学位論文

Axitinib(AG-013736), an Oral Specific VEGFR TKI, Shows Potential Therapeutic Utility Against Cholangiocarcinoma

(胆管癌に対する経口特異的VEGFRチロシンキナーゼ阻害剤 Axitinib(AG-013736)の治療薬としての可能性)

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Original Article

Axitinib (AG-013736), an Oral Specific VEGFR TKI, Shows Potential Therapeutic Utility Against Cholangiocarcinoma

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Objective: Cholangiocarcinoma is a refractory cancer whose incidence has been increasing worldwide in recent years. Neoangiogenesis plays an important role in the growth of various solid cancers, including cholangiocarcinoma. Vascular endothelial growth factor plays an important role in tumor-induced angiogenesis and its expression is associated with the progression and prognosis of cholangiocarcinoma. This study examined whether axitinib (AG-013736, INLYTA®), a potent and selective second-generation inhibitor of vascular endothelial growth factor receptors 1, 2 and 3, could be a potentially useful therapeutic agent for cholangiocarcinoma.

Methods: We performed expression profiling of angiogenesis-related molecules in eight cholangiocarcinoma cell lines and found that three of them showed high vascular endothelial growth factor expression. Among them, we examined the *in vivo* anti-tumor effect of axitinib on NCC-BD1 (a gemcitabine-sensitive extra-hepatic cholangiocarcinoma cell line) and TKKK (a gemcitabine-resistant intra-hepatic cholangiocarcinoma cell line) using subcutaneous xenograft models.

Results: Oral administration of axitinib significantly inhibited the growth of TKKK xenografts at a dose of 6 mg kg^{-1} day^{-1} ($P<0.05$), and the growth of NCC-BD1 xenografts at 30 mg kg^{-1} day^{-1} ($P<0.05$). Treated tumors showed a significant decrease of microvessel density and the tumor cell proliferation index and a mild but significant increase of the apoptotic index in comparison with untreated tumors.

Conclusions: Our results suggest that axitinib should be a promising therapy for vascular endothelial growth factor-expressing cholangiocarcinoma, irrespective of tumor origin and gemcitabine sensitivity.

Key words: cholangiocarcinoma – VEGFR-TKI – axitinib – xenograft model

INTRODUCTION

Cholangiocarcinoma (CC) is a highly malignant tumor arising from the ductular epithelium or cholangiocytes of the intra- and extra-hepatic biliary system. The worldwide incidence of CC and the resulting mortality, especially for intra-hepatic cholangiocarcinoma (IHCC), has increased over the last three decades (1–7). CC is difficult to detect and diagnose because it lacks clinical symptoms, but often shows diffuse spread in the liver parenchyma or through the bile ducts at an early stage. Most patients have unresectable disease at clinical

presentation and a poor prognosis even after curative surgical resection, which offers the only hope of potential eradication of the disease at present (6,8,9). Combinations of adjuvant therapies including chemotherapy and radiation therapy or chemotherapeutic regimens for unresectable and recurrent CC are only minimally useful in terms of anti-tumor effect and improvement of patient survival (7,9–11). Existing Phase II data and a more recent meta-analysis suggest that gemcitabine and gemcitabine-based platinum regimens offer a slight advantage over other regimens (6,12). Recently, a large RCT comparing combined gemcitabine plus cisplatin therapy with gemcitabine treatment alone demonstrated survival benefit of the combined regimen over gemcitabine alone (13,14). As a result, combined gemcitabine plus cisplatin therapy has come to be recognized as standard therapy for unresectable biliary tract cancer. The survival, however, remains dismal, and novel effective therapeutic strategies are urgently required to improve the prognosis of patients with CC.

Neoangiogenesis is one of the most important hallmarks of malignant tumors (15). Various tumor-derived cytokines are involved with this process (16,17). Among them, vascular endothelial growth factor (VEGF) plays a major role in tumor-induced angiogenesis by promoting endothelial cell proliferation, migration and survival. VEGF binds to its receptors (VEGFR1 and VEGFR2) on vascular endothelial cells, and activation of VEGFR2 is mainly sufficient for VEGF-induced mitogenesis, angiogenesis and vascular permeability (18). Overexpression of VEGF has been reported to be of prognostic significance in a wide range of solid cancers including CC (19–24), and therefore VEGF signaling is a potential target for treatment of CC. In this study, we examined whether axitinib (AG-013736, INLYTA), an oral specific VEGFR-1/2/3 tyrosine kinase inhibitor, can exert a potent anti-tumor effect on CC cells *in vivo*.

PATIENTS AND METHODS

Cell Lines

NCC-CC1, NCC-CC3-1, NCC-CC3-2, NCC-CC4-1, NCC-CC4-2 and NCC-CC4-3 cells were established from human IHCC, and NCC-BD1 and NCC-BD2 cells from human extra-hepatic cholangiocarcinoma (EHCC) at the National Cancer Center Research Institute (25). TKKK and OZ cells were purchased from RIKEN Bio Resource Center (Tsukuba, Japan, http://www.brc.riken.jp/lab/cell/) or from the Japanese Collection of Research Bioresources (Osaka, Japan, http://cellbank.nibio.go.jp/). TKKK cells were established from IHCC, and OZ cells from EHCC. The originally established eight CC cell lines were maintained in RPMI with 10% bovine serum. TKKK and OZ cells were maintained in Dulbecco's modified Eagle medium with 10% bovine serum. The identities of these cells were confirmed by analyzing their short tandem repeat profiles using the Cell ID System (Promega, Madison, WI, USA).

Gene Expression Analysis

Total RNA was extracted from the CC cell lines using an RNeasy Mini Kit (Qiagen, Velencia, CA, USA) in accordance with the manufacturer's instructions. The total ribonucleic acid (RNA) yields and purity were determined by measuring the absorbance of aliquots at 260 and 280 nm. Cy3-labeled cRNAs were synthesized using a Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). The labeled cRNA probes were hybridized to an oligonucleotide microarray (Whole Human Genome 44 K Array; Agilent Technologies) covering $>$41 000 human transcripts. Array hybridization and washing were carried out in accordance with the recommended protocols, and the microarrays were scanned using a DNA Microarray Scanner (Agilent Technologies) and analyzed using Gene Spring software (Agilent Technologies).

Axitinib Preparation

Axitinib was provided by Pfizer (CT, USA). It was dissolved as a homogeneous suspension with 0.5% carboxymethyl cellulose (carboxymethyl cellulose sodium salt low viscosity; MP Biomedicals, Solon, OH, USA) and administered orally twice a day at 0.1 ml 10 g^{-1} body weight (b.w.).

Therapeutic Xenograft Model

All animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation, and the experiments were conducted in accordance with the Guidelines for Animal Experiments of the National Cancer Center, Tokyo, Japan. Eight-week-old female C.B-17/Icr-scid (scid/scid) congenitally athymic mice were purchased from CLEA Japan (Tokyo, Japan) and housed under specific pathogen-free conditions at the National Cancer Center Research Institute Animal Center. Eight million cells were suspended in 0.2 ml of culture medium without fetal bovine serum and injected subcutaneously into the right flank of each mouse. At 30 days after injection, the mice were randomly divided into four treatment groups, namely axitinib 60, 30, 6 mg kg^{-1} b.w. per day, or vehicle control. The therapeutic doses of axitinib was decided based on recommended doses by Pfizer. The PK/PD analysis indicate that the administration of 30 mg kg^{-1} b.i.d per day axitinib for mouse is sufficient doses considering a VEGFR inhibitor, and has equal effectiveness to 5–10 mg kg^{-1} b.i.d per day axitinib for human that estimated from the anti-tumor effect on mouse, the non-bonding concentration in plasma on mouse, and the non-bonding concentration in plasma on human (26). Treatment was started from the next day and continued for at least 4 weeks. Tumor volume was calculated using the formula: $(\text{short diameter})^2 \times (\text{long diameter})/2$, and was determined twice a week. All mice were killed at the end of the study period and the subcutaneous tumors were removed completely.

Immunohistochemical Evaluation

Formalin-fixed, paraffin-embedded serial sections (4 μm) of 80 tumor xenograft tissues were prepared on silicone-coated slides for immunohistochemical evaluation. Hematoxylin-eosin sections were observed, and the presence of tumors was confirmed microscopically. Immunohistochemical staining for CD31 and Ki-67 was performed using a polymer-based method (Envision^{TM} + Dual link-system-HRP; Dako, Glostrup, Denmark) in accordance with the manufacturer's instructions. The sections were deparaffinized in xylene, and rehydrated through graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide solution for 30 min. For antigen retrieval, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min. We used a rabbit anti-mouse CD31 polyclonal antibody (sc-1506-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 250 and a rabbit anti-human Ki-67 polyclonal antibody (180191z; Invitrogen, Frederick, MD, USA) at a dilution of 1 : 100. After protein blocking, the sections were incubated for 60 min at room temperature with each primary antibody, followed by incubation with Envision + Dual link reagent at room temperature for 30 min. 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen, and the tissue sections were counterstained with hematoxylin. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) was conducted to assess the degree of apoptosis using an *In Situ* Cell Death Detection Kit, POD (Roche, Basel, Schweiz) in accordance with the manufacturer's instructions. Microvessel density (MVD) was defined as the mean number of microvessels in three fields (original magnification, ×400) containing high levels of CD31-stained microvessels. The Ki-67 proliferation index (PI) and apoptotic index (AI) were defined as the percentage of positive cells among 1000 tumor cells or over in the same fields.

Statistics

All statistical analyses were performed with the Statview 5.0 statistical software package (Abacus Concepts, Berkeley, CA, USA). For the therapeutic protocol, changes in tumor volume were estimated using repeated measures analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Between-group comparisons of the response to axitinib (MVD, PI and AI) were estimated using one-way ANOVA followed by Dunnett's *post hoc* test. All numerical data were presented as mean ± SD Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Expression Profile of the Tumor Angiogenesis-related Pathway in CC Cell Lines

Many soluble factors are known to be associated with tumor angiogenesis (16,17). To elucidate the expression of these angiogenesis-related molecules in CC, microarray gene expression profiling of the tumor angiogenesis pathway was performed in eight CC cell lines (Fig. 1A). We examined the gene expressions of VEGFs, angiopoietins (ANGPTs) and the related protein, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) in these cell lines.

VEGFA, VEGFB, VEGFC and VEGFD are well-validated ligands for VEGFR, and these were highly expressed in three of the CC cell lines (3/8, 37.5%, Fig. 1A and B). Angiopoietin-like protein 2 (ANGPTL2) was abundantly expressed in four CC cell lines. FGF1 and FGF2 were not much expressed in any of the CC cell lines. PDGFC and PDGFD mRNAs were abundant in NCC-CC4-1. Notably high ANGPTL2 expression was observed in cell lines with low VEGF/PDGF expression. The presence of *KRAS* mutation (25) was not correlated with VEGF or ANGPTL2 expression (Fig. 1A). Gemcitabine sensitivity (25) was also not associated with the expression of any of the angiogenesis factors (Fig. 1A).

Based on these expression signatures, we chose NCC-BD1 and TKKK, both of which showed high VEGF expression and low-to-moderate expression of other angiogenesis factors, for the axitinib treatment experiment.

In Vivo Anti-tumor Effect of Axitinib in CC Xenografts

We evaluated *in vivo* anti-tumor effect of axitinib against a gemcitabine-sensitive cell line (NCC-BD1 from EHCC) and a gemcitabine-refractory cell line (TKKK from IHCC) using subcutaneous xenograft models. Following the protocol used in a previous study (27), we measured the change in tumor volume during axitinib administration. *In vivo* growth of both cell lines was significantly inhibited by axitinib treatment relative to the vehicle control (Fig. 2). The TKKK xenograft was significantly suppressed by axitinib treatment at doses of 6 mg kg^{-1} and more (Fig. 2A), whereas significant reduction of the NCC-BD1 xenograft tumor was observed at a dose of 30 mg kg^{-1} and more (Fig. 2B).

Immunohistochemical Evaluation of Axitinib Treatment

To further examine the biological effects of axitinib on CC tissues *in vivo*, we performed immunohistochemical analysis of all xenograft tumors after the treatment protocol (Fig. 3). We first measured the MVD, since it has been used as a common biological endpoint of anti-angiogenesis treatment in previous studies (28,29). As shown in Fig. 3A, MVD was assessed by CD31 staining of microvessels in tumor sections (30). Consistent with the higher sensitivity to axitinib, MVD was significantly decreased in all axitinib-treated groups of TKKK xenografts relative to the vehicle-treated group (Fig. 4A left). In contrast, MVD was significantly reduced in the groups of NCC-BD1 xenografts treated with doses of 30 and 60 mg kg^{-1} (Fig. 4A, right).

We then focused on the effects of axitinib treatment on tumor cells. The PI, defined by the ratio of Ki-67 positive cells (Fig. 3B), was significantly decreased in all axitinib-treated

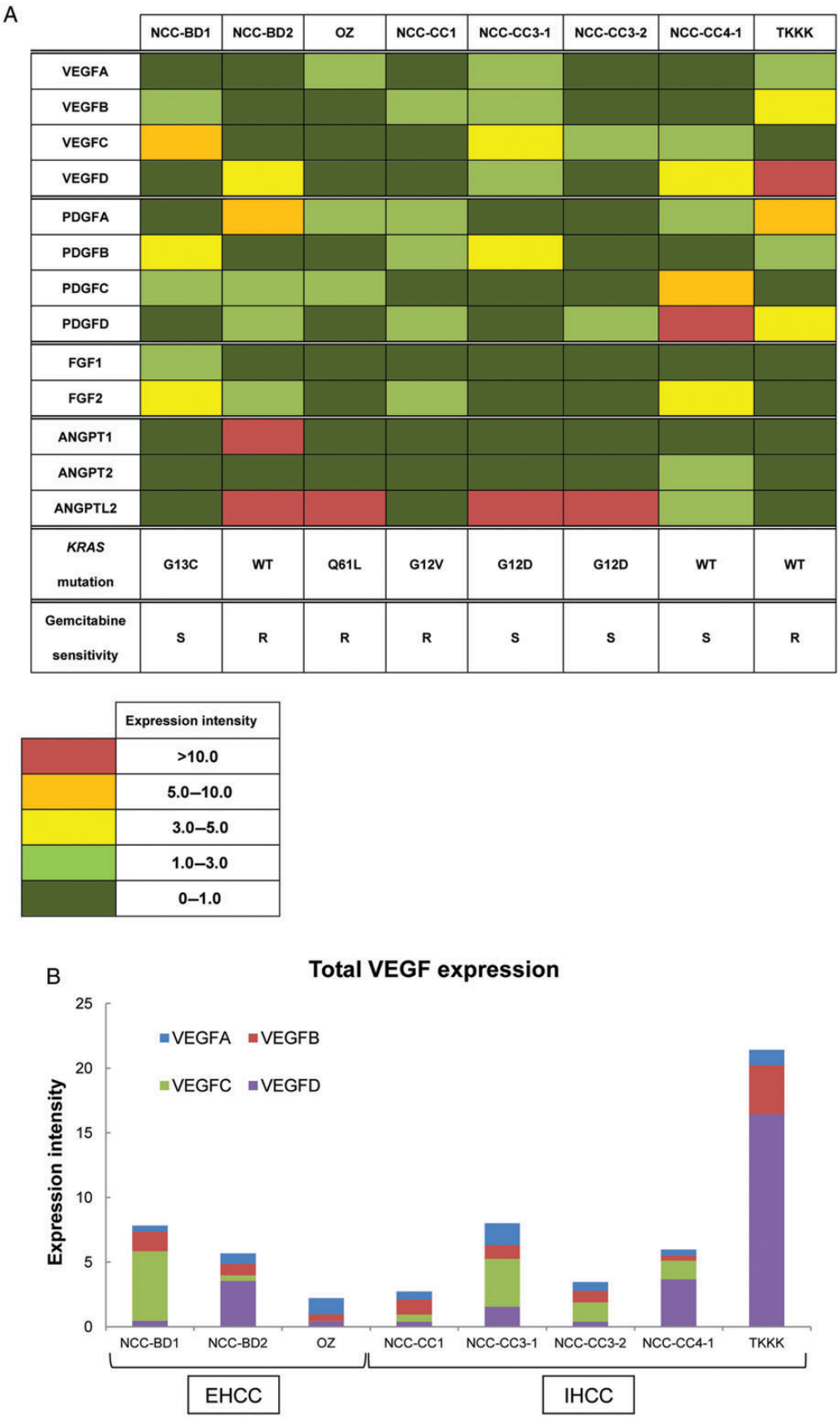

Figure 1. (A) Expression intensity of angiogenesis-related genes in the eight CC cell lines. Normalized expression data are presented in graduated color patterns. Red, >10.0; orange, 5.0–10.0; yellow, 3.0–5.0; pale green, 1.0–3.0; green 0–1.0. Mutation status of the *KRAS* gene and gemcitabine sensitivity (S: sensitive, R: resistance) are indicated at the bottom. (B) Total VEGF expression in eight CC cell lines. EHCC: extra-hepatic CC, IHCC: intra-hepatic CC.

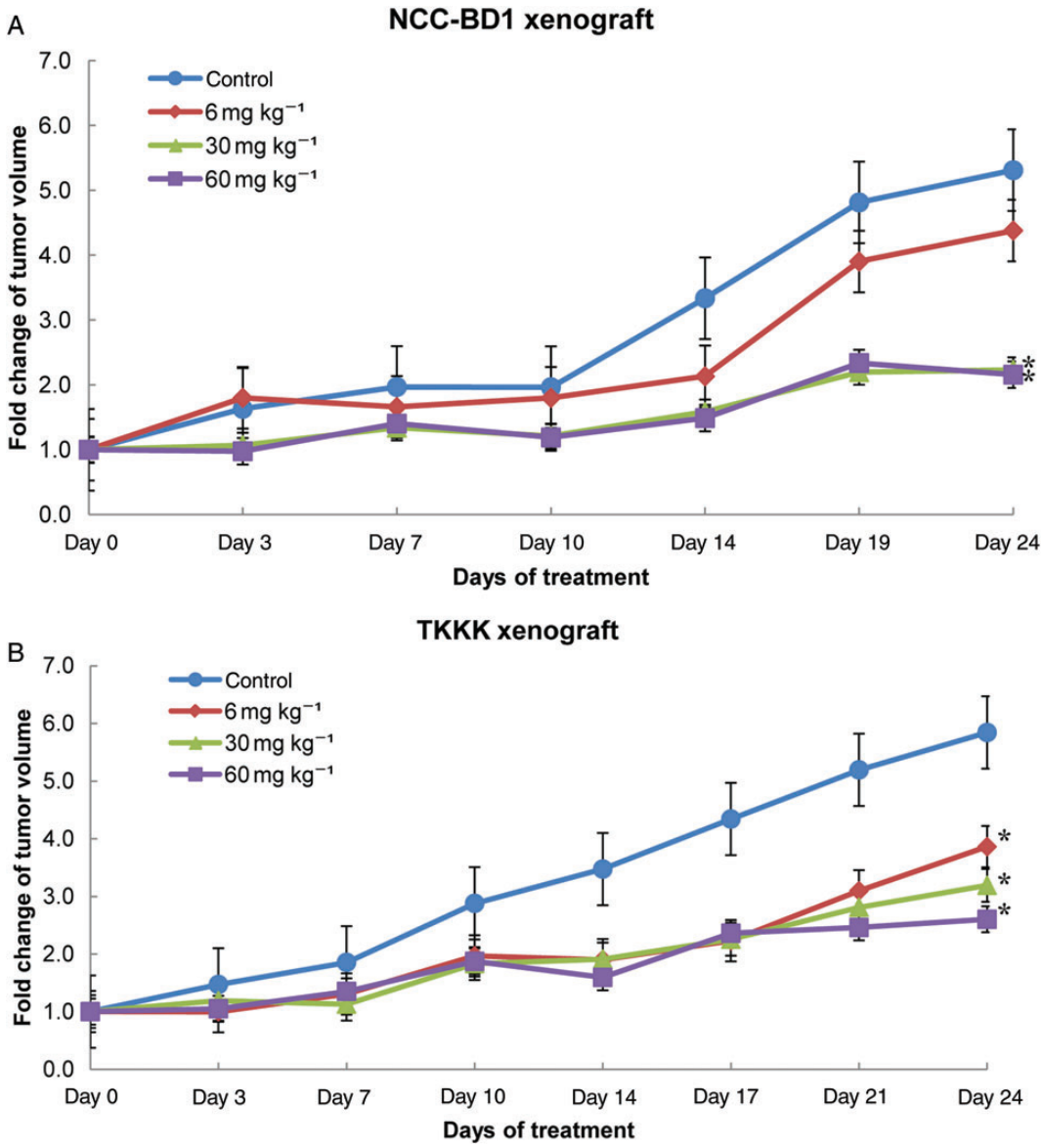

Figure 2. Average fold changes in tumor volume at each measurement point were plotted. (A) NCC-BD1 xenografts ($n = 10$). (B) TKKK xenografts ($n = 10$). All data are presented as mean ± SD *$P < 0.05$ (repeated measures ANOVA and Dunnett's test).

groups of NCC-BD1 xenografts (Fig. 4B left) and the groups in TKKK xenografts treated with 30 and 60 mg kg^{-1} axitinib (Fig. 4B right) relative to the vehicle-treated group. We also measured the frequency of apoptosis in tumor cells, which has been reported to be increased by treatment with other VEGFR inhibitors (29). Although the apoptosis positivity rate was not so marked (Fig. 3C), the AI was significantly increased in all the axitinib-treated groups of NCC-BD1 and TKKK xenografts relative to the vehicle-treated group.

DISCUSSION

In the present study we demonstrated the promising anti-tumor efficacy of axitinib, an orally administered specific VEGFR-1/2/3 tyrosine kinase inhibitor, against CC using xenograft models. Multi-tyrosine kinase inhibitors, including sorafenib and sunitinib, have previously been shown to have inhibitory effects on VEGFR tyrosine kinase, and their anti-neoangiogenesis effects have been demonstrated in various tumors (30–33). However, broad-spectrum TKIs showed low tolerability because of various side effects in clinical trials (34), and specific VEGFR TKIs are expected to be less toxic and better safety in combination with chemotherapy. Axitinib is a highly specific TKI for VEGFR1/2/3 in comparison with other VEGFR TKIs (34). Phase II studies in patients with various cancers (35–39) and a Phase III study (33) of patients with metastatic renal cell carcinoma (40) have shown the effectiveness in axitinib single use or in combination with other anti-tumor agents, but no clinical trials for patients with CC have been performed.

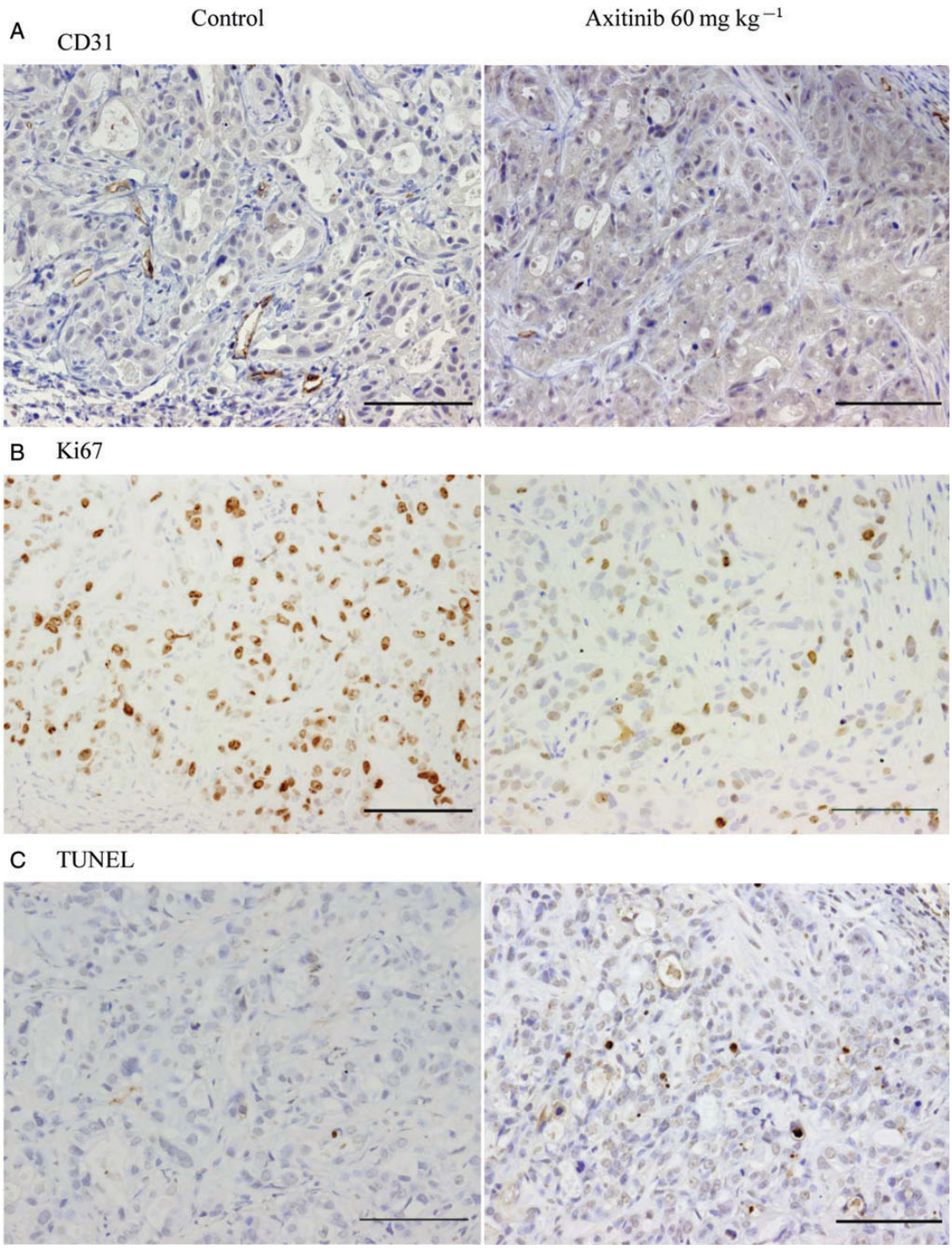

Figure 3. Representative pictures of CD31 (vascular endothelial cell marker, A), Ki-67 (proliferation marker, B) and TUNEL (apoptosis marker, C) in tumors of subcutaneous xenograft models (left: control, right: axitinib-treated). In the groups treated with axitinib 60 mg kg^{-1}, the microvessel density (MVD) and proliferation index (PI) were decreased and the apoptotic index (AI) was increased relative to control tumors. Scale bar indicates 200 μm.

Histological examination of axitinib-treated tumors revealed no induction of massive tumor necrosis. However, neoangiogenesis and tumor cell proliferation were significantly inhibited in both xenograft models. Axitinib treatment did not inhibit the proliferation of NCC-BD1 and TKKK cells *in vitro* (Supplementary data, Fig. S1). These observations confirmed that the anti-tumor effect of axitinib was more indirect, probably mainly involving anti-neoangiogenesis in the tumor microenvironment, being consistent with the highly selective anti-VEGFR activity of axitinib.

We previously reported that tumor cells overexpressed VEGF in ∼60% of primary CC cases (24), indicating that

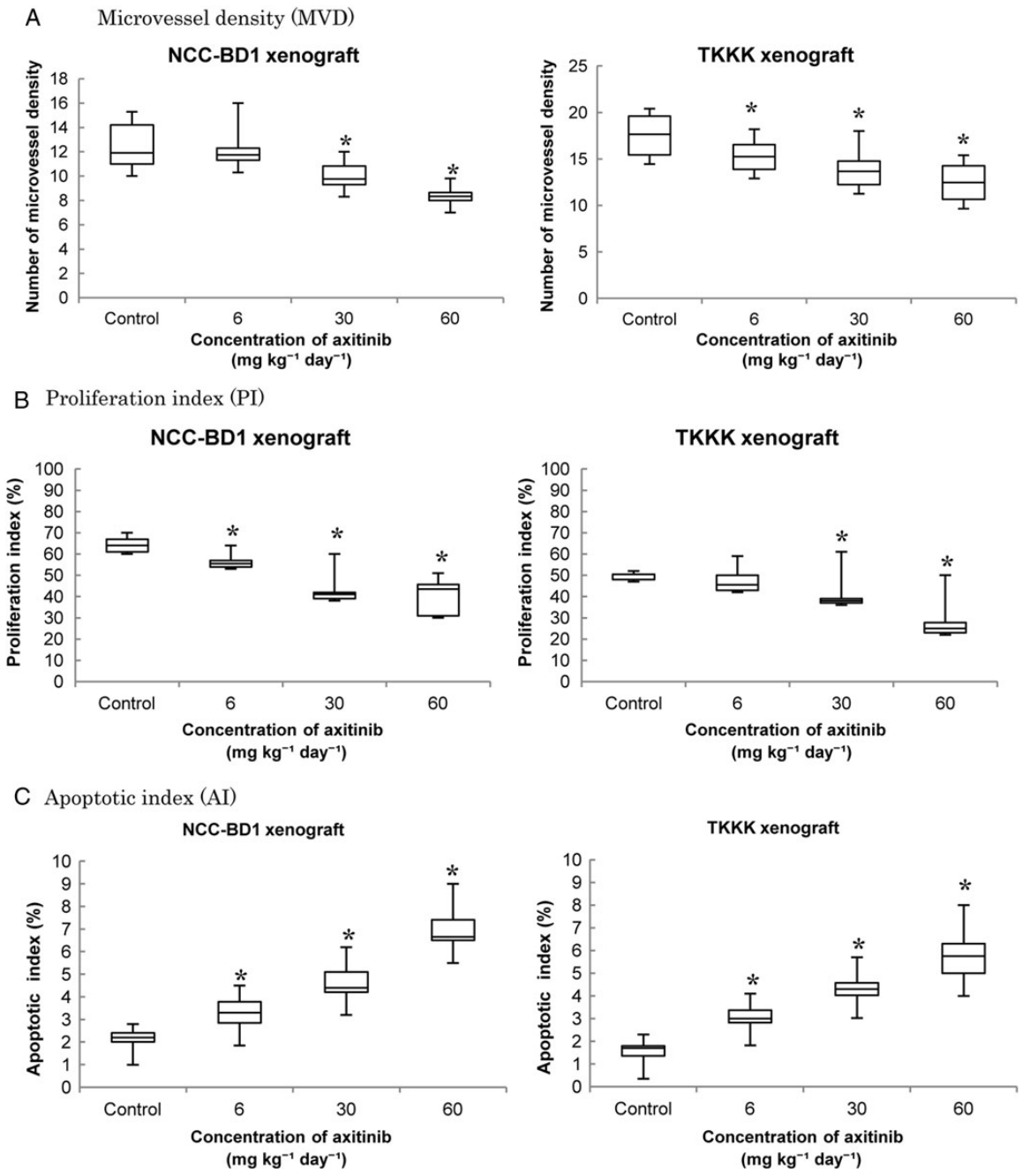

Figure 4. Box plots of MVD (A), PI (B), and AI (C) of control and axitinib-treated xenograft tumors. Upper and under bar means 90th and 10th percentiles, respectively, and the line in the box means the median. *$P < 0.01$ (ANOVA and Dunnett's test).

tumor cells are one of the main VEGF sources in primary CC, and the VEGF mRNA expression corresponds with the VEGF protein expression in CC cell lines (29). Based on gene expression profiling, we selected two CC cell lines that expressed abundant VEGF and did not show high expression of other angiogenesis factors for application to our therapeutic models. No precise biomarker for predicting the efficacy of anti-angiogenesis has yet been defined (17). However, considering the selective activity of axitinib for VEGFRs and our *in vivo* results, it is possible that VEGF expression could be a potential biomarker for axitinib treatment. Interestingly, microarray analysis revealed that expression of ANGPTL2 was complimentary to that of VEGFs and PDGFs in the CC cell lines. Endo *et al.* (41) reported that ANGPTL2 was involved in tumor angiogenesis with low VEGF expression. Therefore, expression of other angiogenesis factors, especially ANGPTL2, could be a negative predictive marker for axitinib treatment. These possibilities should be examined in a future clinical trial.

Although gemcitabine plus cisplatin combination therapy (GC therapy) have been established as a standard treatment for unresectable CC (13,14), most patients treated with GC

therapy have progression. However, there is no consensus regarding alternative treatment or sequential treatments (7,9–12). In this study, we showed that axitinib exerted an anti-tumor effect on both a gemcitabine-resistant cell line (TKKK) and a gemcitabine-sensitive cell line (NCC-BD1). This result suggested that axitinib might be applicable as a second-line treatment after gemcitabine treatment has failed. Small-molecule compounds or antibodies with anti-angiogenesis effects have been used in combination with other chemotherapeutic agents (42), and axitinib has also been used in combination with mFOLFOX6 (43) and Docetaxel (39) in Phase II studies.

In conclusion, our preclinical study has clearly demonstrated that an orally administered selective VEGFR TKI, axitinib, exerts a considerable anti-tumor effect against CC in xenograft models. Our results also suggest that expression of VEGF and other angiogenesis factors may be associated with the efficacy of axitinib treatment, and that axitinib warrants further evaluation in clinical trials such as second line setting in patients with gemcitabine refractory disease.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org

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Conflict of interest statement

None declared.

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