ABSTRACT

Purpose: Paclitaxel is a cytotoxic chemotherapy commonly used in patients with triple negative breast cancer (TNBC); however, the resistance to paclitaxel is a cause of poor response in the patients. The aim of this study was to examine the role of protein phosphatase 1H (PPM1H) in paclitaxel resistance in breast cancer patients.

Methods: To investigate the function of PPM1H in paclitaxel treatment, we conducted in vitro assays and molecular experiments using a stable cell line (MDA-MB-231) in which PPM1H is overexpressed. We also performed molecular analyses on patient tissue samples. Molecular expression related to PPM1H in breast cancer patients was analyzed using TCGA data.

Results: We investigated whether PPM1H was associated with paclitaxel resistance in breast cancer. PPM1H expression was upregulated in breast cancer cells treated with paclitaxel. We also observed that overexpression of PPM1H in breast cancer cells resulted in increased sensitivity to paclitaxel in vitro. Additionally, paclitaxel treatment induced dephosphorylation of cyclin-dependent kinase inhibitor p27 (p27), which was more evident in PPM1H-overexpressing cells. To understand how upregulation of PPM1H increases paclitaxel sensitivity, we determined the levels of p27, phospho-p27, and CDK2, since CDK2 exerts antagonistic effects against PPM1H on p27 phosphorylation. The patient-derived xenograft (PDX) tumors that did not respond to paclitaxel showed increased levels of CDK2 and phospho-p27 and decreased levels of total p27 compared to the other breast tumor tissues. The use of dinaciclib, a selective CDK inhibitor, significantly inhibited tumor growth in the PDX model.

Conclusion: CDK2 kinase activity was significantly upregulated in basal breast cancer tumors and was negatively correlated with p27 protein levels in the TCGA breast cancer dataset, suggesting that targeting CDK2 may be an effective treatment strategy for TNBC patients.

Keywords: Cyclin-dependent kinase 2; Cyclin-dependent kinase inhibitor p27; Paclitaxel; PPM1H protein
INTRODUCTION

Breast cancer is the leading cause of death in women worldwide [1]. Although the treatment outcome for breast cancer patients has substantially improved during the last few decades, more than one third of patients with surgery and systemic adjuvant therapies ultimately develop systemic recurrence after 15 years of follow-up [2]. Patients with distant metastasis often receive cytotoxic chemotherapy to delay disease progression and to maintain their quality of life. However, most patients eventually develop resistance to anti-tumor agents through complex and diverse mechanisms [3].

Paclitaxel, a microtubule stabilizing agent, is one of the most commonly used cytotoxic chemotherapy agents in breast cancer patients. Despite the rapid development of agents targeting breast cancer, efforts to improve taxane delivery and to develop more effective taxanes are still ongoing [4]. P-glycoprotein, an MDR1 gene product, has been reported to be involved in paclitaxel resistance, but other molecular mechanisms regulating the response to paclitaxel are largely unknown [5].

During chemotherapy, the cancer cells evolve in complex and dynamic ways which can be diverse and unique to each tumor. For example, trastuzumab-resistant cells display lower expression of the serine/threonine phosphatase protein phosphatase 1H (PPM1H) [6]. In this study, we investigated how PPM1H responds to paclitaxel treatment in tumor cells, using PPM1H-overexpressing triple negative breast cancer (TNBC) cell lines. Our results present the possibility of a novel mechanism that can overcome tumor cell resistance to paclitaxel treatment. Our results also suggest that a subset of TNBC patients may benefit from targeting the cyclin-dependent kinase (CDK) signaling pathways.

METHODS

Cell culture
MDA-MB-231 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. PPM1H overexpressing MDA-MB-231 cells were cultured in DMEM 10% FBS, 1% penicillin/streptomycin, and 0.05 µg/mL puromycin. Cells were sub-cultured every 3 to 4 days.

PPM1H overexpression
Human PPM1H cDNA was amplified by reverse-transcription polymerase chain reaction (PCR) from SK-BR-3 human breast cancer cell lines obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) using the primers 5’-GCTAGCGCCACCATTGCTCAGTGAAATCT-3’ and 5’-CCGGAATTCCGGTGACAGCTTTGCAT-3’. The PCR product was ligated at the NheI and EcoRI sites of the Pgem-T Easy vector (Promega, Madison, USA). The resulting sequence was inserted into pCDH-CMV-MCS-EF1-RFP-T2A-Puro (System Biosciences, Palo Alto, USA).

Quantitative real-time PCR
RNAs were extracted from the cells lysed by TRIzol (Favorgen, Pingtung, Taiwan). The Prime Script 1st strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan) was used for reverse-transcription of RNA, and then cDNA was amplified using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA). The sequences of the
primers used for amplifying PPM1H are 5′-CCAATTTCATGGGCGGCATC-3′ and 5′-TCCACCTCGTCTGAGACAG-3′.

**Western blotting**

Proteins were harvested with RIPA buffer (Thermo Scientific, Palm Springs, USA) including 1 mM protease and phosphatase inhibitors and 0.5 M ethylenediaminetetraacetic acid. Protein concentration was measured using the BCA Assay kit (Thermo Scientific). Cell lysates were loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk and incubated with primary antibodies against PPM1H, p27 and phospho-p27 (Abcam, Cambridge, UK) overnight at 4°C. A peroxidase-conjugated secondary antibody was used for detection. Bands were detected using an Amersham Imager 600 (GE Healthcare Life Sciences, Pittsburgh, USA).

**Cell proliferation, migration, and invasion assays**

For proliferation assays, cells were seeded in triplicate into 96-well plates at a density of 3,000 cells/well. Proliferation assays were conducted using the CellTiterGlo Luminescent Cell Viability Assay kit (Promega) or MTT solution following the manufacturer’s protocol. For migration assays, 2 × 10^4 cells were seeded in an insert (8 μm pore size) with serum-free media. Media with 10% FBS was added in lower chambers. Cells were incubated for 24 hours, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. For invasion assays, 9.8 µg/mL of Matrigel was added to an insert before seeding cells. Experiments were performed in duplicate.

**Three-dimensional (3D) culture, soft-agar colony formation, and drug sensitivity screening**

The 3D culture for formation of spheroids was performed using growth factor-reduced Matrigel (BD Sciences, San Jose, USA) [7]. Growth in the Matrigel was measured by counting the number of spheroids using ImageJ software (U.S. National Institutes of Health, Bethesda, USA). For 3D drug screening, after coating the plate with Matrigel, the cells were seeded 3 times in 3D 96-well plates at a density of 3,000 cells/well. To test sensitivity to various anti-cancer compounds, cells were treated with 5 µM of each breast cancer chemotherapy drug. After 72 hours of drug treatment, cell viability was measured by Celltiter glo-3D (Promega).

For soft-agar colony formation, 3,000 cells/well were seeded in triplicate into 6-well plates. Paclitaxel (Merck, Darmstadt, Germany) was administered every 4 days. After 2 weeks, colonies were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet.

**Immunohistochemistry (IHC)**

IHC was performed with the IHC Staining kit (Dako, Santa Clara, USA). Tissue sections were deparaffinized in xylene, hydrated in phosphate buffered saline, and blocked with normal goat serum. Slides were incubated with anti-PPM1H (Abcam) at 4°C overnight. The following day, tissue sections were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Sections were developed with diaminobenzidine and hydrogen peroxide, which produces brown precipitate, counterstained with hematoxylin, dehydrated, and mounted.

**Paclitaxel treatment in patient-derived xenograft (PDX) models**

We established TNBC PDX models derived from patients’ primary and recurrent tumors. For each TNBC PDX model, control (n = 5) and experimental (n = 5) groups were treated...
with intraperitoneal injection of PBS (control), paclitaxel (15 mg/kg), or dinaciclib (10 mg/kg) for 4 weeks. After treatment, residual tumor tissues were harvested. The mice were cared for according to the institutional guidelines for animal care. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Seoul National University (No. 14-0016-C0A0). Acquisition of human tissues for establishment of PDX models was approved by the Institutional Review Board (IRB) of Seoul National University Hospital (IRB No. 1402-054-555). All procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the participating patients.

**Statistics**

Data are expressed as mean ± standard deviation using 2-tailed Student's t-tests or Mann-Whitney U tests. A 2-sided p-value < 0.05 was considered statistically significant. GraphPad Prism 8 (GraphPad Software, San Diego, USA) was used for generating graphs and performing statistical tests.

**RESULTS**

**PPM1H overexpression sensitizes breast cancer cells to paclitaxel**

To understand the role of PPM1H in determining paclitaxel sensitivity, we screened PPM1H gene expression levels in breast cancer cell lines and observed that TNBC cells showed lower levels of PPM1H mRNA (Figure 1A). Accordingly, downregulation of PPM1H in TNBC cells was consistently observed in the METABRIC dataset (Figure 1B) [8]. *In vitro* paclitaxel treatment significantly increased PPM1H expression in MDA-MB-231 cells (Figure 1C).

Increased expression of PPM1H was also confirmed in tumor tissue of TNBC patients receiving paclitaxel treatment (Supplementary Figure 1). Next, we established stable MDA-MB-231 cells overexpressing PPM1H (Supplementary Figure 2A), and these cells did not show differences in cell proliferation, migration, or invasion (Figure 1D and E). We also performed 3D cell culture in Matrigel. PPM1H-overexpressing cells developed fewer spheroids (Figure 1F) that were smaller in size compared to those in the control cells (Supplementary Figure 2B).

As PPM1H is reported to be involved in regulating resistance to trastuzumab [6], which shares a common cell cycle-related mechanism of action with paclitaxel, we explored whether PPM1H overexpression was associated with a different response to paclitaxel. PPM1H-overexpressing MDA-MB-231 cells were more sensitive to paclitaxel treatment in 2-dimensional (2D) monolayer culture (Figure 1G). The increased sensitivity to paclitaxel in PPM1H-overexpressing MDA-MB-231 cells was also tested and observed in 3D Matrigel culture systems, since studies have shown that 3D culture systems can reflect drug responsiveness more accurately than 2D culture methods (Figure 1H) [9]. A soft agar colony formation assay, another well-established assay for testing drug sensitivity [10], also identified increased sensitivity to paclitaxel in PPM1H-overexpressing cells (Figure 1I). PPM1H-overexpressing cells were more sensitive to paclitaxel (≥ 40 nM) in soft-agar assays (Supplementary Figure 2C). These data indicate that PPM1H upregulation is associated with increased resistance to paclitaxel.

**PPM1H mediates p27 induction and dephosphorylation by treatment with paclitaxel**

As mentioned above, PPM1H has been identified as phosphatase that impacts p27 stability by dephosphorylation at T187. We tested whether PPM1H overexpression affected p27
dephosphorylation during treatment with paclitaxel. After treatment with paclitaxel, the amount of PPM1H increased in a time-dependent manner (Figure 2A and B), and p27 levels were elevated (Figure 2A and C). After 72 hours of paclitaxel treatment, increased PPM1H levels induced the dephosphorylation of p27 (Figure 2A and D). These data indicate a relationship between PPM1H and p27 during paclitaxel treatment.

**CDK2 activity mediate resistance to paclitaxel in basal-type breast cancers**

Our findings suggest that PPM1H overexpression increases the dephosphorylation of p27, thereby maintaining a high amount of p27 and conferring increased sensitivity to paclitaxel in breast cancer cell lines. After being phosphorylated by CDK2 at T187, the p27 protein
undergoes ubiquitination and proteasomal degradation [11,12]. Therefore, we further investigated the hypothesis that CDK2 is involved in increasing levels of PPM1H and p27. To further verify that paclitaxel increases PPM1H levels in PDX models, we performed IHC of PPM1H (Figure 3A). We observed that the PDX tumor model derived from a TNBC patient, which showed primary resistance to the paclitaxel treatment, showed significantly higher levels of CDK2 and phospho-p27, and downregulation of p27 protein when compared to other primary breast tumors of various subtypes (Figure 3B and C). In the PDX tumor model, despite upregulation of PPM1H by paclitaxel, the cause of resistance is attributed to the phosphorylation of CDK2 and p27. These data suggest that our PDX model is characterized by high CDK2 activity compared to other breast cancer cases. Thus, since our results suggest the presence of active CDK2 signaling in our TNBC PDX model, we tested the anti-tumor efficacy of the selective CDK 1,2,5,9 inhibitor dinaciclib in our PDX model. As shown in Figure 3D, dinaciclib treatment significantly inhibited PDX tumor growth. To further determine the efficacy of dinaciclib compared to other commonly used anti-tumor compounds, we tested the viability of TNBC cells derived from either primary tumors or from PDX tumors when exposed to fixed doses of 28 different anti-cancer drugs in a 3D culture system. Dinaciclib treatment led to the largest reduction in cell viability when compared to the other agents tested (Figure 3E). The TCGA breast cancer dataset shows that CDK2 activity is substantially higher in basal breast tumors compared to other molecular subtypes (Figure 3F). CDK2 activity is negatively correlated with p27 protein levels in TCGA breast cancer cases for which proteomic profiling data was available (Figure 3G). Our data suggest that targeting CDKs can be a potentially effective strategy in patients with basal tumors.
DISCUSSION

Targeting CDKs in breast cancer is a promising therapeutic strategy, considering their recent success in several clinical trials involving hormonal receptor-positive breast cancer patients [13-15]. The use of CDK inhibitors in hormone receptor-positive tumors is a result of the lack of in vitro efficacy of CDK4/6 inhibitors in basal cancer cell lines shown by Finn et al. [16]. Recently, McCurdy et al. [17] have developed an mRNA gene signature that reflects CDK2 kinase activity in multiple tumor types.

While initial studies addressing the efficacy of CDK inhibitors have focused on their use in hormone receptor-positive breast tumors, recent studies have raised the possibility that a subset of TNBC patients can also benefit from CDK-targeting approaches. Horiuchi et al.

**Figure 3.** CDK2 activity in the PDX tumor and basal-type breast cancers. (A) Immunohistochemical analysis of PPM1H in TNBC PDX tumor tissue treated with vehicle or paclitaxel. (B) Western blot showing the expression of p27, phospho-p27, and CDK2 in PDX tumors and primary breast tumor tissues and (C) the corresponding quantifications. (D) The activity of dinaciclib in the paclitaxel-resistant PDX models. (E) Heatmap showing the growth-inhibiting effects of multiple anti-cancer drugs in basal-subtype breast tumors and PDX models. (F) The CDK2 activity signatures in TCGA breast cancer subtypes. (G) The correlation between p27 protein levels and CDK2 signatures in the TCGA breast cancer dataset.

PDX = patient-derived xenograft; LumA = Luminal A type of breast cancer; LumB = Luminal B type of breast cancer; HER2 = human epidermal growth factor receptor 2; TNBC = triple negative breast cancer; p27 = Cyclin-Dependent Kinase (CDK) Inhibitor p27; phospho-p27=phosphorylation of p27; CDK2 = Cyclin-Dependent Kinase 2; TCGA = The Cancer Genome Atlas.

\*p < 0.05; \*p < 0.01; \*p < 0.001; \*p < 0.0001; Student’s t-test.
have suggested that MYC-activated TNBC cases may benefit from CDK inhibitors such as purvalanol or dinaciclib. Asghar et al. [19] have also shown that the luminal androgen receptor subtype of TNBC is characterized by increased sensitivity to the CDK4/6 inhibitor palbociclib. Along with our observations, these data suggest that the potential benefits of CDK inhibitors should also be explored in TNBC patients in addition to its current target population of hormone receptor-positive cases.

Our study carries several limitations. First, we were not able to conduct in vivo experiments using a large set of PDX models. While we have included other TNBC cases in our drug sensitivity screening and additionally analyzed TCGA datasets, our results still need further validation using a larger cohort of breast cancer patients. Second, our suggestion that PPM1H expression may increase paclitaxel sensitivity has not been tested in preclinical models. Third, our drug sensitivity screening experiments used a fixed dose of diverse anti-cancer agents, which may have obscured the efficacy of other drugs at different concentrations.

In conclusion, our data show that PPM1H, a gene which is induced during paclitaxel treatment, sensitizes breast cancer cells to paclitaxel. Despite upregulation of PPM1H, resistance to paclitaxel in the PDX model may be caused by increased expression of CDK2 and p27. There is an inverse relationship between PPM1H and CDK2 in cell cycle regulation. The amount of PPM1H and CDK2 affects cell cycle and anticancer effects. The paclitaxel resistance model suggests that phosphorylation of p27 and CDK2 has a greater effect than PPM1H. Thus, drug screening using multiple TNBC samples and TCGA data analysis suggest that selective CDK inhibitors can be an effective approach in TNBC treatment.

SUPPLEMENTARY MATERIALS

Supplementary Figure 1
Immunohistochemical analysis of PPM1H in primary TNBC breast tumor tissues. Scale bar = 25 μm.

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Supplementary Figure 2
(A) Western blot of PPM1H o/e in MDA-MB-231 cells; (B) 3D Matrigel growth; (C) Soft agar colony formation assay. Scale bar = 100 μm.

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