Molecular signatures of triple-negative breast cancer cells acquiring palbociclib resistance via continuous exposure

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Abstract. Palbociclib, a cyclin-dependent kinase (CDK)-4/6 inhibitor, exhibits therapeutic potential for triple-negative breast cancer (TNBC), for which effective treatments remain limited. However, understanding the mechanisms underlying drug resistance is essential for its clinical application. Although the mechanisms underlying resistance to CDK4/6 inhibitors in hormone receptor-positive breast cancer have been investigated, the mechanisms by which continuous drug exposure induces resistance in TNBC cells remain unclear. Therefore, the present study aimed to establish palbociclib-resistant TNBC cells using a continuous drug exposure model and to elucidate their characteristics. MDA-MB-231 cells, a human TNBC cell type with wild-type retinoblastoma (Rb) protein, were continuously exposed to palbociclib at gradually increasing concentrations (0.01-1 μM) to establish resistant cells, which were named MB231/PalR cells. Drug sensitivity was subsequently evaluated using cell viability assays; gene and protein expression levels were analyzed by quantitative polymerase chain reaction and western blotting, respectively; and P-glycoprotein-mediated efflux capacity was evaluated using rhodamine 123 staining with a flow cytometer. Compared with parental cells, MB231/PalR cells exhibited reduced sensitivity to palbociclib and abemaciclib. Rb protein expression levels were decreased, without affecting *RB1* mRNA expression, in MB231/PalR cells. The expression levels of *CCNE1*, *CDK6*, ATP-binding cassette (*ABC*)*B1* mRNA and cyclin E1 protein were increased, whereas those of *ABCG2* and *CD274* mRNA were decreased in resistant cells. Furthermore, MB231/PalR cells exhibited reduced sensitivity to the CDK2 inhibitor CVT-313, and co-treatment with CVT-313 failed to restore palbociclib sensitivity. Although P-glycoprotein efflux capacity and *ABCB1* mRNA expression were increased in resistant cells, verapamil treatment did not affect palbociclib sensitivity. In conclusion, continuous exposure of MDA-MB-231 cells to palbociclib induced alterations associated with CDK4/6 inhibitor action, resulting in drug resistance. These findings provide mechanistic insights into CDK4/6 inhibitor resistance in TNBC and may support the development of biomarkers and therapeutic strategies to overcome or prevent resistance.

Introduction

Breast cancer is the most prevalent malignancy among women worldwide, accounting for approximately 24% of all newly diagnosed cancer cases in women in 2022 (1). Breast cancer is conventionally classified into three intrinsic subtypes according to estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) expression: Hormone receptor (HR)-positive/HER2-negative, HER2-positive, and triple-negative breast cancer (TNBC) (2). TNBC, which accounts for approximately 10-15% of all breast cancer cases, is associated with a higher risk of recurrence and poorer overall survival than other molecular subtypes (3). However, treatment options for TNBC remain limited because of its lack of HRs and HER2 expression, resulting in lower therapeutic efficacy compared to other breast cancer subtypes (4,5).

Cyclin-dependent kinase 4/6 (CDK4/6) and cyclin D play critical roles in promoting the G1-to-S phase transition of the cell cycle. The primary target of CDK4/6 in complex with cyclin D is retinoblastoma (Rb) protein (6). Rb functions as a key regulator of the cell cycle by binding to E2F transcription factors and suppressing their activity. In the early G1 phase, Rb is maintained in a non-phosphorylated state, thereby inhibiting E2F-mediated transcription. Upon mitogenic stimulation, CDK4/6, activated by cyclin D, phosphorylates Rb, leading to its dissociation from E2F (7). E2F transcription factors subsequently induce the expression of genes required for S-phase entry and promote cell cycle progression (8). To date, only three CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) have been approved by the U.S. Food and Drug Administration for clinical use in HR-positive HER2-negative breast cancer (9,10). Recently, several pre-clinical studies have demonstrated the potential efficacy of CDK4/6 inhibitors for TNBC treatment. Asghar *et al* (11) revealed that the

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luminal androgen receptor TNBC subtype cell lines are more sensitive to palbociclib than basal-like TNBC cell lines and that treatment with palbociclib significantly reduces tumor volume in a xenograft model of luminal androgen receptor TNBC. Furthermore, the combination of palbociclib with paclitaxel (12), phosphoinositide 3-kinase-α inhibitors (13), or mechanistic target of rapamycin kinase inhibitors (14) shows enhanced antitumor efficacy against TNBC. These reports suggest that CDK4/6 inhibitors, including palbociclib, exhibit potential as therapeutic agents for TNBC. However, changes in TNBC cell characteristics upon exposure to CDK4/6 inhibitors remain unclear.

In TNBC, prolonged exposure to anticancer agents induces diverse alterations in cellular characteristics, including acquisition of drug resistance (15), enhancement of stem cell-like properties (16), epithelial-mesenchymal transition (EMT) (17).

In this study, we established an *in vitro* continuous exposure model using the MDA-MB-231 human TNBC cell line to investigate the molecular signatures associated with the changes in cellular characteristics induced by prolonged treatment with palbociclib, a selective CDK4/6 inhibitor. Additionally, we evaluated the associations between changes in cellular characteristics and molecular alterations related to drug resistance.

Materials and methods

Cell lines and reagents. The MDA-MB-231 human TNBC cell line was kindly provided by Dr Tamotsu Sudo of the Hyogo Cancer Center (Hyogo, Japan). The cells were cultured in the Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Tokyo, Japan), 100 U/ml penicillin G, and 100 μg/l streptomycin sulfate (Fujifilm Wako Pure Chemical, Osaka, Japan). Palbociclib and abemaciclib were purchased from Cayman Chemical (Ann Arbor, MI, USA). Verapamil was purchased from Tokyo Chemical Industry (Chuo-ku, Tokyo, Japan). MG-132 and CVT-313 were obtained from MedChemExpress (Monmouth Junction, NJ, USA). Rhodamine 123 was purchased from Molecular Probes Inc. (Eugene, OR, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt and 1-methoxy-5-methylphenazinium methyl sulfate were obtained from Dojindo Laboratories (Kumamoto, Japan).

Establishment of palbociclib-resistant human TNBC sublines. MDA-MB-231 cells were cultured continuously in the RPMI-1640 medium containing palbociclib at stepwise increasing concentrations (0.01-1 μM) to establish resistant cell lines over at least 3 months. Palbociclib-resistant cells were designated as MB231/PalR cells. These resistant cells were maintained under the same conditions as MDA-MB-231 cells, except that the culture medium contained 1 μM palbociclib.

Cell viability assay. MDA-MB-231 and MB231/PalR cells were seeded at a density of 5,000 cells/well and cultured in 100 μl RPMI 1640 medium without palbociclib at 37˚C for 24 h. The culture medium was replaced with fresh medium containing various concentrations of the test drugs. For co-treatment experiments, cells were treated with palbociclib alone or in combination with verapamil (10 μM) or CVT-313 (5 μM) for 72 h. After 72 or 144 h of incubation at 37˚C, the medium was replaced with 110 μl of medium including 10 μl of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt reagent solution. After 3 h, absorbance was measured at 450 nm using SpectraMax iD3 (Molecular Devices, San Jose, CA, USA). The 50% growth inhibitory concentration (IC_{50}) values of the test drugs in MDA-MB-231 and MB231/PalR cells were calculated using the sigmoid inhibitory effect model, $E=E_{max} \times [1-C^{\gamma}/(C^{\gamma} + IC_{50}{}^{\gamma})]$, via non-linear least-squares fitting (Solver; Microsoft Excel). In this equation, E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively, and C and γ represent the drug concentration in the medium and sigmoidicity factor, respectively (18-20).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MDA-MB-231 and MB231/PalR cells were pre-cultured in the RPMI-1640 medium without palbociclib for 48 h, and total RNA was isolated using the Quick-RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). First-strand cDNA was synthesized from 500 ng of total RNA using oligo(dT) primers and the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) on a thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA). Reverse transcription was performed in a 20-μl reaction mixture at 37˚C for 15 min, followed by inactivation at 85˚C for 5 sec and cooling to 4˚C. The cDNA was subsequently used to detect the mRNA expression. qPCR was performed using TB Green Premix Ex Taq II (Takara Bio) on the StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The reaction consisted of an initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The dissociation step was conducted at 95˚C for 15 sec, 60˚C for 1 min, and 95˚C for 15 sec. *ACTB* was used as an internal control. All primers used in this study are listed in Table I. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (21) and normalized to the internal reference gene *ACTB*.

Western blotting. MDA-MB-231 and MB231/PalR cells were pre-cultured in the RPMI-1640 medium without palbociclib for 48 h. For proteasome inhibition experiments, cells were treated with MG-132 (0.25 μM) for 6 h in the absence of palbociclib before protein extraction. Proteins were extracted using the radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid, a protease inhibitor cocktail (Sigma-Aldrich), and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). The extracted proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA). The membrane was blocked with wash buffer containing 20 mM Tris (pH 7.6), 150 mM NaCl, 50 mM KCl, and 0.2% Tween-20 with 3% bovine serum albumin and immunoblotted with antibodies against phosphorylated Rb, total Rb (1:1,000; cat. nos. 30376-1AP and 67521-1-Ig, Proteintech, Rosemont, IL, USA), Cyclin E1 (1:1,000; cat. no. 20808, Cell Signaling

Table I. List of primer sequences used in this study.

Gene	Forward, 5'-3'	Reverse, 5'-3'
RB1	CACTTTGTGAACGCCTTCTGT	CACGTTTGAATGTCTCCTGAACA
E2F1	TCTCGGCCAGGTACTGATG	ACCCTGACCTGCTGCTCTT
CCND1	ACAAACAGATCATCCGCAAACAC	TGTTGGGGCTCCTCAGGTTC
CCND2	TCATTGCTCTGTGTGCCACC	CAGCTCAGTCAGGGCATCAC
CCND3	AGGGATCACTGGCACTGAAG	ACAGGTGTATGGCTGTGACAT
CCNE1	TAGAGAGGAAGTCTGGAAAATCATG	ATATACCGGTCAAAGAAATCTTGTG
CDK4	ATGGCTACCTCTCGATATGAGC	CATTGGGGACTCTCACACTCT
CDK6	CGTGGTCAGGTTGTTTGATG	CAGGCTCTGGAACTTTATCC
ABCB1	TTCCTTCACCCAGGCAATG	ATGAGTTTATGTGCCACCAAGTAG
ABCC1	CAGTGACCTCTGGTCCTTAAACAA	TTGGCGCATTCCTTCTTCC
ABCC2	ACTTGTGACATCGGTAGCATGGA	AAGAGGCAGTTTGTGAGGGATGA
ABCG2	TGACGGTGAGAGAAAACTTAC	TGCCACTTTATCCAGACCT
CD274	ATTTGGAGGATGTGCCAGAG	CCAGCACACTGAGAATCAACA
ACTB	TCATGAAGTGTGACGTGGACATC	TGCATCCTGTCGGCAATG

Technology, Inc., Danvers, MA, USA) and GAPDH (cytosolic marker; 1:2,000; cat. no. 60004-1-Ig, Proteintech). Primary antibody incubation was performed overnight at 4˚C using an immunoreaction enhancer solution (Can Get Signal; Toyobo, Osaka, Japan). GAPDH was used as an internal control. Subsequently, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:2,000; anti-rabbit, cat. no. 7074, Cell Signaling Technology, Inc., anti-mouse, cat. no. ab6728, Abcam, Cambridge, UK) for 1 h at room temperature. An enhanced chemiluminescence detection system (Bio-Rad Laboratories) was used, and protein expression levels were quantified using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Intracellular accumulation of rhodamine 123. MDA-MB-231 and MB231/PalR cells were seeded at a density of $5x10^6$ cells and incubated in the RPMI-1640 medium without palbociclib at 37˚C for 48 h. After washing thrice with warmed Hanks' balanced salt solution (HBSS, Fujifilm Wako Pure Chemical), the cells were incubated in fresh HBSS containing 3 μM rhodamine 123 (Fujifilm Wako Pure Chemical) at 37˚C for 30 or 120 min. The accumulation process was terminated by removing HBSS from the dishes, followed by three washes with ice-cold phosphate-buffered saline (Takara Bio). The cells were collected in ice-cold phosphate-buffered saline and subjected to flow cytometric analysis using FACSAria II (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using the BD FACS Diva software (BD Biosciences).

Statistical analyses. All statistical analyses were performed using the EZR (version 1.7; Jichi Medical University, Tochigi, Japan), a graphical user interface for R (22). Data are represented as the mean ± standard deviation. Comparisons between two groups were performed using an unpaired Student's t-test. For experiments involving two independent variables, two-way ANOVA followed by Tukey's post hoc test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

MDA-MB-231 and MB231/PalR cell sensitivity to CDK4/6 inhibitors. The sensitivity of MDA-MB-231 and MB231/PalR cells to palbociclib and abemaciclib was evaluated using cell viability assays, with IC_{50} values after 72 and 144 h of exposure presented in Table II. Continuous exposure to palbociclib resulted in the establishment of MB231/PalR cells, which exhibited significantly reduced sensitivity to CDK4/6 inhibitors. Compared to those in MDA-MB-231 cells, IC_{50} values of palbociclib increased 2.15- and 9.28-fold at 72 and 144 h, respectively, and those of abemaciclib increased 11.3- and 34.2-fold, respectively, in the resistant cells.

mRNA expression profiles of cell cycle-related genes. Next, we investigated the effects of continuous exposure of MDA-MB-231 cells to palbociclib on the mRNA expression levels of cell cycle-related genes, such as *CCND1*, *CCND2*, *CCND3*, *CCNE1*, *CDK4*, *CDK6*, *RB1*, and *E2F1*, via RT-qPCR. The mRNA expression levels of *CCNE1*, which encodes cyclin E1, and *CDK6* were significantly higher in MB231/PalR cells than in MDA-MB-231 cells (P<0.01, Table III). However, mRNA levels of *CCND2*, which encodes cyclin D2, were significantly lower in MB231/PalR cells (P<0.01). Notably, no significant difference in the mRNA levels of *CDK4*, a key target of palbociclib, was observed between the resistant and parental cells.

Effects of continuous palbociclib exposure on Rb protein expression and phosphorylation. CDK4/6 promotes the cell cycle via Rb phosphorylation. Therefore, we elucidated the expression and phosphorylation status of Rb in MB231/PalR cells via western blotting analysis (Figs. 1A and S1). Expression levels of total and phosphorylated Rb were significantly lower in MB231/PalR cells than in MDA-MB-231 cells (P<0.05, Fig. 1B; P<0.01, Fig. 1C). However, the ratio of phosphorylated Rb to total Rb remained unchanged (Fig. 1D). To investigate the

Table II. IC_{50} values of cyclin-dependent kinase-4/6 inhibitors at 72 and 144 h.

A, Palbociclib

	IC_{50} value, μM		
Exposure time, h	MDA-MB-231	MB231/PalR	R.S.
72	4.90±0.94	10.54±2.25[a]	2.15
144	1.09±0.25	10.13±0.96[a]	9.29

B, Abemaciclib

	IC_{50} value, μM		
Exposure time, h	MDA-MB-231	MB231/PalR	R.S.
72	0.49±0.18	5.57±2.6[a]	11.3
144	0.098±0.03	3.36±1.9[a]	34.2

Each value represents the mean ± standard deviation (n=8). [a]P<0.01 vs. MDA-MB-231 cells (unpaired Student's t-test). IC_{50}, 50% growth inhibitory concentration; R.S., relative sensitivity [calculated by dividing the IC_{50} values in MDA-MB-231/PalR cells (a palbociclib-resistant derivative of MDA-MB-231) by those in parental MDA-MB-231 cells].

Table III. mRNA expression levels of cell cycle-related genes in resistant cells.

Gene	Protein name	mRNA expression levels relative to MDA-MB-231
CCND1	Cyclin D1	0.85±0.22
CCND2	Cyclin D2	0.30±0.06[a]
CCND3	Cyclin D3	1.07±0.50
CCNE1	Cyclin E1	26.2±8.48[a]
CDK4	CDK4	1.43±0.31
CDK6	CDK6	2.46±0.26[a]
RB1	Rb	1.99±1.01
E2F1	E2F	0.86±0.34

Relative mRNA levels of the cell cycle-related genes *CCND1*, *CCND2*, *CCND3*, *CCNE1*, *CDK4*, *CDK6*, *RB1* and *E2F1* in MDA-MB-231 and MB231/PalR cells. The Cq values were used to quantify the PCR products, and relative expression levels of these genes are presented as $2^{-\Delta\Delta Cq}$. ΔCq was calculated by subtracting the Cq of *ACTB* (internal standard) from that of the target gene. Each bar represents the mean ± standard deviation (n=3/group). [a]P<0.01 vs. MDA-MB-231 cells (unpaired Student's t-test). CDK, cyclin-dependent kinase; Rb, retinoblastoma.

Table IV. mRNA expression profiles of ABC transporters in resistant cells.

Gene	Protein name	mRNA expression levels relative to MDA-MB-231
ABCB1	P-glycoprotein	13.1±3.06[a]
ABCC1	MRP1	0.55±0.07[a]
ABCC2	MRP2	1.26±0.30
ABCG2	BCRP	0.23±0.06[a]

Relative mRNA expression levels of the ABC transporters *ABCB1*, *ABCC1*, *ABCC2*, and *ABCG2* in MB231/PalR cells compared to those in the parental MDA-MB-231 cells. The Cq values were used to quantify the PCR products, and relative expression levels of these genes are presented as $2^{-\Delta\Delta Cq}$. ΔCq was calculated by subtracting the Cq of *ACTB* from that of the target gene. Each bar represents the mean ± standard deviation (n=3/group). [a]P<0.01 vs. MDA-MB-231 cells (Student's unpaired t-test). *ABC*, ATP-binding cassette; BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein.

mechanism underlying the post-transcriptional reduction of Rb protein expression, MDA-MB-231 and MB231/PalR cells were treated with 0.25 μM MG-132 for 6 h, a condition that did not induce apparent cytotoxicity, in the absence of palbociclib. However, MG-132 treatment did not restore Rb protein expression in MB231/PalR cells (Fig. S2).

Cyclin E1 protein expression and response to CDK2 inhibition. We performed western blotting to evaluate cyclin E1 protein expression in MB231/PalR cells. Cyclin E1 protein expression was significantly higher in MB231/PalR cells than in MDA-MB-231 cells (P<0.01, Figs. 2A and S3). To investigate the involvement of the cyclin E1-CDK2 axis in palbociclib resistance, cell viability was evaluated using the CDK2 inhibitor CVT-313. MB231/PalR cells exhibited reduced sensitivity to CVT-313 compared with parental cells (IC_{50} values: 9.0±0.5 vs. 4.8±0.2, respectively; P<0.01, Fig. 2B). In addition, co-treatment with 5 μM CVT-313 partially reduced the IC_{50} value of palbociclib in both cells. However, the relative difference in palbociclib sensitivity between the two cell lines was maintained, with MB231/PalR cells remained resistant to palbociclib (IC_{50} values: 5.5±0.7 vs. 2.0±0.7, respectively; P<0.01, Fig. 2C).

mRNA expression profiles of ATP-binding cassette (ABC) transporters and immune checkpoint molecules. To clarify the effects of continuous palbociclib exposure on the expression levels of ABC transporters, we analyzed their mRNA expression profiles. The mRNA levels of ABCB1, which encodes P-glycoprotein, were significantly higher in MB231/PalR cells than in MDA-MB-231 cells (P<0.01, Table IV). In contrast, mRNA levels of ABCC1 and ABCG2, which encode multidrug resistance-associated protein 1 and breast cancer resistance protein (BCRP), respectively, were significantly lower in the resistant cells (P<0.01). Notably, mRNA expression levels of ABCC2, which encodes multidrug resistance-associated protein 2, showed no significant difference between the two cell types. MB231/PalR cells exhibited significantly lower

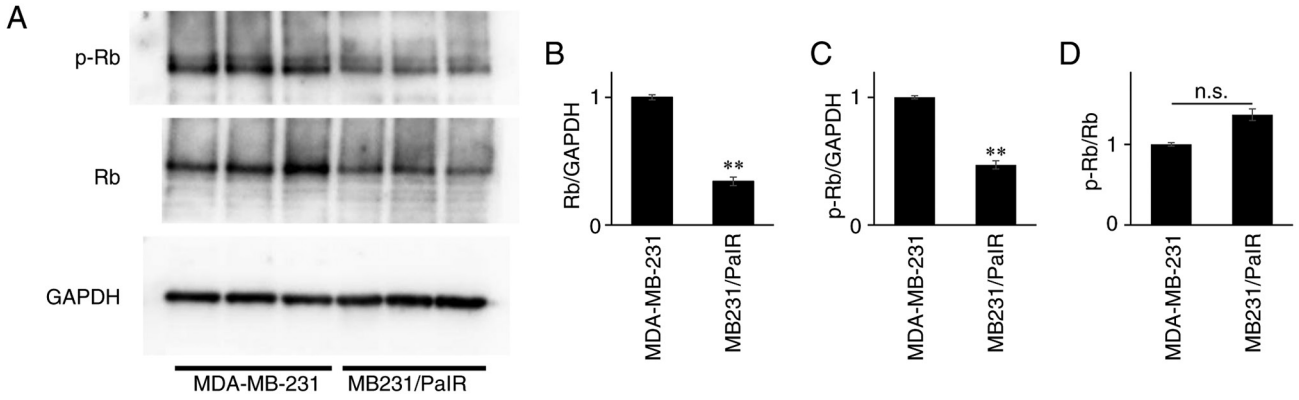

Figure 1. Rb expression and phosphorylation in resistant cells. (A) Western blot analysis of the Rb protein expression and phosphorylation status. GAPDH was used as a loading control. Densitometric semi-quantification of western blot images showing (B) total Rb protein expression, (C) p-Rb protein expression, and (D) the p-Rb/Rb ratio. Data are presented as relative values normalized to MDA-MB-231 sample from a representative experiment. Each bar represents the mean ± standard error of the mean from three independent biological experiments (n=3/group). **P<0.01 vs. MDA-MB-231 cells (unpaired Student's t-test); n.s., not significant. p-, phosphorylated; Rb, retinoblastoma.

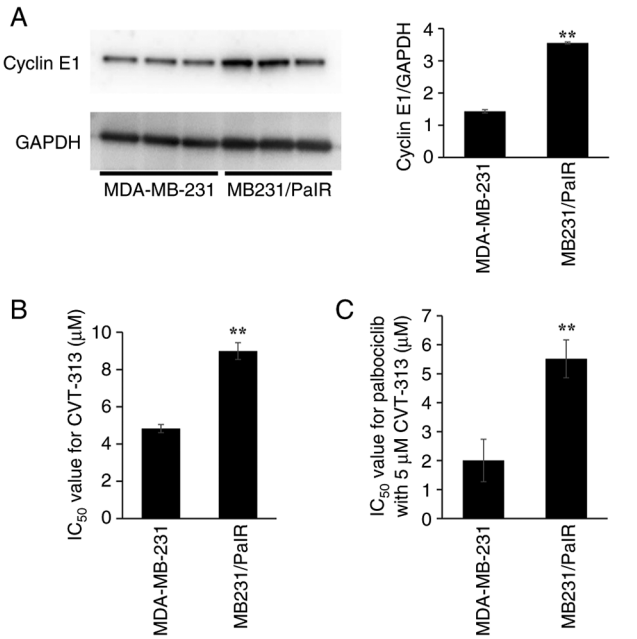

Figure 2. Cyclin E1 expression and effects of CDK2 inhibition in resistant cells. (A) Western blotting of the cyclin E1 protein expression (left panels). GAPDH was used as a loading control. Densitometric semi-quantification of western blot images. Data are presented as relative values normalized to MDA-MB-231 sample from a representative experiment (right graph). Each bar represents the mean ± standard error of the mean from three independent biological experiments (n=3/group). (B and C) Cells were treated for 72 h, and sensitivity was evaluated by MTT assay and IC_{50} values were calculated. (B) Sensitivity to the CDK2 inhibitor CVT-313. (C) Sensitivity to palbociclib in the presence of 5 μM CVT-313. Each bar represents the mean ± standard deviation (n=3-4/group). **P<0.01 vs. MDA-MB-231 cells (unpaired Student's t-test). CDK2, cyclin-dependent kinase; IC_{50}, 50% growth inhibitory concentration.

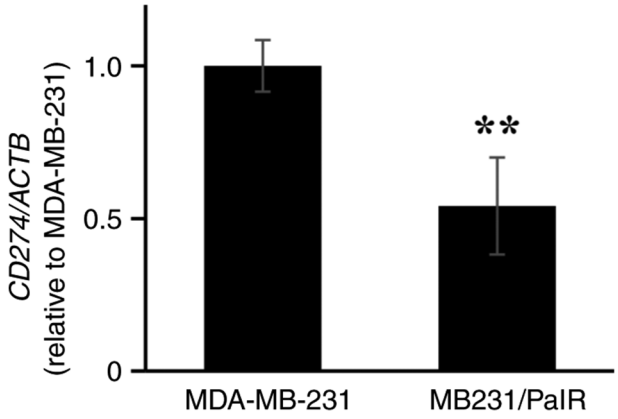

Figure 3. mRNA expression levels of immune checkpoint molecules in resistant cells. Relative mRNA levels of *CD274* in MDA-MB-231 and MB231/PalR cells. The Cq values were used to quantify the PCR products, and relative expression of *CD274* is presented as $2^{-\Delta\Delta Cq}$. ΔCq was calculated by subtracting the Cq of *ACTB* from that of the target gene. Each bar represents the mean ± standard deviation (n=3/group). **P<0.01 vs. MDA-MB-231 (unpaired Student's t-test).

expression levels of CD274, which encodes programmed death ligand 1 (PD-L1), than the parental cells (P<0.01, Fig. 3).

Functional evaluation of P-glycoprotein activity. We evaluated the P-glycoprotein-mediated efflux capacity in MB231/PalR and MDA-MB-231 cells using rhodamine 123, a P-glycoprotein substrate, via flow cytometric analysis. Intracellular accumulation of rhodamine 123 was observed in both cell lines at 30 and 120 min after exposure. The population of cells with low rhodamine 123 accumulation was significantly higher in MB231/PalR cells than in the parental cells at both time points, with a more pronounced difference at 120 min (Fig. 4A and B). To further investigate the contribution of P-glycoprotein to palbociclib resistance, both cell lines were co-treated with palbociclib and the P-glycoprotein inhibitor verapamil. Verapamil co-treatment did not change the IC_{50} values of palbociclib in either cell line (Fig. S4).

Discussion

In this study, we established a palbociclib-resistant TNBC cell line, designated MB231/PalR, by continuous exposure to 1 μM palbociclib. This concentration was selected based on previous studies that established palbociclib-resistant cell lines using 1 μM (23), 4 μM (24,25) palbociclib. Although it is 2.4-fold higher than the clinical C_{max} (0.41 μM) (26), it

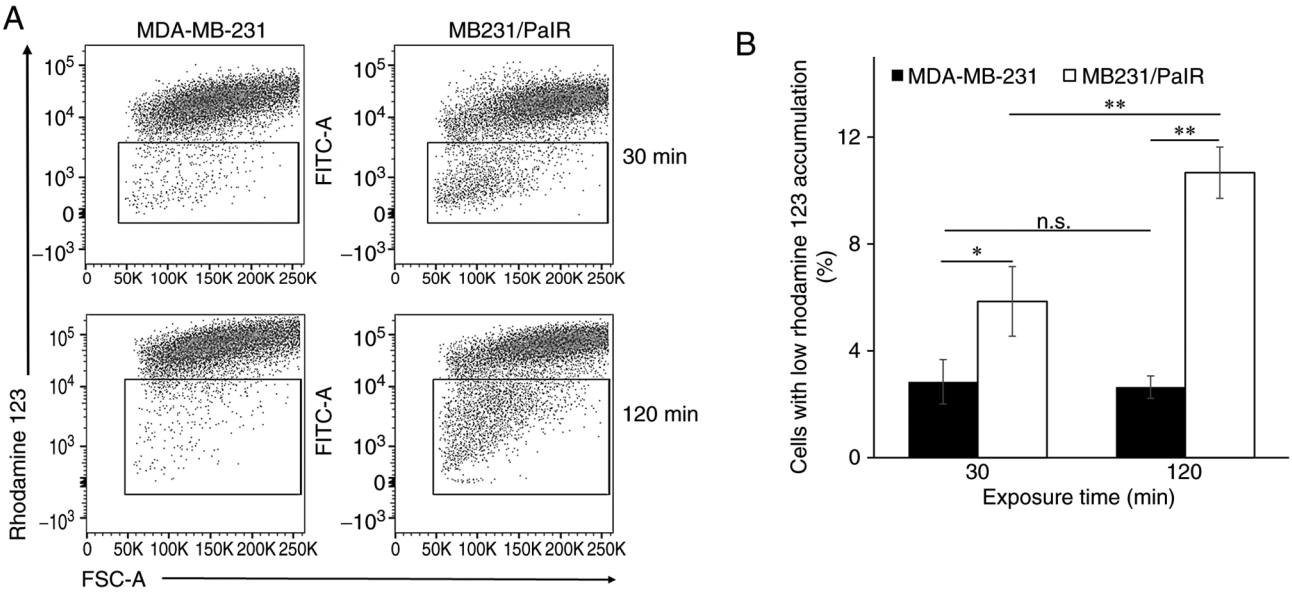

Figure 4. Cells with low rhodamine 123 accumulation detected via flow cytometric analysis. (A) Plots are representative of three independents experiments. (B) Percentages of cells with low rhodamine 123 accumulation at different exposure time points. Each bar represents the mean ± standard deviation (n=3/group). *P<0.05, **P<0.01 (two-way ANOVA followed by the Tukey's post hoc-test); n.s., not significant. FSC-A, forward scatter-area.

was used to provide sufficient selective pressure to induce resistance. The aim of generating resistant sublines is to apply sufficient selective pressure to identify potential adaptive mechanisms, rather than to mimic clinical pharmacokinetics. Nevertheless, the use of a single concentration represents a limitation of this study. We could not determine whether the observed molecular changes occur only upon reaching this concentration or develop in a dose-dependent manner at lower concentrations. Accordingly, it remains unclear whether these changes occur at clinically relevant concentrations, and further validation under such conditions will be required.

Continuous exposure to palbociclib decreased the sensitivity of breast cancer cells to palbociclib. This change was associated with reduced Rb protein levels. In contrast, *RB1* mRNA levels showed no significant changes. These findings suggest that the decrease in protein abundance is regulated at the post-transcriptional or post-translational level. A previous study reported that palbociclib reduces Rb protein levels within one day in breast cancer cell lines (27). Furthermore, a previous study reported that increased ubiquitin-mediated degradation of Rb confers resistance to CDK4/6 inhibitors in breast cancer cells (28). However, Proteasome inhibitor MG-132 treatment did not clearly restore Rb protein expression in MB231/PalR cells, suggesting that mechanisms other than proteasome-dependent degradation may also contribute to the reduction in Rb protein levels. Collectively, these findings suggest that reduced Rb protein levels following long-term palbociclib exposure may contribute to decreased sensitivity to CDK4/6 inhibitors and the acquisition of drug resistance.

In this study, both *CCNE1* mRNA and cyclin E1 protein expression were increased in MB231/PalR cells compared with parental cells. Several studies have indicated that the upregulation of cyclin E1 is a critical mechanism underlying resistance to CDK4/6 inhibition. Palbociclib-resistant cells in HR-positive breast cancer cell lines (MCF-7 and T47D) showed significantly overexpress cyclin E1 which was reversed by a CDK2 inhibitor (29,30). Consistent with these reports, MB231/PalR cells also showed increased cyclin E1 expression. However, in contrast to previous findings, MB231/PalR cells also exhibited resistance to CVT-313. Although co-treatment with CVT-313 reduced the IC_{50} of palbociclib in both parental and resistant cells, the relative difference in palbociclib sensitivity between the two cell lines was maintained. These findings suggest that although cyclin E1 upregulation may contribute to palbociclib resistance, additional molecular alterations may also be involved in MB231/PalR cells. Supporting this interpretation, Guarducci *et al* (31) reported that a common molecular feature associated with palbociclib resistance in HR-positive breast cancer cell lines is the concomitant overexpression of cyclin E1 and downregulation of Rb. These findings are consistent with the characteristics observed in our established MB231/PalR cells, suggesting that reduced Rb protein levels and increased *CCNE1*/cyclin E1 expression contribute to resistance to CDK4/6 inhibitors. However, the precise mechanisms underlying the reduction in Rb protein levels and the upregulation of *CCNE1* following long-term palbociclib exposure remain unclear. Further investigation of these molecular alterations may provide important insights into strategies for overcoming resistance to CDK4/6 inhibitors.

Palbociclib is a substrate of both P-glycoprotein, encoded by *ABCB1*, and BCRP, encoded by *ABCG2* (32). In addition, abemaciclib acts as both a substrate and an inhibitor of P-glycoprotein and BCRP (33,34). Therefore, we evaluated the mRNA expression profiles of ABC transporters and P-glycoprotein efflux capacity in MB231/PalR cells. *ABCB1* mRNA levels were increased, whereas *ABCC1* and *ABCG2* were decreased. Consistently, enhanced P-glycoprotein-mediated rhodamine 123 efflux was observed. However, pharmacological inhibition of P-glycoprotein with verapamil did not change palbociclib sensitivity in either parental or resistant cells. Increased P-glycoprotein or BCRP expression in cancer cells, not limited to breast cancer, contributes to resistance to various

anticancer drugs (35-38). Although ABCB1 overexpression has been reported to reduce sensitivity to palbociclib, and verapamil co-treatment fully reversed this resistance in cells where ABCB1 overexpression was the sole resistance mechanism (39), verapamil did not restore palbociclib sensitivity in MB231/PalR cells. This suggests that the contribution of ABCB1 upregulation to palbociclib resistance in our model may be limited relative to other molecular alterations, such as reduced Rb protein expression and cyclin E1 upregulation. Decreased ABCG2 expression may reduce the efflux of palbociclib and partially counterbalance the effect of increased ABCB1. However, previous studies have shown that ABCG2 overexpression does not significantly contribute to palbociclib resistance (39), and its efflux capacity for palbociclib is lower than that of ABCB1 (40). Therefore, the observed decrease in ABCG2 expression is unlikely to substantially affect drug sensitivity. A previous study reported that CDK6 inhibition leads to *ABCB1* downregulation (41), suggesting that alterations in CDK4/6 signaling partially modulate ABC transporter expression. Continuous exposure to palbociclib may alter the expression profiles of several ABC transporters, as observed in MB231/PalR cells. However, the precise molecular mechanisms underlying these changes remain unclear and require further investigation.

MB231/PalR cells exhibited significantly lower mRNA levels of *CD274*, which encodes PD-L1, than the parental cells. This is notable as the relationship between cyclin D-CDK4/6 and PD-L1 expression remains controversial. Zhang *et al* (42) suggested that cyclin D-CDK4 promotes speckle type BTB/POZ protein-dependent PD-L1 degradation and that CDK4/6 inhibition leads to increased PD-L1 protein levels. In contrast, Shrestha *et al* (43) reported that CDK4/6 inhibitors suppress PD-L1 expression through Rb-mediated inhibition of E2F activation. The reduction in *CD274* mRNA levels in MB231/PalR cells with decreased Rb protein is consistent with the observation of Shrestha *et al* (43), who showed that Rb-mediated suppression of E2F activation regulates *CD274* transcription. However, CDK4/6 also modulates PD-L1 protein degradation via speckle type BTB/POZ protein (42). Therefore, the observed decrease in *CD274* mRNA levels may not directly translate into reduced PD-L1 protein levels on the cell surface. Determining whether this transcriptional decrease translates into reduced surface PD-L1 levels and altered immune interactions is important for the effective combination of CDK4/6 inhibitors and immune checkpoint blockade against TNBC.

MB231/PalR cells exhibited reduced sensitivity to both palbociclib and abemaciclib, indicating the development of cross-resistance to abemaciclib. Moreover, the change in the relative sensitivity of resistant cells was greater for abemaciclib than for palbociclib. Although abemaciclib exhibited stronger antitumor activity than palbociclib, as reflected by its lower IC_{50} value in the parental cells, this difference was more pronounced in the resistant cells. This is possibly because long-term palbociclib exposure induces alterations in the signaling pathways directly associated with the pharmacological action of CDK4/6 inhibitors. Particularly, the reduction in Rb protein levels and increase in *CCNE1* mRNA levels in MB231/PalR cells represent alterations in both the cyclin D-CDK4/6-Rb and cyclin E1-CDK2 pathways, which drive the G1-to-S phase transition (6,44,45). Consistent with our findings, the neoMONARCH Phase II neoadjuvant study in patients with HR-positive breast cancer reported decreased *RB1* mRNA levels and increased *CCNE1* mRNA levels in patients exhibiting resistance to abemaciclib (46). Because abemaciclib more strongly inhibits the cyclin D-CDK4/6 axis than palbociclib, the reduction in drug sensitivity after resistance acquisition may become more pronounced. Collectively, these data suggest that the cellular changes induced by long-term palbociclib exposure reflect alterations in the pathways associated with the pharmacological action of CDK4/6 inhibitors, thereby conferring cross-resistance to both palbociclib and abemaciclib.

Current treatment options for TNBC include chemotherapy, immune checkpoint inhibitors (47), antibody-drug conjugates (48), and PARP inhibitors (49). Despite these advances, drug resistance remains a major clinical challenge. Although CDK4/6 inhibitors are not currently standard treatments for TNBC, they have shown therapeutic potential in preclinical studies of TNBC (11). In this study, we established a palbociclib-resistant TNBC cell line through continuous exposure to palbociclib and characterized its molecular signatures. In addition, the reduction in Rb protein expression observed in resistant cells suggests that resistance may not be fully overcome by strategies targeting the cyclin D-CDK4/6-Rb axis alone. Therefore, combination approaches with agents targeting pathways outside the cell cycle machinery, such as mTOR inhibitors (14) or cytotoxic anticancer drugs (12), may represent rational strategies for overcoming resistance to CDK4/6 inhibitors. Further elucidation of the molecular mechanisms underlying palbociclib resistance may contribute to the optimization of CDK4/6 inhibitor-based therapeutic strategies for TNBC.

A key limitation of this study is that all experiments were performed using a single TNBC cell line (MDA-MB-231). Considering the substantial heterogeneity of TNBC, the extent to which these findings can be generalized to other subtypes remains uncertain. Nevertheless, MDA-MB-231 cells represent a well-characterized basal-like TNBC model with functional Rb signaling, which is particularly relevant for studying mechanisms of resistance to CDK4/6 inhibition. The observed molecular changes, including Rb protein loss, *CCNE1*/cyclin E1 upregulation, and *ABCB1* induction, are biologically plausible and consistent with previously reported resistance-associated pathways in HR-positive breast cancer cell lines (31,39,50). Future studies incorporating additional TNBC cell lines and patient-derived models will be important to validate and extend these findings. Another limitation of this study is that EMT-related changes were not evaluated in the resistant cells. EMT is an important process associated with cancer progression and drug resistance, and evaluation of EMT markers may provide a more comprehensive characterization of the resistant phenotype. However, the present study focused primarily on characterizing the molecular signatures of cells that acquired resistance following prolonged palbociclib exposure. Therefore, whether prolonged palbociclib exposure induces EMT-like phenotypic changes in MB231/PalR cells remains unclear. Future studies evaluating epithelial and mesenchymal markers, such as E-cadherin, N-cadherin, vimentin, and Snail, will be needed to further characterize the phenotypic changes associated with acquired resistance.

In conclusion, the present study established a palbociclib-resistant TNBC cell model and identified molecular alterations associated with acquired resistance, including

reduced Rb protein expression and increased *CCNE1*/cyclin E1 expression. These findings provide insight into the mechanisms underlying CDK4/6 inhibitor resistance and may contribute to the identification of predictive biomarkers and the development of novel therapeutic strategies to overcome resistance in TNBC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding authors.

Authors' contributions

DE and KT conceived and designed the study. DE, YI, NI and HO prepared the experimental materials and conducted the experiments. DE, YI, NI, HO, YU, MM and TS contributed to data acquisition and preliminary data interpretation. DE and KT performed the statistical analysis, interpreted the data and analyzed the results. KT provided supervision, critically revised the manuscript for important intellectual content and approved the final version of the manuscript. DE and KT confirm the authenticity of all the raw data. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the work, ensuring that any questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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