Effect of Bevacizumab on a Human Breast Cancer Model that Exhibited Palbociclib-resistance by RB Knockout

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Abstract. Background/Aim: Although CDK4/6 inhibitors have been increasingly used in combination with hormonal agents to treat hormone-receptor positive and human epithelial growth factor receptor 2-negative breast cancer, the mechanism of CDK4/6 inhibitor resistance and its impact on established therapy for post-resistance, especially bevacizumab combined with chemotherapy, are unclear. Materials and Methods: Sensitivity of RB knockout MCF7 clones to CDK4/6 inhibitors was evaluated in vitro. One RB knockout clone was subcutaneously implanted in nude mice and the effects of bevacizumab on volume and microvessel density (MVD) of tumors were investigated. Results: Palbociclib did not exhibit antitumor efficacy against the RB knockout tumor, in contrast to the parental MCF7 xenograft model. Bevacizumab significantly exhibited antitumor efficacy and suppressed the MVD both in RB knockout and parental MCF7 xenograft models. Conclusion: Bevacizumab inhibited tumor growth by suppressing MVD in the CDK4/6 inhibitor-resistant tumor acquired due to RB loss, suggesting its efficacy also in patients after treatment with CDK4/6 inhibitors.

In the patients with hormone receptor (HR)-positive, human epithelial growth factor receptor 2 (HER2)-negative breast cancer, hormone therapy is prescribed as standard therapy. However, resistance to this therapy eventually develops, and the treatment shifts to chemotherapy. As a standard therapy for 1st or 2nd line chemotherapy, bevacizumab has been used in combination with chemotherapeutic agents such as paclitaxel, capecitabine, and anthracycline-based therapy (1, 2). Recently, it has been reported that CDK4/6 inhibitors, such as, palbociclib, abemaciclib, and ribociclib, in combination with hormone therapies prolong overall survival (OS) or progression-free survival (PFS) compared to hormone therapy alone in patients with advanced HR-positive, HER2-negative breast cancer (3-5). Currently, these combinations are used as a first or second line hormone therapy for patients with HR-positive and HER2-negative metastatic breast cancer (1, 2).

CDK4/6 binds to Cyclin D synthesized in response to stimuli from estrogen receptors and cell mitogens to form a complex (6). This complex collaborates with the cyclin E-Cdk2 complex to phosphorylate and inactivate the retinoblastoma tumor suppressor gene (Rb; encoding RB), a gatekeeper to prevent cell cycle progression from G1 to the S phase, resulting in cell cycle progression (6, 7). The above are part of an important checkpoint mechanism of the cell cycle and are tightly regulated in normal cells. CDK4/6 inhibitors effectively restrict excessive cell cycle progression and inhibit tumor growth by preventing phosphorylation of RB and thus activating RB (8). Hormone therapies in combination with CDK4/6 inhibitors also eventually develop resistance (9-11), therefore research into this particular resistance is also underway. Specifically, in a study examining patients before and after fulvestrant monotherapy and the combination therapy with palbociclib, it was reported that mutations, found only after the combination therapy, occurred only in the Rb locus (9). These Rb mutations appeared in 4.7% of patients, and were either stop codon acquisitions or frameshift deletions, and also were very likely to abrogate the function of RB. Mutations in Rb are rare in primary breast cancer (12). In preclinical studies, phenomena related to RB inactivation such as RB loss, Cyclin E1/E2 amplification (13), and CDK6 amplification...
(14), were observed in resistant tumors after long-term exposure to CDK4/6 inhibitors. Based on the above findings, we focused on the dysfunction of RB located downstream of the CDK-RB axis as a potential mechanism for CDK4/6 inhibitor resistance. To the best of our knowledge, there have been no investigations on whether RB knockout reduces the sensitivity of breast cancer cells to CDK4/6 inhibitors. That is, RB loss has not been directly linked to the cause of resistance to CDK4/6 inhibitors in HR-positive and HER2-negative breast cancers.

However, previously reported clinical trials exhibiting the efficacy of chemotherapy after hormone therapy did not include patients who received hormone therapy in combination with CDK4/6 inhibitors (15-19). The efficacy of chemotherapeutic agents alone after the acquisition of CDK4/6 inhibitor resistance has been reported, albeit in a preclinical study using cells with long-term exposure to CDK4/6 inhibitors (20). However, it has not been investigated whether bevacizumab, which is used in combination with chemotherapeutic agents in clinical settings, is effective after the acquisition of CDK4/6 inhibitor resistance.

This study investigated the effect of RB loss on the acquisition of CDK4/6 inhibitor resistance in HR-positive HER2-negative breast cancer cells sensitive to CDK4/6 inhibitors, and the effect of bevacizumab in a model of such resistance.

Materials and Methods

Antitumor agents. Palbociclib (PD 0332991) isethionate, abemaciclib, and paclitaxel were purchased from LC Laboratories (Woburn, MA, USA), Selleck (Houston, TX, USA), and Tokyo chemical industry (Tokyo, Japan), respectively. Bevacizumab was manufactured by Chugai pharmaceutical (Tokyo, Japan).

Cell lines and culture conditions. The MCF7 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in the Minimum Essential Medium Eagle (Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Nichirei, Tokyo, Japan), 2 mM L-glutamine (Merck KGaA), 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA, USA), 0.1 mM MEM NEAA (Thermo Fisher Scientific), 1.5 g/l sodium bicarbonate (Merck KGaA) and 10 μg/ml insulin solution from bovine pancreas (Merck KGaA).

Establishment of RB knockout cells using CRISPR/CAS9 system. Single guide RNA (sgRNA) was synthesized using the Guide-it sgRNA in vitro transcription kit (Takara Bio, Siga, Japan). The target gene was Rb. Primer sequence for sgRNA production was as follows: 5’- CACCCTCTAACGACTCAGTATTAGGTCGCAGGGGT TCCGGGCTTTTAAAGAGCTATGC-3’. Prior to the transfection of ribonucleoprotein (RNP), the complex of sgRNA and Cas9 protein [Guide-it Recombinant Cas9 (Electroporation-Ready); Takara Bio], into MCF7 cells, it was confirmed by sequence analysis that the sequence of the genome editing target region of MCF7 cells matched the reference sequence (NCBI: NC_000013.11). Sequence analysis was performed as follows: PCR was performed using genomic DNA generated from MCF7 cells using Simple Prep reagent for DNA (Takara Bio) as a template, and the obtained PCR product was cleaned up using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific). Then, sanger sequence analysis was performed. The PCR primer sequences were as follows: forward, 5’-GCCAGGTTTCCGAGTTATTTCTC-3’; Reverse, 5’-CTCAACAGAGGGAACCTGC ACCTG-3’. Using the Neon Transfection System (Thermo Fisher Scientific), RNP was transfected into MCF7 cells under the conditions of 1,200 V, 20 ms, 2 times. The cells were confirmed to have undergone target-specific cleavage by genome editing using the Guide-it Mutation Detection Kit (Takara Bio), and then single-cell cloning was performed. By confirming the target sequence of the obtained clones using sequence analysis, knockout clones of the target gene (Rb) were identified. The sequencing primers used were the same as the PCR primers.

Western blotting analysis. Cell pellets were homogenized with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) containing Complete Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche). The homogenate was centrifuged at 14,000 rpm, 4°C for 5 min. The resultant supernatant was used for the assays. Protein concentration of the supernatant was quantified by using a Direct Detect spectrometer (Merck KGaA). Cell lysates (1–10 μg protein/lane) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using an iBlot 2 Dry Blotting System (Thermo Fisher Scientific). The membrane was blocked with Blocking One (Nacalai Tesque; Kyoto, Japan) and was primarily treated with an antibody against RB (abcam; Cambridge, UK), p107 (Cell Signaling Technology), p130 (Cell Signaling Technology), and β-actin (Cell Signaling Technology). ECL Prime Western Blotting Detection Reagents (GE Healthcare, Chicago, IL, USA) and ChemiDOC Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) were used for detection.

In vitro cell proliferation assay. MCF7 and its RB knockout cell lines, MCF7-RBKO-A02, -B02, and -C03 were plated onto 96-well plates (Corning, Corning, NY USA) (5×10³ cells/well). After overnight incubation (Day 1), palbociclib, abemaciclib or paclitaxel was dissolved in dimethyl sulfoxide (Merck KGaA), added at specific concentrations as shown in Figure 1B, C, and D. After an additional 6-day incubation (Day 7), the remaining (live) cells were estimated using a DNA quantification assay (FluoReporter Blue Fluorometric dsDNA Quantitation kit; Thermo Fisher Scientific). Percentage of live cells was estimated using the following equation: % of live cells=(a-b)/(c-b), where a, b and c are the fluorescence intensity on Day 7, fluorescence intensity on Day 1, and fluorescence intensity of control treatment on Day 7, respectively. The 50% growth-inhibitory concentration (IC₅₀) was calculated using the following equation: IC₅₀=[10^(LOG(A/B)+(50-C)/(D-C)+LOG(B))], where A is the higher concentration derived from the two values located immediately on both sides of the 50% cell viability, B is the lower concentration derived from the same two values, C is the survival rate determined for B, and D is the survival rate determined for A.

Animals. Female 5- or 6-week-old BALB/c-nu/nu mice were purchased from Charles River Japan (Kanagawa, Japan). All
animals were housed in a specific pathogen-free environment under controlled conditions (temperature, 20°C-26°C; humidity, 35%-75%; 12 h light/12 h dark cycle) and allowed to acclimatize and recover from shipping-related stress for more than 6 days prior to the study. Chlorinated water and irradiated food were provided ad libitum. The health of the mice was monitored by daily observation. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd. (approval no. 19-383) and confirmed to the Guide for the Care and Use of Laboratory Animals published by the Institution of Laboratory Animal Research (ILAR).

In vivo tumor growth inhibition studies. BALB/c-nu/nu mice were subcutaneously implanted with 0.72 mg beta-estradiol pellet (Innovative Research of America, Sarasota, FL, USA) in the back. On the next day, MCF7 or its RB knockout cell line, MCF7-RBKO-A02, (5x10^6 cells) in 100 μl 50% Matrigel Basement Membrane Matrix (Corning, Inc.) Minimum Essential Medium Eagle was subcutaneously inoculated into the right flank of these mice. From the day of implanting the beta-estradiol pellets or the next day, to prevent urinary tract disorders or related death due to the pellets, 4 mg/0.1 ml/mouse ceftriaxone sodium salt (Nichikou, Toyama, Japan) was administered subcutaneously to the back approximately twice a
week. Mice with established tumors were randomly allocated to each treatment group (Day 1). For treatment, palbociclib (a CDK4/6 inhibitor) or bevacizumab (a humanized anti-VEGF antibody) were used. Palbociclib at a dose of 50 mg/kg body weight or 50 mM lactate buffer (pH4) as control was administered (10 ml/kg body weight) perorally to the mice for 5 days, with 2 days off. Bevacizumab or human immunoglobulin G (HulG, MP biomedicals, Irvine, CA, USA) was diluted in saline (Otsuka, Tokyo, Japan) and administered intraperitoneally (10 ml/kg body weight) to the mice at a dose of 25 mg/kg weekly from Day 1. After the experiments, all animals not to be sampled for tumor tissue were euthanized by CO2 asphyxiation with a CO2 displacement rate of 20% of the chamber volume per min, followed by cervical dislocation; and the animals to be sampled for tumor tissue were euthanized by exsanguination under 2.0-2.5% isoflurane inhalation anesthesia using isoflurane inhalation solution (Pfizer, New York, NY, USA). Tumor volume was measured once a week. Tumor volume was estimated using the following equation: Tumor volume=ab^2/2, where a and b are the tumor length and width (a>b), respectively.

Immunohistochemistry and quantification of microvessel density (MVD) in tumor tissues. MVD in tumor tissues was evaluated by immunohisostchemical staining of CD31. Tumor samples were collected on Day 39. Fresh frozen blocks were prepared from the collected tumors with Optimal cutting temperature (O.C.T.) compound (Sakura Finetek Japan, Tokyo, Japan). Immunohisostchemical staining was conducted as described previously (21). In brief, immunohistochecmical analysis of CD31 was conducted using a Rat HRP-Polymer 1-Step (mouse adsorbed) system (cat. no. BRR4016; Biocare Medical, Irvine, NC USA) according to the manufacturer’s protocols. As the primary antibody, rat anti-mouse CD31 monoclonal antibody (clone MEC 13.3; dilution, 1:500; cat. no. 553370) was purchased from BD Biosciences (San Jose, CA, USA). MVD (%) was calculated from the ratio of the CD31-positive staining area to the total observation area in the viable region. Positive staining areas were calculated using imaging analysis software (Definiens Tissue Studio, version 3.60, Definiens, Carlsbad, CA, USA).

Statistical analysis. The results are presented as mean±standard deviation. For comparisons between two groups, data were analyzed using the Wilcoxon rank sum test. p<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using JMP software (version 15.0.0; SAS Institute, Cary, NC USA).

Results

RB knockout reduced the sensitivity to CDK4/6 inhibitors. First, RB knockout cells were established from the ER-positive, HER2-negative breast cancer MCF7 cells using the CRISPR/CAS9 system. The expression of RB protein in the representative RB knockout clones, MCF7-RBKO-A02, -B02, and -C03, was determined by western blotting analysis. No expression of RB protein was observed in these clones (Figure 1A). The expression of p107 and p130, which are family proteins of RB, tended to be similar to or higher than that of MCF7 cells. These results indicated the RB-specific knockout in these clones.

Table 1. IC50s of palbociclib, abemaciclib, and paclitaxel in breast cancer cells (mean).

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<tr>
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<th>Palbociclib (nM)</th>
<th>Abemaciclib (nM)</th>
<th>Paclitaxel (nM)</th>
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<tbody>
<tr>
<td>MCF7</td>
<td>56</td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td>MCF7-RBKO-A02</td>
<td>1,326</td>
<td>251</td>
<td>2</td>
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<tr>
<td>MCF7-RBKO-B02</td>
<td>1,377</td>
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<td>2</td>
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<tr>
<td>MCF7-RBKO-C03</td>
<td>1,127</td>
<td>198</td>
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Using these clones, the effects of RB knockout on the sensitivity against CDK4/6 inhibitors were investigated by in vitro proliferation assay. The 50% growth-inhibitory concentrations (IC50s) in MCF7-RBKO clones for palbociclib was approximately 20-25 times higher than those in parental MCF7 cells (Table 1). The IC50s in MCF7-RBKO clones for abemaciclib was approximately 7-16 times higher than those in parental MCF7 cells. The IC50s in MCF7-RBKO clones of PTX were similar to those in parental MCF7 cells. The growth inhibitory curves of these cells are shown in Figure 1B, C, and D. This result indicated that RB knockout reduced the sensitivity against CDK4/6 inhibitors. However, these clones exhibited similar sensitivity against paclitaxel compared with parental MCF7 cells.

MCF7-RBKO-A02 was a CDK4/6 inhibitor-resistant tumor model in vivo. Next, to confirm the resistance against the CDK4/6 inhibitor in the RB knockout model in vivo, the antitumor activity of palbociclib was examined using the xenograft model. Since three RB knockout cells exhibited similar in vitro CDK4/6 inhibitor-sensitivity, MCF7-RBKO-A02 was used in the following experiments. In the MCF7 model on Day 22, palbociclib significantly inhibited tumor growth compared with control treatment (Figure 2A). However, in MCF7-RBKO-A02 model on Day 22, palbociclib did not significantly inhibit tumor growth compared with control treatment (Figure 2B). Thus, MCF7-RBKO-A02 was determined to be a palbociclib-resistant tumor model in vivo.

Bevacizumab exhibited antitumor efficacy in the CDK4/6 inhibitor-resistant MCF7-RBKO-A02 tumor model. To investigate the antitumor efficacy of bevacizumab in the CDK4/6 inhibitor-resistant tumor model, the xenograft model was used. In the parental MCF7 model on Day 39, bevacizumab significantly inhibited tumor growth compared with the control (Figure 3A). In the MCF7-RBKO-A02 model on Day 39, bevacizumab also significantly inhibited tumor growth compared with the control (Figure 3B). These results indicated that bevacizumab exhibited antitumor
Figure 2. Antitumor activity of palbociclib in MCF7 and MCF7-RBKO-A02 tumors. Tumor growth curves. Mice bearing (A) MCF7 or (B) RB knockout MCF7-RBKO-A02 tumors were randomly divided into two groups: control and palbociclib. Data are presented as the mean±SD. (A) n=5/group; (B) n=7/group. *p-Value<0.05 (Wilcoxon rank sum test). n.s.: no significant difference.

Figure 3. Antitumor activity of bevacizumab in MCF7 and MCF7-RBKO-A02 tumors. Tumor growth curves. Mice bearing (A) MCF7 or (B) MCF7-RBKO-A02 tumors were randomly divided into two groups: control and bevacizumab. Data are presented as the mean±SD. (A) n=12/group; (B) n=11/group. The following animals were excluded from data analysis after the date of their death: (A) One mouse in the bevacizumab group on Day 21; (B) One mouse in the control group on Day 27. *p-Value<0.05 (Wilcoxon rank sum test).
Figure 4. Effect of microvessel density (MVD) in MCF7 and MCF7-RBKO-A02 tumors. Tumor microvessels stained immunohistochemically with anti-CD31 in (A) MCF7 or (B) MCF7-RBKO-A02 tumor tissues on Day 39. MVD in (C) MCF7 or (D) MCF7-RBKO-A02 tumor tissue was determined by calculating the ratio of the CD31-positive area to the total observed area. Data are presented as the mean±SD (C) control, n=12/group; bevacizumab, n=11/group; (D) control, n=10/group; bevacizumab, n=11/group. *p-Value<0.05 (Wilcoxon rank sum test).
efficacy not only in the palbociclib-sensitive model but also in the palbociclib-resistant model by RB knockout. 

**Bevacizumab suppressed MVD in the CDK4/6 inhibitor-resistant MCF7-RBKO-A02 tumor model.** To investigate the anti-angiogenic effect of bevacizumab in the CDK4/6 inhibitor-resistant tumor model, the tumor MVD was analyzed on Day 39. In the MCF7 model, bevacizumab significantly suppressed MVD compared to control (Figure 4A and B). Also, in the MCF7-RBKO-A02 model, bevacizumab significantly suppressed MVD compared with the control (Figure 4C and D). These results indicated that bevacizumab suppressed angiogenesis in the tumor regardless of whether the model was palbociclib-sensitive or palbociclib-resistant due to RB knockout.

**Discussion**

In this study, we focused on RB as a candidate for the CDK4/6 inhibitor resistance mechanism in HR-positive and HER2-negative breast cancer cells based on the following: (i) RB is a direct substrate of CDK4/6, which is the target of CDK4/6 inhibitors (8), (ii) mutations leading to loss of RB function were found after clinical treatment with a CDK4/6 inhibitor (9), (iii) the resistance mechanisms of CDK4/6 inhibitors in preclinical studies were associated with the loss of RB function or enhancement of RB inactivating factors (11, 13, 14, 22, 23), (iv) CDK4/6 inhibitors were less/not effective in RB-negative cell lines (8, 14). However, to the best of our knowledge, there are no reports directly verifying that loss of RB function alone is responsible for CDK4/6 inhibitor resistance in HR-positive HER2-negative breast cancer cells. Therefore, first we investigated whether knockout of RB alone causes resistance to CDK4/6 inhibitors in HR-positive HER2-negative breast cancer cells. Several clones of the RB knockout cells were established using the CRISPR/CAS9 system in the MCF7 cell line, which is known to have the following properties: HR-positive and HER2-negative, exhibition of growth in an estrogen-dependent manner both in vitro and in vivo, CDK4/6 inhibitor-sensitive, and RB positive. RB has been reported to form a family with RB-like proteins called p107 and p130, which have high gene sequence homology and partially complement each other’s functions, as gatekeepers of the cell cycle (24). By examining the expression of RB, p107 and p130 proteins, the specific knockout of RB protein was confirmed in these established clones.

In the parental MCF7 cells, both palbociclib and abemaciclib exhibited in vitro growth inhibitory activity as previously reported (25). In all three RB knockout clones, the in vitro growth inhibitory effect of palbociclib and abemaciclib was reduced compared to that in the parent cells. Next, we investigated the in vivo effect of RB knockout on CDK4/6 inhibitor sensitivity using palbociclib in a xenograft model using MCF-RBKO-A02 cells, which is one of RB knockout clones. The MCF7 tumor growth was significantly inhibited by palbociclib in vivo, whereas the MCF-RBKO-A02 tumor growth was not significantly inhibited. This indicated that palbociclib resistance at proliferation levels of breast cancer cells was caused by RB knockout. It has been reported that RB knockout suppresses the inhibition of S phase progression due to palbociclib in the breast cancer cells and reduces sensitivity to palbociclib at the proliferation level in lung cancer cells (26). However, there have been no reports on whether RB knockout reduces the sensitivity to CDK4/6 inhibitors in breast cancer. This study indicated that RB reduced sensitivity to palbociclib at the proliferation level in breast cancer cells in vitro/in vivo. Combined with these results and the report that mutations in the Rb gene appeared only in patients after hormone therapy including palbociclib (9), the loss of RB function was suggested to be one of the resistance mechanisms to CDK4/6 inhibitors in a clinical setting.

There was no previous investigation on the effect of bevacizumab in HR-positive and HER2-negative breast cancer patients who acquire resistance to CDK4/6 inhibitors. Therefore, this study investigated the efficacy of bevacizumab in a CDK4/6 inhibitor resistance model. In the CDK4/6 inhibitor-resistant MCF-RBKO-A02 xenograft, bevacizumab exhibited significant antitumor activity similar to that in the CDK4/6 inhibitor-sensitive parental MCF7 xenograft. This result indicates that bevacizumab is also effective in breast cancer cells that have acquired CDK4/6 inhibitor-resistance because of the loss of RB function.

Two major candidates for the mechanism of CDK4/6 inhibitor resistance have been reported: (i) abnormalities of the CDK-RB axis characterized by up-regulation of CDK6 and Cyclin E/Cdk2, causing the phosphorylation/inactivation of RB, and the loss of RB function due to RB mutation (13, 14), and (ii) abnormalities of survival signals, namely the activation of the PI3K/AKT/mTOR pathway (9, 27). A potential resistance mechanism related to the CDK-RB axis would lead to the loss of function of RB, which is located furthest downstream of the CDK-RB axis and acts as a gatekeeper that negatively regulates cell cycle progression. Considering this, CDK4/6 inhibitor resistance may be caused by the above-mentioned abnormalities of the CDK-RB axis, which exhibit the same characteristics as the resistance caused by RB knockout. Of the two major resistance mechanisms, bevacizumab would at least be generally effective for resistance caused by CDK-RB axis abnormalities.

Bevacizumab significantly suppressed MVD in the MCF7-RBKO-A02 xenograft model, as in the MCF7 model. This indicated that bevacizumab exhibited tumor angiogenesis-suppressing activity, which was its main mechanism, even in breast cancer cells that had acquired CDK4/6 inhibitor resistance because of the loss of RB function.
In summary, as far as we know, this is the first study to indicate that RB knockout confers CDK4/6 inhibitor resistance in HR-positive HER2-negative breast cancer cells. In addition, in the model of CDK4/6 inhibitor resistance caused by RB loss of function, bevacizumab exerted antitumor activity by suppressing MVD, which was similar in the CDK4/6 inhibitor-sensitive model. These results suggest that bevacizumab-containing therapies may be clinically effective in patients who were previously treated with CDK4/6 inhibitor-containing therapy, especially in those who acquired resistance due to abnormalities in the CDK-RB axis.

Conflicts of Interest
All the Authors are employees of Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

Authors’ Contributions
Concept and design; N. Ishikura, M. Sugimoto, and O. Kondoh. Acquisition of data; N. Ishikura, K. Yorozu, and M. Kurasawa. Analysis and interpretation of data (statistical analysis, etc.); N. Ishikura, K. Yorozu, and M. Kurasawa. Writing, review, and/or revision of the manuscript; N. Ishikura, M. Sugimoto, K. Yorozu, and D. Wakita. Study supervision; D. Wakita.

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References
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