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Treatment Effect of Combining Lenvatinib and Vemurafenib for *BRAF* Mutated Anaplastic Thyroid Cancer

Chae Moon Hong[1,2]*, Ji Min Oh[1]*, Prakash Gangadaran[1,3], Ramya Lakshmi Rajendran[1] and Byeong-Cheol Ahn[1,2]

Department of Nuclear Medicine, School of Medicine, Kyungpook National University[1], Department of Nuclear Medicine, Kyungpook National University Hospital[2], BK21 Plus KNU Biomedical Convergence Program, Department of Biomedical Science, School of Medicine, Kyungpook National University[3], Daegu, Korea

Background and Objectives: Even though most of the thyroid cancer shows good prognosis, de-differentiated thyroid cancer is still refractory to conventional treatments. Recently, kinase inhibitors including multi-kinase and BRAF inhibitors are widely used for treatment of de-differentiated thyroid cancers, but resistant to single kinase inhibitor treatment eventually encountered. Therefore, combination therapy may have better therapeutic effect than single therapy for thyroid cancer. In this study, we evaluated therapeutic effect of multi-kinase and BRAF inhibitor combination to anaplastic thyroid cancer cell lines with and without *BRAF* mutation. **Materials and Methods:** We used anaplastic thyroid cancer cell lines with $BRAF^{V600E}$ mutation (8505C) and with *NRAS* mutation (HTh7). Both cell lines were treated with various concentration of multi-kinase inhibitor (lenvatinib) and BRAF inhibitor (vemurafenib). And combination of various concentration of both kinase inhibitors were used to treat both cell lines. Cytotoxic effect was assessed with cell counting kit-8 and therapeutic effect of single kinase inhibitor therapy and the combination therapy was compared. **Results:** Anti-proliferative effect of vemurafenib on 8505C $BRAF^{V600E}$-mutated cells was demonstrated from 0.25 μM concentration. However, HTh7 cells with *NRAS* mutation represented drug resistance up to 4 μM of vemurafenib. In case of lenvatinib treatment as a multi-kinase inhibitor, 8505C and HTh7 cells showed decreased cell viability dose-dependent manner. Combination treatment with vemurafenib and lenvatinib showed synergistic cytotoxic effect in *BRAF* mutated 8505C cell line, even at lower concentrations. **Conclusion:** Combination treatment with multi-kinase inhibitor and BRAF inhibitor showed promising therapeutic results in *BRAF* mutated anaplastic thyroid cancer cell line.

Key Words: Anaplastic thyroid cancer, BRAF, Lenvatinib, Vemurafenib, Combination treatment

Introduction

Anaplastic thyroid cancer (ATC) is a de-differentiated thyroid cancer which accounts for approximately 1% of thyroid cancer, but it contributes about 50% of thyroid cancer associated mortality.[1,2] ATC is aggressive and commonly refractory to conventional treatment strategy, such as radioiodine therapy, external radiotherapy and other systemic therapies.[3]

The mutation of *BRAF* was observed 33-59% of differentiated or poorly differentiated thyroid cancer, and it occurs approximately 45% in ATC.[4] *BRAF* mutation has a critical role for the activation of the MAPK

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Correspondence: Byeong-Cheol Ahn, MD, PhD, Department of Nuclear Medicine, School of Medicine, Kyungpook National University, 130 Dongdeok-ro, Jung-gu, Daegu 41944, Korea
Tel: 82-53-200-5583, Fax: 82-53-200-6447, E-mail: abc2000@knu.ac.kr
*These authors are equally contributed to this article.

signaling pathway and loss of radioactive iodine (RAI) avidity.[5-7] Previous genetic studies of ATC showed that *BRAF* and *RAS* are the predominant drivers, and *TP53*, the *TERT* promoter and other genetic mutations are frequently observed.[1,4,8] These findings suggest that ATC probably evolves from preexisting differentiated thyroid cancers or poorly differentiated thyroid cancers by adding of additional genetic abnormalities.[1,9]

In RAI-refractory thyroid cancer, multi-kinase inhibitors, such as lenvatinib and sorafenib, are widely used, but efficacy in ATC is not much elucidated.[10] Administration of BRAF inhibitors such as vemurafenib (PLX4032) showed promising therapeutic results for RAI-refractory thyroid cancers, but adverse effects and resistance to the drug often limit its usage.[11] Additional mutational events or activation of alternative signaling pathways which reactivate ERK signaling are considered as underlying biology of the resistance.[11]

Due to limited treatment strategies, mean survival of ATC was about 6 months.[1] Recently, the United States Food and Drug Administration approved the combined use of dabrafenib and trametinib for $BRAF^{V600E}$-mutated ATC treatment. This combination treatment showed good response rate but only for a limited duration.[12] Therefore, more effective therapeutic strategies are warranted to overcome incurable ATC. In this study, we treated the multi-kinase inhibitor (lenvatinib) and BRAF inhibitor (vemurafenib) to ATC cell lines with and without *BRAF* mutation.

Materials and Methods

Cell Culture

Cell lines with 8505C harboring $BRAF^{V600E}$ mutation were purchased from Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ; Braunschweig, Germany); HTh7 was gifts given by Dr. Minho Shong (School of Medicine, Chungnam National University). The 8505C cell line was maintained in Roswell Park Memorial Institute-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (HyClone). HTh7 cell line harboring *NRAS* ($BRAF^{V600E}$ wild-type) was maintained in Dulbecco's modified Eagle's medium (DMEM high glucose; HyClone) supplemented with 10% FBS and 1% penicillin-streptomycin.

Chemicals

Lenvatinib as a multi-kinase inhibitor and vemurafenib as a BRAF inhibitor were purchased from Selleckchem (Pittsburgh, PA, USA). A 10mM stock solution of each inhibitor was prepared by dissolving with dimethyl sulfoxide and was stored at −20°C before their usage.

Cell Viability

Cell viability of the thyroid cancer cell lines after lenvatinib and vemurafenib treatment were determined using a cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Kumamoto, Japan). For single treatment, multiple dose of vemurafenib (0, 0.25, 0.5, 1, 2, 4, 8 μM) and lenvatinib (0, 0.78, 1.53, 3.13, 6.25, 12.5, 25 μM) were applied to 8505C and HTh7 cells. Based on single treatment results, 0, 0.25, 0.5 μM of vemurafenib and 0, 0.78, 1.53, 3.13, 6.25, 12.5, 25 μM of lenvatinib were applied to 8505C and HTh7 cells. Firstly, the cells (5×10^3) were seeded in 96-well plates and incubated for 24 hours in a humidified incubator at 37°C with 5% CO_2. On the next day, various concentrations of lenvatinib and vemurafenib were applied to cells, which were incubated for an additional 72 hours in the CO_2 incubator at 37°C. After then, CCK-8 reagent was added in each well, and light absorbance at 450 nm was measured to assess the cell viability. The cell viability test was performed with quintuplicates, and the highest and the lowest values of the tests were excluded for the analysis.

Protein Extraction and Western Blot Analysis

The 8505C cells were exposed at fixed lenvatinib and vemurafenib concentrations for 72 hours. Following the incubation, cell pellets were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing a protease and phosphatase inhibitor cocktail kit

(Thermo Fisher Scientific). Lysed cells were vortexed three times at 10 minutes intervals and then centrifuged at 13,000 g for 20 minutes at 4°C. Next, the supernatants from lysed cells was collected and quantified using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). For western blot analysis, 20 μg proteins were electrophoresed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin (BSA) prepared in Tris-buffered saline containing Tween-20 (TBS-T) and then probed with primary antibodies overnight at 4°C. Next day, membranes were washed using TBS-T and probed with a horseradish peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. Membranes were then washed three times with TBS-T and signals were visualized by using an enhanced chemiluminescence detection reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Protein bands were detected using an X-ray films with imager system. PARP (Cell Signaling, Danvers, MA, USA), cleaved PARP (Cell Signaling), GAPDH (Santa Cruz, Dallas, TX, USA) antibodies were used as primary antibodies with dilution ratio of 1:5,000. Anti-mouse and anti-rabbit HRP-conjugated secondary antibody (Dilution ratio 1:10,000; Cell Signaling) was used.

Immunofluorescence

After confirming the cell viability, suitable concentrations (lenvatinib 12.5 μM, vemurafenib 1 μM) was used for further experiments. The thyroid cancer cells (3×10^4) were cultured for 72 hours in 4-well chamber slides in the humidified incubator at 37°C with 5% CO_2. For γH2A.X, as a DNA damage marker, immunofluorescence imaging, 8505C cells were treated with lenvatinib 12.5 μM or vemurafenib 1 μM alone or with a combination of both kinase inhibitors. Following incubation for 72 hours, cells were fixed with chilled methanol for 10 minutes at −20°C and washed three times with PBS at 10 minutes intervals. Cells were then permeabilized by incubating with 0.5% Triton X-100 for 90 seconds, followed by three washes with PBS at 10 minutes intervals. Cells were blocked with 3% BSA in PBS containing 0.1% Triton X-100 for one hour and were incubated an anti-γH2A.X primary antibody conjugated with Dylight 488 (Abcam) at room temperature for 4 hours. Coverslips were mounted onto chamber slides using Vecta mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). γH2A.X-stained cells were observed using confocal laser microscopy (LSM 5 exciter; Zeiss, Oberkochen, Germany).

Statistical Analysis

All data are expressed as mean±standard deviation. The results from a Student's t-test of the treated cells were compared with those of the control cells using Excel software (Microsoft, Redmond, WA, USA). A p-value of <0.05 was considered to be statistically significant.

Results

Anti-Proliferative Effect of Lenvatinib and Vemurafenib in *BRAF*^{V600E}-Mutated Thyroid Cancer Cells

To investigate the anti-proliferative effect of lenvatinib and vemurafenib, we chose two ATC cell lines with different mutations. Anti-proliferative effect of vemurafenib on 8505C *BRAF*^{V600E}-mutated cells showed from 0.25 μM concentration. However, HTh7 cells with *NRAS* mutation represented drug resistance up to 4 μM and abruptly cell viability was decreased from 8 μM (Fig. 1A). There were statistical differences of cell viability in 0.25 to 4 μM concentration of vemurafenib between two cell lines ($p<0.05$). In case of lenvatinib treatment as a multi-kinase inhibitor, 8505C cells harboring *BRAF* mutation gradually decreased cell viability dose-dependent manner. HTh7 harboring *NRAS* mutation also showed similar pattern of cell viability with 8505C cells after lenvatinib treatment until 12.5 concentration of lenvatinib ($p>0.05$). Only in 25 μM concentration of lenvatinib, there was statistical difference of cell viability between two cell

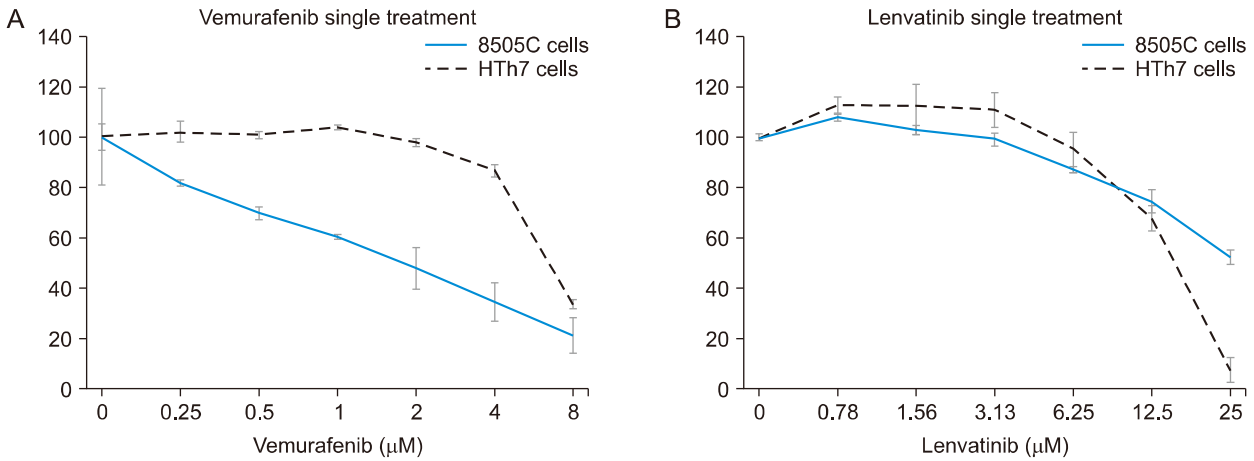

Fig. 1. Anti-proliferative effect of lenvatinib and vemurafenib in HTh7 and 8505C anaplastic thyroid cancer cells. (A) Single treatment of vemurafenib in *BRAFV*^{600E} mutated 8505C cells showed anti-proliferative effect from 0.25 μM concentration. However, HTh7 cells with *NRAS* mutation represented drug resistance up to 4 μM and abruptly cell viability was decreased from 8 μM. (B) Single treatment of lenvatinib in 8505C and HTh7 cells showed dose dependent anti-proliferative effect. Values presented here are mean value±standard deviation.

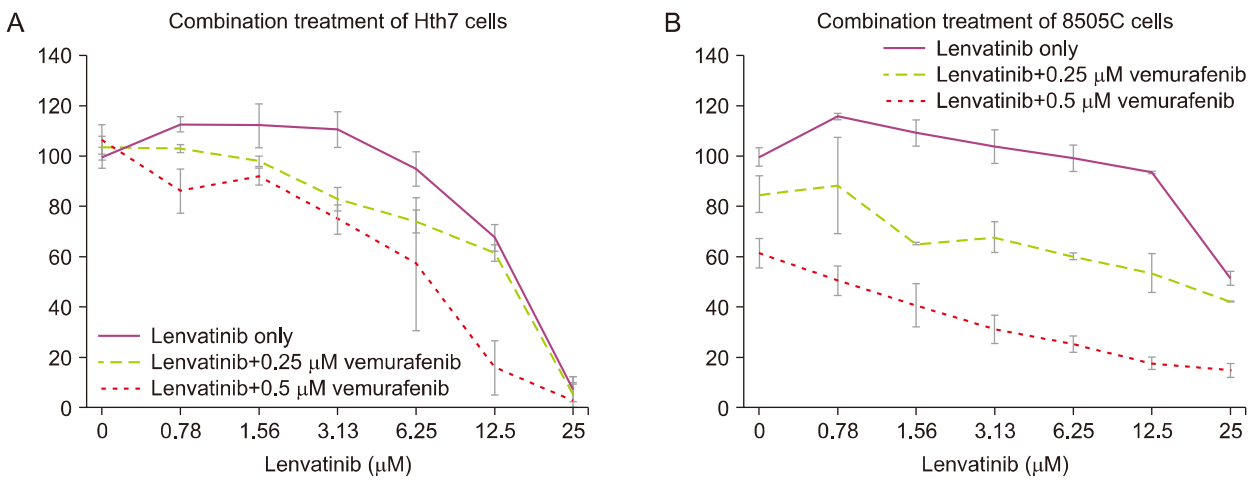

Fig. 2. Combination treatment of lenvatinib and vemurafenib. (A) Combination treatment of lenvatinib and vemurafenib in HTh7 cells showed only additive cytotoxic effect. (B) In 8505C cells, synergistic cytotoxic effect was observed by the combination treatment. Values presented here are mean value±standard deviation.

lines ($p<0.05$) (Fig. 1B). These results represented that vemurafenib as a BRAF inhibitor had an effect for inhibiting of proliferation in *BRAF*-mutated thyroid cancers, but not in *NRAS*-mutated thyroid cancers.

Efficacy of Combination Treatment in Thyroid Cancer Cells

From the results with single treatment, we selected suitable concentrations for evaluating combination treatment. Next, we compared cytotoxic effect between single and combination treatments. HTh7 cells harboring *NRAS* mutation have only some additive effects of vemurafenib compared with lenvatinib single treatment (Fig. 2A). There were statistical differences of cell viability in 3.13 to 6.25 μM of lenvatinib between lenvatnib only and combination of lenvatinib+0.25 μM vemurafenib ($p<0.05$). And there were statistical differences of cell viability in 3.13 to 12.5 μM of lenvatinib between lenvatnib only and combination of lenvatinib+0.5 μM vemurafenib ($p<0.05$).

However, 8505C cells harboring *BRAF*^{V600E} mutation showed the synergistic anti-proliferative effect with

lenvatinib and vemurafenib (Fig. 2B). There were statistical differences of cell viability in 1.56 to 12.5 μM of lenvatinib between lenvatnib only and combination of lenvatinib+0.25 μM vemurafenib ($p<0.05$). And there were statistical differences of cell viability in every concentration of lenvatinib between lenvatnib only and combination of lenvatinib+0.5 μM vemurafenib ($p<0.05$). Interestingly, 1.56 μM concentration of lenvatinib with 0.25 μM concentrations of vemurafenib demonstrated a synergistic effect.

DNA Damage from Combination Treatment in *BRAF*^{V600E}-Mutated Thyroid Cancers

Based on previous results, we pursued to monitor DNA damage via lenvatinib and vemurafenib combination treatment in 8505C cells harboring *BRAF*^{V600E} mutation. To perform the immunofluorescence experiment, 12.5 μM concentration of lenvatinib and 1 μM concentration of vemurafenib were selected. Treating 8505C cells with lenvatinib alone triggered the DNA damage trivially in nucleus. However, vemurafenib single treatment induced the DNA damage substantially. Moreover, combination treatment with vemurafenib and lenvatinib markedly enhanced the DNA damage suggesting the synergistic effect of the two kinase inhibitors in *BRAF*^{V600E}-mutated thyroid cancer cells (Fig. 3).

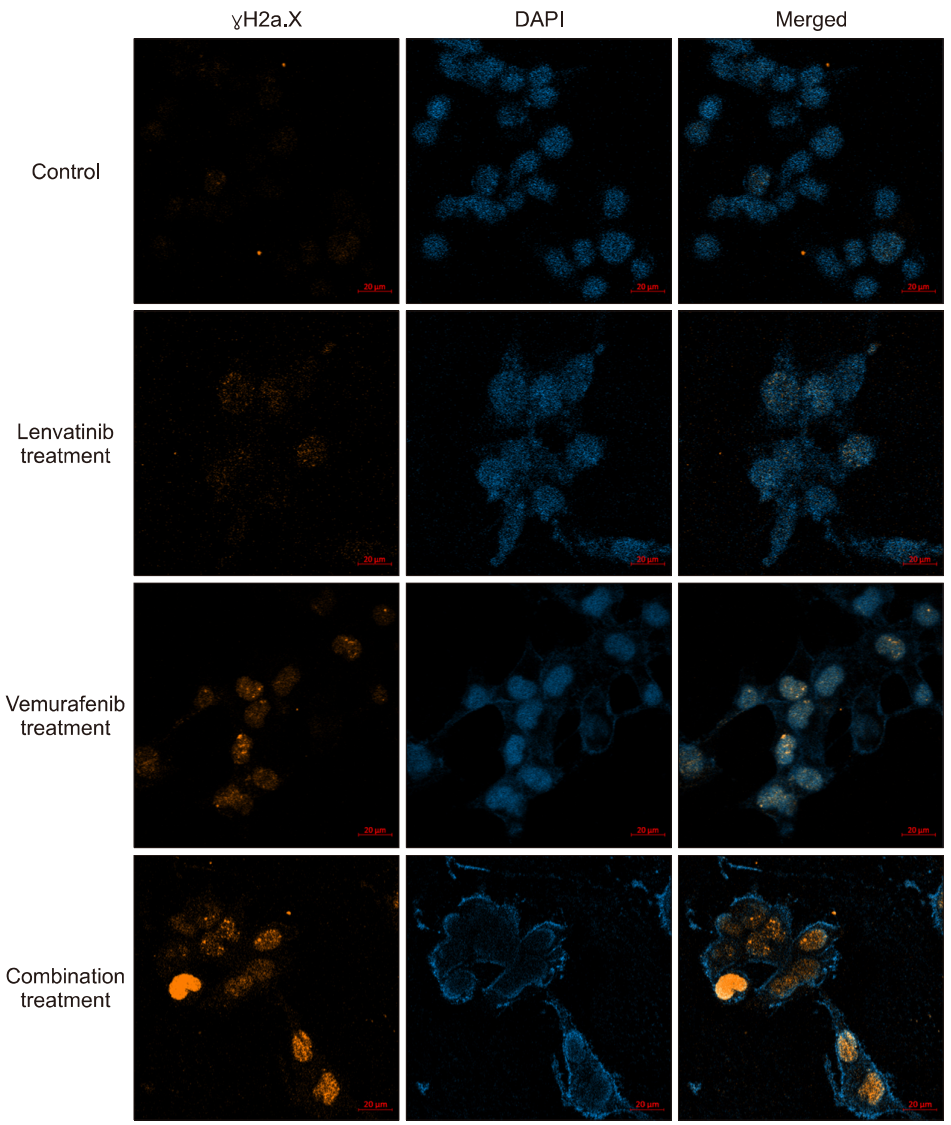

Fig. 3. DNA damage analysis using γH2A.X stain. After 72 hours of treatment, γH2A.X stain was significantly increased in the combination treatment, compared with the control and the single treatments.

Induction of Apoptosis by Lenvatinib and Vemurafenib Combination Treatment in *BRAF*^{V600E}-Mutated Thyroid Cancers

After investigating the effects of lenvatinib or/and vemurafenib on DNA damage, we evaluated the change of apoptosis induction in thyroid cancer harboring *BRAF*^{V600E} mutation. Poly (ADP-ribose) polymerase (PARP), as a nuclear enzyme, is involved in various cellular processes, including DNA repair, apoptosis. As shown Fig. 4, we confirmed both expression of PARP and cleaved-PARP after the treatments. Although total PARP expression was not changed by the treatments, cleaved PARP was higher in both lenvatinib and vemurafenib single treatments and was highest in combination treatment. These results suggested that lenvatinib and vemurafenib combination treatment could induce apoptosis strongly than the single drug treatments.

Discussion

Our study showed that *BRAF* mutated 8505C cells were more resistant to lenvatinib only treatment than to vemurafenib treatment. Lenvatinib is one of the widely used multi-kinase inhibitors for treatment of the RAI-refractory thyroid cancer, but it does not target for the *BRAF* mutation.[13] Previous studies showed that the *BRAF* mutation is closely associated with impairment of thyroid-specific genes, activation of MAPK signaling pathway and RAI-refractoriness.[6,14-17] Therefore, the additional strategy is needed to enhance the treatment effect of lenvatnib in *BRAF* mutated ATC.

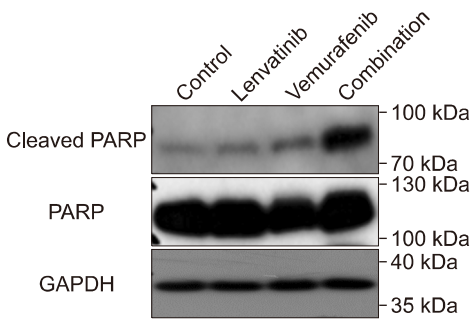

Fig. 4. Western blot analysis of apoptosis pathway. Western blot analysis showed significantly increased cleaved PARP by the combination treatment compared with the control and the single treatments.

Ofir et al.[18] reported therapeutic effect of vemurafenib in patients with RAI-refractory thyroid cancer, however, the disease progressed again by induction of resistance to vemurafenib. They suggested the drug resistance is associated with a new genetic mutation in *NRASQ61K*. Montero-Conde et al.[19] reported that inhibition of *BRAF* mutation induces overexpression of human epidermal growth factor receptor 3 (HER3) which re-induces the activation of both ERK and AKT pathways. Therefore, overcoming the resistance to BRAF inhibitors is warranted to improve prognosis of the patients.

The combination treatment of BRAF/MEK inhibitor and HER inhibitor was applied for redifferentiation of *BRAF* mutated papillary thyroid cancer.[20] Song et al.[21] demonstrated that a combination of vemurafenib and MEK inhibitor could overcome a transient vemurafenib response in *BRAF*-mutated thyroid cancers. And they also demonstrated synergistic effects following combination treatment with BRAF inhibitor and MEK inhibitor to increase NIS expression and RAI uptake in *BRAF*-mutated papillary thyroid cancer.[22]

In this study, the combination treatment with vemurafenib and lenvatinib showed synergistic cytotoxic effect in *BRAF* mutated 8505C cell line, but showed only trivial additive cytotoxic effect in *NRAS* mutated HTh7 cell line. Immunofluorescence imaging for DNA damage and western blot results for apoptosis support these results. As BRAF inhibitor (vemurafenib) and multi-kinase inhibitor (lenvatinib) have different targets,[13] authors considered that the combination of the two inhibitors showed synergistic effect in *BRAF*-mutated ATC only.

Interestingly, combination treatment using below therapeutic drug concentration (0.25 μM of vemurafenib and 1.56 μM of lenvatinib) also showed significant cytotoxic effects. Even though, *BRAF*-mutant melanoma cell line were sensitive to vemurafenib, but *BRAF* mutated thyroid and colorectal cancer cell lines were relatively refractory to the drug and required higher dose than malignant melanoma.[19] In the current study, relatively high concentration of vemur-

afenib was used for the *in vitro* experiments by the relative resistance of the thyroid cancers to the drug, therefore, pleotropic effect should be considered for clinical translation. However, combination of lenvatinib and vemurafenib can effectively reduce viability of thyroid cancers with lower drug concentration, therefore, the combination therapy may reduce the side effects and pleotropic effects of the drug in thyroid cancer patients. These findings suggest that combination treatment of vemurafenib and lenvatinib using low dose would be effective for *BRAF* mutated ATC and may not induce serious adverse effects of each drug.

In conclusion, combination treatment of multi-kinase inhibitor and BRAF inhibitor showed promising therapeutic results in *BRAF* mutated ATC.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Orcid

Chae Moon Hong: https://orcid.org/0000-0002-5519-6982
Ji Min Oh: https://orcid.org/0000-0001-8474-7221
Prakash Gangadaran: https://orcid.org/0000-0002-0658-4604
Ramya Lakshmi Rajendran:
https://orcid.org/0000-0001-6987-0854
Byeong-Cheol Ahn: https://orcid.org/0000-0001-7700-3929

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