NEOPLASIA
www.neoplasia.com

Volume 20 Number xx Month 2018 pp. 197–206 197

Anti-cancer Effects of HNHA and Lenvatinib by the Suppression of EMT-Mediated Drug Resistance in Cancer Stem Cells[1,2]

Yong Sang Lee[*,†,3], Seok-Mo Kim[*,†,3], Bup-Woo Kim[*,†], Ho Jin Chang[*,†], Soo Young Kim[*,†], Cheong Soo Park[*,†], Ki Cheong Park[*,‡,3] and Hang-Seok Chang[*,†,3]

[*]Thyroid Cancer Center, Gangnam Severance Hospital, Department of Surgery, Yonsei University College of Medicine, Seoul 120-752, Korea; [†]Gangnam Severance Hospital, Department of Surgery Yonsei University College of Medicine 211 Eonjuro, Gangnam-gu, Seoul 135-720, Korea; [‡]Department of Surgery, Yonsei University College of Medicine, 50-1, Yonsei-ro, Seodaemun-gu, Seoul, 120-752 Korea

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.

Neoplasia (2018) 20, 197–206

Abbreviations: ATC, anaplastic thyroid cancer; EMT, epithelial-mesenchymal transition; PTC, papillary thyroid cancer; TKI, tyrosine kinase inhibitors.

Address all correspondence to: Hang-Seok Chang, M.D., Ph.D., Gangnam Severance Hospital, Department of Surgery, Yonsei University College of Medicine, 211 Eonjuro, Gangnam-gu, Seoul 135-720, Korea. E-mail: SURGHSC@yuhs.ac or Ki Cheong Park., Ph. D., Department of Surgery, Yonsei University College of Medicine, 50-1, Yonsei-ro, Seodaemun-gu, Seoul 120-752, Republic of Korea. Email: ggiru95@yuhs.ac

[1] Funding: This research was supported by he "SEBANG" Faculty Research Assistance Program of Yonsei University College of Medicine 6-2013-0160 and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B03029716).

[2] Conflicts of interest: The authors declare that they have no competing interests.

[3] These authors contributed equally to this work.

Received 16 September 2017; Revised 12 December 2017; Accepted 14 December 2017

1476-5586
https://doi.org/10.1016/j.neo.2017.12.003

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 6×10^3 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 10× 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 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,