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# Antiproliferative Effect of Lenvatinib on Human Liver Cancer Cell Lines *In Vitro* and *In Vivo*

SACHIKO OGASAWARA[1], YUTARO MIHARA[1], REIICHIRO KONDO[1], HIRONORI KUSANO[1], JUN AKIBA[2] and HIROHISA YANO[1]

[1]*Department of Pathology, Kurume University School of Medicine, Kurume, Japan;*
[2]*Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Japan*

**Abstract.** *Background/Aim: Lenvatinib is a potent inhibitor of receptor tyrosine kinases, targeting vascular endothelial growth factor receptors (VEGFR1-3), fibroblast growth factor receptors (FGFR1-4), KIT, and RET. Here, we investigated the antiproliferative effects of lenvatinib in liver cancer cells in vitro and in vivo. Materials and Methods: Eleven hepatocellular carcinoma cell lines and two combined hepatocellular/cholangiocarcinoma cell lines were treated with 0-30 μM lenvatinib. Cell growth, apoptosis and the expression of FGFR1-4, FGF19, fibroblast growth factor receptor substrate (FRS)2α and RET were examined. Two HCC cell lines were subcutaneously implanted on nude mice and mice were treated with 3, 10, 30 mg/kg/day of lenvatinib or vehicle for 14 consecutive days. Tumor volume was measured every 3 days. Mice were sacrificed on day 15 and tumors were processed for histological examination. Blood vessels, microvessel density, necrosis, and apoptosis were also examined. Results: Lenvatinib dose- and time-dependently inhibited growth of all cell lines; however, sensitivity to lenvatinib varied. Apoptosis was not observed in any cell line, and expression of FGFR1, -2, -3 and -4, FGF19, FRS2α, and RET were observed in these cell lines. Cell lines with high expression of these factors showed higher response to lenvatinib. In mice, lenvatinib dose-dependently suppressed tumor growth. Blood vessels and microvessel density were significantly reduced and the rate of necrosis was significantly increased by lenvatinib; apoptosis was not observed. Conclusion: Antiproliferative effects of lenvatinib on liver cancer cells were observed in vitro and in vivo. Lenvatinib may suppress tumor formation by inhibiting angiogenesis, and via an additional direct antiproliferative effect in some liver cancer cells.*

*Correspondence to:* Sachiko Ogasawara, Department of Pathology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan. E-mail: sachiko@med.kurume-u.ac.jp

*Key Words:* Liver cancer, tyrosine kinase inhibitor, lenvatinib, angiogenesis, fibroblast growth factor.

Liver cancer is one of the most prevalent and leading causes of cancer-related mortality worldwide, and has a high incidence in Asia (1). Hepatocellular carcinoma (HCC) accounts for approximately 90% of primary liver cancer (2). The major risk factors for HCC are chronic viral hepatitis (type B and C), excessive alcohol consumption, and exposure to aflatoxin and the contributions of these factors may vary depending upon geographical location.

The progression of HCC is associated with growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (3). Sorafenib, an oral multi-kinase inhibitor targeting RAF kinase, VEGF receptors (VEGFR1-3), PDGF receptor (PDGFR), fms-like tyrosine kinase 3, RET, and KIT (4, 5), was approved for first-line treatment of advanced HCC in 2007. However, the objective response rate (ORR) is low, and several studies have reported substantial toxicities associated with sorafenib (6, 7). Therefore, alternative therapies that are less toxic and more efficacious are necessary. Although several tyrosine kinase inhibitors (TKIs) have been compared to sorafenib in clinical studies of patients with advanced HCC, the clinical trials did not show non-inferiority (8-10) or superiority (11) in prolonging survival when used as first-line treatment of HCC.

Lenvatinib is an oral multi-kinase inhibitor that targets VEGFR1-3, FGFR1-4, PDGFR, RET, and KIT and has shown antitumor activity against advanced solid tumors (5, 12, 13). Lenvatinib has shown promising antitumor effect for the treatment of radioiodine-refractory differentiated thyroid cancer and is approved in more than 50 countries including the United States, Japan, and Europe.

The phase III trial of lenvatinib demonstrated an effect on the overall survival (OS) of 954 patients with previously untreated unresectable HCC tumors by statistical confirmation of non-inferiority when compared to sorafenib

(14). In this study, lenvatinib demonstrated statistically significant superiority in progression-free survival, time to progression, and ORR and was approved for the treatment of HCC in Japan, the United States, Europe, South Korea and China in 2018.

In this study, we examined: i) The antiproliferative effects of lenvatinib on liver cancer cell lines *in vitro* and *in vivo*; ii) the protein expression of growth-related factors in liver cancer cells; and iii) the mechanism of action of lenvatinib in liver cancer cells.

## Materials and Methods

*Cell lines and reagents*. This study utilized 11 HCC cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, HAK-1B, HAK-2, HAK-3, HAK-4, HAK-5 and HAK-6) and two combined hepatocellular-cholangiocarcinoma cell lines (KMCH-1 and KMCH-2). These cell lines were originally established in our laboratory, and each cell line retains the morphological and functional features of the original tumor as described elsewhere (15-22). The cells were grown in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Co., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56˚C, 30 min) fetal bovine serum (Bioserum, Victoria, NSW, Australia), 100 u/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL/Life Technologies, Inc., Gaithersburg, MD, USA) and 12 mmol/l sodium bicarbonate, in an incubator with humidified atmosphere containing 5% $CO_2$ at 37˚C. Lenvatinib was supplied from Eisai, Tokyo Japan.

*Effects of lenvatinib on the proliferation of liver cell lines in vitro*. The cells ($2\text{-}10\times10^3$ cells per well) were seeded in 96-well plates (Thermo Fisher Science, Roskilde, Denmark), cultured for 24 h, and the culture medium was changed to a new medium with or without lenvatinib at 1.875, 3.75, 7.5, 15 and 30 μM. After culturing for 24, 48, or 72 h, the number of viable cells was examined using the MTT cell growth assay kit (International Inc., Temecula, CA, USA). The 50% inhibitory concentration ($IC_{50}$) for each cell line was estimated after 72 h of culture with lenvatinib.

*Quantitative analysis of lenvatinib-induced apoptosis in vitro*. Cells cultured with or without lenvatinib (7.5 and 30 μM) for 48 h were stained using the Annexin V-EGFP Apoptosis Detection Kit (Medical & Biological Laboratories, Co., Ltd, Nagoya, Japan), according to the manufacturer's protocol. After staining, cells were analyzed using the FACS Aria II flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and the annexin V-EGFP-positive apoptotic cells were quantified.

*Expression of growth-related factors in liver cancer cell lines*. Western blot analysis was performed according to previously described methods (23). To examine the expression of growth-related factors, a sample (20 μg total protein per lane) was resolved by Any kD™ Mini-PROTEAN TGX Precast acrylamide gels (Bio-Rad, Hercules, CA, USA). The proteins were transferred to PVDF membranes (Merck, Darmstadt, Germany), which were incubated with primary antibodies against the following: FGFR1 (D8E4, 1:1000; Cell Signaling, Beverly, MA, USA), FGFR2 (D4L2V, 1:1000; Cell Signaling), FGFR3 [EPR2305(3), 1:10000; Abcam, Cambridge, MA, USA], FGFR4 (D3B12, 1:1000; Cell Signaling), FRS2 (462910, 1:500; R&D Systems, Minneapolis, MN, USA), FGF19 (D1N3R, 1:1000; Cell Signaling), RET (E1N8X, 1:1000; Cell Signaling) and β-actin (AC-15, 1:2000; Sigma-Aldrich, St. Louis, MO, USA). Blots were detected with ImmunoStar LD (Wako Pure Chemical Ind., Tokyo, Japan). Immunoreactive bands were visualized by using FluorChem™ FC3 (ProteinSimple, San Jose, CA, USA).

Effects of lenvatinib on tumor formation of HCC cell lines in nude mice. KYN-2 cells ($8\times10^6$ cells/100 μl) or HAK-1B cells ($8\times10^6$ cells/100 μl) were transplanted subcutaneously into the backs of 4-week-old female BALB/c nude mice. After tumor formation was confirmed, the mice were divided into four groups (n=6 per group) and treated with 3, 10, or 30 mg/kg/day of lenvatinib or vehicle (control) administered orally for 14 consecutive days. The tumor size was measured in two directions using calipers (until day 15), and tumor volume ($mm^3$) was estimated using the equation [length×$(width)^2$×0.5] on every 3 days. On day 15, the mice were sacrificed and the resected tumors were weighed before formalin fixation. Paraffin sections were prepared for histological analysis.

*Histological analysis*. Paraffin sections were stained with hematoxylin-eosin. The terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling (TUNEL) technique (Apoptag Peroxidase In Situ Apoptosis Detection kit; Chemicon International Inc., Temecula, CA, USA) was used to detect apoptotic cells. Immunohistochemical staining with anti-mouse CD34 (Rat monoclonal, MEC14.7, 1:50 dilution; Abcam) was performed by standard avidin-biotin-peroxidase complex method and 3,3'-diaminobenzidine solution was used for color development. To quantify the number of blood vessels and microvessel density (MVD), the slides were stained with antibody to CD34. Under microscopy, five areas were selected at a high power (×200), and the number of blood vessels and the MVD in each specimen were measured using the WinROOF software package (version 6.1; Mitani Corporation, Fukui, Japan). The slides stained with hematoxylin-eosin were photographed using a loupe in the image and necrosis in the entire tumor was analyzed using the WinROOF software package.

*Statistical analysis*. Data are expressed as the mean±standard deviation (SD). Comparisons between groups were performed using Student's *t*-test and two-way factorial analysis of variance (ANOVA); $p<0.05$ was considered significant.

*Ethics statement*. This study was approved by the Ethics Committee of Kurume University (approval 2017#319). Animal experiments for this study were approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine (approval 2017#217), according to guidelines created on the basis of the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

## Results

*Effects of lenvatinib on the proliferation of liver cell lines in vitro*. In all cell lines, a time-dependent antiproliferative effect was observed to various degrees upon treatment with lenvatinib for 24, 48 and 72 h in comparison to the control (Figure 1A). A decrease in cell viability of at least 20% was

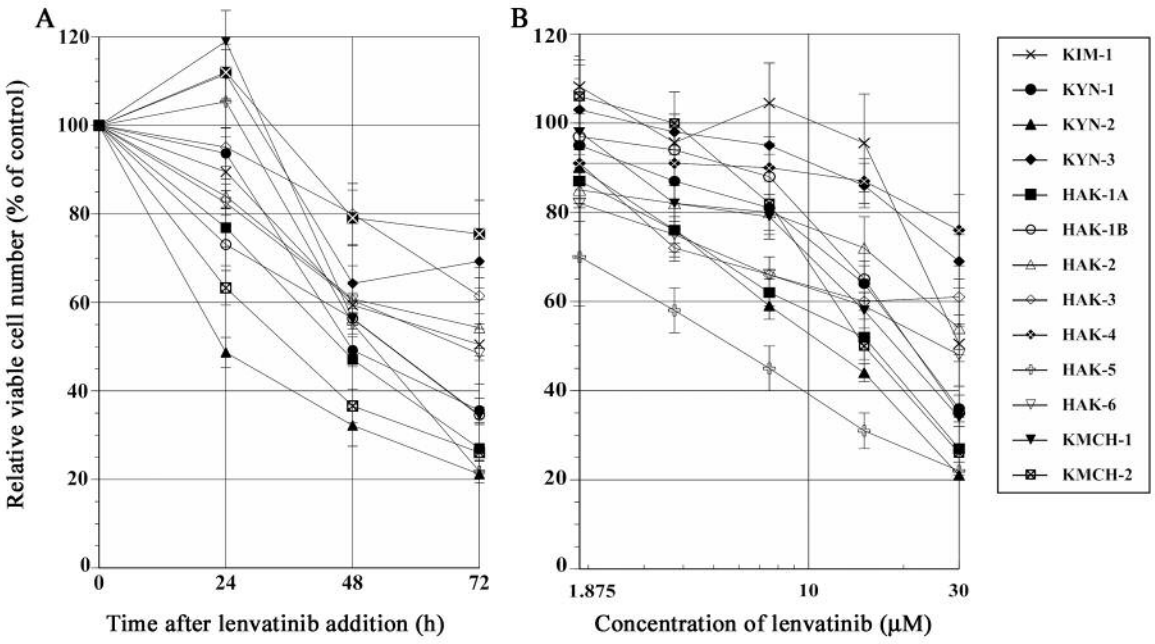


Figure 1. *Antiproliferative effect of lenvatinib. A: Chronological changes in relative viable cell number (% of the control) after adding 30 μM of lenvatinib. B: Relative viable cell number 72 h after adding 1.875, 3.75, 7.5, 15, or 30 μM of lenvatinib. Figures represent the average±SD.*

observed in all 13 cell lines after 72 h of 30 μmol/l lenvatinib treatment. After 72 h, the relative number of viable cells decreased in every cell line in a dose-dependent manner (Figure 1B). The number of viable cells at 72 h decreased to <50% of the control in eight cell lines and the $IC_{50}$ of lenvatinib was 5.8 μM for HAK-5, 10.4 μM for KYN-2, 12.5 μM for HAK-1A, 15.4 μM for KMCH-2, 18.2 μM for KMCH-1, 20.3 μM for KYN-1, 20.4 μM for HAK-1B, and 28.5 μM for HAK-6.

*Quantitative analysis of lenvatinib-induced apoptosis in vitro*. In all cell lines, quantitative analysis of apoptosis revealed that lenvatinib did not induce an increase in the amount of apoptosis *in vitro* (Figure 2).

*Expression of growth-related factors in liver cancer cell lines*. The protein expression of FGFR1-4 and FRS2α varied among cell lines (Figure 3). The expression of FGF19 was observed only in KYN-2 and HAK-6, and the expression of RET was observed in HAK-5.

The protein expression of FGFR1, FGFR2, FGFR3, FGFR4, FGF19, FRS2α, and RET was clearly observed in 11 cell lines (all except KYN-1 and HAK-1A), five (KIM-1, KYN-2, HAK-1A, HAK-1B, and KMCH-2), nine (all except KIM-1, KYN-1, HAK-2, and HAK-6), 10 (except HAK-1A, HAK-3, and HAK-4), two (KYN-2 and HAK-6), 13, and 1 (HAK-5), respectively, of the 13 cell lines analyzed.

*Effects of lenvatinib on tumor formation of HCC cell line in nude mice*. Lenvatinib dose-dependently suppressed tumor growth in nude mice subcutaneously implanted with KYN-2 and HAK-1B HCC cell lines. The average volume of each KYN-2 tumor in mice receiving 3, 10, and 30 mg/kg of lenvatinib decreased to 37% ($p<0.05$), 29% ($p<0.01$), and 20% ($p<0.01$), respectively, compared to the control (Figure 4A). Tumor weights after 3, 10, and 30 mg/kg of lenvatinib also decreased by 50%, 27% ($p<0.05$), and 22% ($p<0.05$), respectively (Figure 4B). In addition, the average volume of each HAK-1B tumor in mice receiving 3, 10, and 30 mg/kg of lenvatinib decreased to 61%, 32% ($p<0.01$), and 17% ($p<0.01$), respectively (Figure 4A) with tumor weights also decreasing by 74%, 42% ($p<0.01$), and 40% ($p<0.01$), respectively (Figure 4B). Lenvatinib administration did not affect the body weights of mice (data not shown).

*Histological analysis*. Lenvatinib did not induce apoptosis of KYN-2 and HAK-1B HCC cells *in vivo* (Figure 5). The number of blood vessels and the MVD per unit area decreased as the concentration of lenvatinib increased (Figure 6). In the KYN-2 cell transplant model, the number of blood vessels in mice receiving 3, 10, and 30 mg/kg of lenvatinib decreased to 72% ($p<0.01$), 39% ($p<0.001$), and 21% ($p<0.001$) , respectively (Figure 7A) compared to the control and the MVD under the same treatment condition decreased to 90%, 14% ($p<0.01$), and

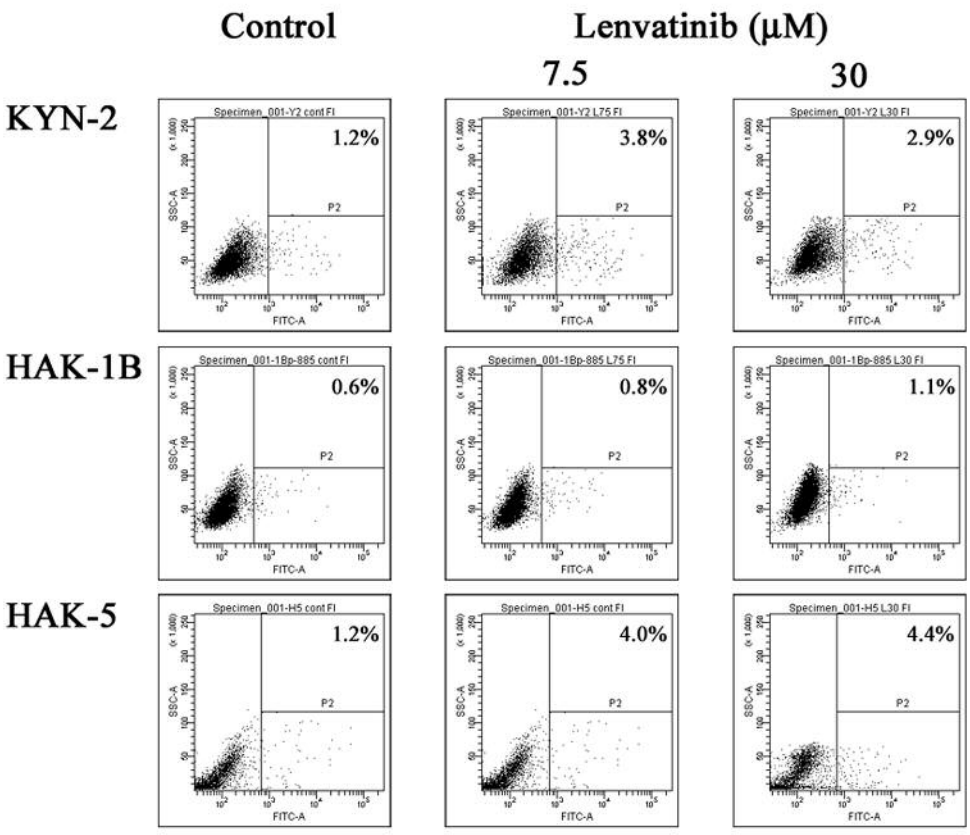


Figure 2. *Quantitative analysis of lenvatinib-induced apoptosis cells in vitro. Representative data of three cell line experiments are shown.*

6% ($p<0.01$), respectively (Figure 7B). The rate of necrosis was significantly increased by lenvatinib in a dose-dependent manner in mice implanted with KYN-2 cells and treated with lenvatinib (control *vs.* 10 mg/kg or 30 mg/kg, $p<0.05$) (Figure 7C). In HAK-1B-transplanted mice, the number of blood vessels in mice receiving 3, 10, and 30 mg/kg of lenvatinib decreased to 59% ($p<0.01$), 29% ($p<0.001$), and 27% ($p<0.001$) , respectively (Figure 7A), and the MVD under the same treatment decreased to 48% ($p<0.05$), 19% ($p<0.01$), and 1% ($p<0.01$), respectively (Figure 7B). The rate of necrosis of transplanted HAK-1B cells increased by more than two-fold with 3, 10, and 30 mg/kg lenvatinib treatment ($p<0.05$) compared with the control (Figure 7C).

## Discussion

In this study, we examined the effect of lenvatinib on cell proliferation *in vitro* using 13 different cell lines of liver cancer. There was a suppression of cell proliferation in response to lenvatinib in all the cell lines tested. However, the sensitivity varied among the cell lines. The $IC_{50}$ values

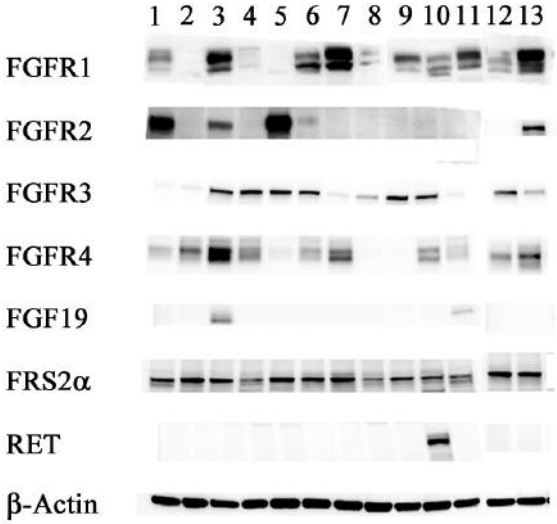


Figure 3. *Western blot analysis of fibroblast growth factor receptor 1-4, FGF19, fibroblast growth factor receptor substrate 2α, and RET in different liver cancer cell lines. Lane 1: KIM-1, 2: KYN-1, 3: KYN-2, 4: KYN-3, 5: HAK-1A, 6: HAK-1B, 7: HAK-2, 8: HAK-3, 9: HAK-4, 10: HAK-5, 11: HAK-6, 12: KMCH-1, 13: KMCH-2.*

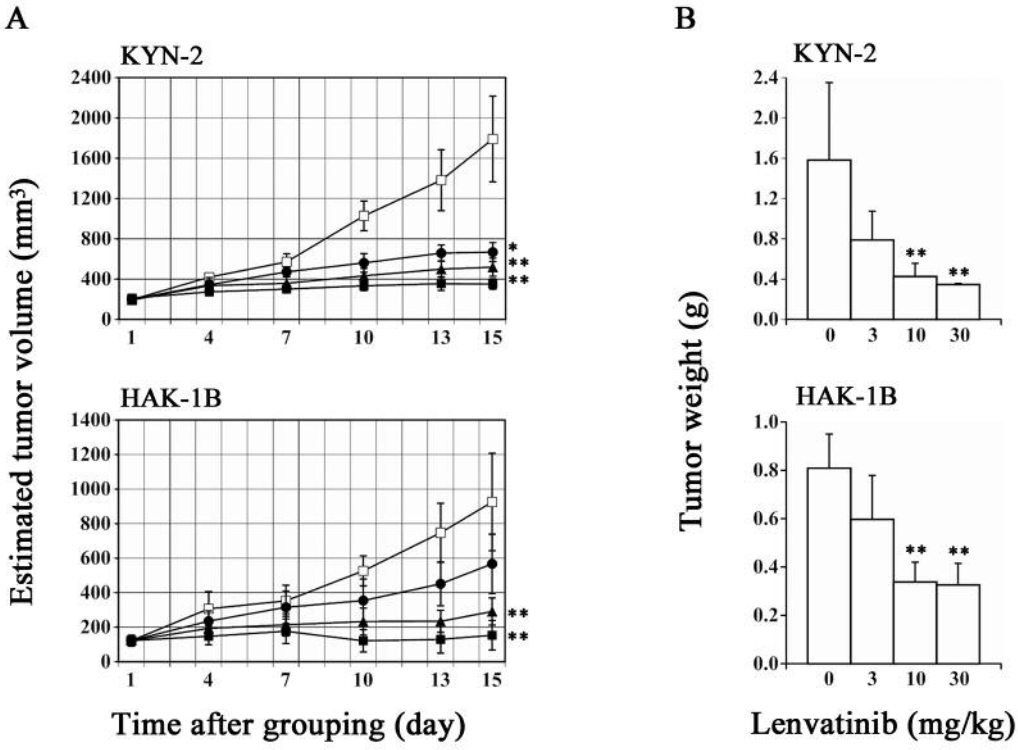


Figure 4. *Antitumor effect of lenvatinib on tumors developed after subcutaneous transplantation of KYN-2 and HAK-1B cells in nude mice. A: Estimated volume of tumors generated by subcutaneously implanted HCC cells over a time course. The mice received 3 (●), 10 (▲), or 30 mg/kg/mouse/day (■), lenvatinib or vehicle (control) (□). B: All mice were sacrificed on day 15, and tumor weight was measured. Figures represent the average±SD. Significantly different at *p<0.05 or **p<0.01 vs. control.*

ranged between 5.8 and 28.5 μM in 8/13 cell lines, however $IC_{50}$ values could not be determined in the remaining five cell lines. We previously examined the effect of sorafenib in the same liver cancer cell lines and observed $IC_{50}$ values ranging from 2.1 to 4.3 μM [(24) for KIM-1 and HAK-1B, data not shown for others]. Yamamoto *et al*. also examined the suppressive effect of lenvatinib on cell proliferation *in vitro* using lung cancer and colon cancer cell lines, in which the $IC_{50}$ values ranged from approximately 20 to 30 μM (13); in addition, the $IC_{50}$ values for lenvatinib were relatively higher than those for sorafenib.

Our analysis on the expression of growth-related factors indicated that cell lines showing high response to lenvatinib expressed growth-related factors in abundance including RET, FGFRs and FGF19. Because lenvatinib is a potent inihibitor of tyrosine receptor kinases including RET and FGFRs, the inhibitory action of lenvatinib may depend upon the expression of growth-related factors. RET was most highly expressed in HAK-5 cells among the cell lines studied, whose $IC_{50}$ value was the lowest in response to treatment of lenvatinib. Lenvatinib was shown to be effective in thyroid cancer (25), where RET is highly expressed and is known to be highly mutated (26, 27). However, in contrast to some studies reporting that RET is highly mutated in thyroid cancer, Kato *et al*. reported that in the analysis of aberrations including *RET* mutations and amplifications, aberrations were only observed in 88 (1.8%) of 4,871 cases of other cancer types and of them, aberrations were observed only in one of 44 types of HCC (28). The frequency of *RET* aberrations is low in other types of cancer and the effect of lenvatinib *via* RET inhibition may be limited to some cases. The expression of RET protein was observed in response to lenvatinib; therefore, analyses of both mutations and protein levels of RET need to be performed.

In our previous study using six out of these 13 cell lines, FGF2 and FGFRs expression was detected and cell proliferation was suppressed by an FGF2-neutralizing antibody (23); therefore, FGF2 and FGFRs have been shown to be involved in the proliferation of HCC cells. The proliferation of liver cancer cells may have been suppressed by the direct interaction of lenvatinib with cancer cells *via* FGFR *in vitro*. The growth of some HCC is considered to be related to the signal transduction system of FGFs and FGFRs (29, 30). It has been reported that the expression of FGFR4,

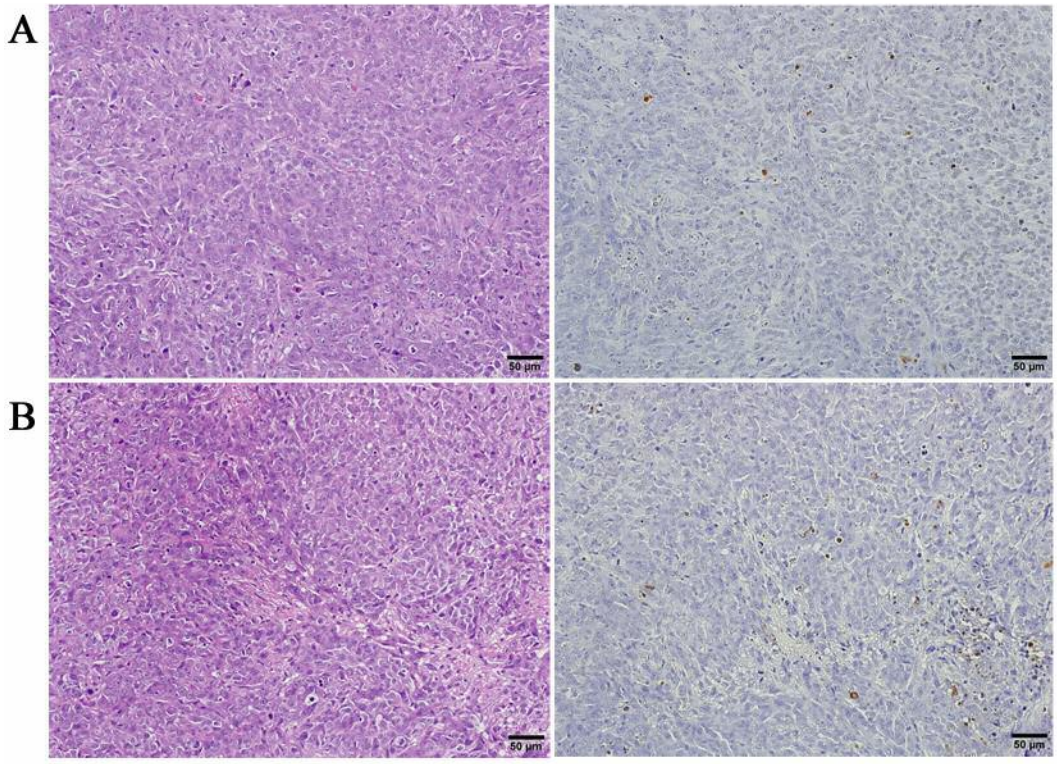


Figure 5. *Analysis of lenvatinib-induced apoptosis in human hepatocellular carcinoma tumor HAK-1B subcutaneously transplanted in a nude mouse that received vehicle (A) and in a mouse that received 30 mg/kg of lenvatinib (B). Left panel: Hematoxylin and eosin staining. Right panel: Staining by the TUNEL technique. Scale bar=50 μm.*

which is involved in angiogenesis, is found in one-third of HCC cases (31-33). Since there are few inhibitors of FGFR4 (34), drugs such as lenvatinib that reliably inhibit FGFR are useful for the treatment of HCC.

Although lenvatinib did not induce apoptosis of liver cancer cells *in vitro* or *in vivo* in this study, it has been reported to do so in thyroid cancer (35) and nasopharyngeal carcinoma (36). Lenvatinib-induced apoptosis appears to be cancer type specific.

We observed suppression of tumor growth and angiogenesis with lenvatinib treatment and an increase in the rate of necrosis of KYN-2 and HAK-1B HCC cells implanted in mice *in vivo*. In our previous study on sorafenib in the same liver cancer cell lines, $IC_{50}$ values were obtained for all 13 cell lines and apoptosis was induced in 8/13 cell lines *in vitro* [(24) for KIM-1and HAK-1B, data not shown for others]. Both tumor volume and tumor weight only decreased to 50% with sorafenib administration in mice transplanted with KYN-2 cells (37); whereas, a decrease to below 30% was observed with lenvatinib administration. The vascular density did not decrease to below 50% with sorafenib administration, but decreased to 14% with lenvatinib administration. We found the effect of lenvatinib to be inferior to that of sorafenib in the suppression of cell growth *in vitro*, but superior in tumor-growth suppression *in vivo*. Type I to V kinase inhibitors binding to VEGFR2 have been reported, of which sorafenib is a type II (38) and lenvatinib a type V inhibitor (39). Type V inhibitors are reported to possess faster binding and higher affinity for VEGFR2, making their kinase-inhibitory activities stronger compared to type II inhibitors. FGFR signaling increases VEGF as well as VEGFR, and when VEGF and VEGFR are suppressed, FGF production is activated to enhance FGFR signaling, which causes VEGFR and FGFR to cooperatively promote tumor angiogenesis (40). Therefore, we presume that the suppressive effect of lenvatinib on angiogenesis was exerted by quickly binding to factors (such as VEGFR and FGFR) involved in tumor angiogenesis with increased affinity, which led to the highly suppressive effect on tumor growth *in vivo*, likely by the indirect activity of lenvatinib rather than its direct activity.

In conclusion, our study suggests that lenvatinib exhibits an antitumor effect mainly by suppressing angiogenesis, but it may directly suppress cell proliferation in cells with high FGFR expression.

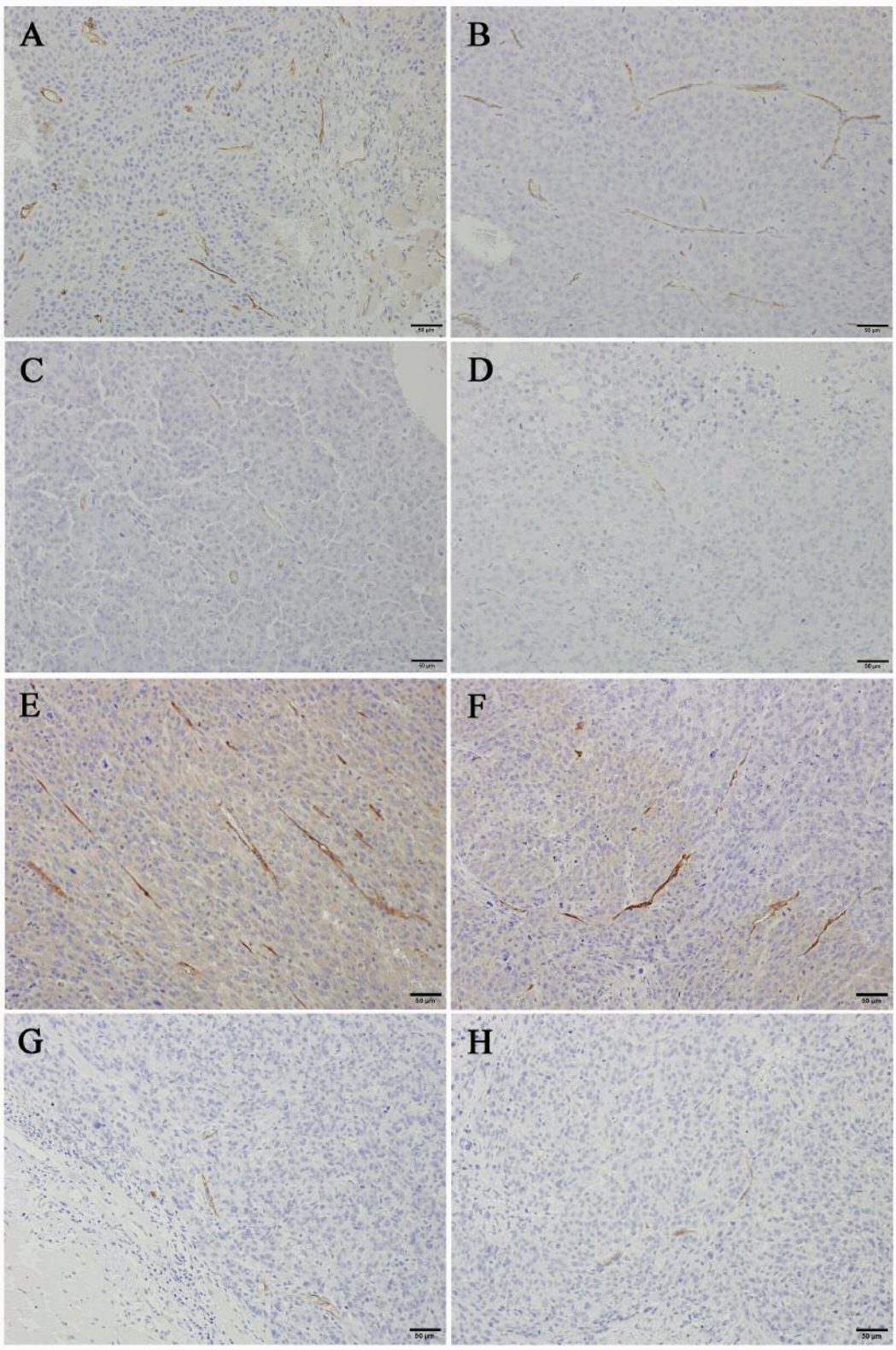


Figure 6. *Effect of lenvatinib on suppression of angiogenesis in tumors developed after subcutaneous transplantation of hepatocellular carcinoma cells. Immunohistochemical staining of CD34 in KYN-2 cell tumors (A-D) and HAK-1B cell tumors (E-H). Scale bar=50 μm. Mice received vehicle (control) (A, E), or lenvatinib of at 3 (B, F), 10 (C, G), or (D, H) 30 mg/kg/mouse/day.*

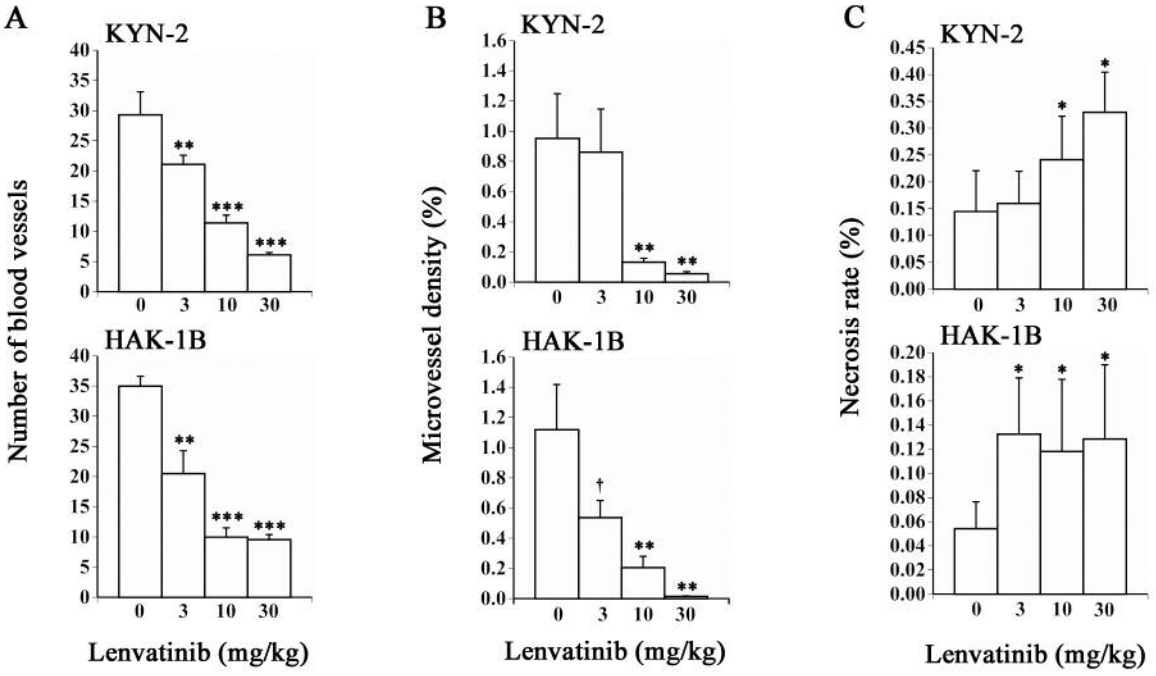


Figure 7. *Effect of lenvatinib on blood vessel frequency (A), microvessel density (B), and necrosis rate of tumors (C) in nude mice. Figures represent the average±SD. Significantly different at *p<0.05, **p<0.01, or ***p<0.001 vs. control.*

## Conflicts of Interest

The Authors have no conflicts of interest to disclose.

## Authors' Contributions

Sachiko Ogasawara: Performed the entire experiment. Article preparation and submission. Yutaro Mihara: Cooperation in animal experiments. Reiichiro Kondo: Cooperation in examination of tissue specimens. Hironori Kusano: Cooperation in examination of tissue specimens. Jun Akiba: Input in evaluating results. Hirohisa Yano: Input in evaluating results.

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