

ORIGINAL ARTICLE

Epidermal growth factor receptor activation confers resistance to lenvatinib in thyroid cancer cells

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Funding information

This work was supported by a Grant-in-aid for Scientific Research (#20K08953) from the Japanese Society for the Promotion of Science

Abstract

Thyroid cancer is the most common endocrine malignancy. A multitargeted tyrosine kinase inhibitor, lenvatinib, has been used for the treatment of advanced thyroid cancer. To elucidate the mechanism of resistance to lenvatinib in thyroid cancer cells, we established lenvatinib-resistant sublines and analyzed the molecular mechanisms of resistance. Two thyroid cancer cell lines (TPC-1 and FRO) were used, and resistant sublines for lenvatinib (TPC-1/LR, FRO/LR) were established. In TPC-1/LR, the phosphorylation of epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK), and Akt was enhanced whereas in FRO/LR, the phosphorylation of EGFR and downstream signal transduction molecules was not enhanced. The addition of epidermal growth factor decreased sensitivity to lenvatinib in TPC-1 and FRO. The combination of EGFR inhibitors lapatinib and lenvatinib significantly inhibited the growth of TPC-1/LR in both in vitro and mouse xenograft models. Short-term exposure to lenvatinib enhanced the phosphorylation of EGFR in six thyroid cancer cell lines regardless of their histological origin or driver gene mutations; however, phosphorylation of ERK was enhanced in all cells except TPC-1. A synergistic growth-inhibitory effect was observed in three thyroid cancer cell lines, including intrinsically lenvatinib-resistant cells. The results indicate that signal transduction via the EGFR pathway may be involved in the development of lenvatinib resistance in thyroid cancer cells. The inhibition of the EGFR pathway simultaneously by an EGFR inhibitor may have therapeutic potential for overcoming lenvatinib resistance in thyroid cancer.

KEYWORDS

drug resistance, EGFR, lenvatinib, signal transduction, thyroid cancer

1 | INTRODUCTION

Thyroid cancer is the most common endocrine malignancy worldwide, with rising incidence in developed countries, partly due to the greater use of imaging methods.¹ According to GLOBOCAN,

an estimated 19.3 million new carcinoma cases and nearly 10 million cancer deaths were reported in 185 countries globally in 2020. Among these, thyroid cancers were reported to occur in 586,202 (3.0%) cases, with related deaths of 43,646 (0.4%), indicating a better prognosis than other carcinomas.² About 90% of thyroid cancers

Abbreviations: ATC, anaplastic thyroid carcinoma; Bax, Bcl-2-associated X protein; DTC, differentiated thyroid carcinoma; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FTC, follicular thyroid carcinoma; KIT, stem cell factor receptor; MKI, multitargeted kinase inhibitor; NTRK, neurotrophic tyrosine kinase receptor gene; PDGFR, platelet-derived growth factor receptor; PTC, papillary thyroid carcinoma; RET, rearranged during transfection; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; TPC-1/LR, lenvatinib-resistant TPC-1 cells; FRO/LR, lenvatinib-resistant FRO cells.

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are differentiated thyroid carcinomas (DTCs), including papillary and follicular thyroid carcinomas (PTC and FTC) originating from follicular epithelial cells and anaplastic thyroid carcinoma (ATC) accounting <2%.³ Ten-year disease-specific survival for DTC is 96%.⁴ Despite a favorable prognosis by standard treatment, such as surgery, with/without radioactive iodine therapy, some cases exhibit refractory aggressive behavior to radioactive iodine, approximately 6%–20% relapse as distant metastases, which becomes life-threatening in the patients, dropping the 10-year overall survival rate to <50%.⁵

Although mutations in oncogenes *BRAF*, *RAS*, or *RET/PTC* rearrangement frequently occur in thyroid cancer, the activation of the MAPK signaling pathway is observed regardless of the presence or absence of these gene mutations.^{6,7} Since vascular endothelial growth factor (VEGF)-driven angiogenesis and other molecular pathways of tumor growth and maintenance, including the mutated driver genes described above, fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR), is shown to associate with metastasis and aggressiveness of thyroid cancer,^{8–13} multitargeted tyrosine kinase inhibitors (TKI) have been developed for the treatment of radioiodine refractory thyroid cancer.^{14–19} Sorafenib and lenvatinib-tyrosine kinase inhibitors (TKIs) have also advanced as the mainstay of treatment in these cases, improving the prognosis of advanced or metastatic DTC.^{14,20} Lenvatinib is an oral TKI targeting VEGFRs 1–3, FGFRs 1–4, RET, KIT, and PDGFR.^{21,22} In a phase 3 study involving patients with radioiodine refractory progressive DTC, lenvatinib demonstrated prolonged progression-free survival and a good response rate than placebo.²⁰

However, no therapeutic effect was observed in some cases from lenvatinib initiation. Additionally, the emergence of resistance during treatment remains a serious problem in clinical practice. Therefore, it is essential to identify biomarkers that predict lenvatinib susceptibility and develop treatment strategies after acquiring resistance to lenvatinib. To date, no study on the molecular mechanism of lenvatinib resistance in thyroid cancer is reported. In this study, to elucidate this mechanism of lenvatinib used for thyroid cancer, we established lenvatinib-resistant sublines in thyroid cancer cell lines and investigated the molecular mechanisms involved in the development of resistance to lenvatinib.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

The PTC cell line TPC-1 and ATC cell line FRO were donated by Dr. Yamashita from Nagasaki University.²³ The FTC cell line, FTC-133, was donated by Dr. Takeda from Shinshu University School of Medicine.²⁴ The ATC cell line, ACT-1, which was established by Dr. Ohata from Tokushima University,²⁵ OCUT-1F, and OCUT-3 cells were donated by Dr. Onoda from Osaka City University.²⁶ The genetic information for these cell lines was provided by the researchers who gave us these cell lines. We outsourced the genetic analysis of FRO cells during this study. The cell lines were cultured in RPMI (Sigma-Aldrich) supplemented with 10% FBS at 37°C under 5% CO₂.

Lenvatinib-resistant sublines were established by stepwise increase in lenvatinib for more than 6 months. We subsequently selected several resistant clones for each thyroid cancer cell line and used one representative clone in subsequent experiments. The established lenvatinib-resistant clones were maintained in lenvatinib-containing media throughout the experiments. We maintained lenvatinib-resistant TPC-1 (TPC-1/LR) cells in a medium containing 15 nM lenvatinib and lenvatinib-resistant FRO (FRO/LR) cells in a medium with 30 nM lenvatinib. All cells were examined regularly for *mycoplasma* contamination. Lenvatinib and lapatinib were purchased from Selleck Biotech Co., Ltd. Human recombinant EGF was purchased from the FUJIFILM Wako Pure Chemical Corporation. In the experiments to examine the dose-dependent effects of EGF, the FBS concentration in the medium was reduced to 1% and cells were incubated for 24 h before addition of EGF. To analyze the biology of cancer cells on acquisition of sufficient resistance to lenvatinib, we removed lenvatinib 24 h before each assay to examine the response of lenvatinib-resistant sublines to drugs other than lenvatinib.

2.2 | WST assay for cell proliferation

The growth-inhibitory effects of lenvatinib and lapatinib were measured using a tetrazolium salt-based proliferation assay (WST assay; FUJIFILM Wako Pure Chemical Corporation).²⁷ Each experiment was independently performed and repeated in triplicate.

2.3 | Western blotting

Proteins were isolated from cells as previously described and used for western blot analyses (4.0–10 µg/lane).^{27,28} The membranes were probed with various antibodies (Table S1). Each experiment was repeated at least thrice. The protein levels were quantified on ChemiDoc XRS (Bio-Rad Laboratories). Protein levels corresponding to each band were quantified based on band intensity using the ImageJ program (version 1.52u).²⁹

2.4 | Experimental mouse model

The Institutional Animal Care and Use Committee of Shinshu University reviewed and approved all animal experimental procedures (approval number: 020102), conducted according to the recommendations of the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, NIH, Department of Health and Human Services, Bethesda, MD, USA). Six-week-old BALB/c-nu nude female mice weighing 15–18 g were purchased from Charles River Laboratories Japan, Inc. and maintained under pathogen-free conditions. Water and food were provided ad libitum. Animals were observed for tumor growth, activity, feeding, and pain according to the guidelines of the Harvard Medical Area Standing Committee on Animals. First, 5 × 10⁶ of TPC-1 or TPC-1/LR cells with Corning Matrigel Matrix were

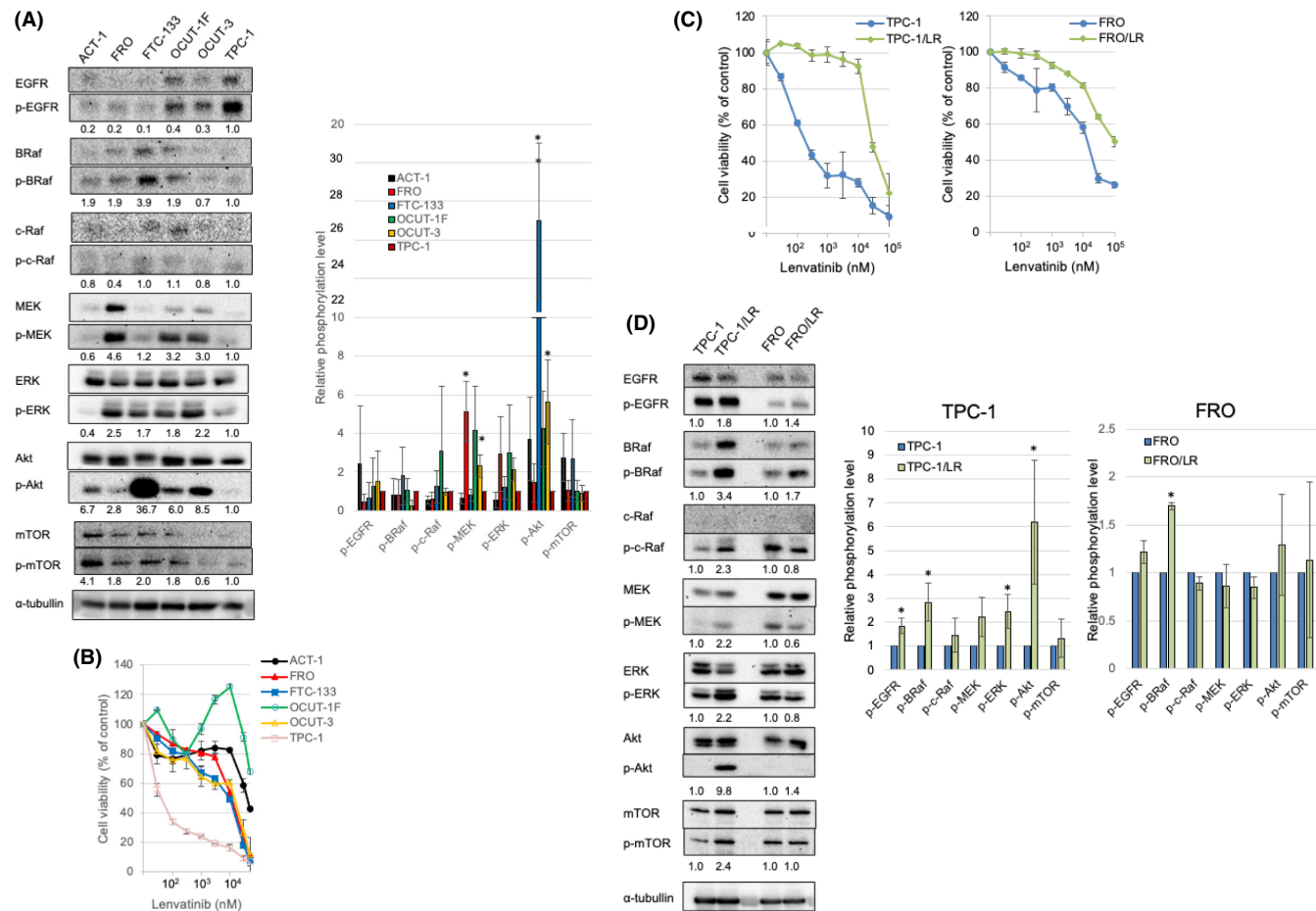


FIGURE 1 Status of signal transduction molecules and sensitivity to lenvatinib in six thyroid cancer cell lines and establishment of lenvatinib-resistant cells in TPC-1 and FRO cells. (A) Expression and phosphorylation of signal transduction related proteins at steady state in thyroid cancer cell lines. Left panel: Representative image of western blots. Six micrograms of protein was loaded on each lane. The protein expression levels were normalized against α -tubulin and phosphorylation levels against the level of expression of the corresponding protein. The relative densitometry values are stated below each protein band. Relative levels to TPC-1 were calculated and demonstrated. Right panel: Quantitative data (relative phosphorylation levels) from triplicate experiments are presented (* $P < 0.05$). (B) The sensitivity to lenvatinib in six thyroid cancer cell lines was determined using the WST assays. The error bars represent the standard error of the value obtained in the experiments performed in triplicate. (C) Sensitivity to lenvatinib in the parental and resistant (TPC-1/LR, FRO/LR) cells (left, TPC-1; right, FRO) was assayed using the WST assays. (D) Expression and phosphorylation of signal transduction related proteins in parental and TPC-1/LR and FRO/LR cells were analyzed by western blotting. Left: Representative image of western blots. Six micrograms of protein was loaded on each lane. The protein expression levels were normalized against α -tubulin and phosphorylation levels against the level of expression of the corresponding protein. The relative phosphorylation is disclosed below each protein band. Right: quantitative data (relative phosphorylation levels) from triplicate experiments are presented (* $P < 0.05$)

TABLE 1 Characteristics and IC_{50} of lenvatinib in various thyroid cancer cell lines determined using WST assay

Cell line	ACT-1	FRO	FTC133	OCUT1F	OCUT3	TPC-1
Origin	Anaplastic	Anaplastic	Follicular	Anaplastic	Anaplastic	Papillary
Driver gene mutation ^a	-	<i>BRAFV600E</i>	<i>PTEN</i> , <i>TP53</i>	-	-	<i>RET/PTC1</i>
IC_{50} (μ M)	35.1 ± 4.6	12.2 ± 3.0	7.8 ± 2.8	N.R.	14.0 ± 1.3	0.1 ± 0.0

Note: Data represent the means \pm SD.

Abbreviations: IC_{50} , half-maximal inhibitory concentration; NR, not reached.

^aWe obtained information for the following representative driver genes: *BRAFV600E*, *Ras*, *RET fusions*, *PAX8/PPARG*, *TP53*, and *PTEN*.

injected subcutaneously into dorsal region. To test the effect of lenvatinib, tumor-bearing mice were divided randomly into four groups ($n = 3$ or 4 per group) when the tumor volume was approximately

200–300 mm³. Lenvatinib and/or lapatinib were orally administered to mice. Each group of mice was administered lenvatinib (3 or 6 mg/kg daily) dissolved in 3 mM HCl diluted water, lapatinib (100 or

Cell line	TPC-1		FRO	
	P	LR	P	LR
Parental or lenvatinib-resistant	P	LR	P	LR
IC ₅₀ (μM) (mean ± SD)	0.2 ± 0.1	28 ± 2.9 [*]	14 ± 1.5	74 ± 4.3 [*]
Relative resistance ratio ^a	1.0	140	1.0	5.3

Abbreviations: IC₅₀, half-maximal inhibitory concentration; LR, lenvatinib-resistant cell; P, parental cell.

^aRelative resistance ratio = IC₅₀ of lenvatinib-resistant cells/IC₅₀ of parental cells.

^{*}*P* < 0.05 (vs. IC₅₀ for parental cells).

200 mg/kg daily) dissolved in 1% Tween 80 diluted water, and vehicle orally using an orogastric probe once-daily for 9 days. When 6 mg/kg lenvatinib was administered orally, its blood concentration was approximately expected at 17.3 μM. Each mouse weight was measured daily. Tumor diameters were measured with a slide caliper every day and tumor volume was calculated using the following formula: volume = major length (mm) × minor length (mm) × minor length (mm)/2. Relative tumor volume (%) was calculated using the following formula: tumor volume on the measuring day/tumor volume on day 1 × 100. Adverse events were judged by body weight (BW) change, which was calculated using the following formula: BW change (%) = [(BW of day *n* - BW of the classified day)/BW of classified day] × 100.

2.5 | Immunohistochemistry

Sections (3 μm) of paraffin-embedded tumor samples were used for immunohistochemical analysis. The slides were heated for antigen retrieval in 10 mM sodium citrate (pH 6.0). Sections were subsequently exposed to specific antibodies for TUNEL (#MK500; Takara Bio Inc.) or Ki-67 (#M7240; DAKO). These were then incubated with Histofine Simple Stain MAX-PO (MULTI) (Nichirei Biosciences Inc.). Staining was revealed using diaminobenzidine (Nichirei Biosciences Inc.) and the slides were counterstained with aqueous hematoxylin. After counterstaining the cells, the slides were analyzed under a microscope (BX700; KEYENCE). Then, immunostaining-positive cells were counted and quantified using the BZ-X analysis application (KEYENCE).

2.6 | Statistical analysis

Statistical significance was tested by performing unpaired Student's *t*-tests or one-way ANOVA with Tukey's multiple comparisons; a

TABLE 2 IC₅₀ and relative resistance ratio for lenvatinib in the parental and lenvatinib-resistant TPC-1 and FRO cells

P value < 0.05 was considered statistically significant, calculated using StatFlex ver. 6 (Artech Co., Ltd.).

2.7 | Evaluation of synergy of growth-inhibitory effect by two drugs

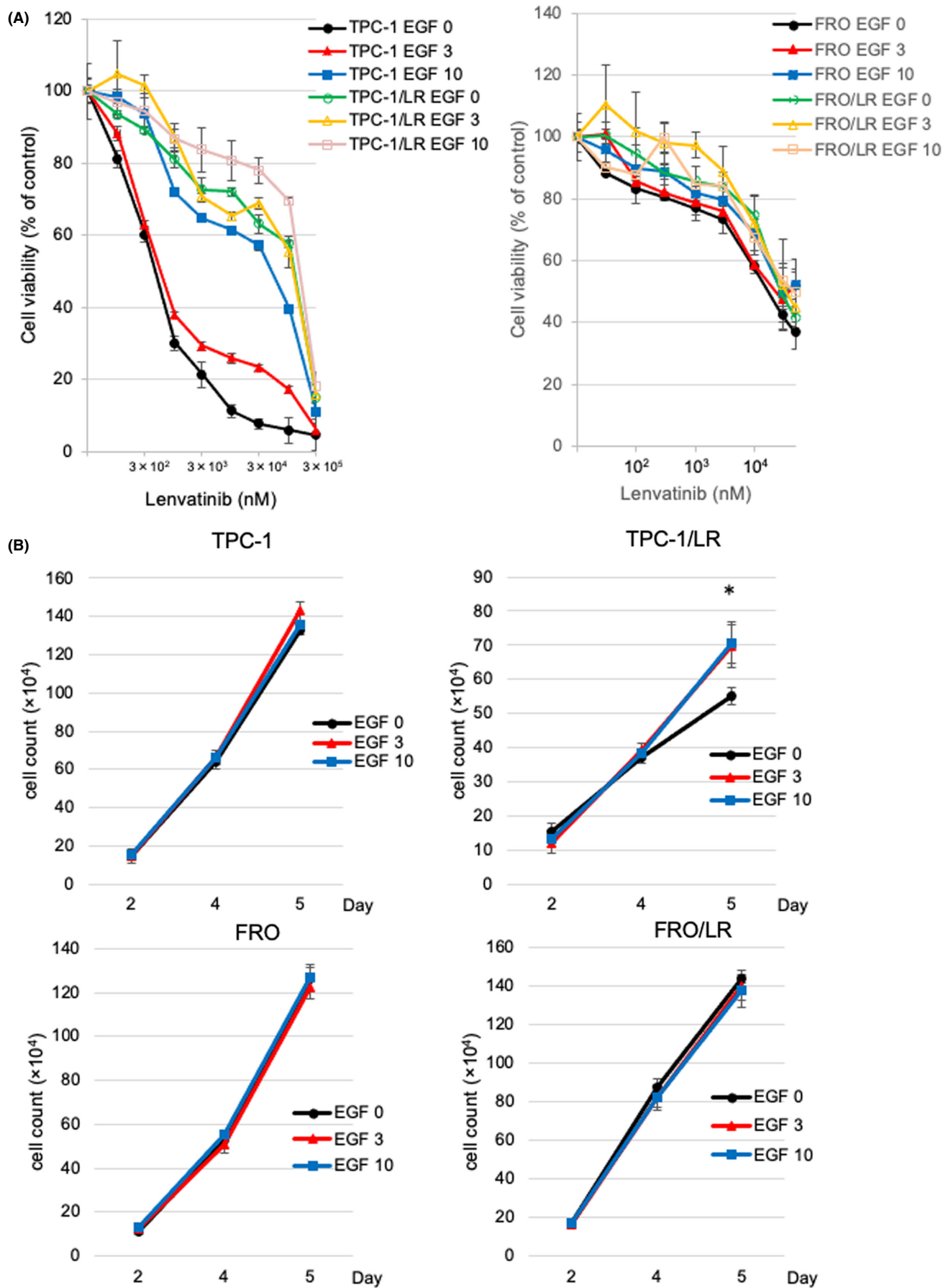
The synergy was evaluated using the Bliss independence model according to a previous study.³⁰ The Bliss independence model was applied to the quantification data of the WST assays. Briefly, Bliss independence was calculated as follows: when drug A at dose *a* inhibited *Ya*% and drug B at dose *b* inhibited *Yb*% of the tumor growth, the combined percentage inhibition *Yab,P* could be predicted using complete additivity in probability theory as $Yab,P = Ya \times Yb - YaYb$. The observed combined percentage inhibition *Yab,O* was then compared with *Yab,P*. If $Yab,O > Yab,P$, the combination effect was considered synergistic.³¹

3 | RESULTS

3.1 | Establishment of lenvatinib-resistant cells in TPC-1 and FRO cells, and protein expression in parental and lenvatinib-resistant TPC-1 and FRO cells

Initially, we examined the expression and phosphorylation of molecules in the signal transduction pathway at steady state in six thyroid cancer cell lines (Figure 1A). The phosphorylated molecules in the signal transduction pathway in steady state were different among cell lines. The phosphorylation of either or both MEK-ERK and Akt-mTOR pathways was observed in five cell lines except for TPC-1. In contrast, phosphorylation of EGFR was elevated in TPC-1 relative to the other cell lines.

FIGURE 2 Effect of EGF on the sensitivity to lenvatinib in parental and lenvatinib-resistant TPC-1 and FRO cells. (A) Sensitivity of parental (TPC-1 and FRO) and TPC-1/LR and FRO/LR cells in the presence of EGF (0, 3, 1, and 10 ng/ml) were analyzed using WST assay (left, TPC-1; right, FRO). (B) The effects of EGF on proliferation in parental and TPC-1/LR and FRO/LR cells were evaluated by cell proliferation assay. The growth of TPC-1, TPC-1/LR, FRO, and FRO/LR cells in the presence of EGF (0, 3, 10 ng/ml) was measured by direct cell count for 5 days. The error bars represent the standard deviations of the values (**P* < 0.05). (C) Expression and phosphorylation of EGFR, ERK, and Akt in parental and TPC-1/LR and FRO/LR cells 10 min after the addition of EGF were analyzed by western blot. α-tubulin was used as a loading control and 10 μg/lane proteins were loaded. Representative image of western blots (upper panels). The relative phosphorylation levels are disclosed below each protein band. Quantitative data (relative phosphorylation levels) from three independent experiments are presented as a bar chart (lower panels) (**P* < 0.05 vs. 0 ng/ml of EGF)



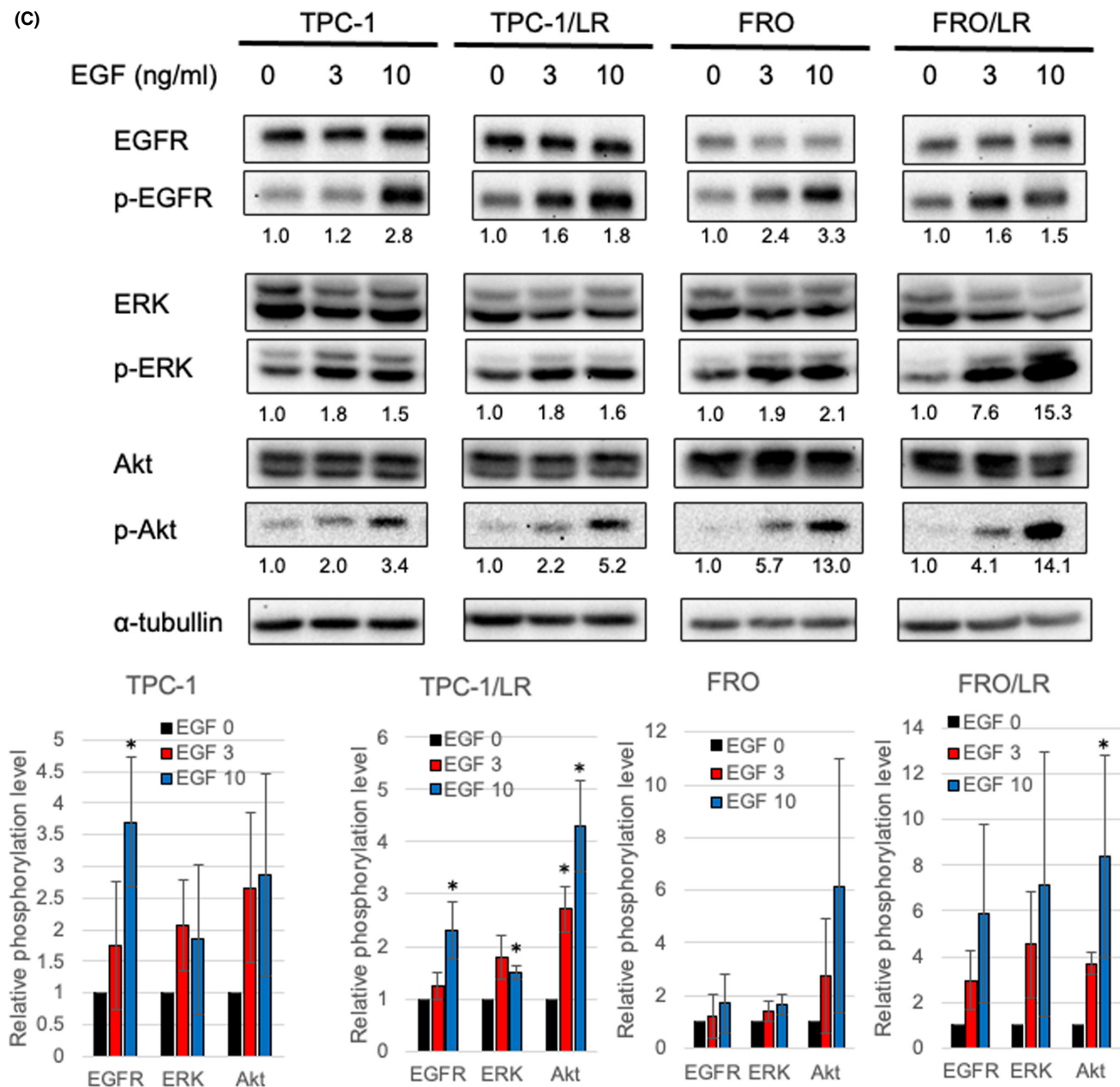


FIGURE 2 (Continued)

TABLE 3 Sensitivity to lenvatinib in the parental and lenvatinib-resistant TPC-1/FRO cells in the presence of EGF

Cell line	TPC-1			TPC-1/LR		
EGF concentration (ng/ml)	0	3	10	0	3	10
IC ₅₀ (μ M) (mean \pm SD)	0.1 \pm 0.0	7.4 \pm 5.3	8.3 \pm 2.5 [*]	15.1 \pm 3.5	13.1 \pm 1.1	16.7 \pm 1.1
Relative resistance ratio ^a	1.0	106	119	1.0	0.9	1.1
Cell line	FRO			FRO/LR		
EGF concentration (ng/ml)	0	3	10	0	3	10
IC ₅₀ (μ M) (mean \pm SD)	18.0 \pm 2.9	20.5 \pm 2.7	31.2 \pm 2.7 [*]	23.5 \pm 3.3	23.5 \pm 8.0	29.4 \pm 13.3
Relative resistance ratio ^a	1.0	1.1	1.7	1.0	1.0	1.3

Abbreviations: EGF, epidermal growth factor; IC₅₀, half-maximal inhibitory concentration; LR, lenvatinib-resistant.^aRelative resistance ratio = IC₅₀ of cells with various concentration of EGF/IC₅₀ of cells without EGF.^{*}*P* < 0.05 (vs. IC₅₀ for 0 ng/ml of EGF).

Thereafter, we tested the growth-inhibitory effects of lenvatinib in these cell lines (Figure 1B and Table 1). TPC-1 showed the highest sensitivity to lenvatinib, while ACT-1 and OCUT-1F showed little lenvatinib sensitivity. As the cell lines exhibited intrinsic resistance to lenvatinib were not considered suitable for developing resistant sublines, we established lenvatinib-resistant sublines in TPC-1 and FRO cells with a stepwise increase in lenvatinib concentration for more than 6 months. The lenvatinib-resistant cell lines were designated as TPC-1/LR and FRO/LR, respectively (Figure 1C). As demonstrated in Table 2, TPC-1/LR and FRO/LR exhibited over 140- and 5.3-fold higher lenvatinib resistance than their parental cells, respectively.

Phosphorylation of EGFR, B-Raf, c-Raf, and their downstream signaling molecules MEK and ERK in the MAPK pathway were enhanced in TPC-1/LR (Figure 1D). Phosphorylation of Akt and mTOR was also enhanced in TPC-1/LR. In FRO, EGFR phosphorylation slightly increased in FRO/LR, that of BRaf was also increased in FRO/LR; however, phosphorylation of downstream signal transduction molecules was not enhanced in FRO/LR.

We measured the EGF in serum-free conditioned media in TPC-1 and TPC-1/LR to determine whether an increase in autocrine EGF was involved in the enhancement of EGFR phosphorylation in TPC-1/LR (Figure S1). Although EGF in the conditioned media was slightly increased in TPC-1/LR compared to TPC-1, no significant difference was observed.

3.2 | Enhanced EGFR phosphorylation by EGF confers lenvatinib resistance in TPC-1 and FRO cells

Since enhanced EGFR phosphorylation was observed in lenvatinib-resistant TPC-1/LR cells, we tested whether lenvatinib resistance is

TABLE 4 Doubling time of the parental and lenvatinib-resistant TPC-1/FRO cells in the presence of EGF

Cell lines/EGF (ng/ml)	0	3	10
TPC-1	23.0 ± 2.6	21.8 ± 2.6	23.5 ± 2.4
TPC-1/LR	42.5 ± 1.5	30.9 ± 7.6	28.2 ± 5.2 [*]
FRO	19.1 ± 1.9	19.0 ± 0.9	20.2 ± 1.3
FRO/LR	34.1 ± 4.7	31.6 ± 3.8	34.6 ± 8.8

Abbreviations: EGF, epidermal growth factor; IC₅₀, half-maximal inhibitory concentration; LR, lenvatinib-resistant.

*P < 0.05 (vs. IC₅₀ for 0 ng/ml of EGF).

TABLE 5 IC₅₀ and relative resistance ratio for lapatinib in the parental and lenvatinib-resistant TPC-1 and FRO cells

Cell line	TPC-1	TPC-1/LR	FRO	FRO/LR
IC ₅₀ (μM) for lapatinib	4.1 ± 0.7	1.6 ± 0.1 [*]	4.0 ± 0.7	5.2 ± 0.3
Relative resistance ratio ^a	1.0	0.4	1.0	1.3

Abbreviation: IC₅₀, half-maximal inhibitory concentration.

^aRelative resistance ratio = IC₅₀ of lenvatinib-resistant cells/IC₅₀ of parental cells.

*P < 0.05 (vs. IC₅₀ for parental cells).

induced by activation of the EGFR pathway in the presence of 3 or 10 ng/ml of EGF in TPC-1 and FRO cells. Addition of 10 ng/ml exogenous EGF significantly increased the IC₅₀ for lenvatinib in parental TPC-1 and FRO cells (Figure 2A and Table 3). The enhancement of lenvatinib resistance by 10 ng/ml EGF was more prominent in TPC-1 than in FRO while not affecting the sensitivity to lenvatinib with addition of exogenous EGF to TPC-1/LR and FRO/LR.

Next, the effect of exogenous EGF on cell proliferation was investigated in these cells (Figure 2B). Although cell growth was not enhanced by 3 or 10 ng/ml of EGF in parental TPC-1 and both parental and lenvatinib-resistant FRO, these amounts of EGF significantly increased the growth of TPC-1/LR. Both TPC-1/LR and FRO/LR cells showed a longer doubling time as compared to the parental cells, but the addition of EGF significantly shortened the doubling time only in TPC-1/LR cells (Table 4). These results indicate a greater dependence of cellular proliferation on the EGFR pathway in TPC/LR cells as compared to other cell lines.

Western blotting showed that the addition of EGF enhanced the phosphorylation of EGFR in a dose-dependent manner in all cells after 10 min of EGF stimulation, but only TPC-1/LR cells showed a significant increase in phosphorylation of EGFR, ERK, and Akt (Figure 2C). These data suggest that both growth promotion and alteration of signal transduction via the activated EGFR pathway might contribute to the development of lenvatinib resistance in TPC-1.

3.3 | Effect of EGFR inhibitor, lapatinib on growth, and protein expression in parental and lenvatinib-resistant TPC-1 and FRO cells

To investigate whether the EGFR signaling pathway was involved in the acquisition of resistance to lenvatinib in TPC-1 cells, we examined the inhibitory effect of the EGFR signaling pathway using lapatinib in TPC-1 and FRO cells. The IC₅₀ values for lapatinib in each cell line are shown in Table 5. As the IC₅₀ for lapatinib in TPC-1/LR (1.6 μM) was less than half of that in the parental TPC-1 cells, TPC-1/LR showed a significantly higher sensitivity to lapatinib than parental TPC-1 cells (Figure 3A). However, sensitivity to lapatinib was slightly decreased in FRO/LR cells compared to that in the parental FRO cells.

When these cells were treated with lapatinib, lapatinib inhibited the phosphorylation of EGFR in both TPC-1 and TPC-1/LR cells; however, the phosphorylation of downstream signaling molecules was different between these cells. Lapatinib did not inhibit the

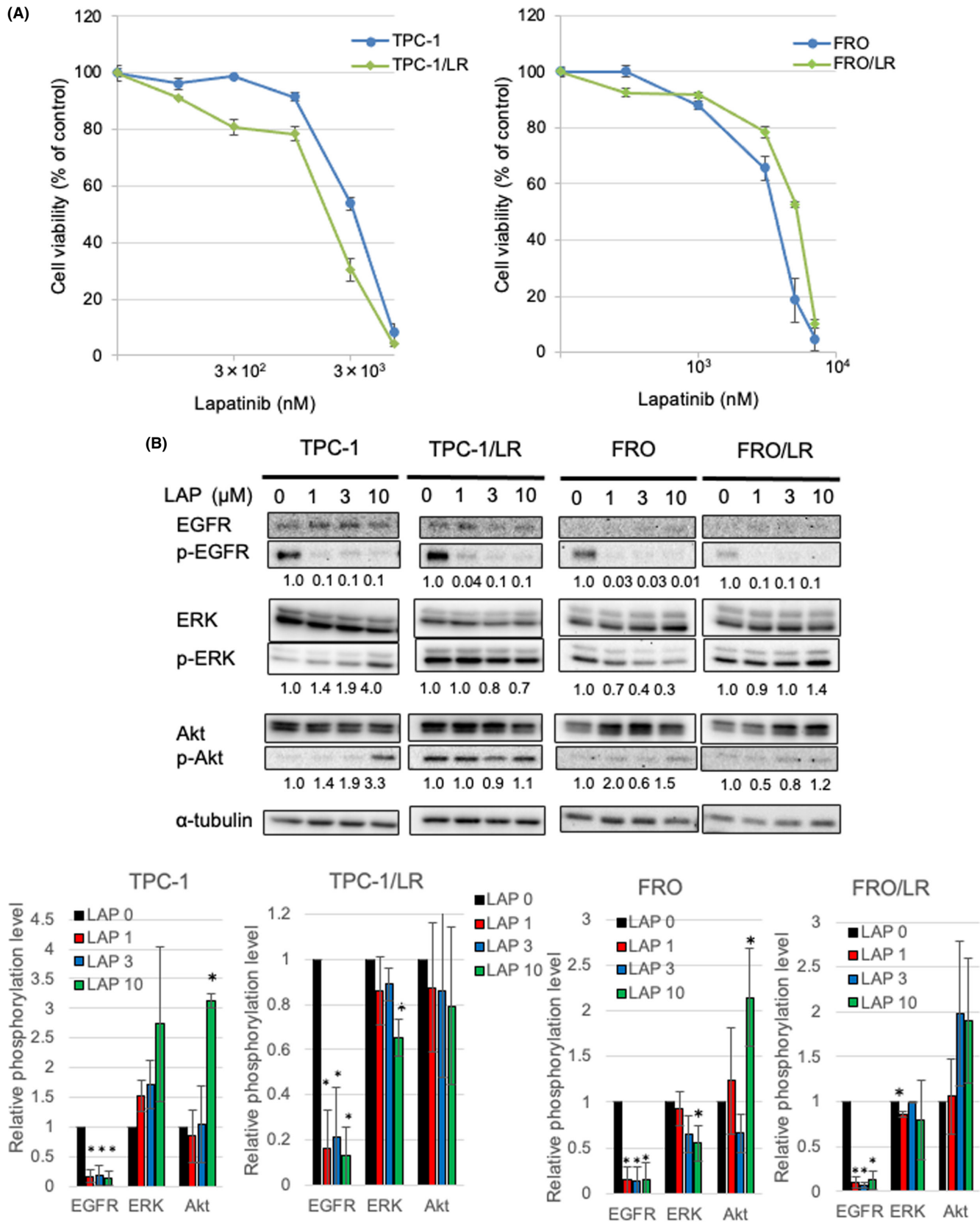


FIGURE 3 Effect of EGFR inhibitors, lapatinib on growth and protein expression in parental and lenvatinib-resistant cells. (A) Sensitivity to lapatinib in parental (TPC-1 and FRO) and TPC-1/LR and FRO/LR cells was verified using the WST assays. Error bars represent the standard error. (B) Expression and phosphorylation of EGFR, ERK, and Akt in parental and TPC-1/LR and FRO/LR cells 6 h after treatment with lapatinib (0, 1, 3, and 10 μM) were analyzed by western blot. α-tubulin was used as a loading control and 8 μg/lane proteins were loaded. Representative image of western blots (upper panels). The relative phosphorylation levels are disclosed below each protein band. Quantitative data (relative phosphorylation levels) from triplicate experiments are presented as a bar chart (lower panels) (* $P < 0.05$)

FIGURE 4 Effect of combination of lenvatinib and lapatinib on lenvatinib-resistant TPC-1 cells. (A) Expression and phosphorylation of EGFR in TPC-1/LR cells 6 h after treatment with lenvatinib were analyzed by western blot. α -tubulin was used as a loading control and 14 μ g/lane proteins were loaded. Representative image of western blots (left). The relative phosphorylation levels are disclosed below each protein band. Quantitative data (relative phosphorylation levels) from triplicate experiments are presented as a bar chart (right) (* $P < 0.05$). (B) Sensitivity to lenvatinib in combination with lapatinib in TPC-1/LR was evaluated by WST assay. The concentrations of lapatinib used simultaneously with lenvatinib were described in the graph. The error bars represent the standard error. (C) Bliss independence model evaluating the synergistic effect between lenvatinib with lapatinib in TPC-1/LR cells. The green background indicates the synergistic effect with $P < 0.05$ and when the reduction in viability is $>10\%$. (D) Expression and phosphorylation of EGFR, ERK, and Akt in TPC-1/LR cells 6 h after treated with lenvatinib in combination with lapatinib were analyzed by western blotting. α -tubulin was used as a loading control and 9 μ g/lane proteins were loaded. Representative image of western blots (upper panel). The relative phosphorylation levels are disclosed below each protein band. Quantitative data (relative phosphorylation levels) from triplicate experiments are presented as a bar chart (lower panel) (* $P < 0.05$, ** $P < 0.01$)

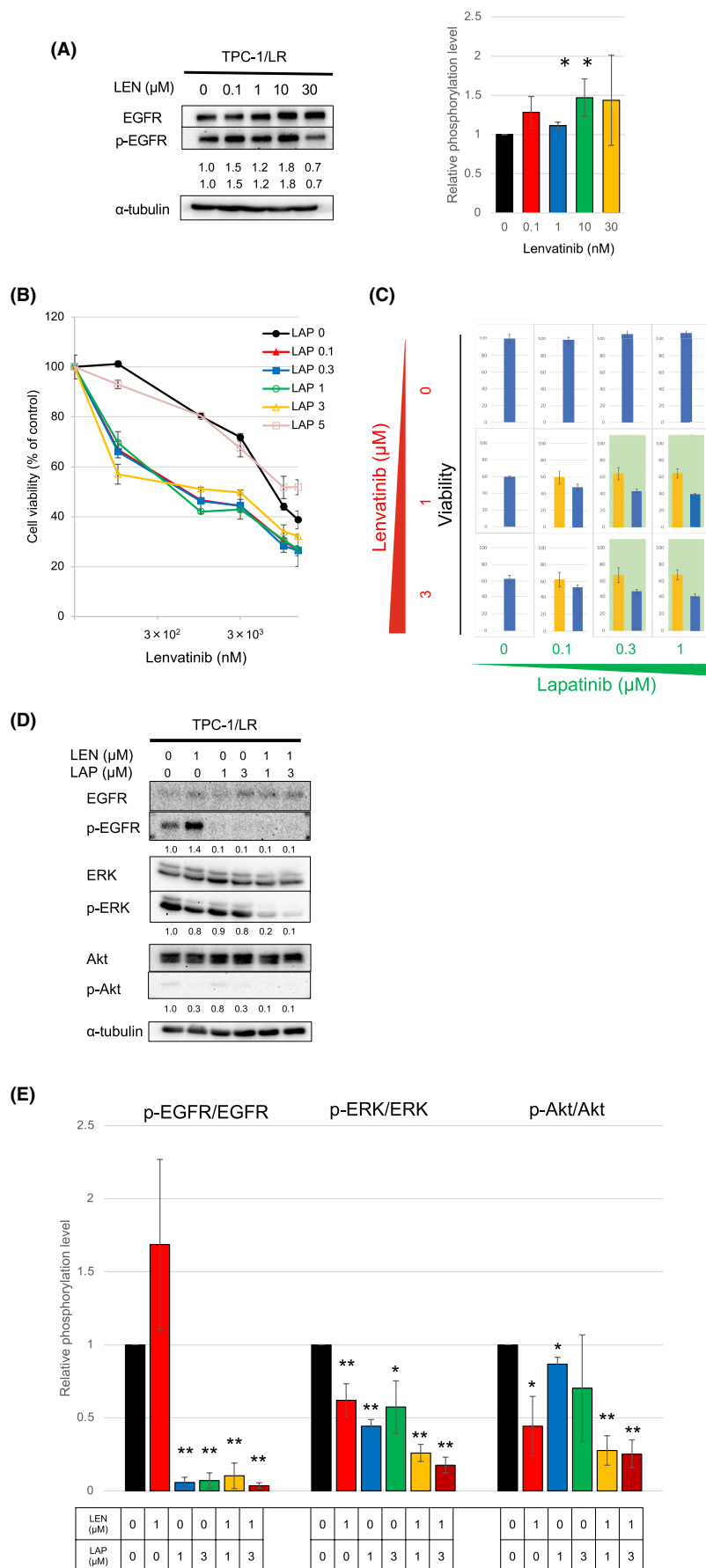


TABLE 6 IC₅₀ and relative resistance ratio for lenvatinib in combination with lapatinib in TPC-1/LR cells

Lapatinib (μM)	0	0.1	0.3	1	3	5
IC ₅₀ for lenvatinib (μM)	10.5 ± 2.4	0.7 ± 0.1 [*]	0.4 ± 0.2 [*]	0.3 ± 0.2 [*]	1.0 ± 1.0 [*]	NR
Relative resistance ratio ^a	1.0	0.06	0.04	0.02	0.10	-

Abbreviations: IC₅₀, half-maximal inhibitory concentration; LR, lenvatinib-resistant; NR, not reached.

^aRelative resistance ratio = IC₅₀ for lenvatinib in TPC-1/LR in the presence of lapatinib/IC₅₀ for lenvatinib without lapatinib.

^{*}*P* < 0.05 (vs. IC₅₀ for TPC-1/LR cells without lapatinib).

phosphorylation of ERK and Akt in TPC-1 cells, while it inhibited the phosphorylation of ERK and Akt in TPC-1/LR cells (Figure 3B).

In FRO and FRO/LR cells, phosphorylation of Akt increased, although that of EGFR was inhibited by lapatinib. These findings suggest the possibility that dependence on the EGFR pathway for survival could be enhanced during the acquisition of resistance to lenvatinib in TPC-1/LR.

3.4 | Effect of combination of lenvatinib and lapatinib on lenvatinib-resistant thyroid cancer cells

As we observed increased sensitivity to lapatinib in lenvatinib-resistant TPC-1 (TPC-1/LR) cells, we next tested the combined effects of lenvatinib and lapatinib in TPC-1/LR cells. First, we confirmed that 0.1–10 μM of lenvatinib enhanced EGFR phosphorylation in TPC-1/LR cells 6 h after treatment with lenvatinib, while 30 μM lenvatinib reduced EGFR phosphorylation (Figure 4A).

When lapatinib and lenvatinib were used in combination, 0.1 μM of lapatinib significantly increased the growth-inhibitory effect (Figure 4B and Table 6). Additionally, when the combined effect of lapatinib and lenvatinib was evaluated using the Bliss independence model, a synergistic growth-inhibitory effect was confirmed in the presence of 0.3 μM of lapatinib, for which the concentration was <20% of IC₅₀ of lapatinib monotherapy for TPC-1/LR (Figure 4C and Table 5).

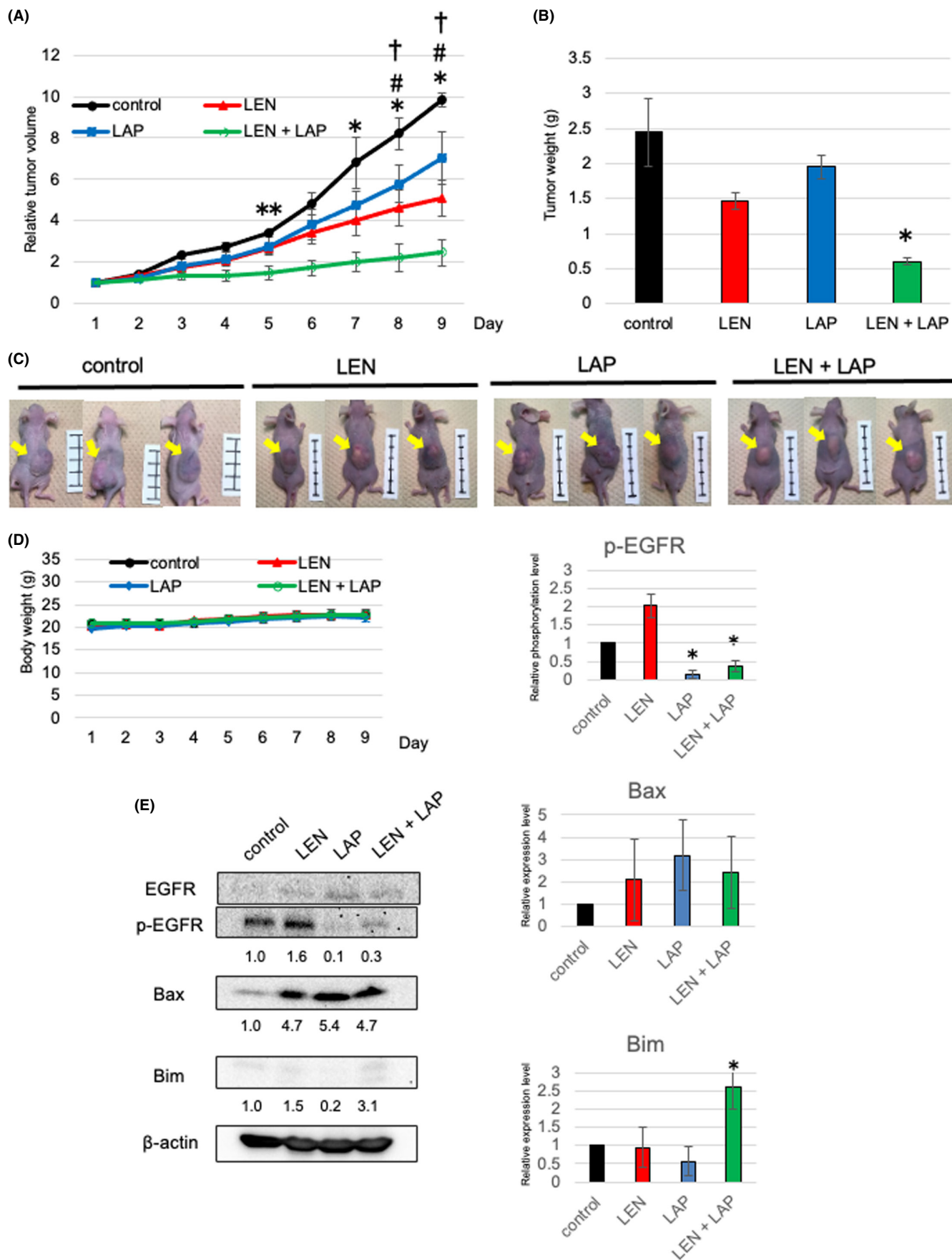
Although 1 and 3 μM of lapatinib inhibited the phosphorylation of EGFR at 6 h after treatment, the inhibition of phosphorylation of ERK was observed only in the presence of lenvatinib (Figure 4D).

3.5 | Effect of simultaneous administration of lenvatinib and lapatinib in lenvatinib-resistant TPC-1 in vivo

Next, we tested the effects of lenvatinib and lapatinib combination treatment in a TPC-1 tumor xenograft model in vivo. First, we tested whether lenvatinib-resistant TPC-1 (TPC-1/LR) cells maintained lenvatinib resistance in mouse xenograft models. Oral administration of lenvatinib (6 mg/kg/day) completely inhibited the growth of the parental TPC-1-tumors grown in the mouse xenograft models (Figure S2), while lenvatinib-resistant TPC-1 (TPC-1/LR) grew even after the administration of 6 mg/kg/day of lenvatinib, confirming that TPC-1/LR cells maintained lenvatinib resistance in the mouse xenograft model (Figure 5A). To verify the combined effect of lenvatinib and lapatinib, lenvatinib (6 mg/kg) monotherapy, lapatinib (200 mg/kg) monotherapy, or both were orally administered to mice bearing TPC-1/LR tumors daily for 9 days (Figure 5A). Although monotherapy with lenvatinib or lapatinib significantly inhibited tumor growth compared to the control group, lenvatinib and lapatinib simultaneous administration significantly reduced the tumor weight compared to monotherapy alone (Figure 5B,C). Figure 5D illustrates body weight changes were not seen in four groups.

Furthermore, we performed western blot analysis using TPC-1/LR-tumor specimens obtained from the xenograft models 9 days after treatment (Figure 5E). Compared to the tumors obtained from control mice, the tumors from mice treated with lenvatinib showed increased phosphorylation of EGFR. Administration of lapatinib decreased the phosphorylation of EGFR in the TPC-1/LR tumors regardless of the presence or absence of lenvatinib. This

FIGURE 5 Effect of simultaneous administration of lenvatinib and lapatinib on TPC-1/LR in mouse xenograft model. (A) The average relative tumor volumes were plotted from day 1 to day 9. Closed circles (●) indicate control, closed red triangles (▲) indicate 6 mg/kg of lenvatinib (LEN), closed blue squares (■) indicate 200 mg/kg of lapatinib (LAP), and opened green circles (○) indicate 6 mg/kg of lenvatinib and 100 mg/kg of lapatinib groups. **P* < 0.01 (● or ▲ or ■ vs. ○ group), ***P* < 0.01 (▲ vs. ○ group), #*P* < 0.05 (■ vs. ○ group), †*P* < 0.05 (● vs. ▲ or ■) using unpaired Student's *t*-tests. (B) Comparison of average tumor weights removed 9 days after the initiation of treatment. LEN, 6 mg/kg of Lenvatinib; LAP, 200 mg/kg of lapatinib; LEN + LAP, 6 mg/kg of lenvatinib and 100 mg/kg of lapatinib. **P* < 0.05 (control vs. LEN + LAP). (C) Representative photographs of mice bearing TPC-1/LR-tumor in each treatment group on day 9. Each scale bar represents 1 cm. (D) The average body weights were plotted from day 1 to day 9. Closed circles (●) indicate control, closed triangles (▲) indicate lenvatinib (LEN), closed squares (■) indicate lapatinib (LAP), and opened circles (○) indicate lenvatinib and lapatinib groups. (E) Western blot analysis of tumors obtained from the xenograft models 9 days after treatment. Expression and phosphorylation of EGFR, Bax and Bim expression was analyzed. β-actin was used as a loading control and 20 μg/lane proteins were loaded. Representative image of western blots (left). The relative densitometry values (β-actin-corrected) are disclosed below each protein band. Quantitative data (relative expression or phosphorylation levels) from triplicate experiments are presented as bar chart (right) (**P* < 0.05). (F) Immunohistochemical analysis of TPC-1/LR tumors. Representative pictures of TUNEL and Ki67 staining on day 9. Scale bar = 100 μm. Quantification of percentage of TUNEL-positive (lower left) and Ki67-positive (lower right) cells in the tumor. (A) Values are mean ± SEM. **P* < 0.05



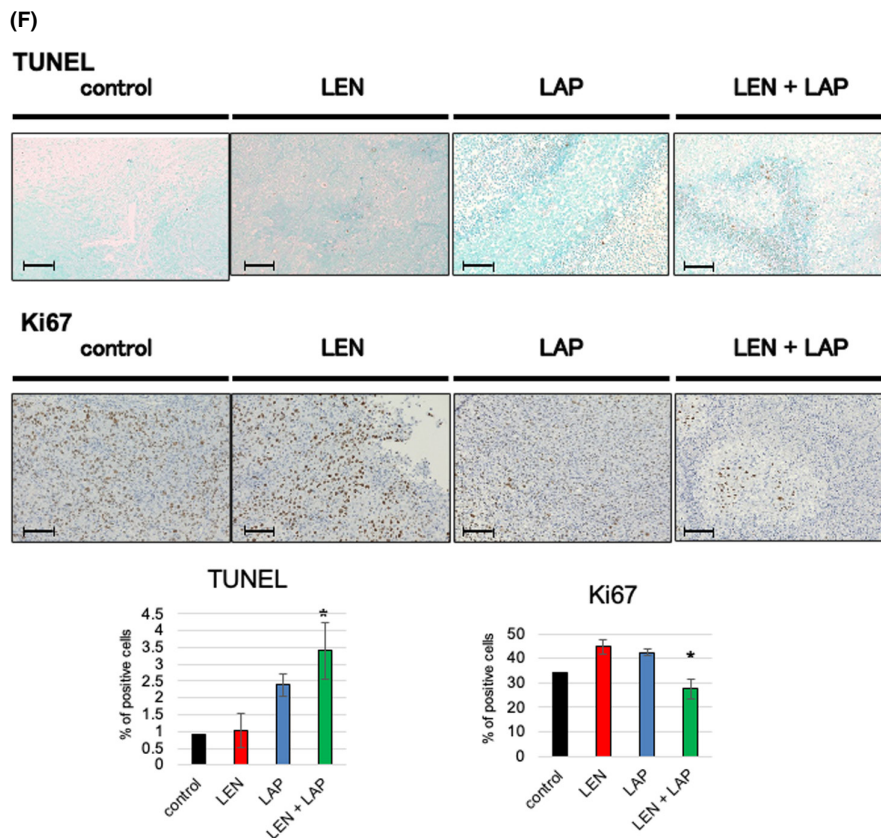


FIGURE 5 (Continued)

was consistent with in vitro experiment results. In addition, the expression of proapoptotic proteins Bax and Bim in the tumor was increased by both monotherapy and simultaneous administration of these agents (Figure 5E).

Significantly more TUNEL-positive cells by TUNEL staining were observed in the tumors treated with the simultaneous administration of lenvatinib and lapatinib than those treated with lenvatinib monotherapy ($p < 0.05$) (Figure 5F). In contrast, the number of Ki67 positive cells was significantly lower in the tumors treated with a combination of lenvatinib and lapatinib than with monotherapy alone. Thus, simultaneous administration enhanced the antitumor effect on lenvatinib-resistant TPC-1 (TPC-1/LR) tumors in vivo and in vitro.

3.6 | Effects of lenvatinib on the signal transduction pathway in various thyroid cancer cell lines

Results of lenvatinib-resistant TPC-1 cells suggest that lenvatinib might activate the EGFR pathway in thyroid cancer cells. To investigate whether short-term exposure to lenvatinib alters the EGFR pathway in thyroid cancer cells, we exposed six different thyroid cancer cell lines with lenvatinib for 24 h. The effect of lenvatinib on the expression or phosphorylation of EGFR and its downstream signal transduction molecules in these thyroid cancer cell lines was

examined by western blotting (Figure 6). A low dose around the IC_{50} and a high dose of lenvatinib were administered to each cell line for 24 h. EGFR phosphorylation was enhanced by lenvatinib in all thyroid cancer cell lines. In all except TPC-1 cells, lenvatinib enhanced ERK phosphorylation, while Akt phosphorylation was inhibited in OCUT-1F and OCUT-3 cells. Thus, activation of EGFR by short-term exposure to lenvatinib was observed in multiple thyroid cancer cell lines regardless of their histological origin or driver gene mutations, although molecules activated in the downstream pathway varied.

3.7 | Effect of simultaneous administration of lenvatinib and lapatinib in various thyroid cancer cell lines

Enhanced phosphorylation of EGFR by lenvatinib was observed in six thyroid cancer cell lines. We analyzed whether the growth-inhibitory effect of lenvatinib is enhanced during simultaneous administration with lapatinib in OCUT-1F cells. This showed least sensitivity to lenvatinib monotherapy (Table 1). The combined use of lapatinib $\geq 0.1 \mu\text{M}$ significantly reduced the IC_{50} of lenvatinib (Figure 7A and Table 6).

In the analyses of signal transduction pathway by western blotting in OCUT-1F, lapatinib monotherapy suppressed EGFR phosphorylation (not Akt), however, the combined use suppressed

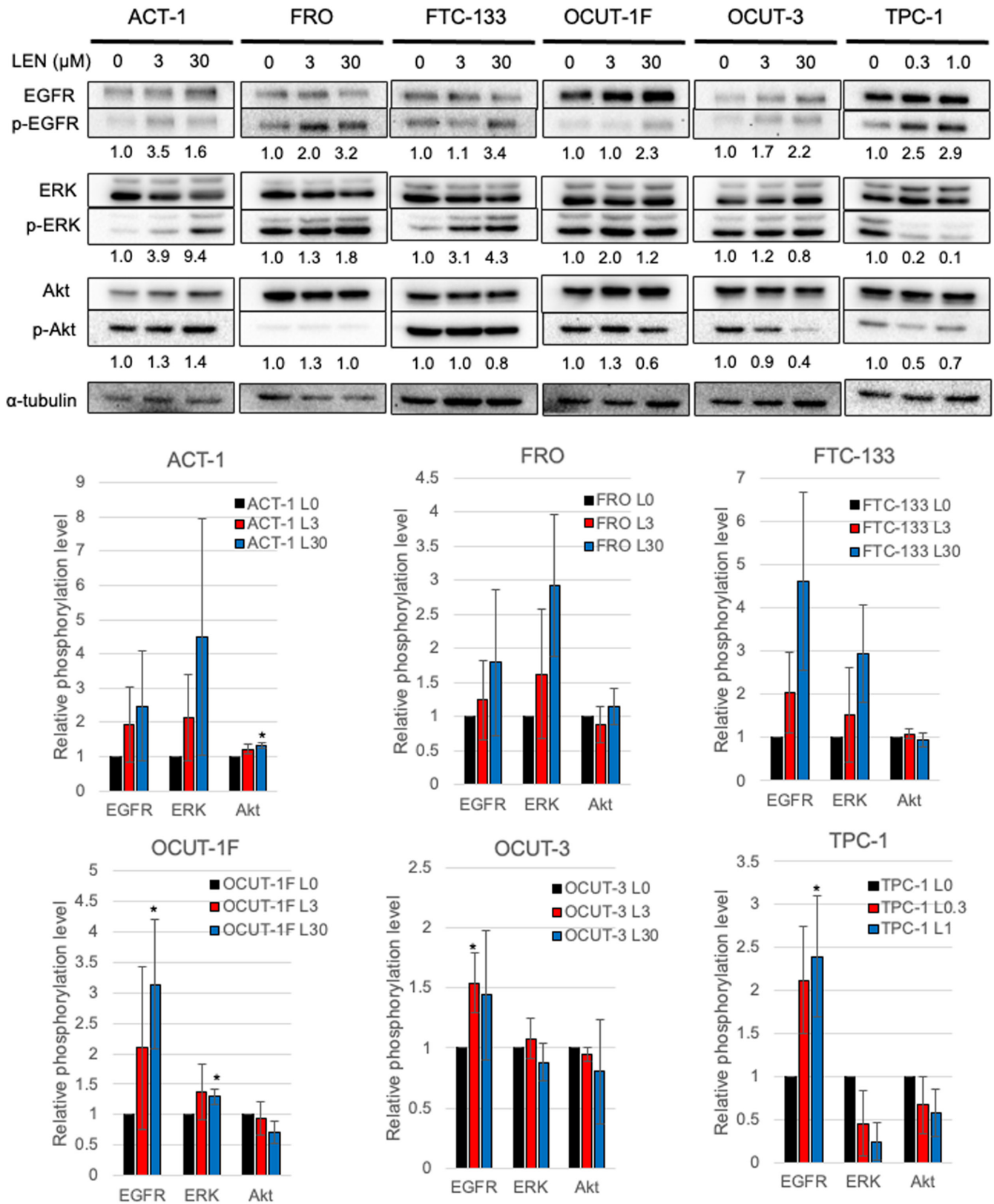


FIGURE 6 Effects of lenvatinib on signal transduction pathway in various thyroid cancer cell lines. Expression and phosphorylation of EGFR, ERK, and Akt in six thyroid cancer cell lines 24h after treatment with indicated concentrations of lenvatinib were analyzed by western blot. α -tubulin was used as a loading control and 10 μ g/lane proteins were loaded. Representative images of western blots (upper panels). The relative phosphorylation levels are disclosed below each protein band. Quantitative data (relative phosphorylation levels) from triplicate experiments are presented as bar chart (lower panels) (* $P < 0.05$)

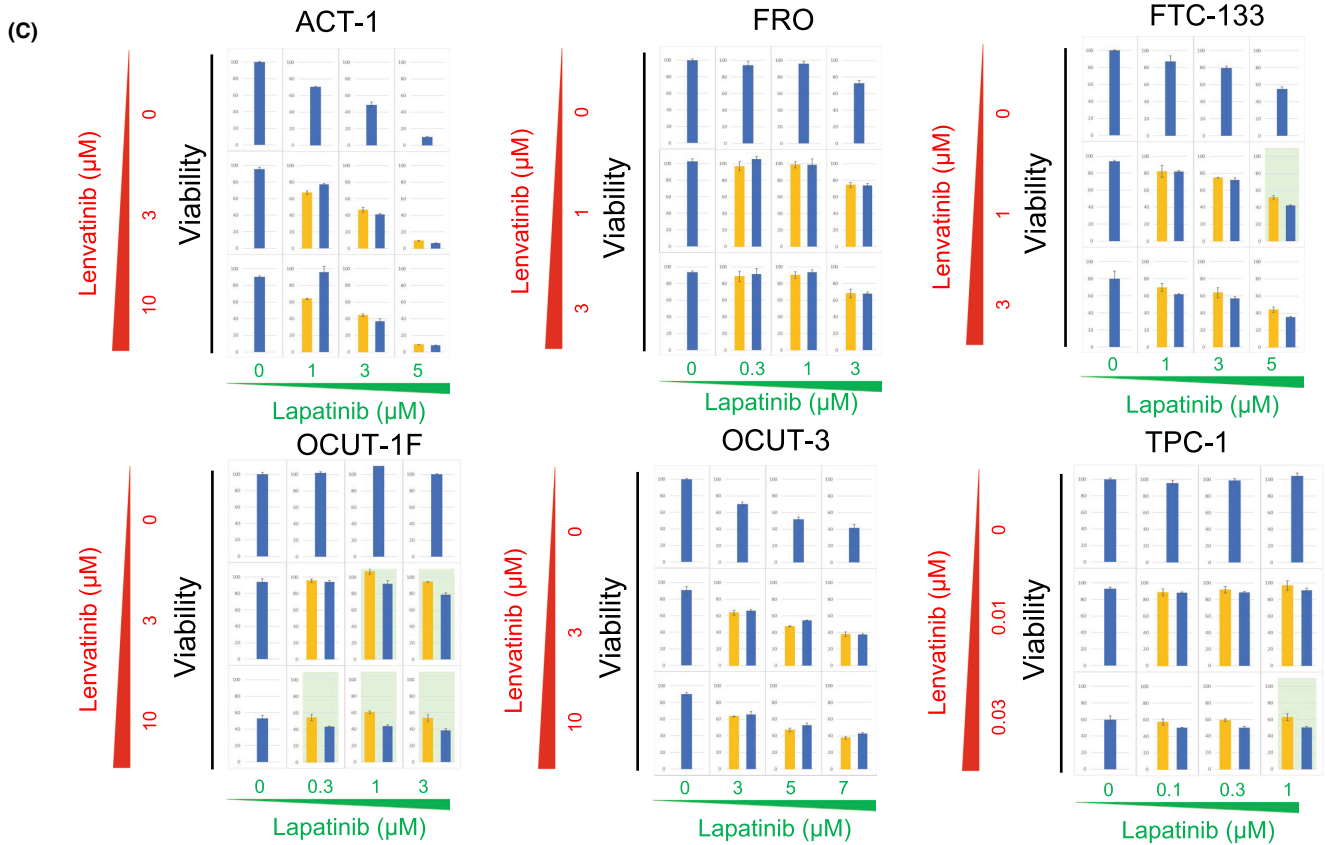
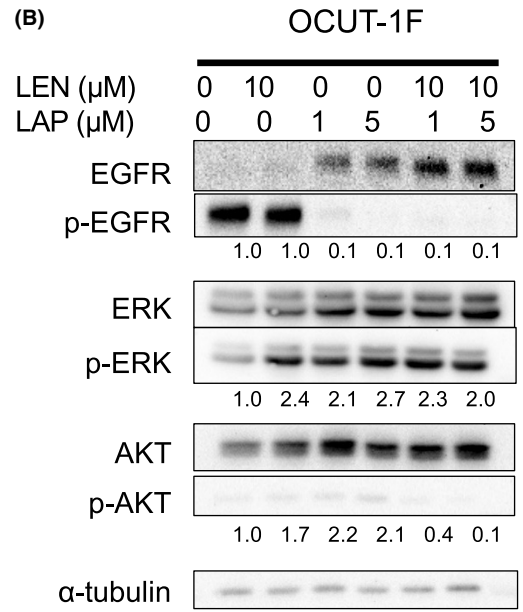
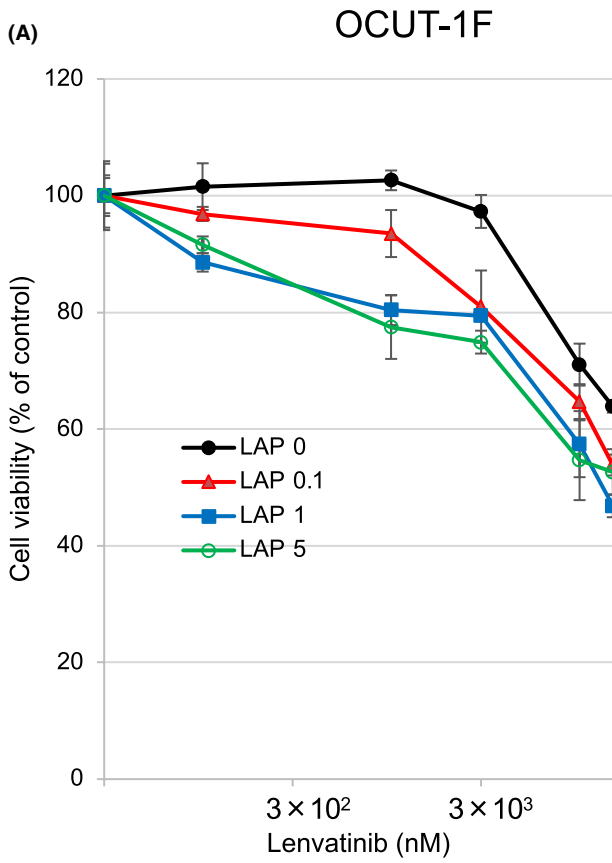


FIGURE 7 Effect of combination of lenvatinib and lapatinib on thyroid cancer cell lines. (A) Sensitivity to lenvatinib in combination with lapatinib (0, 0.1, 1, and 5 μ M) in OCUT-1F cells was evaluated by WST assay. (B) Expression and phosphorylation of EGFR, ERK, and Akt in OCUT-1F cells simultaneously treated with lenvatinib and lapatinib for 24 h. α -tubulin was used as a loading control and 6 μ g/lane proteins were loaded. The relative phosphorylation levels are disclosed below each protein band. (C) Bliss independence model evaluating the synergistic effect between lenvatinib with lapatinib in thyroid cancer cells. The green background indicates the synergistic effect with $P < 0.05$ and when the reduction in viability is $>10\%$

the phosphorylation of Akt, which was similar to that observed in lenvatinib-resistant TPC-1/LR cells (Figure 7B).

Furthermore, when the combined effect was analyzed using the Bliss score in six thyroid cancer cell lines, it demonstrated a synergistic growth-inhibitory effect on FTC-133, OCUT-1F, and TPC-1 (Figure 7C). In contrast, this combination attenuated the effect of lenvatinib on ACT-1, FRO, and OCUT3 cells, indicating that inhibition of the EGFR pathway with an EGFR inhibitor may enhance lenvatinib susceptibility to a subset of thyroid cancer cells that are intrinsically resistant to lenvatinib.

4 | DISCUSSION

Since the favorable results of the phase 3 trial for radioiodine refractory DTC were reported in 2015,²⁰ lenvatinib has been widely used for advanced or recurrent thyroid cancer. However, no precise molecular mechanism of lenvatinib resistance in thyroid cancer has been reported so far. Here, we show that short-term administration of lenvatinib induces increased EGFR phosphorylation in multiple thyroid cancer cell lines and activation of the EGFR pathway contributes to lenvatinib resistance in a subset of thyroid cancer cells. Furthermore, we have shown that the combined administration of an EGFR inhibitor and lenvatinib could restore lenvatinib susceptibility to these cells that acquired resistance to lenvatinib in *in vitro* and *in vivo* mouse model systems. To our knowledge, this is a novel study analyzing the molecular mechanisms of lenvatinib resistance in thyroid cancer cells.

Lenvatinib acts as a multi-tyrosine kinase inhibitor and has a strong anti-angiogenic effect. Its pharmacological action has made it a potent therapeutic agent for various malignancies that share similar biological characteristics.²² Currently, lenvatinib is used worldwide for thyroid cancer, hepatocellular carcinoma (HCC), and renal cell carcinoma.^{32,33} Furthermore, it has recently been approved for unresectable thymic cancer in Japan.³⁴ However, few reports analyze the molecular mechanisms of lenvatinib resistance development. Recently, the inhibition of receptor tyrosine kinases, such as those belonging to the VEGFR and FGFR families, by lenvatinib has been shown to induce activation of signaling through the receptor tyrosine kinases that are not targeted by lenvatinib, thereby conferring lenvatinib-resistance in both HCC and RCC cells.^{30,33,35} Based on this, the therapeutic potential of a simultaneous dual inhibition strategy of administration of lenvatinib and another molecular-targeted agent that suppresses the alternate pathway activated by lenvatinib while overcoming lenvatinib resistance has been demonstrated in these cancers.

As for thyroid cancer, the molecular mechanisms involved in lenvatinib resistance have not been elucidated. Here, we observed that one of the thyroid cancer cell lines, TPC-1, activated EGFR when developing lenvatinib resistance. Subsequently, short-term administration of lenvatinib transiently enhanced EGFR phosphorylation in all analyzed thyroid cancer cell lines, regardless of their histological origin or driver gene mutations. Thus, it is anticipated that the receptor tyrosine kinase-mediated pathway that was not targeted by lenvatinib may be activated for survival in thyroid cancer under the potent inhibition of receptor tyrosine kinases by lenvatinib. As HGF/c-MET axis is activated by lenvatinib in HCC, we examined whether or not this axis was activated in TPC-1/LR cells using phosphorylation arrays (Figure S3). c-Met was instead suppressed in these thyroid cancer cells. The results suggested that signal transduction pathways activated for survival in the presence of lenvatinib may depend on the nature of each cancer cell line.

EGFR has been reported to be expressed at higher levels in thyroid cancer tissues than in normal thyroid tissue with a poor prognostic factor for thyroid cancer.³⁶⁻⁴⁰ Previous preclinical studies *in vitro* have demonstrated that EGF stimulates thyroid follicular cell proliferation and enhances migration and invasion of PTC cells,^{36,41,42} playing an important role in thyroid tumor development.⁴³ These findings suggest that the molecular blockade of EGFR activation may have therapeutic potential in advanced thyroid cancer. In a study using ATC cell lines, Schiff et al.⁴⁴ showed the growth-inhibitory effect of the EGFR inhibitor gefitinib on the ATC cell line with enhanced EGFR phosphorylation *in vitro* and *in vivo*. However, they also showed that EGFR activation occurred only in a subset of ATC cell lines. Subsequently, in a phase 2 trial for gefitinib against ATC, although reductions in tumor volume were observed in one-third of patients, none met the criteria for a partial response.⁴⁵ These results indicate that although the EGFR pathway is indispensable for the survival of thyroid cancer cells, inhibiting EGFR alone is insufficient to suppress cell growth. Younes et al. demonstrated that simultaneous blockade of EGFR and VEGFR signaling pathways by a dual specific EGFR and VEGFR inhibitor effectively suppressed the two downstream targets, Akt and MAPK, and the growth of FTC cells *in vitro* and in a mouse model, thus indicating the usefulness of simultaneous suppression of pathways mediated by EGFR and VEGFR.⁴⁶

Herein, some thyroid cancer cell lines showed a strong intrinsic resistance to lenvatinib. However, a synergistic enhancement in growth inhibition was observed when lapatinib was used in combination with lenvatinib in these cell lines. In clinical practice, some thyroid cancers show resistance to lenvatinib from the initial treatment or gain resistance within a short period. Among these

lenvatinib-resistant thyroid cancer cells, EGFR may have a crucial function in survival and simultaneous use of EGFR inhibitors may be a promising therapeutic strategy against these lenvatinib-resistant thyroid cancers.

Although short-term exposure to lenvatinib induced increased EGFR phosphorylation in six thyroid cancer cell lines, the established lenvatinib-resistant FRO (FRO/LR) line did not show enhanced EGFR phosphorylation, as observed in lenvatinib-resistant TPC-1 (TPC-1/LR) cells, resulting in a limitation as we did not investigate other mechanisms underlying lenvatinib resistance apart from EGFR activation. Various mechanisms may be involved in the development of resistance to lenvatinib in thyroid cancer cells, thus an extensive analysis is warranted for their elucidation.

Driver genetic alterations are frequently observed in thyroid cancer.⁶ Clinical trials conducted for advanced or recurrent thyroid cancer have already demonstrated the clinical usefulness of targeted therapies based on genetic alterations in *BRAF*, *RET*, and *NTRK*.⁴⁷⁻⁴⁹ Hence, molecular-targeted therapies based on gene alterations will be introduced earlier than MKIs such as lenvatinib or sorafenib to treat advanced thyroid cancer.^{50,51} However, approximately 20%–30% of thyroid cancers do not have driver gene mutations, thus MKIs such as lenvatinib will still be in use. Additionally, lenvatinib is anticipated as a second-line treatment for cases that have acquired resistance to *BRAF*, *RET*, or *NTRK* inhibitors, therefore it is crucial to develop strategies to enhance the antitumor effects of lenvatinib on thyroid cancer cells.

A synergistic effect can be achieved using multiple molecular-targeted drugs to inhibit signaling molecules from being activated by one drug.⁵² For *BRAF*V600E mutation-positive ATC, a simultaneous inhibition of MEK, which is activated in the presence of *BRAF* inhibitor, shows a higher antitumor effect which has not been obtained with any other conventional treatment strategies.⁴⁷ Although the mechanisms of activation of EGFR in the presence of lenvatinib observed in the present study require further elucidation, we think that the synergistic growth inhibitory effect obtained with lenvatinib in combination with lapatinib may be associated with an increased signaling transduction through EGFR, as has been proven by the combination of *BRAF* and MEK inhibitors.

One of the limitations of this study is that we did not analyze the effects of lenvatinib on the tumor microenvironment *in vivo*. Lenvatinib is a potent inhibitor of VEGFRs 1–3 and FGFRs 1–4, and inhibition of angiogenesis in the tumor microenvironment is reportedly responsible for its antitumor effects *in vivo*.⁵³ In this study, we did not evaluate whether there were differences in the expression of angiogenic factors such as VEGF and FGF between parental and lenvatinib-resistant TPC-1 cells. To elucidate the mechanism underlying lenvatinib resistance in thyroid cancer *in vivo*, it is necessary to analyze the effects of lenvatinib not only on cancer cells but also on the tumor microenvironment, including angiogenesis inhibition and immunomodulation. Therefore, we are currently attempting to construct an experimental system to evaluate the effects of lenvatinib *in vivo*.

In conclusion, we demonstrated the possibility of the involvement of activation of the EGFR pathway in the development of lenvatinib resistance in thyroid cancer cells. We also showed that a simultaneous inhibition of the EGFR pathway may have therapeutic potential for overcoming lenvatinib resistance in thyroid cancer *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

We thank Ms. Yukiko Kanai and Ms. Hiromi Fujita for their technical assistance.

DISCLOSURE

The authors have no conflict of interest.

ETHICAL APPROVAL

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: The Institutional Animal Care and Use Committee of Shinshu University reviewed and approved all animal experimental procedures (approval number: 020102), conducted according to the recommendations of the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, NIH, Department of Health and Human Services, Bethesda, MD, USA).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ohno K, Shibata T, Ito K-i. Epidermal growth factor receptor activation confers resistance to lenvatinib in thyroid cancer cells. *Cancer Sci.* 2022;113:3193-3210. doi: [10.1111/cas.15465](https://doi.org/10.1111/cas.15465)