cells, the combination was more efficient than either drug alone (Figure 4C).

CDK4/6 inhibitor palbociclib impairs growth of fulvestrant-resistant breast cancer cell lines

To evaluate the potential use of combination therapy with fulvestrant and CDK4/6 inhibitors in fulvestrant-resistant breast cancer cells, we examined the effect of palbociclib, fulvestrant, and the combination of the two drugs on the growth of the two MCF-7-derived fulvestrant-resistant cell line models. Treatment of fulvestrant-resistant cell lines with palbociclib alone significantly inhibited growth ($p < 0.05$) compared with untreated cells, while fulvestrant, as expected, had no marked effect on growth (Figure 5A). The combined treatment of palbociclib and fulvestrant was significantly more effective in inhibiting growth of fulvestrant-resistant cell lines ($p < 0.05$) than each of the single agents used separately. For the fulvestrant-sensitive cells, palbociclib and fulvestrant as single agents significantly ($p < 0.05$) reduced cell growth compared with
untreated cells. Combined palbociclib and fulvestrant treatment inhibited growth of fulvestrant-sensitive MCF-7/S0.5 cells more effectively than with either drug alone ($p < 0.05$). Moreover, Rb protein exhibited decreased phosphorylation at S780, S807/811, T826 and T821 residues following CDK4/6 inhibition with palbociclib in both fulvestrant-resistant and -sensitive cell lines (Figure 5B), suggesting that the effect of CDK4/6 inhibition on cell growth is mediated by phosphorylation of Rb in several phosphorylation sites.

**CDK6 levels in ER+ metastatic lesions of breast cancer patients treated with fulvestrant significantly correlates with clinical outcome**

To evaluate the clinical relevance of our findings, we analyzed the expression of CDK6 using immunohistochemistry in full sections of distant metastatic tumor tissues from postmenopausal patients with ER+ breast cancer who had received fulvestrant in the advanced setting following disease progression (N=45). We also examined a second cohort of advanced ER+ patients treated with fulvestrant (N=46) to validate the results from the initial cohort. In addition, distant metastatic tumors from postmenopausal ER+ breast cancer patients who did not receive fulvestrant, but were treated with tamoxifen or/and aromatase inhibitors (N=68), were also examined for CDK6 expression. Clinical and pathological characteristics of the patient cohorts are shown in Supplementary Table S2. $\chi^2$ tests of clinicopathological characteristics according to CDK6 expression levels in all patients identified no differences relative to age (primary tumor), tumor size, nodal status, tumor grade, HER2 status, site of relapse, time to recurrence and therapy at time of biopsy (Supplementary Table S3). ER status in primary
tumors and metastasis is shown in Supplementary Table S4. Of all patients in the three cohorts (N=159), only two had an ER- metastatic biopsy. For patients treated with fulvestrant, Kaplan-Meier curves showed that high levels of CDK6 in the metastatic tumor significantly correlated with shorter PFS of fulvestrant treatment compared with patients with tumors exhibiting lower levels of CDK6 (initial cohort: median time to progression of 2.5 vs. 8.2 months, respectively, p=0.0006; validation cohort: median time to progression 3.4 vs. 8.9 months, respectively, p=0.018) (Figures 6A and 6B). In contrast, survival analysis of these cohorts showed no significant association between CDK6 levels and PFS of tamoxifen/AI treatment administered as second-/third- (N=39) or first-line (N=56) endocrine therapy in the advanced setting (second-/third-line tamoxifen/AI: median time of 12.0 vs. 9.8 months, p=0.578; first-line tamoxifen/AI: median time to progression of 21.2 vs. 20.1 months, p=0.887) (Figures 6C and 6D). Moreover, no significant association between CDK6 levels and PFS was observed in a cohort of patients who did not receive fulvestrant treatment, but were treated only with second-/third- (N=34) or first-line (N=52) tamoxifen or/and aromatase inhibitors (second-/third-line tamoxifen/AI: median time to progression of 5.3 vs. 6.6 months, p=0.511; first-line tamoxifen/AI: median time to progression of 15.4 vs. 28.3, p=0.135) (Figure 6E and 6F) indicating CDK6 as predictive marker of fulvestrant responsiveness in this clinical setting. Detailed information on the number of patients receiving tamoxifen/AI as first- or/and second-/third-line therapy in the metastatic setting is shown in Supplementary Table S2. Representative micrographs of breast cancer section showing no staining or CDK6 staining at increasing intensity (1-3+) are shown in Figures 6G-J, and a comparison of CDK6 staining between representative clinical samples and FFPE
breast cancer cell lines is shown in Supplementary Figure S3. Cox proportional hazard regression analysis of PFS according to CDK6 levels and clinicopathological characteristics, including age at metastatic disease and site of relapse (Table 1), showed that CDK6 was an independent prognostic factor of PFS of fulvestrant treatment in both fulvestrant-treated cohorts (initial cohort: HR 3.5 and $p=0.001$; validation cohort: HR 2.4 and $p=0.03$). Although univariate analysis showed a significant correlation between age at metastasis and CDK6 expression (Supplementary Table S3), no significant association was observed between age at metastasis and PFS in cox multivariate analysis (Table 1).
Discussion

The successful clinical use of CDK4/6 inhibitors in combination with endocrine therapy in ER+ breast cancer requires understanding the role of the cyclin D/CDK4-6/p16\textsuperscript{INK4a}/Rb pathway in endocrine-resistant tumors. There are currently no biomarkers that distinguish subgroups of patients who will benefit from therapy with CDK4/6 inhibitor in combination with a specific endocrine drug. We found CDK6 to be highly upregulated in two MCF-7-based fulvestrant-resistant cell line models that maintain some ER expression [21], but not in a T47D-based fulvestrant-resistant cell model in which ER expression was lost [24]. As shown in our study and by others, most tumors initially responsive to endocrine therapy retain ER expression at recurrence on antiestrogen therapy [32,33], suggesting that the MCF-7-derived fulvestrant-resistant cell line model best resembles the majority of metastatic breast tumors.

Previously, other members of the cyclin D/CDK4-6/Rb pathway have been shown to play a role in endocrine resistance, such as cyclin D1 [34,35] and Rb [36,37]. Moreover, the CDK4/Rb/E2F transcriptional axis has been reported to control estrogen-independent growth of breast cancer cells and mediate resistance to AI [38]. However, we observed reduced or no altered expression of the other regulators of the cyclin D/CDK4-6/Rb pathway in our endocrine-resistant cell line models.

CDK6 downregulation affected the growth of our MCF-7-derived fulvestrant-resistant cells both in the presence and absence of fulvestrant, suggesting that CDK6 functions independently of ER, similar to growth factor signaling pathways in fulvestrant-resistance, which eventually act as
ER-independent drivers of tumor growth [39-41]. In contrast, CDK6 knockdown did not affect the growth of the parental fulvestrant-sensitive cell line. Our results are consistent with a study that implicated CDK6 in fulvestrant resistance in breast cancer cell lines and showed that treatment with CDK4/6 inhibitor reduced growth of resistant cells [42], although this study did not determine whether that effect was mediated by the inhibition of CDK4 or CDK6 or both. Furthermore, we observed increased apoptosis following reduction of CDK6 in fulvestrant-resistant cells, but not in the parental fulvestrant-sensitive cell line. The role of CDK6 in the control of cell growth and survival observed in our study is in line with previous reports showing decreased proliferation and enhanced apoptosis in response to chemotherapy following CDK6 knockdown in glioma cells expressing high levels of CDK6 [43], and inhibited proliferation and induced apoptosis by miR-211 through repression of cyclin D1 and CDK6 expression in epithelial ovarian cancer cells [44]. The changes observed in our study in apoptosis following CDK6-specific knockdown were more substantial than the effect in cell proliferation, suggesting a more important role for CDK6 in the control of fulvestrant-resistant cells survival rather than proliferation.

Cell cycle analysis using propidium iodide staining of nuclear DNA and flow cytometry showed that CDK6-specific knockdown resulted in measurable accumulation of fulvestrant-resistant cells in the G1 phase fraction. The modest growth arrest observed at the G1/S checkpoint can be explained by the partially redundant role of CDK4 and CDK6 in controlling cell cycle progression through the G1 phase into the DNA duplication phase [45]. We also observed that
CDK6 knockdown impaired growth and survival selectively in fulvestrant-resistant cells, and resulted in alteration of Rb phosphorylation levels at several different residues. However, as the changes in Rb phosphorylation levels were not consistent between all resistant cell lines, the results suggest that CDK6 may function independently of the Rb-phosphorylation pathway to induce the phenotypic changes observed in fulvestrant-resistant cells. CDK6 activity independent of Rb-phosphorylation has been shown previously in a model of lymphoid leukemia where CDK6 was found to be part of a transcription complex that enhances proliferation and stimulates angiogenesis [46]. By performing activity-based kinase assay following CDK6 knockdown, we identified Akt as downstream kinase of CDK6 activity. Akt acts as an anti-apoptotic signaling molecule either by transcription regulation or direct phosphorylation of a range of targets involved in apoptosis [47]. In our study, treatment with Akt inhibitor re-sensitized all fulvestrant-resistant cells to fulvestrant and more effectively decreased the growth of resistant cells when administered in combination with palbociclib, suggesting that increased CDK6 expression affects fulvestrant resistance by stimulating the Akt pathway.

In contrast to what we observed following CDK6-specific knockdown, CDK4/6 inhibition with palbociclib showed a consistent decrease in Rb-phosphorylation levels in all MCF-7-based fulvestrant-resistant cells, as shown in other studies [15,42]. These results suggest that inhibition of both CDK4 and CDK6 are necessary to consistently alter phosphorylated-Rb levels and induce substantial changes in cell cycle progression in breast cancer cell lines, while CDK6
alone may control cell growth and survival through Rb-independent pathways. Combined treatment with palbociclib and fulvestrant resulted in significantly decreased proliferation in fulvestrant-resistant and -sensitive MCF-7 cells compared with the effect of either drug alone. These results suggested that combined palbociclib and fulvestrant therapy reduces growth of tumor cells expressing high levels of CDK6 that may not respond, or have a weak response, to fulvestrant therapy alone. Our results concur with the report of Finn et al [15] showing that treatment of tamoxifen-resistant MCF-7 cells with palbociclib enhanced sensitivity to tamoxifen. Furthermore, results from a clinical study in ER+ breast cancer patients showed that combined palbociclib and letrozole therapy resulted in improved PFS compared with letrozole alone [48]. However, not all patients are expected to benefit from combined endocrine and palbociclib therapy, and CCND1 (cyclin D1) amplification or overexpression or loss of CDKN2A (p16) have been evaluated for patient selection without indication of any preferential benefit [48], highlighting the need for novel predictive biomarkers.

To investigate the potential of CDK6 to predict response to fulvestrant in advanced ER+ breast tumors, we evaluated CDK6 expression in metastatic lesions of ER+ patients treated or not with fulvestrant. High expression of CDK6 was significantly associated with a shorter PFS of fulvestrant treatment in two independent cohorts of advanced disease patients treated with fulvestrant, whereas no significant association was observed between CDK6 expression and PFS of first- or second-/third-line tamoxifen/AI treatment in these two cohorts and in an independent cohort of patients who only received tamoxifen/AI in the metastatic setting. This
suggests that high expression of CDK6 is associated with a reduced efficacy of fulvestrant and is supported by our in vitro findings showing enhanced expression of CDK6 in fulvestrant-resistant cell lines and restoration of sensitivity to fulvestrant following CDK6 knockdown and inhibition. To our knowledge, this is the first study to investigate the potential of CDK6 as a predictive biomarker of clinical responsiveness to fulvestrant. Furthermore, our in vitro findings showed that the CDK4/6 inhibitor palbociclib was particularly effective in inhibiting growth of ER+ breast cancer cells with high expression of CDK6 that responded poorly to fulvestrant alone.

In conclusion, our data show upregulation of CDK6 in breast cancer cell lines resistant to fulvestrant and suggest an important role for CDK6 in the survival of fulvestrant-resistant cells. Other mechanisms may, however, be observed in some fulvestrant-resistant tumors. The decreased PFS found in fulvestrant treated ER+ breast cancer patients with metastatic lesions expressing high level of CDK6 strongly suggests the potential of this protein as a predictive biomarker of fulvestrant treatment response, and for selection of patients who may benefit from combined targeted therapy with CDK4/6 inhibitors and fulvestrant.

Acknowledgements

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References


Table 1. Cox multivariate regression analysis of PFS according to CDK6 levels and clinicopathological characteristics in the two cohorts of patients with ER+ advanced breast cancer treated with fulvestrant.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (initial cohort)</th>
<th>P (initial cohort)</th>
<th>Hazard ratio (validation cohort)</th>
<th>P (validation cohort)</th>
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<tr>
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<td>Age</td>
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<td>0.390</td>
<td>0.576</td>
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<td>Site of relapse</td>
<td>1.149</td>
<td>0.537</td>
<td>0.988</td>
<td>0.965</td>
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Figure Legends

Figure 1. CDK6 is highly expressed in fulvestrant-resistant vs. parental MCF-7/S0.5 breast cancer cell lines. (A) Validation of the expression of CCND1, CDK4, CDK6, CDKN2A and Rb in two MCF7-based fulvestrant-resistant cell line models, 182R and 164R, compared with parental MCF-7/S0.5 cell line by RT-qPCR. (B) Cyclin D1, CDK4, CDK6 and Rb protein expression in fulvestrant-resistant and -sensitive cell lines using Western blotting. A representative of two separate experiments is shown. (C) Immunocytochemistry analysis of FFPE fulvestrant-resistant and MCF-7/S0.5 cells for CDK6 protein (x40 magnification). Evaluation of CDK6 expression in T47D-based fulvestrant-resistant cell lines (T47D-R1 and T47D-R2) compared with parental T47D-S5 cells by RT-qPCR (D) and Western blotting (E). Gene expression was normalized using PUM1 and depicted as relative expression in fulvestrant-resistant vs. parental cells, mean ± standard deviation. β-actin was used as loading control in Western blotting experiments.

Figure 2. siRNA-mediated knockdown of CDK6 inhibits cell growth and increases apoptosis in fulvestrant-resistant breast cancer cells. Fulvestrant-resistant 182R and 164R and parental MCF-7/S0.5 cell lines were transfected with siRNAs specific for CDK6 leading to a reduction of mRNA (A) and protein (B) expression levels compared with cells transfected with control siRNA, evaluated by RT-qPCR and Western blotting, respectively. PUM1 was used for normalization of RT-qPCR data. β-actin was used as loading control in Western blotting. Effect of CDK6 knockdown on cell growth was measured using crystal violet-based colorimetric assay, both in the presence (C) and absence of fulvestrant (D). (E) BrdU incorporation assay was performed to
confirm the effect of CDK6 reduction in cell proliferation by measuring the incorporation of BrdU in the new DNA synthesized. (F) Percentage of apoptotic cells following CDK6 knockdown was determined by evaluating the presence of nucleosomes in cells cytoplasm. Two siRNAs (siRNA CDK6-5 and siRNA CDK6-6) were used individually and in combination. A representative of three separate experiments performed in triplicate is shown. * p < 0.05. Data is shown with error bars representing mean ± standard deviation.

Figure 3. siRNA-mediated CDK6 knockdown induces alterations in the distribution of the cell cycle phase fractions of fulvestrant-resistant breast cancer cells. (A) Evaluation of cell cycle phase distribution by flow cytometry of propidium iodide-stained MCF-7/S0.5 and fulvestrant-resistant (182R-6) cells, following transfection with control siRNA or CDK6-specific siRNA. (B) Column diagram showing the percentage of cells in each phase of cell cycle following CDK6 knockdown in all fulvestrant-resistant cells and MCF-7/S0.5 cells. (C) Rb phosphorylated at different residues and total Rb protein were evaluated by Western blotting following transfection with control siRNA or CDK6-specific siRNA. β-actin was used as loading control. A representative of two separate experiments is shown.

Figure 4. Reduction of CDK6 expression by siRNA decreased the activity of Akt in fulvestrant-resistant breast cancer cell lines. (A) Heat map showing fold change in peptide substrate phosphorylation using PamGene arrays. Red tones indicate increased phosphorylation and blue tones indicate decreased phosphorylation in four MCF-7-based fulvestrant-resistant and
parental MCF-7/S0.5 cell lines transfected with CDK6 siRNA compared to the same cell line treated with control siRNA. (B) Protein expression levels of Akt, S6K and its downstream target S6, by Western blotting with the indicated antibodies. β-actin was used as loading control. A representative of two separate Western blots is shown. (C) Growth of fulvestrant-resistant and parental MCF-7/S0.5 cell lines following treatment with 120 nM of Akt inhibitor, 100 nM of palbociclib, 10^{-7}M of fulvestrant, combination of Akt inhibitor with the other drugs or no drug (control). Cell growth was determined by crystal violet colorimetric assay. A representative of three independent experiments performed in triplicates is shown. Error bars representing mean ± standard deviation.

Figure 5. CDK4/6 inhibitor palbociclib impairs growth of fulvestrant-resistant breast cancer cell lines. (A) Growth of fulvestrant-resistant and parental MCF-7/S0.5 cell lines following treatment with 100 nM of palbociclib, 10^{-7}M of fulvestrant, the two drugs in combination or no drug (control). Cell growth was determined by crystal violet colorimetric assay. A representative of three independent experiment performed in triplicates is shown. * p < 0.05. Error bars representing mean ± standard deviation. (B) Rb phosphorylated at different residues and total Rb protein were evaluated by Western blotting following treatment with 10^{-7}M of fulvestrant, 200 nM of palbociclib, the two drugs in combination or no drug (control). β-actin was used as loading control. A representative of two separate Western blots is shown.
Figure 6. Survival curves from Kaplan-Meier estimates showing the association of PFS with CDK6 expression in metastatic lesions of ER+ breast cancer patients treated or not with fulvestrant in the advanced disease setting. Kaplan-Meier plots evaluating PFS of fulvestrant treatment according to expression of CDK6 protein in ER+ metastatic breast tumors from two cohorts of patients with advanced disease treated with fulvestrant, an initial cohort (A) and a validation cohort (B). PFS of second-/third- (C) and first-line (D) tamoxifen/AI treatment in the same fulvestrant-treated patient cohorts combined, for the patients who also received tamoxifen/AI treatment in addition to fulvestrant. PFS according to expression of CDK6 protein in ER+ metastatic breast tumors from a cohort of patients not treated with fulvestrant, but only with tamoxifen and/or aromatase inhibitors administered as second-/third- (E) or first-line (F) treatment in the metastatic setting. Twenty-two patients from the two fulvestrant-treated patient cohorts and 18 patients from the cohort treated only with tamoxifen/AI received tamoxifen and/or AIs as both first- and second-/third-line therapy in the metastatic setting. A two-sided $p$ value ($*p < 0.05$) was calculated using log-rank testing. Representative micrograph of breast cancer metastasis sections showing no CDK6 staining (G) or CDK6 staining at increasing intensity (1-3+) (H-J), 20x magnification.
Figure 1
Figure 2
Figure 3

A. 

- Control siRNA
- CDK6 siRNA

<table>
<thead>
<tr>
<th>Count</th>
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<th>182R-6</th>
<th>164R-1</th>
<th>164R-4</th>
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<tr>
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<td>G2-M</td>
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<td>10.88</td>
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B. 

DNA content (propidium iodide)

<table>
<thead>
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<th>Relative cell count (%)</th>
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<tr>
<td>&lt; 2N</td>
</tr>
<tr>
<td>+</td>
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C. 

- CDK4
- CDK6
- pRb-S780
- pRb-S807/811
- pRb-T821
- pRb-T826
- Rb
- β-actin

<table>
<thead>
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<th>Control siRNA</th>
<th>CDK6 siRNA</th>
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<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
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</tbody>
</table>
Figure 4
Figure 5

A

Control | Fulvestrant | Palbociclib | Palbociclib+Fulvestrant

Growth (OD 570 nm)

MCF-7/10.5 | 182R-1 | 182R-6 | 164R-1 | 164R-4

B

CDK4

CDK6

p-Rb S780

p-Rb S807/811

p-Rb T821

p-Rb T826

Rb

β-actin

Palbociclib

Fulvestrant

- + - + - + - + - + - + - + - + - + - + - + - + - + - +
Figure 6

A and B: Kaplan-Meier plots showing progression-free survival (PFS) for CDK6 low and CDK6 high groups. The p-values are 0.0006 and 0.018, respectively.

C and D: Kaplan-Meier plots showing similar survival data with a p-value of 0.578 and 0.887, respectively.

E and F: Additional survival plots with p-values of 0.511 and 0.135, respectively.

G, H, I, and J: Representative images of tissue sections stained for CDK6 expression.
High CDK6 protects cells from fulvestrant-mediated apoptosis and is a predictor of resistance to fulvestrant in estrogen receptor-positive metastatic breast cancer

Carla L Alves, Daniel Elias, Maria B Lyng, et al.

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