Anti-cancer Effects of HNHA and Lenvatinib by the Suppression of EMT-Mediated Drug Resistance in Cancer Stem Cells\textsuperscript{1,2}

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**Abstract**

Anaplastic thyroid cancer (ATC) constitutes less than 2\% of total thyroid cancers but accounts for 20–40\% of thyroid cancer-related deaths. Cancer stem cell drug resistance represents a primary factor hindering treatment. This study aimed to develop targeted agents against thyroid malignancy, focusing on individual and synergistic effects of HNHA (histone deacetylase), lenvatinib (FGFR), and sorafenib (tyrosine kinase) inhibitors. Patients with biochemically and histologically proven papillary thyroid cancer (PTC) and ATC were included. Cell samples were obtained from patients at the Thyroid Cancer Center, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. PTC and ATC cells were treated with lenvatinib or sorafenib, alone or in combination with HNHA. Tumor-bearing mice (10/group) were administered 10 mg/kg lenvatinib (p.o.) or 40 mg/kg sorafenib (p.o.), alone or in combination with 25 mg/kg HNHA (i.p.) once every three days. Gene expression in patient-derived PTC and ATC cells was compared using a microarray approach. Cellular apoptosis and proliferation were examined by immunohistochemistry and MTT assays. Tumor volume and cell properties were examined in the mouse xenograft model. HNHA-lenvatinib combined treatment induced markers of cell cycle arrest and apoptosis and suppressed anti-apoptosis markers, epithelial-mesenchymal transition (EMT), and the FGFR signaling pathway. Combined treatment induced significant tumor shrinkage in the xenograft model. HNHA-lenvatinib combination treatment thus blocked the FGFR signaling pathway, which is important for EMT. Treatment with HNHA-lenvatinib combination was more effective than either agent alone or sorafenib-HNHA combination. These findings have implications for ATC treatment by preventing drug resistance in cancer stem cells.

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Introduction
Thyroid cancer represents more than 90% of all endocrine cancer cases, and its incidence has increased over the past three decades [1,2]. Thyroid cancer encompasses a broad scope of tumors derived from follicular cells that range from well-differentiated papillary (PTC) and follicular cancer (FTC), which generally have a favorable prognosis, through anaplastic thyroid cancer, a clinically aggressive form with poor prognosis, including poorly differentiated (PDTC) and undifferentiated thyroid cancer [3–5]. In particular, the advanced cancer subtype (ATC) has a poor prognosis owing to its resistance to treatment and aggressive behavior [5,6]. The total median survival is only a few months [6,7].

Poorly differentiated cancers are often resistant to anti-cancer drugs; moreover, effective clinical guidelines for ATC are currently lacking [7]. However, recent evidence has shown that induction of the epithelial-mesenchymal transition (EMT) in cancer cells not only results in metastasis, but also serves as a major contributing factor in drug resistance [8,9]. Nevertheless, the mechanisms of EMT-mediated drug resistance remain unclear. EMT is a physiological process in which epithelial cells exhibit collapse of cell–cell junctions and temporary or permanent transition to a condition that is characteristic of migratory cells [10]. Although EMT constitutes a fundamental aspect of resistance to ErbB-targeting compounds, a lack of knowledge regarding the molecular mechanisms underlying this process has prevented progress in the development of therapeutic approaches targeting this drug-resistant state [11,12]. Previous research has shown that fibroblast growth factor receptor 1 (FGFR1) expression is significantly induced during TGF-β-mediated EMT and plays a crucial role in metastatic cancer [13–15]. These previous studies suggest that the drug resistance of poorly differentiated cancer stem cells (CSCs) is related to EMT, which is mediated by the FGFR signaling pathway [16,17]. Although various molecules and mechanisms are closely associated with poor clinical outcomes for advanced thyroid cancer [18,19], we focused on EMT and drug resistance in CSCs to explain these poor clinical results [8,20]. In particular, in this study we examined the mechanisms underlying drug resistance, including FGFR signaling and EMT, in response to current treatments and methods to address the issues associated with resistance.

Materials and Methods

Patients and Tissue Specimens
Fresh tumors were obtained from patients with biochemical and histologically proven PTC and ATC who were treated at the Thyroid Cancer Center, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Fresh tumors were acquired during surgical resection of thyroid cancer primary and metastatic sites. Several patients with thyroid cancer were chosen depending on cancer subtype. The research protocol was approved by the Institutional Review Board of the Thyroid Cancer Center, Gangnam Severance Hospital, Yonsei University College of Medicine (IRB Protocol: 3–2016-0076).

Tumor Cell Isolation and Primary Culture
After resection, tumors were kept in normal saline with antifungal and antibiotics and moved to the laboratory. Normal tissue and fat were removed and the tissues were rinsed with 1× Hank’s Balanced Salt Solution. Tumors were minced in a tube with dissociation medium containing DMEM/F12 with 20% fetal bovine serum supplemented with 1 mg/ml collagenase type IV (Sigma, St. Louis, MO; C5138). Minced and suspended tumor cells were filtered through sterile nylon cell strainers with 70-micron pores (BD Falcon, Franklin Lakes, NJ, USA), rinsed with 50 ml of 1× Hank’s Balanced Salt Solution, and centrifuged at 220 g for 5 minutes. Cells were resuspended in RPMI-1640 (Hyclone, South Logan, UT) medium with 10% fetal bovine serum (Hyclone) and 2% penicillin/streptomycin solution (Gibco, Grand Island, NY, USA). Cell viability was determined using the trypan blue dye exclusion method.

Cell Culture
The patient-derived PTC, ATC and resistance to sorafenib ATC cells were isolated and grown in RPMI-1640 medium with 10% fetal bovine serum (cells were authenticated by short tandem repeat profiling, karyotyping, and isoenzyme analysis).

Cell Viability Assay
Cell proliferation was measured using the MTT assay. Cells were seeded in 96-well plates at 6 × 10³ cells per well and incubated overnight to achieve 80% confluency. The indicated drugs were added to achieve final concentrations of 0–100 μM. Cells were incubated for the indicated times prior to the determination of cell viability using the MTT reagent according to the manufacturer’s protocol (Roche, Basel, Switzerland; 11,465,007,001). Absorbance was measured at 550 nm. Viable cells were counted by trypan blue exclusion. Data were expressed as a percentage of the signal observed in vehicle-treated cells and are shown as the means ± SEM of triplicate experiments.

Microarray Experiment and Data Analysis
RNA purity and integrity were evaluated using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA labeling and hybridization were performed by using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). Briefly, 100 ng of total RNA from each sample was linearly amplified and labeled with Cy3-dCTP. The labeled cRNAs were purified using an RNAeasy Mini Kit (Qiagen, Venlo, The Netherlands). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using the NanoDrop ND-1000. Then, 600 ng of each labeled cRNA was fragmented by adding 5 μl 10x blocking agent and 1 μl of 25× fragmentation buffer, and then heated at 60 °C for 30 minutes. Finally, 25 μl of 2× GE hybridization buffer was added to dilute the labeled cRNA. Hybridization solution (40 μl) was dispensed into the gasket slide and assembled to the Agilent SurePrint G3 Human GE 8X60K, V3 Microarrays (Agilent†). Raw data were extracted using Agilent Feature Extraction Software (v11.0.1.1). The raw data for each gene were then summarized automatically in an Agilent Feature Extraction Software (v11.0.1.1). The raw data for each gene were then summarized automatically in an Agilent feature extraction protocol to generate the raw data text file, providing expression data for each gene probed on the array. Gene-enrichment and functional annotation analysis for the significant probe list was performed using gene ontology (www.geneontology.org) and Kyoto Encyclopedia for Genes and Genomes (KEGG) (http://kegg.jp) analyses. All data analysis and visualization of differentially expressed genes were conducted using R 3.1.2 (www.r-project.org).

Immunofluorescence Analysis and Confocal Imaging
The expression of β-catenin was analyzed by immunofluorescence staining. Cells grown on glass-bottomed dishes (MatTek, Ashland,
MA were fixed with 4% formaldehyde solution (R&D Systems, Abingdon, UK) for 10 minutes and permeabilized with 0.5% TritonX-100 in phosphate buffered saline (PBS) for 10 minutes. Slides were air-dried, washed with PBS, and incubated with anti-β-catenin (1:25; Abcam, Cambridge, UK) in 3% bovine serum albumin in PBS. After being washed with PBS, slides were incubated with Alexa 488 (1:200; Molecular Probes, Eugene, OR) Nuclei were stained with Hoechst 33,342 (Life Technologies, Grand Island, NY) for visualization. Images were observed under a confocal microscope (LSM Meta 700; Zeiss, Oberkochen, Germany) and were analyzed using Zeiss LSM Image Browser, version 4.2.0121.

**Immunoblot Analysis**

Cells were washed twice with cold PBS and lysed on ice with protein extraction buffer (Pro-Prep, iNtRON Biotechnology, Seoul, Korea) following the manufacturer’s protocol. Protein concentrations were determined by a BCA assay (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (20 μg) were separated on 8–10% sodium dodecyl sulfate-polyacrylamide gels; the resolved proteins were electro-transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were subsequently blocked with 5% nonfat milk in TBS-T for 1 hour at room temperature and incubated overnight at 4 °C. The membranes were then rinsed 3 times with TBST and probed with the corresponding secondary antibodies (all from Abcam, Cambridge, UK). The membranes were subsequently blocked with 5% nonfat milk in TBS-T for 1 hour at room temperature and incubated with appropriate concentrations of primary antibodies against Ki-67, Vimentin, E-cadherin, Snail, Zeb1 (all from Abcam), Cyclin D1, CDK4, p21, p53, p-ERK 1/2, ERK 1/2, p-NFκB, Bcl-2, Caspase 3, and β-actin (all from Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. The membranes were then rinsed 3–5 times with TBST and probed with the corresponding secondary antibodies conjugated to horse radish peroxidase (Santa Cruz) at room temperature for 1 hour. After rinsing, blots were developed with ECL reagents (Pierce) and exposed using Kodak X-OMAT AR Film (Eastman Kodak, Rochester, NY) for 3–5 minutes.

**Flow Cytometry Analysis of the Cell Cycle**

Cells were treated with sorafenib and lenvatinib alone or in combination in RPMI-1640 medium containing 10% fetal bovine serum for 4 hours, harvested by trypsinization, and fixed with 70% ethanol. Cells were stained for total DNA using PBS containing 40 μg/ml propidium iodide and 100 μg/ml RNase I for 30 minutes at 37 °C. The cell cycle distribution was then analyzed using the FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA). The proportions of cells in the sub-G0/G1, G0/G1, S, and G2/M phases were analyzed using FlowJo v8 for MacOSX (Tree Star, Ashland, OR). This experiment was repeated in triplicate and the results were averaged.

**Human Thyroid Cancer Cell Xenografts**

The patient-derived PTC and ATC cells (3.5 × 10^6 cells/mouse) were cultured in vitro and then injected subcutaneously into the upper left flank region of female BALB/c nude mice. After 11 days, tumor-bearing mice were assigned to groups randomly (n = 10/group) and administered 10 mg/kg lenvatinib (p.o.) and 40 mg/kg sorafenib (p.o.), either alone or in combination with 25 mg/kg HNHA (given i.p.) once every 2 days. Tumor size was measured every three days using calipers. Tumor volume was estimated using the following formula: \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \) (L, longest diameter; S, shortest diameter). Animals were maintained under specific pathogen-free conditions. All experiments were approved by the Animal Experiment Committee of Yonsei University.

**Immunohistochemistry**

All tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin wax following standard protocols. Tissue sections (5 μm) were dewaxed, and antigen retrieval was performed in citrate buffer (pH 6), using an electric pressure cooker set at 120 °C for 5 minutes. Sections were incubated for 5 minutes in 3% hydrogen peroxide to quench endogenous tissue peroxidase. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Immunohistochemistry results were evaluated by ANOVA followed by Bonferroni post-hoc tests. Values are expressed as the means ± SD. P < .05 indicated statistical significance.

**Results**

**FGFR Signaling Pathway and EMT Marker Expression are Higher in PDTC than in DTC**

Given that gene expression is largely dependent on the cancer subtype, we considered that CSCs achieve a stem-like phenotype by transcriptional reprogramming. We performed a gene expression microarray analysis to compare PDTC (GSA1 and GSA2) and DTC (GSP1 and GSM1) patient-derived thyroid cancer cells (Figure 1A). Many genes were significantly differentially expressed between PDTC and DTC cells, suggesting that multiple biological processes were reprogrammed depending on cancer cell differentiation. Genes related to FGFR and EMT were particularly induced in PDTC (Figure 1B). Consequently, we focused on the mechanism by which drug resistance is acquired via the FGFR signaling pathway and EMT. Patient-derived GSA1/2, GSP1, and GSM1 cells were used to analyze the expression of genes related to human diseases (Figure 1C). Expression patterns in patient-derived thyroid cancer cells were not significantly different from those of other thyroid cancers, including endometrial cancer. Together, these data show that genes related to the FGFR signaling pathway and EMT are expressed more highly in PDTC than in DTC.

**Synergistic Effects of HNHA and Lenvatinib on Cancer Cell Proliferation in Patient-Derived Thyroid Cancer Cells**

To investigate the synergistic anticancer effects of sorafenib or lenvatinib with HNHA on patient-derived PTC and ATC, we assayed GSP1, GSA1, and GSA2 (Table 1, Information for PTC and ATC from Gangnam Severance Hospital) cell proliferation in the presence and absence of these compounds by MTT assays (Figure 2A, C, and E). IC_{50} was lowest for the combination of HNHA and lenvatinib among all treatment groups for GSP1, GSA1, and GSA2 (Table 2). Further characterization of the synergistic effect of HNHA and lenvatinib on GSP1, GSA1, and GSA2 cell viability showed that the combination reduced the viability of PTC and ATC cells to a greater extent than the reduction observed for either agent alone or the combination of HNHA and sorafenib. The combination of HNHA and lenvatinib suppressed cell proliferation more effectively than either agent used singly or the combination of HNHA and sorafenib (Figure 2A, C, and E); moreover, this effect was concentration-dependent (Figure 2B, D, and F). Collectively, these results imply that the synergistic effect of HNHA and lenvatinib is more potent that the effects of either agent alone or the combination of HNHA and sorafenib in patient-derived PTC and ATC.
HNHA and Lenvatinib in Combination Induced Apoptosis and Cell Cycle Arrest in Patient-Derived Thyroid Cancer Cells

Combined treatment with HNHA and lenvatinib showed the most significant induction of the sub-G0G1 population, resulting in the induction of cell death in GSP1, GSA1, and GSA2 cells (Figure 2G–I). The synergistic effect of HNHA and lenvatinib most potently induced the sub-G0G1 population, leading to apoptosis, cell cycle arrest, and strong inhibition of GSP1, GSA1, and GSA2 cell viability. Immunoblot analyses of protein levels in GSP1, GSA1, and GSA2 cell lines indicated that the HNHA and lenvatinib combination induced the most marked increases in the levels of p53 and p21, which are well-known arrestors of the cell cycle, and decreases in the levels of cyclin D1 and CDK 4, which are positive regulators of the cell cycle, as compared with responses to either agent alone or the combination of HNHA and sorafenib (Figure 2J–L). Of note, the proliferation marker (Ki-67) and anti-apoptotic (phosphorylated NF-κB, p65, and Bcl-2) markers were most highly suppressed in the HNHA and lenvatinib combination treatment group compared with groups treated with either agent alone or the combination of HNHA and sorafenib (Figure 2J–L). The expression of apoptotic markers (Apaf-1 and cleaved-caspase 3) were most highly induced in the HNHA and lenvatinib combination treatment group compared with groups treated with either agent alone or the combination of HNHA and sorafenib (Figure 2J–L). Together, these data indicate that the HNHA and lenvatinib combination effectively suppresses PDTC as well as DTC.

Drug Resistance of Cancer Stem Cells was Related to EMT Induction Mediated by FGFR Signaling Pathway Activation in GSP1, GSA1, and GSA2

We investigated whether the treatments inhibited EMT activation by the prevention of FGFR signaling transduction. The FGFR signaling...
pathway is an evolutionarily conserved signaling cascade involved in various biological processes, including drug resistance. The combination of HNHA and lenvatinib had the greatest suppressive effects on the FGFR signaling pathway (PKC, MEK, and p-ERK1/2), and led to the inhibition of EMT (vimentin, snail, and zeb1) and dramatic increases in E-cadherin compared to either agent alone or the combination of HNHA and sorafenib in GSP1, GSA1, and GSA2 (Figure 2M–O). With respect to ligand binding, FGFR induced a cascade of downstream signaling pathways including mitogen-activated protein kinase enzymes MEK and ERK (in the MAPK pathway) and PKC, via PLCγ activation (Figure 2M–O).

These results suggest that more advanced cancer cells, CSCs, were more resistant to drugs through EMT induction mediated by FGFR signaling pathway activation. Synergistic effects of the HNHA and lenvatinib combination effectively suppressed EMT induction via the inhibition of the FGFR signaling pathway in GSP1, GSA1, and GSA2.

**Figure 2.** Synergistic anti-cancer effect of HNHA and lenvatinib on patient-derived thyroid cancer cells compared with the effects of each agent alone or HNHA and sorafenib in combination. Cell viability and proliferation assay of HNHA and lenvatinib combined, each agent alone, or HNHA and sorafenib combined in patient-derived thyroid cancer cells (GSP1, (A and B); GSA1, (C and D); GSA2, (E and F)). Points indicate mean % of the value observed in the solvent-treated control. All experiments were repeated at least 3 times. Data represent means ± SD. *P < .05 vs. control, **P < .01 vs. control, ***P < .005 vs. control. Cell cycle arrest induced by the combination of HNHA and lenvatinib (G, H and I). Cells were exposed to the indicated inhibitors, harvested, and stained with propidium iodide before analysis by flow cytometry and FlowJo v8. Immunoblot analysis about markers of cell cycle arrest, apoptosis, E-cadherin (negatively related to EMT), proliferation, cell cycle, anti-apoptosis activity, FGFR signaling pathway and EMT in patient-derived thyroid cancer cells (GSP1, (J and M); GSA1, (K and N); GSA2, (L and O)). GSP1, GSA1 and GSA2 were exposed to the indicated inhibitors for 24 hours prior to the analysis of the expression of Ki-67 (cell proliferation), Cyclin D1 and CDK4 (cell cycle), p21 and p53 (cell cycle arrest), Apaf-1 and cleaved-caspase 3 (apoptosis), p-NFκB and Bcl-2 (anti-apoptosis), PKC, MEK and phosphorylated ERK1/2 (FGFR signaling pathway), Vimentin, Snail, and Zeb1 (EMT), E-cadherin (loss of E-cadherin function promotes EMT) by immunoblotting.
### G) GSP1

| Status              | Sub-G1 | G1,G0 | S     | G2/M   |
|---------------------|--------|-------|-------|--------|
| Control             | 2.4 ± 0.01 | 35.9 ± 0.03 | 34.8 ± 0.02 | 26.9 ± 0.02 |
| Sorafenib only      | 18.4 ± 0.03 | 43.4 ± 0.04 | 22.6 ± 0.05 | 15.6 ± 0.02 |
| Lenvatinib only     | 25.2 ± 0.02 | 45.5 ± 0.01 | 19.4 ± 0.02 | 9.9 ± 0.05  |
| HNHA                | 33.4 ± 0.04 | 48.7 ± 0.01 | 11.4 ± 0.05 | 6.5 ± 0.05  |
| HNHA+Sorafenib      | 61.4 ± 0.01 | 27.8 ± 0.06 | 6.8 ± 0.03  | 4.0 ± 0.03  |
| HNHA+Lenvatinib     | 69.4 ± 0.02 | 23.7 ± 0.01 | 4.6 ± 0.01  | 2.3 ± 0.04  |

### H) GSA1

| Status              | Sub-G1 | G1,G0 | S     | G2/M   |
|---------------------|--------|-------|-------|--------|
| Control             | 1.3 ± 0.02 | 45.5 ± 0.01 | 30.2 ± 0.03 | 23.0 ± 0.03 |
| Sorafenib only      | 14.2 ± 0.01 | 46.3 ± 0.02 | 20.0 ± 0.02 | 19.5 ± 0.03 |
| Lenvatinib only     | 19.5 ± 0.04 | 49.5 ± 0.02 | 15.8 ± 0.05 | 15.2 ± 0.06 |
| HNHA                | 26.8 ± 0.05 | 46.7 ± 0.02 | 15.9 ± 0.05 | 10.6 ± 0.01 |
| HNHA+Sorafenib      | 51.8 ± 0.05 | 31.5 ± 0.04 | 11.5 ± 0.02 | 5.2 ± 0.06  |
| HNHA+Lenvatinib     | 67.2 ± 0.05 | 21.3 ± 0.05 | 8.2 ± 0.04  | 3.3 ± 0.02  |

### I) GSA2

| Status              | Sub-G1 | G1,G0 | S     | G2/M   |
|---------------------|--------|-------|-------|--------|
| Control             | 0.7 ± 0.05 | 41.2 ± 0.02 | 41.9 ± 0.02 | 16.2 ± 0.04 |
| Sorafenib only      | 4.5 ± 0.02 | 40.8 ± 0.01 | 39.7 ± 0.03 | 15.0 ± 0.01 |
| Lenvatinib only     | 15.9 ± 0.02 | 48.4 ± 0.05 | 26.2 ± 0.03 | 9.5 ± 0.01  |
| HNHA                | 23.4 ± 0.01 | 51.3 ± 0.03 | 20.5 ± 0.01 | 4.8 ± 0.04  |
| HNHA+Sorafenib      | 45.7 ± 0.02 | 35.4 ± 0.01 | 14.8 ± 0.05 | 4.1 ± 0.02  |
| HNHA+Lenvatinib     | 59.3 ± 0.02 | 30.7 ± 0.03 | 7.9 ± 0.02  | 2.1 ± 0.01  |

Figure 2. (continued).
Figure 3. Lenvatinib prevents EMT through repression of β-catenin nuclear translocation in GSA1 and GSA2 cancer stem-like cells. Immunofluorescence cytochemical staining showed that β-catenin nuclear localization was greater in GSA1 (B) and GSA2 (C) cancer stem-like cells, than in GSP1 (A). However, these cells were sensitive to EMT inhibition via β-catenin nuclear localization by lenvatinib.
β-Catenin, an EMT Marker, Plays a Key Role in EMT Induction by Nuclear Localization on Advanced Thyroid Cancer Cells

β-Catenin constitutes a well-known, conserved regulatory factor with a crucial role in a tumorigenesis that is correlated with a poor prognosis. β-Catenin nuclear localization acts synergistically to promote the expression of target genes related to drug resistance. PDTC CSCs acquired drug resistance via EMT activation mediated by β-catenin nuclear localization. In GSA1 and 2, patient-derived anaplastic thyroid cancer 1 and 2, β-catenin nuclear localization was greater than that in patient-derived papillary thyroid cancer 1, GSP1 (Figure 3A–C, each control panel). β-Catenin nuclear localization in GSA1 and 2 was significantly inhibited by the FGFR inhibitor lenvatinib as opposed to non-FGFR inhibitors, HNHA or sorafenib (Figure 3A–C). These results indicate that the disruption of EMT activation via FGFR signaling pathway inhibition contributed to the efficacy against drug resistance by lenvatinib.

Significant Tumor Shrinkage was Induced by the Combination of HNHA and Lenvatinib in a Xenograft Model

To investigate the synergistic anti-cancer effect of the combination of HNHA and lenvatinib in vivo, we developed a mouse xenograft tumor model using patient-derived papillary thyroid cancer cells, GSP1, GSA1, and GSA2. Each agent used alone and the combination of HNHA and sorafenib did not significantly suppress GSP1, GSA1, and GSA2 cell xenograft tumors; however, the combination of HNHA and lenvatinib resulted in tumor suppression (Figure 4A, D, and G). Moreover, there was no evidence for systemic toxicity or treatment-related death in any group. Mouse body weight was not significantly influenced by treatment with sorafenib, lenvatinib, or HNHA (Figure 4B, E, and H). The HNHA and lenvatinib combination treatment group had significantly smaller tumor volumes than those of mice treated with each agent used alone or the combination of HNHA and sorafenib (Figure 4C, F, and I). As anti-apoptotic activity is a fundamental factor in the evaluation of tumorigenesis and Bcl-2 serves as an important marker of anti-apoptotic activity, we identified this marker by immunohistochemistry analysis of GSP1, GSA1, and GSA2 cell xenograft tumors. This demonstrated that the HNHA and lenvatinib combination treatment group showed the strongest decrease in Bcl-2 expression among all groups (Figure 4J–L). Accordingly, the HNHA and lenvatinib combination treatment was considered to have potent anti-cancer effects in CSCs of PDTC as well as in the DTC xenograft model.

Discussion

EMT is linked to cancer growth, including metastasis, therapeutic resistance, and recurrence. CSCs, which are found in poorly differentiated tumors and have stem cell-like features [21], have the capacity for self-renewal and are associated with metastases and therapeutic resistance [22]. Recent studies have demonstrated a link between EMT, drug resistance, and CSCs [23]. In particular, CSCs and EMT-type cells, which share some molecular characteristics with CSCs, are believed to perform crucial roles in drug resistance and cancer metastasis, as observed in some malignant cancers in humans [23]. EMT is associated with the gain of stem cell-like properties and
is sufficient to provide differentiated normal and cancer cells with stem cell properties. Furthermore, CSCs frequently also exhibit EMT properties. Numerous studies have examined the relationship between EMT and drug resistance in CSCs. In the present study, we investigated EMT-mediated drug resistance via the FGFR signaling pathway in patient-derived anaplastic thyroid cancer cells, a type of cancer stem-like cells. These cells exhibited high expression of markers of the EMT and FGFR signaling pathway. Some studies have shown that FGFR2 and β3 integrin are part of an EMT signature that contributes to FGFR1-mediated drug resistance and metastatic progression [15]. Drug resistance of CSCs was dependent on the EMT-associated FGFR signaling pathway. Consequently, we focused on the effect of inhibition of the FGFR signaling pathway by tyrosine kinase inhibitors (TKIs) on CSCs.

TKIs are suggested in patients with radiiodine-refractory DTC with metastatic, rapidly progressive, symptomatic, and/or imminently threatening disease that is not otherwise amenable to local control with other approaches. Systemic therapeutics resulted in improved progression-free survival in three randomized, double-blinded, placebo-controlled clinical trials, i.e., vandetanib, sorafenib, and lenvatinib [24–26]. Sorafenib inhibits RAF-1, a member of the RAF/MEK/ERK signaling pathway, BRAF activity, and VEGFR-2, VEGFR-3, PDGFR-β, and c-KIT [27]. Lenvatinib has a potent inhibitory effect on VEGFR-2, VEGFR-3, PDGFRα/β, KIT, and RET, as well as, unlike sorafenib, FGFR 1–4. The most important difference between lenvatinib and other drugs is the ability to inhibit FGFR 1, establishing it as an effective drug in cases in which resistance to VEGFR inhibitors develops [28–30]. Although both lenvatinib and sorafenib show good results in phase III trials and are the first-line treatment in radiiodine-refractory DTCs, most patients eventually stop responding to these agents, and many are not able to continue medication owing to their toxicity. In patients who exhibit disease progression during initial kinase inhibitor therapy, without prohibitive adverse effects, only lenvatinib may be used as a second-line treatment [24]. Several mechanisms explain TKI resistance, such as receptor autophosphorylation, autophagy, hypoxia-inducing factor, epigenetic regulation, and EMT [31,32]. There are several EMT-inducing cytokines, e.g., TGF-β, FGF, HGF, insulin-like growth factor, and IL-6 [33,34]. EMT in thyroid cancer is induced in more aggressive forms, with increased expression of ZEB1, which can promote drug resistance via EMT-dependent and EMT-independent mechanisms [35–37]. Studies have shown that the down-regulation of ZEB1 expression could restore drug sensitivity [38,39]. For example, sorafenib inhibits EMT in hepatocellular carcinoma, attenuates HGF secretion in polarized macrophages, and decreases plasma HGF. Sorafenib also abolishes polarized-macrophage-induced activation of the HGF receptor Met [40]. Notably, reversion of EMT overcomes drug resistance in lung adenocarcinoma [33].

The present study demonstrated that the drug resistance of CSC-like cancer cells, i.e., sorafenib-resistant-patient derived thyroid cancer cells, was inhibited by combination therapy with HNHA and lenvatinib through the inhibition of the EMT-mediated FGFR signaling pathway. Synergistic effects of HNHA and lenvatinib were more efficient than the effects of either agent used singly or the combination of HNHA and sorafenib. The combined treatment effectively induced markers of cell cycle arrest and apoptosis and reduced anti-apoptosis markers, EMT, and the FGFR signaling pathway. In addition to cell culture studies, in vivo studies using a xenograft model indicated significant tumor shrinkage in the HNHA and lenvatinib combined treatment group. We propose that these effects might be explained by reduced EMT-mediated drug resistance in CSCs. Specifically, the combination of HNHA and lenvatinib blocked the FGFR signaling pathway, which is important for EMT and metastasis [41]. Notably, these findings indicate that the combination of HNHA and lenvatinib is a potentially effective new clinical approach for the care of patients with CSCs with drug-resistant properties.

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References
[1] Nikiforov YE (2008). Thyroid carcinoma: molecular pathways and therapeutic targets. Mod Pathol 21(Suppl 2), S37–43.
[2] Ferrari SM, Fallahi P, Politi U, Materazzi G, Baldini E, Ullise S, Miccoli P, and Antonelli A (2015). Molecular Targeted Therapies of Aggressive Thyroid Cancer. Front Endocrinol (Lausanne) 6, 176.
[3] Saias et al., Floor S, Tarabichi M, Dom G, Hebrant A, van Staveren WC, and Maenhaut C (2012). Thyroid cancer cell lines: an overview. Front Endocrinol (Lausanne) 3, 133.
[4] Xing M (2013). Molecular pathogenesis and mechanisms of thyroid cancer. Nat Rev Cancer 13, 184–199.
[5] Xing M (2009). Identifying genetic alterations in poorly differentiated thyroid cancer: a rewarding pursuit. J Clin Endocrinol Metab 94, 4661–4664.
[6] Cherkanaoi GS, Guerra S, Taleb S, Idris MA, Touil N, Bensoussan A, Baroudi Z, and Chikhaoui N (2015). Poorly differentiated thyroid carcinoma: a retrospective clinicopathological study. Pan Afr Med J 21, 137.
[7] Abe I, Karasaki S, Matsuda Y, Sakamoto S, Nakashima T, Yamamoto H, Kawate H, Ohnaka K, Nakashima H, and Kobayashi K, et al (2015). Complete remission of anaplastic thyroid carcinoma after concomitant treatment with docetaxel and radiotherapy. Case Rep Endocrinol 2015, 726085.
[8] Gryglewicz P, Dymek B, Bukaj A, Gunerpa P, Stanczak A, Lamparska-Przybysz M, Wieczorek M, Dzwonek K, and Zdralik D (2016). Epithelial-mesenchymal transition confers resistance to selective FGFR inhibitors in SNU-16 gastric cancer cells. Gastric Cancer 19, 53–62.
[9] Housman G, Byler S, Heereboth S, Lapinska K, Longacre M, Snyder N, and Sarkar S (2014). Drug resistance in cancer: an overview. Cancers (Basel) 6, 1769–1792.
[10] Wendt MK, Tian M, and Schiemann WP (2012). Deconstructing the mechanisms and consequences of TGF-beta-induced EMT during cancer progression. Cell Tissue Res 347, 85–101.
[11] Stuhlmiller TJ, Miller SM, Zawistowski JS, Nakamura K, Beltran AS, Duncan JS, Angus SP, Collins KA, Granger DA, and Reuther RA, et al (2015). Inhibition of Laptapitin-Induced Kinome Reprogramming in ERBB2-Positive Breast Cancer by Targeting BET Family Bromodomains. Cell Rep 11, 390–404.
[12] Singh A and Settleman J (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 29, 4741–4751.
[13] Wendt MK, Taylor MA, Schiemann BJ, Sossey-Alaoui K, and Schiemann WP (2014). Fibroblast growth factor receptor splice variants are stable markers of oncogenic transforming growth factor beta signaling in metastatic breast cancers. Breast Cancer Res 16, R24.
[14] Warzecha CC, Jiang P, Amirkian K, Dittmar KA, Lu H, Shen S, Guo W, Xing Y, and Castrens RP (2010). An ESRR-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. EMBO J 29, 3286–3300.
[15] Brown WS, Tan L, Smith A, Gray NS, and Wendt MK (2016). Covalent Targeting of Fibroblast Growth Factor Receptor Inhibits Metastatic Breast Cancer. Mol Cancer Ther 15, 2096–2106.
[16] Brown WS, Akhand SS, and Wendt MK (2016). FGFR signaling maintains a drug persistent cell population following epithelial-mesenchymal transition. Oncotarget 7, 83424–83436.
[17] Shibue T and Weinberg RA (2017). EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol 10, 611–629.
Bernet V and Smallridge R (2014). New therapeutic options for advanced forms of thyroid cancer. *Expert Opin Emerg Drugs* **19**, 225–241.

Ma R, Minsky N, Morshed SA, and Davies TF (2014). Stemness in human cancer and derived cell lines: the role of asymmetrically dividing cancer stem cells resistant to chemotherapy. *J Clin Endocrinol Metab* **99**, E400-09.

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, and Shipston M, et al (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704–715.

Liu X and Fan D (2015). The epithelial-mesenchymal transition and cancer stem cells: functional and mechanistic links. *Curr Pharm Des* **21**, 1279–1291.

Borah A, Raveendran S, Rochani A, Maekawa T, and Kumar DS (2015). Targeting self-renewal pathways in cancer stem cells: clinical implications for cancer therapy. *Oncogene* e177.

Sarkar FH, Li Y, Savard A, Coudert L, Khandjian EW, and Marrouti R (2015). Sorafenib, a multikinase inhibitor, induces formation of stress granules in hepatocarcinoma cells. *Oncotarget* **6**, 43927–43943.

Kurimoto R, Iwasa T, Ishiwata T, Sekine I, Tada Y, Tatsumi K, Koide S, Iwama A, and Takiguchi Y (2016). Drug resistance originating from a TGF-beta/FGF-2-driven epithelial-to-mesenchymal transition and its reversion in human lung adenocarcinoma cell lines harboring an EGFR mutation. *Int J Oncol* **48**, 1825–1836.

Qi L, Song W, Li L, Cao L, Yu Y, Song C, Wang Y, Zhang F, Li Y, and Zhang B, et al (2016). FG4 induces epithelial-mesenchymal transition by inducing store-operated calcium entry in lung adenocarcinoma. *Oncotarget* **7**, 74015–74030.

Montemayor-Garcia C, Hardin H, Guo Z, Larrain C, Buchler D, Aoioli S, Chen H, and Lloyd RV (2015). The role of epithelial mesenchymal transition markers in thyroid carcinoma progression. *Endocr Pathol* **24**, 206–212.

Lan L, Luo Y, Cui D, Shi BY, Deng W, Huo LL, Chen HL, Zhang GY, and Deng LL (2013). Epithelial-mesenchymal transition triggers cancer stem cell generation in human thyroid cancer cells. *Int J Oncol* **43**, 113–120.

Zhang P, Sun Y, and Ma L (2015). ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell Cycle* **14**, 481–487.

Meidhof S, Brabletz S, Lehmann W, Preca BT, Mock K, Ruh M, Scherler J, Berthold M, Weber A, and Burk U, et al (2015). ZEB1-associated drug resistance in cancer cells is reversed by the class I HDAC inhibitor mocetinostat. *EMBO Mol Med* **7**, 831–847.

Zhou G, Zhang F, Guo Y, Huang J, Xie Y, Yue S, Chen M, Jiang H, and Li M (2017). miR-200c enhances sensitivity of drug-resistant non-small cell lung cancer to gefitinib through suppression of PI3K/Akt signaling pathway and inhibits cell migration via targeting ZEB1. *Biomed Pharmacother* **85**, 113–119.

Deng YR, Liu WB, Lian ZX, Li X, and Hou X (2016). Sorafenib inhibits macrophage-mediated epithelial-mesenchymal transition in hepatocellular carcinoma. *Oncotarget* **7**, 38292–38305.

Ryu SH, Heo SH, Park EY, Choi KC, Ryu JW, Lee SH, and Lee SW (2017). Selumetinib Inhibits Melanoma Metastasis to Mouse Liver via Suppression of EMT-targeted Genes. *Anticancer Res* **37**, 607–614.