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The Anti-tumor Effect of Cabozantinib on Ovarian Clear Cell Carcinoma *In Vitro* and *In Vivo*

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Abstract. *Background: Several reports have shown that the overexpression of the MET proto-oncogene, receptor tyrosine kinase (MET), was more frequently observed in clear cell carcinoma (CCC) than in non-CCC. We evaluated the antitumor activity of cabozantinib, that targets MET. Materials and Methods: A gene expression analysis of tumors from human ovarian cancers was carried out by transcriptome sequencing. An in vitro 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay (MTT assay) and in vivo experiments were performed to determine the activity of cabozantinib. Results: The MET levels were higher in tumors with CCC than high-grade serous carcinoma (2.2-fold). Cabozantinib inhibited cell viability and phosphorylation of AKT and MAPK under the treatment of hepatocyte growth factor in RMG-I CCC cells. The tumors removed from mice given cabozantinib of 10 mg/kg weighed 70% less than control on day 15, and the immunohistochemical reactivity of phosphorylated MET was reduced compared with control mice. Conclusion: Cabozantinib contributes to tumor reduction, and phosphorylated MET represents an attractive target of CCC.*

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Ovarian cancer is the leading cause of death among malignancies of the female reproductive system, resulting in approximately 125,000 deaths annually (1). Clear cell carcinoma (CCC) of the ovary was first recognized by the World Health Organization as a distinct histologic subtype in 1973 (2). There are marked geographic differences in the prevalence of ovarian CCC, and the incidence of ovarian CCC in women living in United States is 4.8% in whites, 3.1% in blacks, and 11.1% in Asians (3). Improvements in the clinical outcomes of CCC patients have been poor because CCC is less sensitive to platinum-based first-line chemotherapy than other histological subtypes, including high-grade serous carcinoma or endometrioid carcinoma. The effect of standard combination chemotherapy with paclitaxel and carboplatin (TC) is not satisfactory for CCC (around 50% for primary tumors), and other agents have failed to demonstrate superiority to TC (4-6). In addition, there is no effective chemotherapy for recurrent disease after treatment with platinum-based chemotherapy (7). The detailed molecular mechanisms that may contribute to drug resistance in CCC are still not fully understood, and new therapeutic strategies to overcome drug resistance need to be established.

The hepatocyte growth factor (HGF) signaling pathway may be a viable target for the development of directed therapeutic regimens for the treatment of ovarian cancer. HGF is the ligand of the HGF receptor, the MET proto-oncogene, receptor tyrosine kinase (MET), which has been reported to be expressed in approximately 60% of ovarian cancer cases (8). Overexpression of MET has been reported to be associated with a poor clinical outcome in ovarian cancer (8-11) and with chemoresistance of ovarian cancer cells (12, 13). Additionally, recent studies have reported that MET inhibitor could be used to overcome the resistance to cytotoxic agents in ovarian cancer (13, 14). These results

prompted our hypothesis that MET inhibitor may be an active agent for a subset of CCC.

In this study, we searched for mechanisms involved in the effect of cabozantinib, a multi-receptor tyrosine kinase inhibitor for MET, ret proto-oncogene (RET), and kinase insert domain receptor (VEGFR2), that is already approved for progressive medullary thyroid cancer by the US Food and Drug Administration (15). We found that cabozantinib inhibited cell growth *in vitro* and tumor formation in nude mice bearing CCC.

Materials and Methods

Comprehensive gene expression analysis. Total RNAs were extracted from tumor tissues derived from patients with CCC or high-grade serous carcinoma (HGSC) using the RNeasy Mini kit (Qiagen, Tokyo, Japan). Extracted total RNA was quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries for transcriptome were prepared using an Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific). A total of 10 ng of total RNAs were used to prepare the barcoded libraries. The prepared libraries were purified using AMPure XP (Beckman Coulter, Brea, CA, USA) and quantified using an Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific), then diluted to 50 pM and pooled equally with eight samples per pool. Emulsion polymerase chain reaction was performed using the Ion Chef™ System. The templated libraries were then sequenced on Ion Proton™ system using the Ion P1 Hi-Q Chef Kit and Ion P1 Chip Kit v3 (Thermo Fisher Scientific).

Cell lines. The fully verified human ovarian CCC cell lines RMG-I (16), OVTOKO (17) and OVISE and the human ovarian mesonephroid adenocarcinoma cell line RMG-II (18) were purchased from JCRB Cell Bank (Osaka, Japan). The human ovarian CCC cell line ES-2 (19) was purchased from ATCC (Manassas, VA, USA). RMG-I cells were cultured in Ham's F12 with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. OVTOKO and OVISE cells were cultured in RPMI 1640 with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. ES-2 cells were cultured in Dulbecco's modified Eagle's medium DMEM with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin.

Reagents. Cabozantinib (Selleck Chemicals, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) and an equal volume of DMSO was used as control. Recombinant human hepatocyte growth factor (HGF) (Peprotech, Rocky Hill, NJ, USA) was dissolved in double distilled water DDW with 0.9% NaCl.

Analysis of MET signaling pathway in RMG-I cells. A total of 5×10^5 cells were inoculated onto 60-mm dishes. Six hours after attachment of the cells, the medium was changed to serum-free medium and cultured overnight at 37˚C. Cells were treated with 20 nM of cabozantinib. After 1.5-h incubation, HGF (50 ng/ml) was added for 30 min. At the end of treatment, the cells were collected and stored at –80˚C until western blot analyses.

Immunoblotting. Cells were lysed with lysis buffer (10mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, PMSF). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride filter (Millipore, Billerica, MA, USA) by the standard method. Filters were incubated in TBS containing 2% skim milk with rabbit polyclonal antibody against MET (Cell Signaling Technology, Danvers, MA, USA) with 1:1,000 dilution, RET (Cell Signaling Technology) with 1:1000 dilution, VEGFR2 (Cell Signaling Technology) with 1:250 dilution, and α-tubulin (Santa Cruz Biotechnology, Dallas, TX, USA) with 1:2,000 dilution. For the detection of phospho-MET (p-MET, Tyr1234/1235, Cell Signaling Technology), phospho-AKT (p-AKT, Ser473, Cell Signaling Technology), and phospho-MAPK (p-MAPK, Thr202/Tyr204, Cell Signaling Technology), filters were incubated in TBS containing 5% bovine serum albumin and 1% tween-20 with rabbit polyclonal antibody against p-MET (Cell Signaling Technology) with 1:500 dilution, p-AKT (Cell Signaling Technology) with 1:500 dilution, and p-MAPK (Cell Signaling Technology) with 1:500 dilution. All filters were incubated overnight at 4˚C and then with peroxidase-labeled secondary antibodies with 1:5,000 dilution for 1 h at room temperature. Proteins were visualized using a Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, Carlsbad, CA, USA) and quantified using a Lumino Image Analyzer (LAS1000; Fuji Film, Tokyo, Japan).

Measurement of drug sensitivity by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay (MTT assay). The MTT assay was performed for drug sensitivity assays using Cell Proliferation Kit I (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 5×10^3 cells were seeded onto 96-well plates in 100 μl culture medium with reagents. An equal volume of DMSO was used as a control. The wavelength to measure the absorbance of the formazan product was 550 nm, and the reference wavelength was 655 nm.

Analysis of tumor-forming potential in vivo. All experiments were conducted in accordance with guidelines authorized by the Animal Research Committee Hokkaido University. Six-week-old BALB/c nude mice (Clea, Tokyo, Japan) were injected subcutaneously into their abdomen with 5×10^6 cells in 200 μl of matrigel (BD Biosciences, San Jose, CA, USA) and 50 μl of normal culture medium. The tumors reached 100 mm^3 after 4 days. Then, the mice were randomly assigned to one of four treatment groups, receiving any of three concentrations (3 mg/kg, 10 mg/kg, 30 mg/kg) of cabozantinib. An equal volume of DMSO was used as control. Oral cabozantinib administration was started once a day for 14 days. The tumor size was measured every three days. All mice were killed on day 15, and the tumor weight was measured and photos taken. Xenograft tumors were fixed in formalin and embedded in paraffin. They were used for immunohistochemistry later.

Immunohistochemistry. Tissue sections were deparaffinized in xylene and rehydrated through a series of graded ethanol washes. Heat-induced antigen retrieval was carried-out in high-pH antigen retrieval buffer (Dako, Glostrup, Denmark). Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 5 min. The primary antibodies against MET and p-MET (SP44 and SP58, respectively; Spring Bioscience, Pleasanton, CA, USA) were applied for 30 min. These sections were visualized by the HRP-labeled polymer method (EnVision FLEX system in human tissues and EnVision+ system for

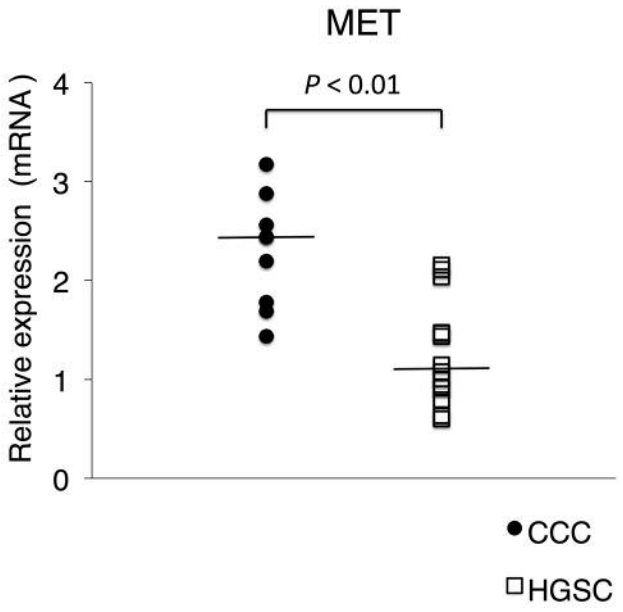

Figure 1. *MET mRNA expression in CCC and HGSC. Transcriptome sequencing of MET mRNA in 9 CCC tumors and 14 HGSC tumors. The Mann-Whitney U-test was used for statistical analysis. The horizontal lines demonstrate the median.*

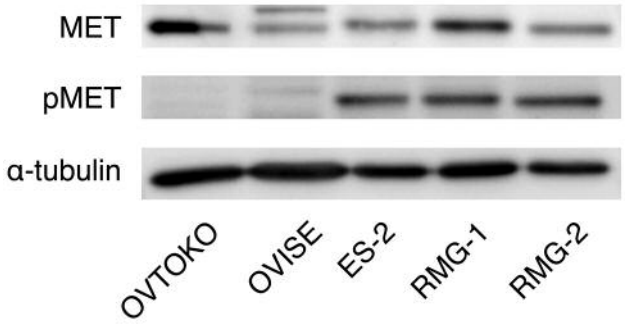

Figure 2. *The basal level of expression of MET and p-MET in CCC cell lines. Immunoblot of MET, phosphorylated-MET (pMET) and α-tubulin in OVTOKO, OVISE, ES-2, RMG-1 and RMG-2 cells.*

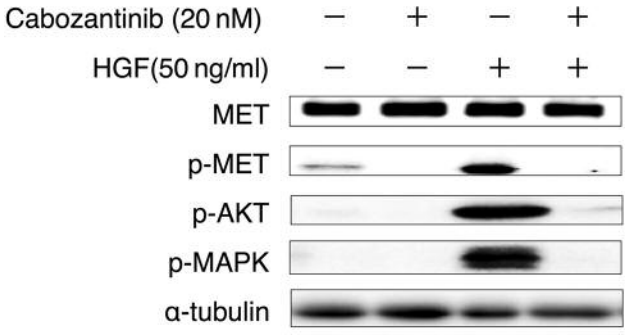

Figure 3. *Effect of cabozantinib on the MET signaling pathway in RMG-I cells. Immunoblot of MET, phosphorylated-MET (pMET), phosphorylated-AKT (pAKT), phosphorylated-MAPK (pMAPK) and α-tubulin in RMG-I cells treated with or without cabozantinib.*

Rabbit in mouse xenograft tissues, respectively; Dako). Immunostained sections were counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene.

Statistical analyses. For *in vitro* and *in vivo* studies, continuous variables were compared using nonparametric Mann-Whitney *U*-test or Student's *t*-test if normally distributed. Data are presented as the medians or means±standard error (SE). For animal experiments, the tumor weight was presented as the means±SE. All statistical tests were two-sided, and *p*-values less than 0.05 were considered significant. Only two-tailed values were reported.

Results

mRNA expression of MET in clinical tumor tissue samples. We first examined the mRNA expression of MET in frozen clinical tissue specimens from 9 of CCC and 14 of HGSC by transcriptome sequencing. MET mRNA expression level was upregulated in CCC than HGSC (2.2-fold), suggesting that CCC might be more strongly dependent on MET signaling pathway for tumor progression than HGSC, and MET could be a good therapeutic target for CCC (Figure 1).

Analysis of the MET signaling pathway with or without cabozantinib treatment in RMG-I cells (CCC cell line). Given the MET activation found in CCC tumor specimens, we evaluated the basal level of expression of MET and p-MET in a CCC cell lines. Among five fully-verified CCC cell lines, both MET and p-MET levels were highest in RMG-I cells by a western blot analysis (Figure 2). The expression of both RET and VEGFR2 were low in RMG-I cells (data not shown). The MET expression in RMG-I cells was not changed with the treatment of HGF (50 ng/ml), a ligand for MET, while the expression of p-MET was induced. HGF also induced the phosphorylation of AKT and MAPK, which are the downstream targets of MET in RMG-I cells (Figure 3). These findings suggested that RMG-I was most suitable for the present study.

We next examined the expression of MET and p-MET under the treatment of cabozanitinib (20 nM) in RMG-I cells. Cabozanitnib did not affect the expression of MET but significantly suppressed the expression of p-MET without HGF treatment. The expression of p-MET, p-AKT, and

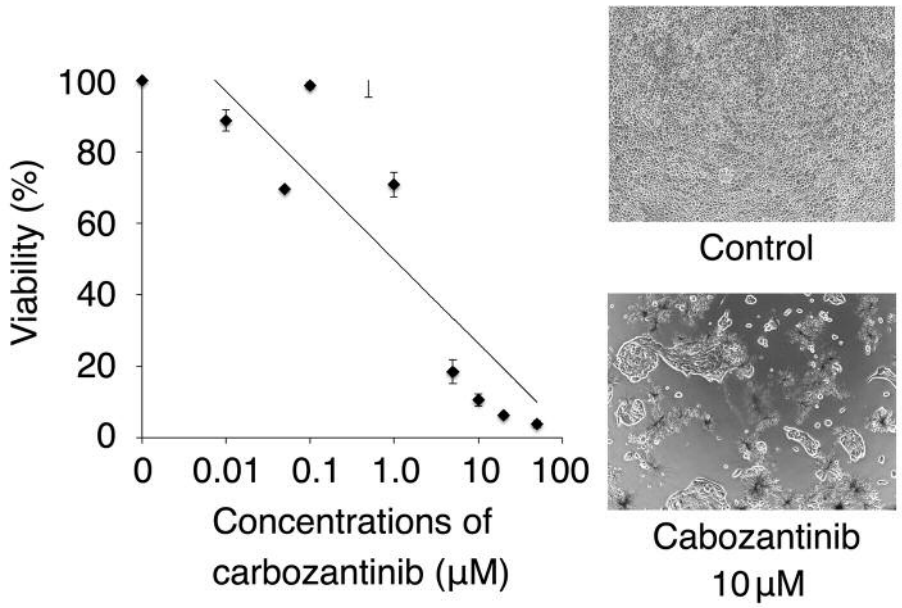

Figure 4. *Anti-proliferative effect of cabozantinib on RMG-I cells in vitro. Direct effects of cabozantinib on RMG-I viability. Left: Viability at each concentration. Data are presented as the means±SE. Right: Representative images of control and 10 μM of cabozantinib.*

p-MAPK induced by HGF (50 ng/ml) was subsequently inhibited by the treatment of cabozantinib (Figure 3).

Cabozanitinib inhibited viability of RMG-I cells in vitro. We next examined the inhibitory effect of cabozantinib on the viability of RMG-I cells *in vitro*. We investigated the anti-proliferative effect of cabozantinib by an MTT assay. Cabozantinib showed anti-proliferative effect on RMG-I cells, and we found that the IC_{50} of cabozantinib was 1.0 μM for RMG-I cells (Figure 4).

Cabozanitinib inhibited tumor growth of RMG-I cells in vivo. To further examine the *in vivo* growth inhibitory effect of cabozantinib, we employed a mouse xenograft model, in which athymic mice were inoculated subcutaneously with RMG-I cells. Drug treatment was well-tolerated except in 1 mouse treated with 30 mg/kg of cabozantinib that died of aspiration. 3 mg/kg, 10 mg/kg and 30 mg/kg of cabozantinib significantly inhibited tumor formation after day 15 (Student's *t*-test $p<0.01$, respectively) compared to vehicle-treated tumors (Figure 5A and B). The tumors removed from mice given cabozantinib of both 10 mg/kg (177 mg) and 30 mg/kg (177 mg) weighed 72% less than control (623 mg) on day 15.

Immunohistochemical expression of MET and p-MET in the mouse xenograft tissues of RMG-I cells with or without cabozantinib treatment. We examined the expression of MET and p-MET in the tissue sections of mouse xenografts of RMG-I cells. To investigate the cancer cell-specific expression of MET and phospho-MET, we evaluated the expression of MET and phospho-MET microscopically. Immunohistochemistry showed that the MET expression was not altered by cabozantinib treatment. In contrast, the expression of p-MET was suppressed in tumors treated with cabozaninib (10 mg/kg and 30 mg/kg) compared to those without cabozantinib treatment (Figure 6), suggesting that the anti-tumor activity of cabozantinib was mainly dependent on the inhibition of phosphorylation of MET.

Discussion

Previous reports have suggested several molecular mechanisms underlying the resistance of CCC to conventional chemotherapy that included the HNF-1β pathway, PI3K/AKT/mTOR pathway, and MET pathway (7). For the PI3K/AKT/mTOR pathway, the clinical efficacy of the mTOR inhibitor temsirolimus has been evaluated for advanced-stage CCC in a phase II trial (GOG268) in both the US and Japan (45 patients each). However, temsirolimus failed to show a significant survival benefit for advanced-stage CCC in combination with TC and in consolidation as first-line chemotherapy (20). Another mTOR inhibitor, everolimus, is expected to be less effective than cabozantinib because a recent phase III trial demonstrated that

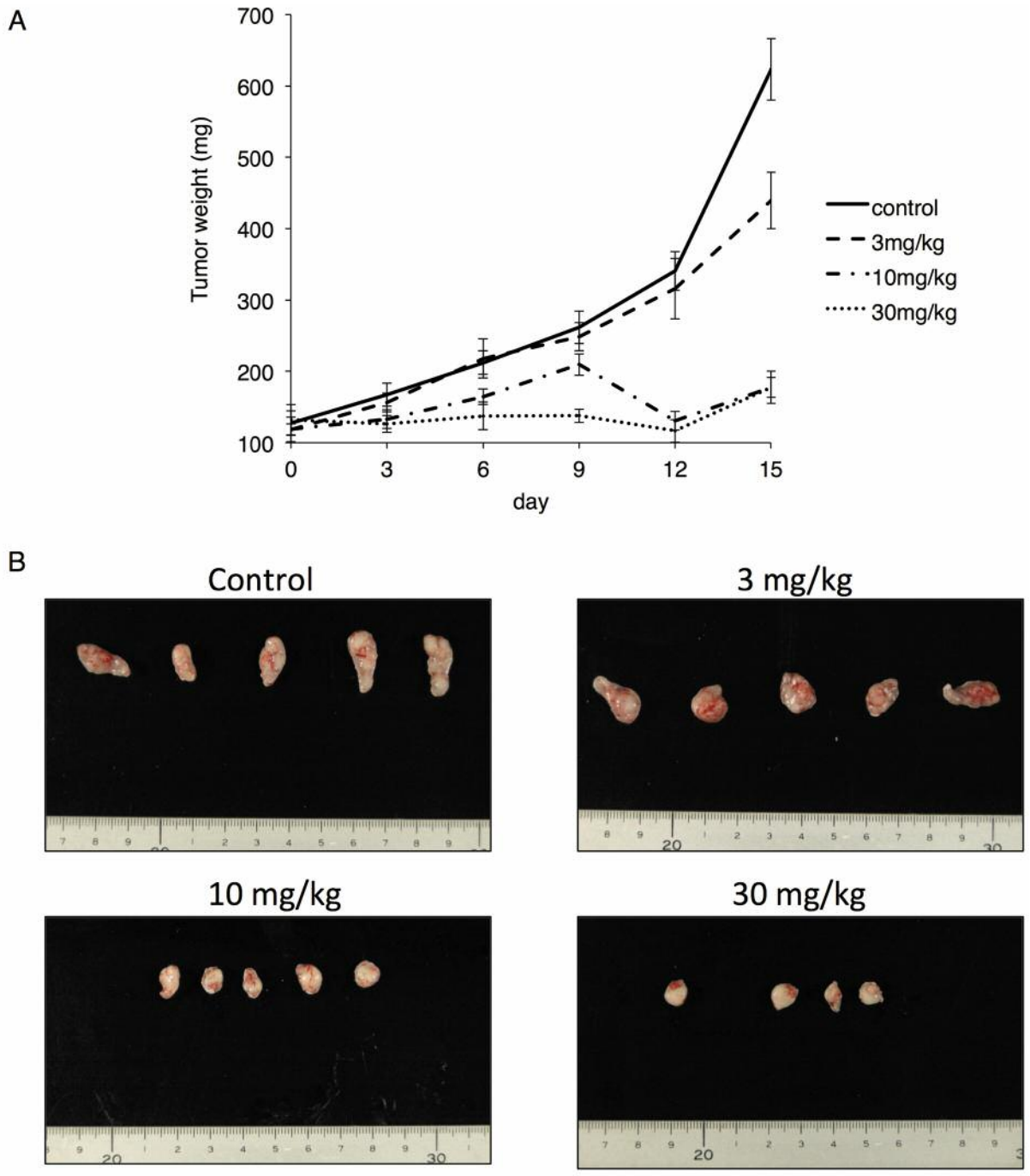

Figure 5. *Sensitization of RMG-I tumors to cabozantinib in vivo. (A) Tumor weights of tumor nodules in the control and cabozantinib groups of mice. Data are presented as the means±SE. (B) Representative images of the subcutaneous tumors in the control and cabozantinib groups of mice.*

cabozantinib significantly improved the survival of patients with advanced-stage renal CCC compared to everolimus (21).

Several reports have shown that the amplification and/or overexpression of MET, which is a target of cabozantinib (22), was quite common and more frequently observed in CCC of the ovary than in non-CCC based on the findings from immunohistochemistry and/or *in situ* hybridization. In addition, this overexpression is associated with a poor

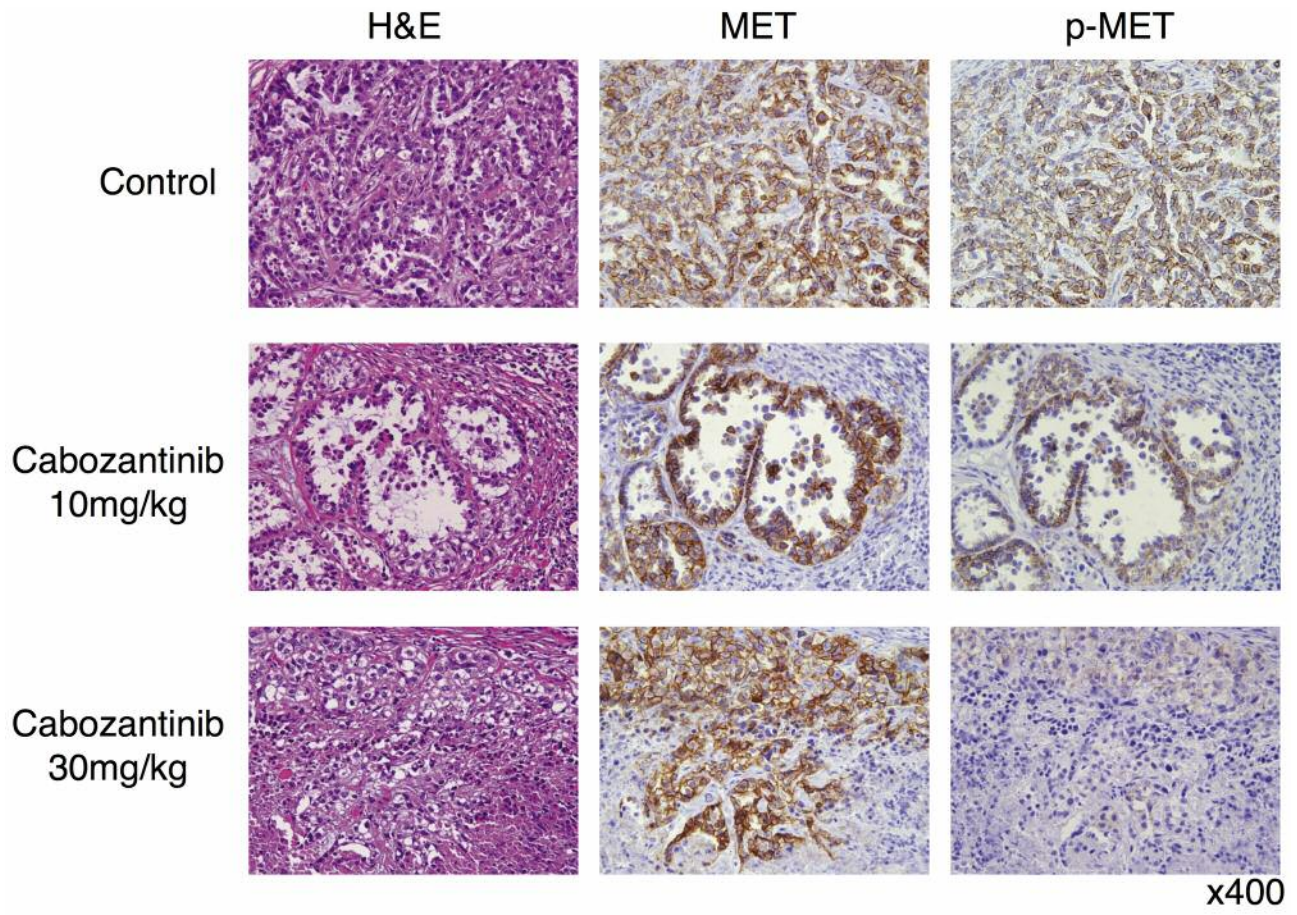

Figure 6. *Cabozantinib therapy suppresses the phosphorylated-MET expression in CCC in vivo. An immunohistochemical analysis of MET and pMET expression in sections of control and cabozantinib-treated RMG-I tumors.*

prognosis of patients with CCC (11, 23). Cabozantinib was tested in a phase II trial for 70 cases of recurrent ovarian cancer (24). In that trial, the response rate of cabozantinib for refractory/resistant disease (recurrence within 6 months after the last chemotherapy session) and sensitive disease (recurrence over 6 months after the last chemotherapy session) was 20.6% (7/34) and 27.8% (10/36), respectively. The disease control rate (complete response + partial response + stable disease) for refractory/resistant disease and sensitive disease was 38.2% (13/34) and 66.7% (24/36), respectively. Thus, cabozantinib seems a promising agent for recurrent ovarian cancer. However, its efficacy for CCC has not been fully evaluated, as only 3 patients with recurrent CCC (4.3%, 3/70) were enrolled in the previous phase II study. We, therefore, conducted the current experiments to investigate the anti-tumor effect of cabozantinib for CCC of the ovary. In our *in vivo* study, tumor reduction was the same for both 10 mg/kg and 30 mg/kg, suggesting the existence of dose limitation of cabozantinib in terms of clinical response to CCC. In the previous human clinical trials, the dose range was from 60 to 100 mg daily (21, 25-27). Although the concentration was high in our *in vivo* study, the results of the current study suggest that the anti-MET activity of cabozantinib should be prospectively tested for CCC patients with advanced-stage or recurrent disease with a poor prognosis. Indeed, the NRG oncology group has been conducting phase II trial testing of cabozantinib for advanced-stage ovarian CCC patients with recurrent disease (NRG-GY-001) (28). For further basic investigations of the effect of cabozantinib, the establishment of a human ovarian CCC cell line expressing RET and VEGFR2 will be needed.

Conclusion

Our findings suggest that cabozantinib might be an active agent for a CCC cell line through *in vitro* and *in vivo* studies. Ongoing phase II trials may reveal that cabozantinib is effective for advanced-stage recurrent CCC patients and will

be approved for recurrent advanced-stage ovarian CCC in patients with a poor prognosis to improve their survival. Cabozantinib should be prospectively tested for ovarian CCC patients with a poor prognosis.

Conflicts of Interest

The Authors report no conflicts of interest in this work.

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