Title

Combination Therapy with Lenvatinib and Radiation Significantly Inhibits Thyroid Cancer Growth by Uptake of Tyrosine Kinase Inhibitor

Authors: Kensuke Suzuki[a], Hiroshi Iwai[a*], Keita Utsunomiya[b], Yumiko Kono[b], Yoshiki Kobayashi[a], Dan Van Bui[a], Shunsuke Sawada[a], Yasutaka Yun[a], Akitoshi Mitani[a], Naoyuki Kondo[c], Tayo Katano[d], Noboru Tanigawa[b], Tomoya Akama[e], and Akira Kanda[a]

Affiliations

[a]Department of Otolaryngology, Head and Neck Surgery, Kansai Medical University, 2-5-1 Shin-machi, Hirakata, Osaka, 573-1010, Japan

[b]Department of Radiology, Kansai Medical University, 2-5-1 Shin-machi, Hirakata, Osaka, 573-1010, Japan

[c]Department of Molecular Genetics, Kansai Medical University, 2-5-1 Shin-machi, Hirakata, Osaka, 573-1010, Japan

[d]Department of Medical Chemistry, Kansai Medical University, 2-5-1 Shin-machi, Hirakata, Osaka, 573-1010, Japan

[e]Department of Pharmacology, Kansai Medical University, 2-5-1 Shin-machi, Hirakata, Osaka, 573-1010, Japan

***Corresponding author:**

Hiroshi Iwai

Department of Otolaryngology, Head and Neck Surgery, Kansai Medical University, 2-5-1 Shin-machi, Hirakata, Osaka, 573-1010, Japan

Tel: +81 72 804 0101

Fax: +81 72 804 2547

E-mail: iwai@hirakata.kmu.ac.jp

Abstract

Although surgical treatment cures >90% of differentiated thyroid cancer (DTC) patients, the remaining patients, including advanced DTC cases, have poor clinical outcomes. These patients with inoperable disease have only two choices of radioactive iodine therapy and tyrosine kinase inhibitors such as lenvatinib, which have a high incidence of treatment-related adverse events and can only prolong progression free survival by approximately 5–15 months.

In this study, we investigated the antitumor effects of combination therapy with lenvatinib and radiation (CTLR) for DTC. CTLR synergistically inhibited cell replication and colony formation *in vitro* and tumor growth in nude mice without apparent toxicities and suppressed the expression of proliferation marker (Ki-67). CTLR also induced apoptosis and G2/M phase cell cycle arrest. Moreover, quantitative analysis of the intracellular uptake of lenvatinib using liquid chromatography and mass spectrometry demonstrated that intracellular uptake of lenvatinib was significantly increased 48 h following irradiation. These data suggest that increased membrane permeability caused by irradiation increases the intracellular concentration of levatinib, contributing to the synergistic effect.

This mechanism-based potential of combination therapy suggests a powerful new therapeutic strategy for advanced thyroid cancer with fewer side effects and might be a milestone for developing a regimen in clinical practice.

Keywords: thyroid cancer; lenvatinib; radiation; combination therapy; membrane permeability

Abbreviations: DTC, differentiated thyroid cancer; OS, overall survival; RAI, radioactive iodine; TKI, tyrosine kinase inhibitor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptors; PFS, progression-free survival; CCRT, concurrent chemoradiation therapy; HNSCC, head and neck squamous cell carcinoma; EBRT, external beam radiation therapy; CTLR, combination therapy with lenvatinib and radiation; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; RR, replication rate; LC-MS/MS, liquid chromatography and mass spectrometry; SEMs, standard errors of the mean

Introduction

Thyroid cancer is the most common type of malignant endocrine tumor [1], and its incidence has been increasing over the last four decades [2]. More than 90% of all thyroid cancers are the follicular or papillary types, known as differentiated thyroid cancer (DTC) [3]. DTC patients with advanced disease have poor clinical outcomes, while 90% of patients with DTC survive for at least 10 years [4]. Shaha et al. reported that the 20-year overall survival (OS) rate of DTC patients in the high-risk group including cases with locally advanced and distant metastasis, was only 57% [5].

These patients with locally advanced or metastatic DTC that is surgically unresectable have only two choices of radioactive iodine (RAI, ^{131}I) therapy and tyrosine kinase inhibitors (TKIs). RAI therapy is thyroid-specific and exerts its effect both systemically and locally, it is a well-accepted modality of adjuvant therapy after surgery or for the treatment of distant metastases. Mazzaferri et al. reported that ^{131}I ablation after total thyroidectomy significantly reduced tumor recurrence [6]. However, many advanced DTC patients do not respond or become refractory to RAI [7]. The median survival of RAI-refractory DTC is only 2.5–3.5 years from the diagnosis of distant metastases [8, 9]. Recently, some novel TKIs have increased the life span of thyroid cancer patients [10, 11]. Lenvatinib, a multi-targeted TKI, selectively inhibits vascular endothelial growth factor receptors (VEGFR) 1–3, fibroblast growth factor receptors (FGFR) 1–4, platelet-derived growth factor receptor-alpha, KIT, and RET [12-14]. In a phase III study of 392 patients with RAI-refractory DTC (SELECT trial), lenvatinib was shown to improve progression-free survival (PFS) and response rate [11]. However, TKIs, including lenvatinib, have a high incidence of treatment-related adverse events and can only prolong PFS by approximately 5-15 months [10, 11], and most patients discontinue

them owing to unresponsiveness [15]. Moreover, TKIs are only reserved for RAI-refractory patients with rapid tumor progression and severe, life-threatening symptoms, according to current treatment guidelines [7, 16].

Concurrent chemoradiation therapy (CCRT) has become one of the standard initial treatments for advanced-stage head and neck squamous cell carcinoma (HNSCC) in recent years [17, 18]. Bernier et al. reported that postoperative CCRT improved the 5-year PFS and OS of locally advanced HNSCC compared with radiotherapy alone (EORTC trial 22931) [19]. Cetuximab, the first molecular targeted agent applied to the treatment for HNSCC, also improved the treatment outcomes in combination with radiotherapy in a phase III study (Bonner trial) [20]. In contrast, no established regimen and few basic studies of combination with irradiation plus chemotherapy or molecular targeted therapy have been documented in thyroid cancer.

Interestingly, some clinical studies have reported cases with rapid tumor regression after concurrent combination therapy of lenvatinib and irradiation [21, 22]. These reported cases were successfully treated by combination with lenvatinib and external beam radiation therapy (EBRT) without any serious complications. Notably, sorafenib, a TKI also applied for unresectable thyroid cancer, showed synergistic effects with irradiation against head and neck cancer [23], renal cell carcinoma, and breast cancer [24]. These reports support the clinical application of combined therapy with lenvatinib and irradiation for thyroid cancer treatment. However, little is known about the synergistic effect of lenvatinib and irradiation, and no report has investigated its mechanisms. Therefore, we hypothesized that this new therapeutic strategy will contribute to the enhancement of treatment effects and reduce drug toxicities. Thus, in this study, we are the first to assess the antitumor effects of combination therapy with

lenvatinib and radiation (CTLR) against DTC through a series of *in vitro* and *in vivo* studies. Moreover, we also investigate the mechanisms of this combination therapy.

Materials and methods

Cell culture

The human DTC cell lines, both K1 (papillary thyroid cancer, PTC cell line) and FTC-133 (follicular thyroid cancer, FTC cell line), were obtained from ECACC (Wiltshire, U.K., EC92030501-F0, EC94060901-F0, respectively). K1 cells were cultured in a mixture of Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Missouri, U.S.A., D6429), Ham's F12 medium (Sigma-Aldrich, N6658), and MCDB 105 medium (Sigma-Aldrich, M6395) (2:1:1, v/v/v) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Massachusetts, U.S.A., 10270106). FTC-133 cells were cultured in a mixture of DMEM and Ham's F12 medium (1:1, v/v) with 10% FBS. All cells were grown at 5% CO_2 and 37˚C. The media was replaced twice per week.

Irradiation and treatment with lenvatinib

The cell suspensions of K1 and FTC-133, in a canister 105 mm in height and 312 mm in diameter, were ɣ-ray irradiated using the Gammacell 40 Exactor (Nordion International, Ontario, Canada) at a central dose rate of 0.84 Gy/min; irradiations were 0, 3, 6, 9, and 12 Gy. Then, cells were seeded in plates or dishes and cultured overnight, and the media was replaced with vehicle (0.1% DMSO in media) or 3, 10, and 20 μM lenvatinib mesilate (lenvatinib; supplied from Eisai Co., Ltd., Tokyo, Japan), followed by the functional *in vitro* experiments. Protocol schema for *in vitro* experiments is shown in **Fig. S1A**.

Replication study in vitro

The replication studies were conducted using the modified version of that described in a previous report [25]. Briefly, 0.05×10^6, 0.1×10^6, and 0.2×10^6 cells were seeded in the 25 cm^2 flask following 0 and 3, 6 and 9, and 12 Gy irradiation, respectively. These cells were cultured for 1, 2, 4, or 7 days. Then, cells were harvested using Accutase (Innovative Cell Technologies, Inc., California, U.S.A., AT-104) at each time point, and the cell numbers were counted by staining with trypan blue. The replication rate (RR) at each time point after irradiation (1, 2, 4, and 7 days) was calculated using the following equation: $RR = N/N_0$, where N is the number of cells after irradiation and N_0 is the number of cells before irradiation. The replication studies were performed in triplicate.

Colony formation assay

A colony formation assay was performed using monolayer culture. Control (0 Gy) and irradiated (3 Gy) cells were seeded into 6-well plates (K1 and FTC-133 cells, 2×10^4 cells per well) and allowed to attach overnight. Cells were then treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib in culture medium for 6 days (7 days after irradiation). Surviving colonies (≥50 cells per colony) were fixed with acetic acid and methanol (1:7, v/v), stained with 0.5% crystal violet, and counted. Merged images on bright field were obtained by using the BZ-9000 microscope (KEYENCE, Tokyo, Japan). Each experiment was performed in triplicates.

Apoptosis assay and cell cycle analysis

For the apoptosis assay, cells with/without 3 Gy irradiation were seeded in the 25 cm^2 flask and allowed to attach overnight. Cells were then treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib in culture medium for 6 days (7 days after irradiation) and then evaluated with the FITC Annexin V Apoptosis Detection Kit (BD Bioscience, 559763) using flow cytometry according to the manufacture's protocol.

Early and late apoptotic cells were counted by the exhibition of Annexin V-FITC+/7-AAD− and Annexin V-FITC+/7-AAD+ staining patterns, respectively. They were collectively called apoptotic cells. Each experiment was performed in triplicate.

For cell cycle analysis, cells with/without 3 Gy irradiation were seeded in the 25 cm^2 flask and allowed to attach overnight. Cells were then treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib in culture medium for 48 h and were fixed and stained for total DNA using the BD Cycletest Plus DNA Kit (BD Biosciences, California, U.S.A., 340242). Then, cell cycle distributions were measured using flow cytometry (FACSCalibur; BD Bioscience), and the proportions of cells in the sub-G0/G1, S, and G2/M phases were analyzed using FlowJo v10. This experiment was performed in triplicate.

Immunofluorescence analysis and immunohistochemistry

For immunofluorescence, cells with/without 3 Gy irradiation were grown on a glass-bottomed 8-well Slide & Chamber (WATSON Bio Lab, Tokyo, Japan, 192-008) and treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib for 48 h. These cells were subsequently fixed with 4% paraformaldehyde solution (Wako, Tokyo, Japan, 163-20145) for 5 min and permeabilized with 0.3% Triton X-100 in PBS for 10 min. Samples were incubated with anti-Ki-67 antibodies (Santa Cruz Biotechnology, Inc., Texas, U.S.A., sc-23900). The nuclei were detected with Hoechst 33342 (Dojindo, Kumamoto, Japan, 346-07951). The expressions were monitored by confocal microscopy (LSM700; Zeiss, Oberkochen, Germany).

For immunohistochemistry, sections were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. These sections were deparaffinized, and antigen

retrieval was performed in sodium citrate buffer (pH 6) using an autoclave under 105ºC for 30 min. The sections were incubated for 30 min in 3% H_2O_2 to quench endogenous tissue peroxidase. Cell proliferation ability was assessed by Ki-67 staining. Immunostaining was done with the FLEX Monoclonal Mouse Anti-Human Ki-67 antibody (Ready-to-Use) (Agilent, California, U.S.A., IR62661-2) according to a standard protocol using Simple Stain MAX PO (Nichirei Bioscience, Tokyo, Japan, 424131). All tissue sections were counterstained with hematoxylin, dehydrated, and mounted.

Human thyroid cancer cell xenografts

All animal experiments were approved by the Animal Experiment Committee of Kansai Medical University (19-016), and all methods involving animals were performed in accordance with the relevant guidelines and regulations. Nude mice (CAnN.Cg-Foxnlnu/CrlCrlj, female, 5 weeks old) were obtained from Charles River Laboratories Japan (Kanagawa, Japan). Mice were maintained under specific pathogen-free conditions and housed in barrier facilities on a 12 h light/dark cycle, with food and water *ad libitum*. K1 or FTC-133 cells were resuspended in PBS and Matrigel (Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix; Thermo Fisher Scientific, Inc., Massachusetts, U.S.A., A1413201) (2:1, v/v). Then, 5×10^6 cells were inoculated subcutaneously into the dorsal right shoulder. Three weeks after transplantation, tumor volumes of K1 and FTC-133 xenografts reached almost 75 mm^3 and 48 mm^3, respectively. Then, mice were randomly divided into four groups: vehicle (sterile distilled water), lenvatinib alone, irradiation alone, and CTLR (n = 5 per group). Protocol schema for *in vivo* experiments is shown in **Fig. S1B**. In the irradiation alone and CTLR groups, mice were irradiated at a fractionated dosage totaling 9 Gy (3 Gy ×

3) every second day during the first week of treatment. Lenvatinib (10 mg/kg) was orally administered once daily. The dose of lenvatinib was decided based on a previous study for *in vivo* experiments using lenvatinib [14]. The tumor sizes were measured every two days using a caliper, and tumor volumes were estimated using the following formula: tumor volume (mm^3) = 1/2 L × S^2 (L, longest diameter; S, shortest diameter). The change in tumor volume in the treated group relative to that in the control group was calculated according to the following formula: $\Delta T/C = (\Delta T/\Delta C) \times 100\%$, where ΔT and ΔC are the changes in tumor volume in the treated and vehicle control groups, respectively. Subsequently, the tumors were harvested and weighted.

Quantification of intracellular lenvatinib by liquid chromatography and mass spectrometry

K1 cells seeded on 6-well plates with/without irradiation were cultured overnight and then treated with medium containing 20 μM lenvatinib for 24, 48, or 72 h. After removing the medium, the cells were washed twice with PBS and then lysed with 100 µL of M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, 78501). Fifty µL of each cell lysate was added to 200 µL of cold acetone (Wako, 016-00346) for protein precipitation, and the samples were placed at –20ºC for 1 hour and then centrifuged for 10 min at 15,000 rpm. The supernatants were transferred to clean vials for liquid chromatography and mass spectrometry (LC-MS/MS) analysis. LC was performed using the Prominence HPLC system consisting of an LC-20AD pump, SIL-20AC autosampler, and CTO-20AC column oven (Shimadzu Co., Kyoto, Japan), and the chromatographic system was operated using Analyst software 1.7.1 (SCIEX, Framingham, Massachusetts, U.S.A.). Chromatographic separations were conducted using a C18 column (SCIEX, 4374841, dimensions 5 µm, 4.6 mm × 150

mm) at 40ºC. The mobile phase was composed of two solvents (solvent A, 0.1% trifluoroacetic acid (Wako, 206-10731) in ultrapure water; solvent B, 0.1% trifluoroacetic acid in acetonitrile (LC-MS grade, Merck, Darmstadt, Germany, 1.00029.1000)). Ten µL of the samples were injected into the column and separated at a flow rate of 0.5 mL/min using the following elution program: 20% solvent B in solvent A for 5 min, a linear gradient of 20% to 100% solvent B in solvent A for 20 min, and 100% solvent B for 5 min. Mass spectrometry was performed using the API3200 LC-MS/MS System (SCIEX). A standard sample of lenvatinib (molecular weight 426.85 g/mol) was observed as a precursor ion at m/z 427.090 and a product ion at 370.100, and this Q1/Q3 product was eluted at a retention time of 8.7 min under the elution condition described above. The intracellular concentration of lenvatinib was quantified as counts of peak area on the intended retention time of lenvatinib and was normalized by protein amounts of the cell lysate.

Statistical analysis

Data are presented as means ± standard errors of the mean (SEMs). Statistical significance was evaluated using ANOVA by the software GraphPad Prism, version 8 (GraphPad, San Diego, U.S.A.). The threshold of significance was set at $p < 0.05$ for all tests.

Results

Inhibition of thyroid cancer cell growth using CTLR in vitro

To investigate the efficacy of CTLR in the thyroid cancer cell lines, K1 and FTC-133, in comparison to monotherapy of irradiation or lenvatinib, a replication study was performed *in vitro*. First, we found that these RR suppressions were induced by

monotherapy of irradiation or lenvatinib in a dose-dependent manner, and the most efficacious timing of RRs was at 7 days after these treatments (**Fig. S2A-D**). As the monotherapy of irradiation at the dose of 12 Gy totally suppressed RR in both cell lines, 12 Gy irradiation was eliminated in the following experiments with combination therapy. Next, we found that the most efficacious of RR suppressions by this combination therapy was in the manner of 3 Gy irradiation with 20 μM lenvatinib in both K1 (**Fig. 1A**) and FTC-133 (**Fig. 1B**) cells. Of note, these RR suppressions plateaued in the 9 Gy high-dose irradiation group. Moreover, similar results were observed in the colony formation assay of K1 (**Fig. 1C**,**E**) and FTC-133 cells (**Fig. 1D**,**F**). Thus, these results suggested that the combination doses of 3 Gy irradiation and 20 μM lenvatinib *in vitro* were most effective.

Induction of apoptosis and G2/M phase cell cycle arrest by CTLR

To examine the effects of lenvatinib and/or irradiation on apoptosis in K1 and FTC-133 cells, we performed an apoptosis assay using flow cytometry. Apoptosis was significantly induced in K1 and FTC-133 cells treated with CTLR compared with control, whereas induction of apoptosis in cells in the lenvatinib or irradiation monotherapy groups were not significant (**Fig. 2A**,**B**, and **S3A**).

Next, we evaluated the effects of lenvatinib and irradiation, individually or in combination, on cell cycle. For cell cycle analysis by flow cytometry, the percentage of K1 cells in the G2/M phase was significantly increased with CTLR compared with control, whereas those treated with lenvatinib or irradiation monotherapy were not significant (**Fig. 3A**,**B**), suggesting a synergistic effect of CTLR induces apoptosis and G2/M phase cell cycle arrest. In contrast, cell cycle distribution among groups did not change significantly in FTC-133 cells (**Fig. S3B**).

Inhibition of proliferation activity by CTLR

To investigate the proliferation abilities of K1 and FTC-133 by CTLR, immunofluorescence with Ki-67 was performed. There was significant suppression of Ki-67 expression in the K1 (**Fig. 4A**,**B**) and FTC-133 (**Fig. S4A**,**B**) cells following treatment with CTLR compared with the control groups, suggesting that the proliferation activity of cancer cells was inhibited using this combination therapy. In contrast, monotherapy with lenvatinib or irradiation resulted in minimal suppression of proliferation.

In vivo *synergistic anticancer effects by CTLR in thyroid cancer xenografts*

The next step was to study whether CTLR has antitumor effects *in vivo*. We used tumor xenografts of K1 and FTC-133 cells murine model, following treatment with monotherapy or the combined therapy. As shown in **Fig. 5A**,**B**,**C**, there were significant differences in the suppression of tumor growth between CTLR and control groups, and this suppression effect was more pronounced than that of monotherapy. Moreover, similar results were found with tumor weight (**Fig. 5D**,**E**), indicating that the combined therapy has synergistic effects. Notably, body weight, an indicator of the health of the animals, was not significantly different among the treatment groups (**Fig. 5F**,**G**), suggesting there were no emergence of toxicity effects by lenvatinib administration. Immunohistochemical examination of Ki-67 revealed that CTLR dramatically decreased Ki-67 expression levels in the tumor compared to monotherapy (**Fig. 5H**,**I)**. Together, these results conclusively show that CTLR exerts potent anticancer activity in the DTC xenograft models.

Intracellular uptake of lenvatinib was significantly increased by irradiation through membrane permeability

Finally, to clarify the mechanism of synergistic effects of CTLR, we investigated the intracellular uptake of lenvatinib after irradiation using LC-MS/MS. The intracellular concentration of lenvatinib was successfully quantified as counts of peak area on the retention time of approximately 8.7 min and peaked at 48 h in both irradiated and nonirradiated cells. Interestingly, we found that the intracellular uptake of lenvatinib was significantly increased in irradiated cells compared with nonirradiated controls at 48 h and 72 h after administration of lenvatinib (**Fig. 6**). These findings suggest that the uptake of lenvatinib into cancer cells was raised up through increased cell membrane permeability by irradiation, demonstrating that the synergistic antitumor effects of CTLR were achieved.

Discussion

Comparing with HNSCC treatment using combination with chemotherapy and irradiation, DTC therapy has been behind in therapy without such measure. On the basis of durable response by combination therapy with lenvatinib and irradiation, as documented in some clinical case reports [21, 22], we made a hypothesis that it could be applied to the treatment for DTC. In this study, we first determined the antitumor effects of CTLR in both human DTC cells and xenograft models. The combined therapy synergistically inhibited cell replication and colony formation *in vitro* and tumor growth in nude mice. Moreover, proliferative activity, represented by Ki-67 expression, was also significantly decreased after CTLR both *in vitro* and *in vivo*. Interestingly, we found increased uptake of TKI through membrane permeability 48 h following irradiation. These findings suggest that CTLR could be a novel approach for patients with advanced thyroid cancer.

In a previous study, Jing *et al*. showed that lenvatinib monotherapy was less effective for colony formation and tumor growth in anaplastic thyroid cancer cell lines [26]. Ogasawara *et al.* reported that lenvatinib monotherapy had no effect on apoptosis in human liver cancer cell lines [27]. Regarding radiation therapy, RAI is considered the gold standard in the treatment of metastatic DTCs. However, many DTC patients with advanced disease do not respond or become refractory to RAI [7]. For EBRT, its role and indications are controversial, as there are no good prospective data to guide decision-making [28]. Recently, several papers have reported improvement in the outcome of patients with HNSCC following combination therapy with chemotherapy and radiation (6.5% 5-year absolute survival benefit of CCRT) [17, 18]. Thus, this evidence suggests that the synergistic effects of combination therapy might be expected in patients with thyroid cancer and sheds light on the new therapeutic option to improve treatment outcomes for this advanced disease. Indeed, we found that apoptosis and G2/M phase cell cycle arrest were significantly induced after CTLR, suggesting that this combination treatment has synergistic effects on the inhibition of DTC growth. Supporting our results, some authors reported that G2/M checkpoint is a promising target for improved efficacy of radiation therapy [29, 30]. Jing *et al*. reported that lenvatinib enhanced apoptosis and G2/M phase cell cycle arrest of anaplastic thyroid cancer cells in combination with paclitaxel [26].

Clinically, lenvatinib has been associated with a high frequency of adverse effects (75.9%) in RAI-refractory DTCs, while PFS was extended to 18.3 months in a phase III trial[11]. Notably, lenvatinib occasionally induces serious side effects such as hemoptysis and aerodigestive fistula [31, 32]. Our data suggested that CTLR can reduce the dose of lenvatinib through synergistic effects, resulting in decreasing drug toxicity.

Moreover, our results of synergistic treatment effects by CTLR also suggest that it has potential application with lenvatinib and RAI, systemic radiation, in the treatment of patients with DTCs. Sheu *et al*. reported two patients with advanced DTC successfully rescued by lenvatinib treatment complementary with RAI in the durable response [33]. Several authors reported that some TKIs elevated RAI uptake and enhanced the therapeutic effect of RAI [34, 35]. The iodine avidity of tumor cells should be considered for potential application of combined therapy with lenvatinib and RAI. Further investigation is required to identify the pharmacodynamics and toxicology of combined treatment of lenvatinib and irradiation, including RAI as well as EBRT in clinical trials.

Interestingly, we found that the membrane permeability of cancer cells was increased by irradiation, resulting in the synergistic inhibition of cancer cell growth due to an increased intracellular concentration of lenvatinib. Although some authors reported the methodology of quantitative analysis of lenvatinib in human plasma using LC-MS/MS [36, 37], intracellular uptake of lenvatinib has not been investigated. In this report, we developed a methodology of quantitative analysis of intracellular uptake of lenvatinib using LC-MS/MS. Thus, our approach suggests that this method could be applied to assess molecularly targeted agents, including other TKIs as well as lenvatinib.

The potential limitations of thyroid cancer cell lines as preclinical models have been previously reported [38]. This study also has some limitations. First, only two different DTC cell lines (K1 derived from PTC and FTC-133 derived from FTC) were used in this study. The concentrations of lenvatinib used in *in vitro* experiments were much higher than the concentrations of lenvatinib that inhibit kinase activity, such as VEGFR, FGFR, RET, and KIT [12, 14] because K1 and FTC-133 cells used in this study are

resistant to lenvatinib *in vitro* as a previous study has reported that the IC_{50} values of K1 and FTC-133 cells were 22 and 24 μM, respectively [14]. Further studies using other cell lines, such as RO82W1 and TT, which are sensitive to lenvatinib *in vitro* [14], are needed to clarify whether lower concentrations of lenvatinib combined with radiation inhibit cell growth and induce apoptosis. Second, the antiangiogenic activity of CTLR is unclear in this study. Yamamoto et al. reported the antitumor effects of lenvatinib based on inhibition of tumor angiogenesis [12]. Some authors reported that other antiangiogenic agents enhanced responsiveness to radiotherapy through vascular normalization in brain tumor [39, 40]. Increased intracellular concentrations of lenvatinib with CTLR in this study may also enhance antiangiogenic activity of lenvatinib, which contributes synergistic antitumor effects with CTLR. A more detailed investigation on tumor angiogenesis after CTLR is required to reveal the antiangiogenic effect of this combined therapy.

In conclusion, our data demonstrate that CTLR demonstrated synergistic antitumor effects against DTC cells and xenograft models. This mechanism-based potential of combination therapy suggests a powerful new treatment strategy for advanced thyroid cancer patients with fewer side effects.

Data availability

The authors declare that all the relevant data supporting the findings of this study are available within the article or from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal

relationships that could have influenced the work reported in this paper.

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Figure legends

Fig. 1. Inhibition of thyroid cancer cell growth using CTLR *in vitro*. (**A**,**B**) Replication rates (RRs) of K1 and FTC-133 cells treated with vehicle (0.1% DMSO in media) or 3, 10, and 20 μM lenvatinib for 6 days following 0–9 Gy irradiation (7 days after irradiation) (n = 3). RRs were compared with the vehicle control in each irradiation dose. Images (**C**,**D**) and quantification (**E**,**F**) of colony formation assay indicate K1 and FTC-133 cells treated with vehicle or 20 μM lenvatinib for 6 days following 0 or 3 Gy irradiation (7 days after irradiation) (n = 3). Each experiment was performed in triplicate. Data represent means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control.

Fig. 2. Apoptosis assay in thyroid cancer cells following CTLR. (**A**) Flow cytometric analysis and (**B**) quantification of the apoptosis analysis in K1 cells treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib for 6 days following 0 or 3 Gy irradiation (7 days after irradiation) (n = 3). Apoptotic cells were included with both Annexin V-FITC$^+$/7-AAD$^-$ and Annexin V-FITC$^+$/7-AAD$^+$. Each experiment was performed in triplicate. Data represent means ± SEM. *$p < 0.05$ vs. control. **Le**, lenvatinib.

Fig. 3. G2/M phase cell cycle arrest in thyroid cancer cell following CTLR. K1 cells treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib for 48 h following 0 or 3 Gy irradiation. (**A**) Representative flow cytometric histograms of cell cycle analysis and (**B**) the percentage of K1 cells in each cell cycle phase (n = 3). DNA contents were determined by cell cycle fraction. Each experiment was performed in triplicate. Data represent means ± SEM. ***$p < 0.001$ vs. control. **Le**, lenvatinib.

Fig. 4. Inhibition of proliferation activity in thyroid cancer cells following CTLR. K1 cells treated with vehicle (0.1% DMSO in media) or 20 μM lenvatinib for 48 h following 0 or 3 Gy irradiation. (**A**) Images for Ki-67 immunofluorescence staining of K1 cells after treatment. Ki-67 and DAPI were stained with red and blue, respectively. (**B**) Graphs of the percentage of Ki-67 positive cells from five microscopic fields in each group. *$p < 0.05$, ***$p < 0.001$ vs. control. **Le**, lenvatinib.

Fig. 5. Synergistic anticancer effects in xenografts following CTLR. A total of 5×10^6 K1 (**A**,**D**,**F**,**H** and left panel of **C**) and FTC-133 cells (**B**,**E**,**G**,**I** and right panel of **C**) were subcutaneously transplanted in nude mice, and the mice were treated daily with lenvatinib (10 mg/kg/d) or vehicle (sterile distilled water) through oral gavage with/without irradiation (3 Gy × 3). (**A**,**B**) Time course of xenograft tumor growth. (**C**) Change in tumor volume in the treated group relative to that in the control group. ΔT/C (%) was calculated as (ΔT/ΔC) × 100%, where ΔT and ΔC are the changes in tumor volume for the treated and vehicle control groups, respectively. (**D**,**E**) Weights of the dissected xenograft tumors. (**F**,**G**) Animal weight in each group. (**H**,**I**) Immunohistochemical analysis of Ki-67 in paraffin-embedded tumor tissues. Data are represented as mean ± SEM (n = 5/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control.

Fig. 6. Upregulation of cell membrane permeability following irradiation. LC-MS/MS analysis of the intracellular uptake of lenvatinib in K1 cells was shown (n = 3). K1 cells with/without 3 Gy irradiation were treated with 20 μM lenvatinib for 24, 48,

or 72 h. The intracellular concentration of lenvatinib was quantified as counts of peak area on the specific retention time of lenvatinib (approximately 8.7 min). Data were normalized by protein amounts of the cell lysate. Each experiment was performed in triplicate. Data represent means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.