Draw the anti-cancer effect *via* gene expression profiles between well-differentiated and undifferentiated thyroid cancer cell derived from patient

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Directed by Professor Hang-Seok Chang

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Yong Sang Lee

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ABSTRACT

Draw the anti-cancer effect *via* gene expression profiles between well-differentiated and undifferentiated thyroid cancer cell derived from patient

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Thyroid gland presents a broad scope of tumors derived from follicular cells that range from well-differentiated cancer including papillary and follicular cancer (PTC and FTC), generally carrying a favorable prognosis, to the clinically aggressive, undifferentiated thyroid cancer (UTC). It is mostly recognized that UTC arise progress from a pre-existing well-differentiated cancer *via* a various procedure of genetic and epigenetic alterations that bring about clonal expansion and neoplastic progression. Mutations and epigenetic changes in UTC are not so clear. This presumes, of course, that UTC may derive from well-differentiated thyroid cancer (WDTC), it is look forward to some UTC would shelter genetic changes that are usual of PTC and FTC**.** It is the instance for several markers (BRAF, NRAS) that are existent in WDTC and UTC. The p53 genes is usually identified in less- and undifferentiated thyroid cancer, confirming a diagnosis of UTC. Especially, UTC are unusual but extremely aggressive malignancies with a greatly short survival that was acquired to multiple anticancer drug-resistant.

This study suggested that therapeutic approaches to undifferentiated thyroid cancer (UTC) *via* the gene expression profile.

Key words: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), well-differentiated thyroid cancer (WDTC), undifferentiated thyroid cancer (UTC), gene expression profile, drug resistance

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I. INTRODUCTION

1. Thyroid cancer

Thyroid cancer is a cancer originating from follicular or parafollicular thyroid cells. These cells give rise to both well-differentiated cancers – papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) – and anaplastic thyroid cancer (ATC), whose anaplastic cells are poorly differentiated. The second cell type, the C or parafollicular cell, produces the hormone calcitonin and is the cell of origin for medullary thyroid cancer (MTC)[1]. The most effective management of aggressive thyroid cancers is surgical removal of thyroid gland (thyroidectomy) followed by radioactive iodine ablation and TSH-suppression therapy. Chemotherapy or radiotherapy may also be used in cases of distant metastases or advanced cancer stage. Five-year survival rates are 98.1% in the United States [NIH, Cancer Statistical Summaries].

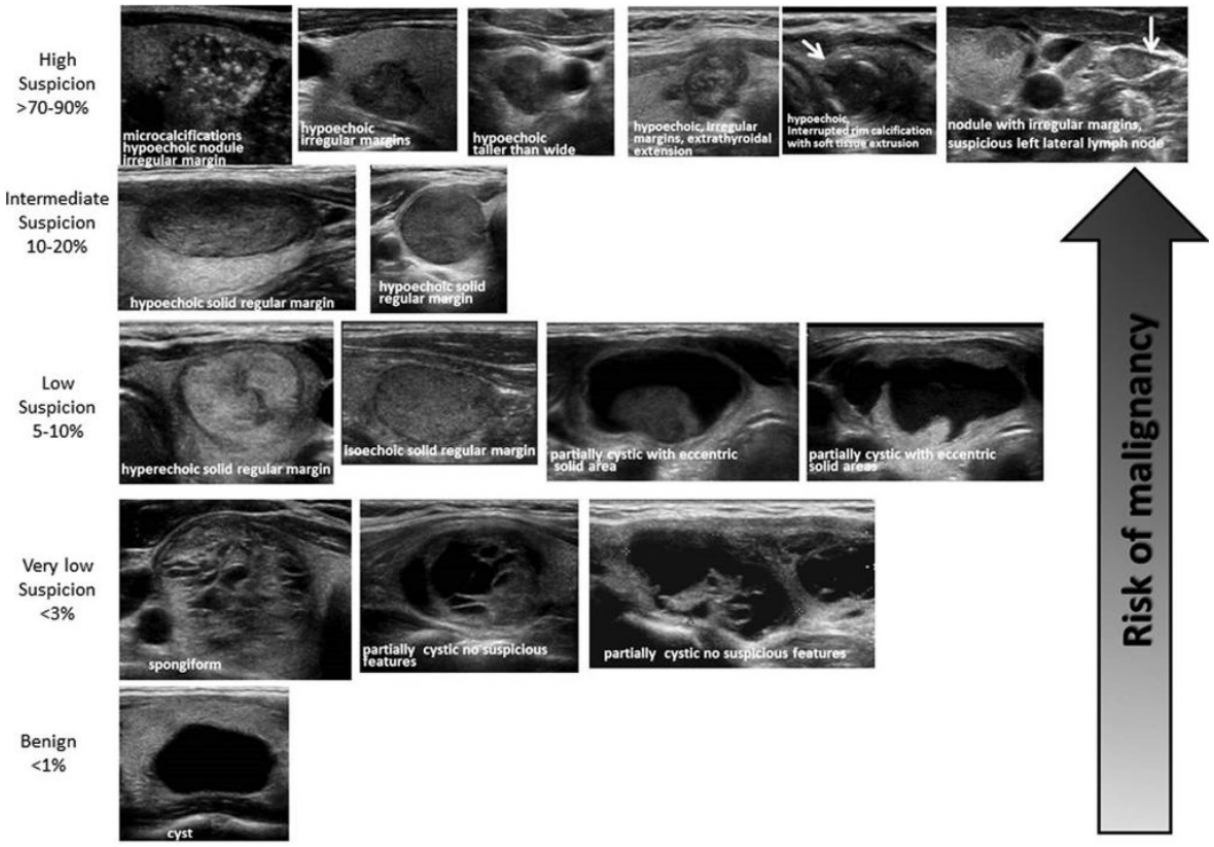

Figure 1. ATA nodule sonographic patterns and risk of malignancy (*Thyroid. 2016 Jan 1; 26(1): 1–133*).

2. Signs and symptoms

Most often the first symptom of thyroid cancer is a nodule in the thyroid region of the neck[2]. However, many adults have small nodules in their thyroids, but typically under 5% of these nodules are found to be cancerous[3,4]. Sometimes the first sign is an enlarged lymph node. Later symptoms that can be present are pain in the anterior region of the neck and changes in voice due to an involvement of the recurrent laryngeal nerve[5]. Thyroid cancer is usually found in a euthyroid patient, but symptoms of hyperthyroidism or hypothyroidism may be associated with a large or metastatic well-differentiated tumor. Thyroid nodules are of particular concern when they are found in those under the age of 20.

The presentation of benign nodules at this age is less likely, and thus the potential for malignancy is far greater[3].

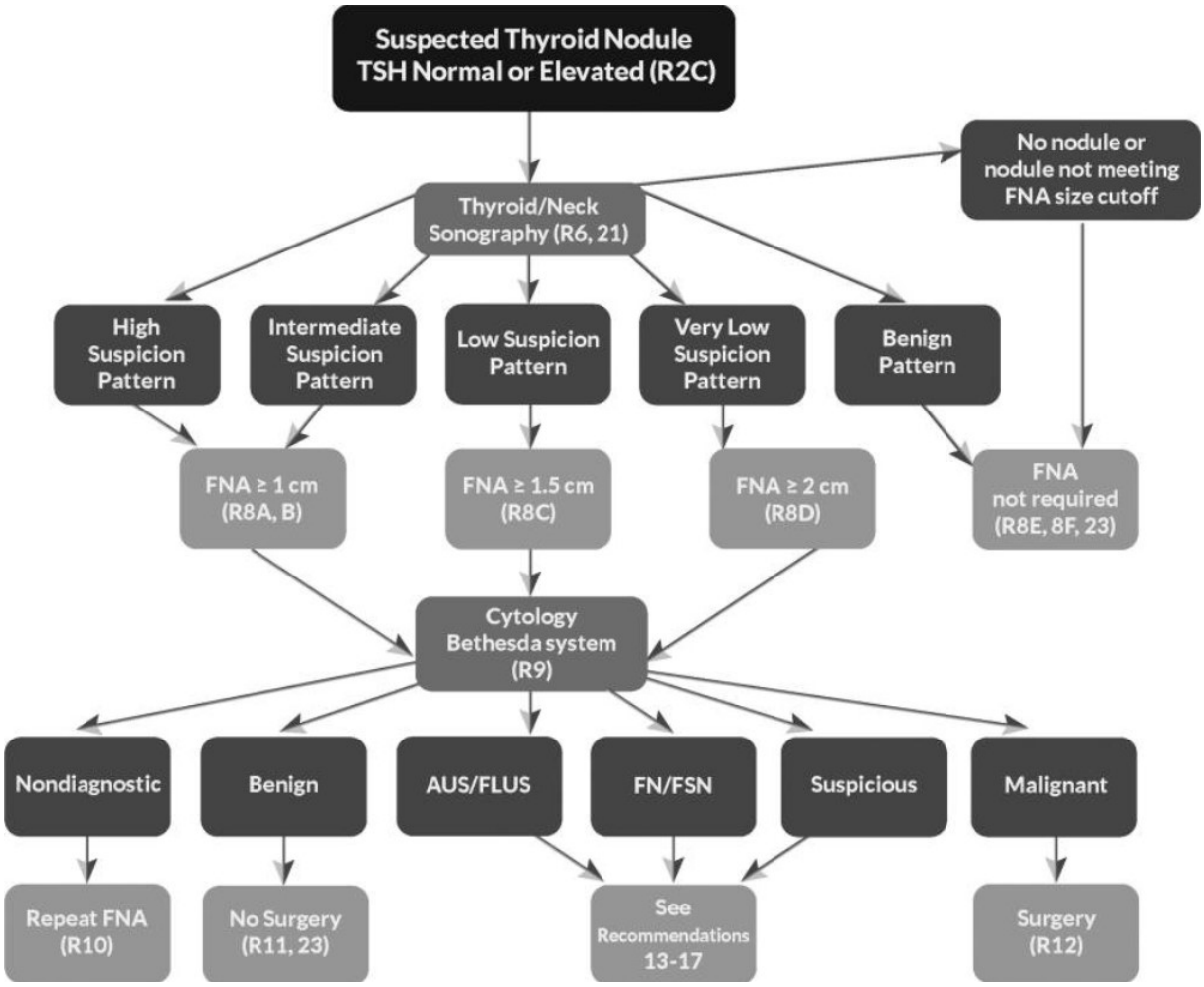

Figure 2. Algorithm for evaluation and management of patients with thyroid nodules based on US pattern and FNA cytology (*Thyroid. 2016 Jan 1; 26(1): 1–133*).

3. Causes

Thyroid cancers are thought to be related to a number of environmental and genetic predisposing factors, but significant uncertainty remains regarding their causes. Environmental

exposure to ionizing radiation from both natural background sources and artificial sources is suspected to play a significant role, and there are significant increased rates of thyroid cancer in those exposed to mantlefield radiation for lymphoma, and those exposed to iodine-131 following the Chernobyl[6], Fukushima, Kyshtym, and Wind scale[7] nuclear disasters. Thyroiditis and other thyroid diseases also predispose to thyroid cancer. Genetic causes include multiple endocrine neoplasia type 2 which markedly increases rates, particularly of the rarer medullary form of the disease[8].

4. Diagnosis

After a thyroid nodule is found during a physical examination, a referral to an endocrinologist or a thyroidologist may occur. Most commonly an ultrasound is performed to confirm the presence of a nodule and assess the status of the whole gland. Measurement of thyroid stimulating hormone and anti-thyroid antibodies will help decide if there is a functional thyroid disease such as Hashimoto's thyroiditis present, a known cause of a benign nodular goiter[9]. Measurement of calcitonin is necessary to exclude the presence of medullary thyroid cancer. Finally, to achieve a definitive diagnosis before deciding on treatment, a fine needle aspiration cytology test is usually performed and reported according to the Bethesda system. In adults without symptoms screening for thyroid cancer is not recommended[10].

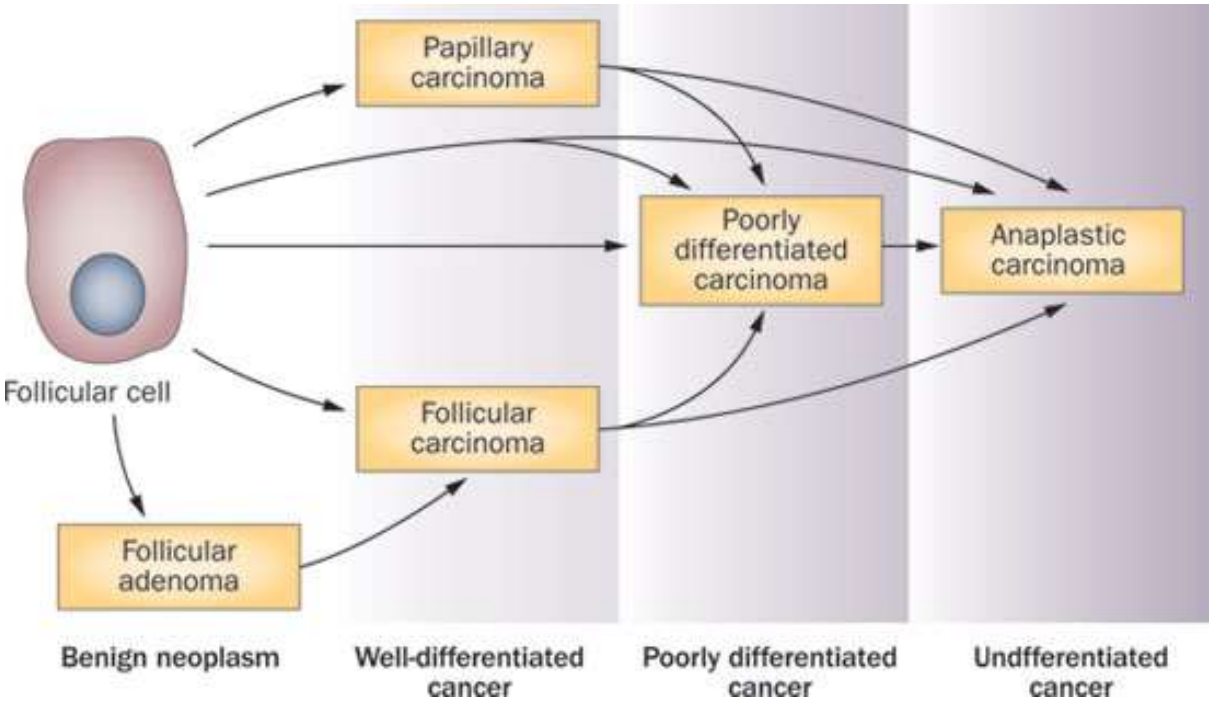

Figure 3. Scheme of step-wise dedifferentiation of follicular cell-derived thyroid cancer (*Nikiforov, Y. E. & Nikiforova, M. N. (2011) Molecular genetics and diagnosis of thyroid cancer Nat. Rev. Endocrinol*).

4.1 Classification

Thyroid cancers can be classified according to their histopathological characteristics[11].

4.1.1 Papillary thyroid cancer (75% to 85% of cases) – often in young females – excellent prognosis. May occur in women with familial adenomatous polyposis and in patients with Cowden syndrome[12].

4.1.2 Newly reclassified variant: noninvasive follicular thyroid neoplasm with papillary-like nuclear features is considered an indolent tumor of limited biologic potential.

4.1.3 Follicular thyroid cancer (10% to 20% of cases) – occasionally seen in patients with Cowden syndrome[13].

4.1.4 Medullary thyroid cancer (5% to 8% of cases) – cancer of the parafollicular cells, often part of multiple endocrine neoplasia type 2[14].

4.1.5 Poorly differentiated thyroid cancer is aggressive pattern of thyroid cancer.

4.1.6 Anaplastic thyroid cancer (less than 5% of cases) is not responsive to treatment and can cause pressure symptoms[15]. The follicular and papillary types together can be classified as "differentiated thyroid cancer"[16]. These types have a more favorable prognosis than the medullary and undifferentiated types[17]. Papillary microcarcinoma is a subset of papillary thyroid cancer defined as measuring less than or equal to 1 cm[18]. The highest incidence of papillary thyroid microcarcinoma in autopsy series was reported by Harach et al. in 1985, who found 36 of 101 consecutive autopsies were found to have an incidental microcarcinoma[19]. Michael Pakdaman et al. report the highest incidence in a retrospective surgical series at 49.9% of 860 cases[20]. Management strategies for incidental papillary microcarcinoma on ultrasound (and confirmed on FNAB) range from total thyroidectomy with radioactive iodine ablation to observation alone. Harach et al. suggest using the term "occult papillary tumor" to avoid giving patients distress over having cancer[19]. It was Woolner et al. who first arbitrarily coined the term "occult papillary carcinoma" in 1960, to describe papillary carcinomas ≤ 1.5 cm in diameter[21].

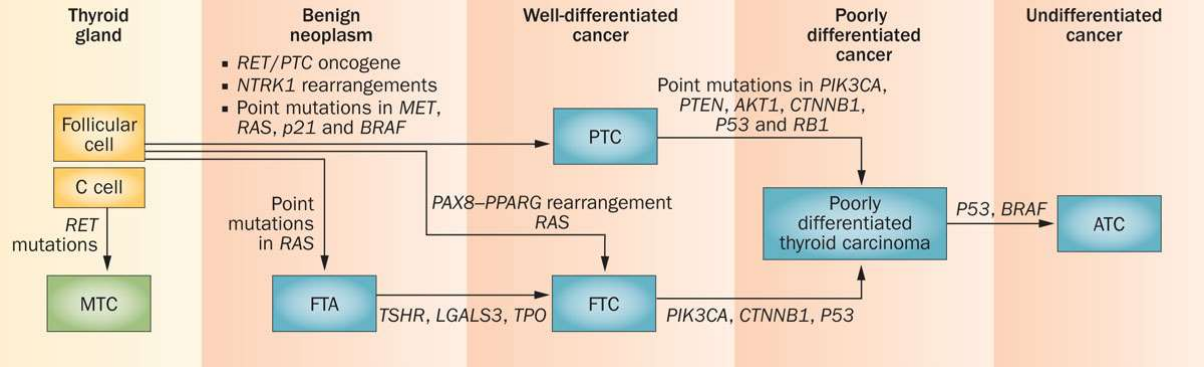

Figure 4. Classification of human thyroid carcinomas and subtype-specific genetic alterations (*Pallante, P. et al. (2013) Deregulation of microRNA expression in thyroid neoplasias Nat. Rev. Endocrinol*).

5. Treatment

Thyroidectomy and dissection of central neck compartment is initial step in treatment of thyroid cancer in majority of cases. Thyroid-preserving operation may be applied in cases, when thyroid cancer exhibits low biological aggressiveness (e.g. well-differentiated cancer, no evidence of lymph node metastases, low MIB-1 index, no major genetic alterations like BRAF mutations, RET/PTC rearrangements, p53 mutations etc.) in patients younger than 45 years[22]. If the diagnosis of well-differentiated thyroid cancer (e.g. papillary thyroid cancer) is established or suspected by FNA the surgery is indicated, whereas watchful waiting strategy is not recommended in any evidence-based guidelines[22]. Watchful waiting reduces overdiagnosis and overtreatment of thyroid cancer among old patients. Radioactive Iodine-131 is used in patients with papillary or follicular thyroid cancer for ablation of residual thyroid tissue after surgery and for the treatment of thyroid cancer[23]. Patients with medullary, anaplastic, and most Hurthle cell cancers do not benefit from this therapy. External irradiation may be used when the cancer is unresectable, when it recurs after resection, or to relieve pain from bone metastasis. Sorafenib and sunitinib, approved for other indications show promise for thyroid cancer and are being used for some patients who do not qualify for clinical trials[24]. Numerous agents are in phase II clinical trials and XL184 has started a phase III trial[24].

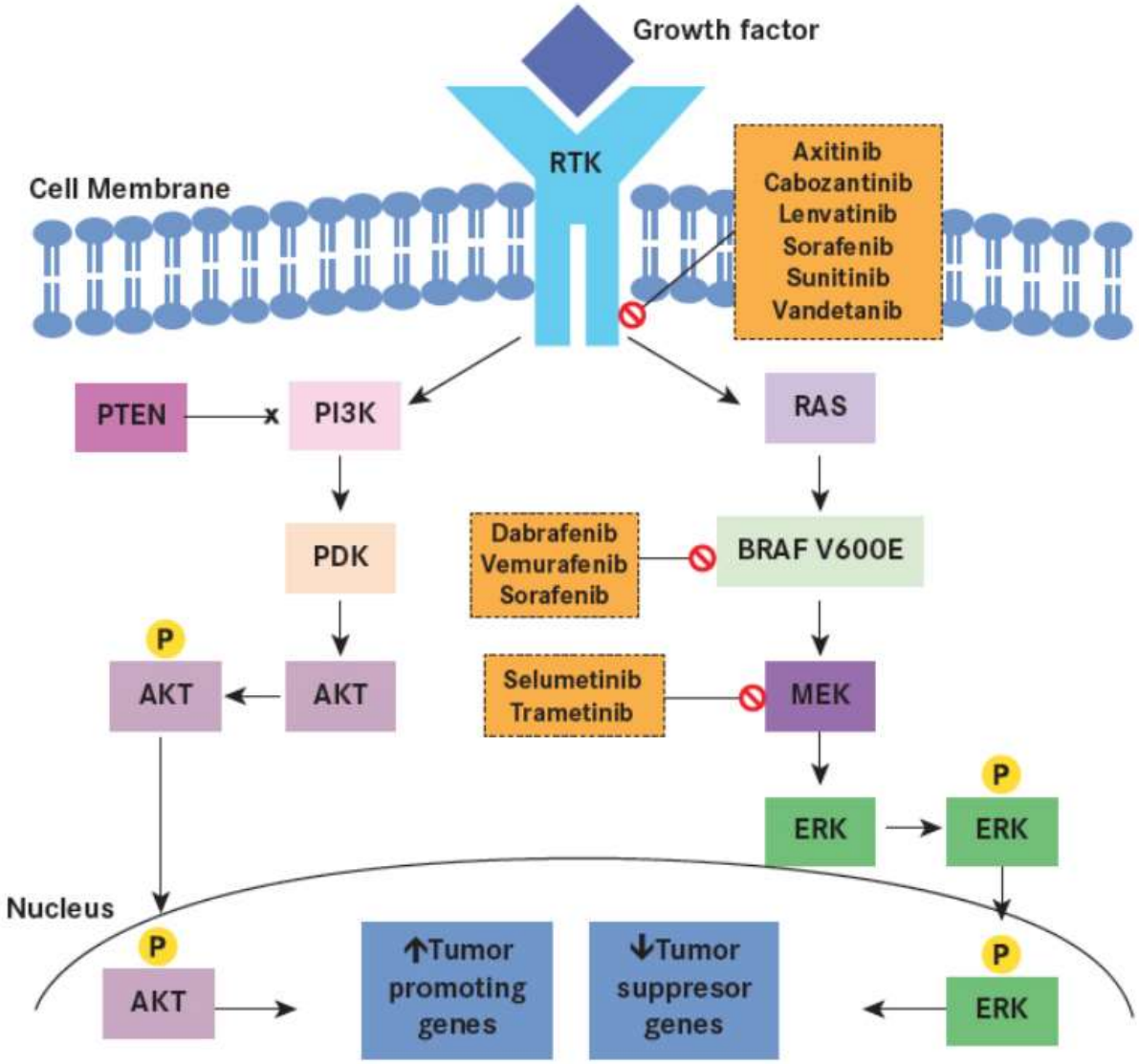

Figure 5. The PI3K-AKT pathway also plays a significant role in sporadic thyroid tumorigenesis. As with the MAPK pathway, an extracellular stimulus activates RTK at the cell membrane, and subsequently PI3K, ultimately leading to phosphorylation and activation of AKT.13 Activated AKT then enters the nucleus to upregulate tumor-promoting genes. Within the cytoplasm, activated AKT also activates other signaling molecules, including the mTOR pathway and phosphorylation of glycogen synthase kinase 3β. Common genetic alterations implicated in induction of the PI3K-AKT pathway include RAS and PTEN mutation or deletion, PI3KCA mutation or amplification, AKT1 mutation, and amplifications of the RTK genes (*Thyroid Cancer: Molecular Pathogenesis, Tyrosine Kinase Inhibitors,*

and Other New Therapies).

6. Prognosis

The prognosis of thyroid cancer is related to the type of cancer and the stage at the time of diagnosis. For the most common form of thyroid cancer, papillary, the overall prognosis is excellent. Indeed, the increased incidence of papillary thyroid carcinoma in recent years is likely related to increased and earlier diagnosis. One can look at the trend to earlier diagnosis in two ways. The first is that many of these cancers are small and not likely to develop into aggressive malignancies. A second perspective is that earlier diagnosis removes these cancers at a time when they are not likely to have spread beyond the thyroid gland, thereby improving the long-term outcome for the patient. There is no consensus at present on whether this trend toward earlier diagnosis is beneficial or unnecessary. The argument against early diagnosis and treatment is based on the logic that many small thyroid cancers (mostly papillary) will not grow or metastasize. This viewpoint holds the overwhelming majority of thyroid cancers are over diagnosed (that is, will never cause any symptoms, illness, or death for the patient, even if nothing is ever done about the cancer). Including these overdiagnosed cases skews the statistics by lumping clinically significant cases in with apparently harmless cancers. Thyroid cancer is incredibly common, with autopsy studies of people dying from other causes showing that more than one-third of older adults technically has thyroid cancer, which is causing them no harm. It is easy to detect nodules that might be cancerous, simply by feeling the throat, which contributes to the level of overdiagnosis. Benign (non-cancerous) nodules frequently co-exist with thyroid cancer; sometimes, it is a benign nodule that is discovered but surgery uncovers an incidental small thyroid cancer. Increasingly, small thyroid nodules are discovered as incidental findings on imaging (CT scan, MRI, ultrasound) performed for another purpose ; very few of these people with accidentally discovered, symptom-free thyroid cancers will ever have any symptoms, and treatment in such patients has the potential to cause harm to them, not to help them[25]. Thyroid cancer is three times more common in

women than in men, but according to European statistics, the overall relative 5-year survival rate for thyroid cancer is 85% for females and 74% for males. There is general agreement that stage I or II papillary, follicular or medullary cancer have a good prognosis, it is not possible when evaluating a small thyroid cancer to determine which ones will grow and metastasize and which will not. As a result, once a diagnosis of thyroid cancer has been established (most commonly by a fine needle aspiration), it is likely that a total thyroidectomy will be performed. This drive to earlier diagnosis has also manifested itself on the European continent by the use of serum calcitonin measurements in patients with goiter to identify patients with early abnormalities of the parafollicular or calcitonin-producing cells within the thyroid gland. As multiple studies have demonstrated, the finding of an elevated serum calcitonin is associated with the finding of a medullary thyroid carcinoma in as high as 20% of cases. In Europe where the threshold for thyroid surgery is lower than in the United States, an elaborate strategy that incorporates serum calcitonin measurements and stimulatory tests for calcitonin has been incorporated into the decision to perform a thyroidectomy; thyroid experts in the United States, looking at the same data sets have, for the most part, not incorporated calcitonin testing as a routine part of their evaluation, thereby eliminating a large number of thyroidectomies and the consequent morbidity. The European thyroid community has focused on prevention of metastasis from small medullary thyroid carcinomas; the North American thyroid community has focused more on prevention of complications associated with thyroidectomy. As demonstrated in the Table below, individuals with stage III and IV disease have a significant risk of dying from thyroid cancer. While many present with widely metastatic disease, an equal number evolve over years and decades from stage I or II disease. Physicians who manage thyroid cancer of any stage recognize that a small percentage of patients with low-risk thyroid cancer will progress to metastatic disease.

Fortunately for those with metastatic thyroid cancer, the last 5 years has brought about a renaissance in thyroid cancer treatment. The identification of some of the molecular or DNA abnormalities for thyroid cancer has led to the development of therapies that target these molecular defects. The first of these agents to negotiate the approval process is vandetanib, a tyrosine kinase

inhibitor that targets the RET proto-oncogene, 2 subtypes of the vascular endothelial growth factor receptor, and the epidermal growth factor receptor. More of these compounds are under investigation and are likely to make it through the approval process. For differentiated thyroid carcinoma, strategies are evolving to use selected types of targeted therapy to increase radioactive iodine uptake in papillary thyroid carcinomas that have lost the ability to concentrate iodide. This strategy would make it possible to use radioactive iodine therapy to treat "resistant" thyroid cancers. Other targeted therapies are being evaluated, making it possible that life will be extended over the next 5–10 years for those with stage III and IV thyroid cancer.

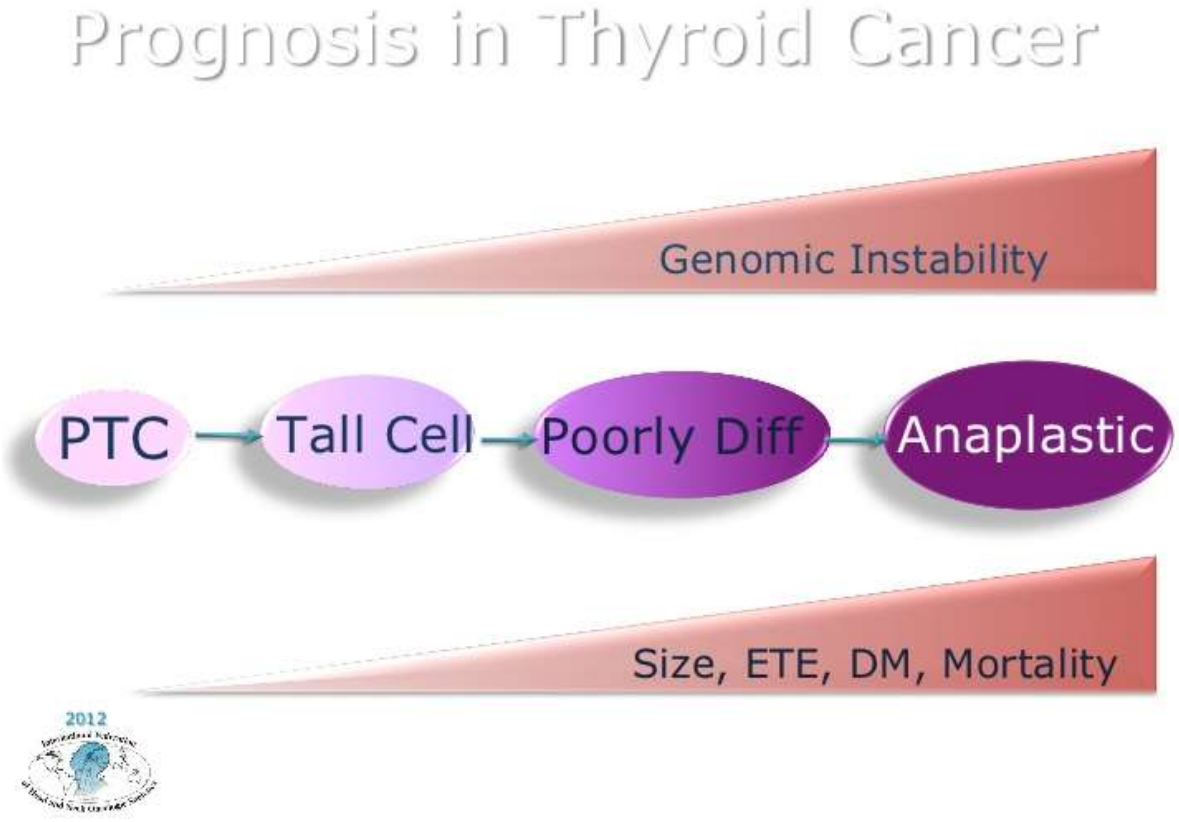

Figure 6. The prognosis system in current therapy of the thyroid cancer. *Thyroid cancer by J. Shah.*

7. Targeted therapy for undifferentiated thyroid cancer

UTC are rare but highly aggressive malignancies with an extremely short survival. Poor prognosis is due to their unlimited growth, invasion, migration and resistance to common anticancer therapies. Advances in understanding the molecular alterations in thyroid carcinomas led to development of new therapeutic strategies such as kinase inhibitors [Resistance to Kinase Inhibitors in Poorly Differentiated and Anaplastic Thyroid Cancer: Preclinical In vitro Evidences].

8. Therapy of undifferentiated thyroid cancer

The advanced cancer subtype, include ATC, was that has poor prognosis due to its resistance to treatment and aggressive behavior. The total median survival is only a few months. Poorly differentiated cancers usually have an ability of the resistant to anti-cancer drug, and presently there is not propose the effective clinical guidelines for the therapy of ATC. One of the most well-known that drug resistance in cancer is the induction of epithelial-mesenchymal transition (EMT). Nevertheless, the mechanisms of EMT-mediated drug resistance dwell incompetently defined.

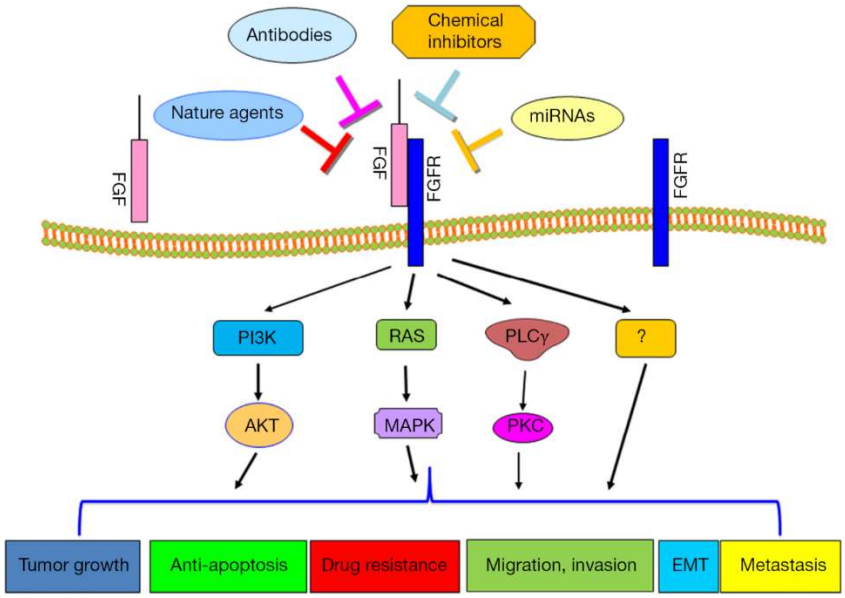

Figure 7. Drug resistance was mediated by FGFR signaling pathway.

Epithelial-mesenchymal transition (EMT) is a physiological procedure depend on epithelial cells collapse cell-cell junctions and temporary or eternally transition on a condition that is more delegate of migratory cells. The morphological lead to EMT is a fundamental aspect of resistance to ErbB-targeting compounds a deficiency in knowledge of the molecular mechanisms of this incident has inhibited the progress of therapeutic approach of targeting this drug resistant state. Previous well-known research have proved that expression of fibroblast growth factor receptor 1 (FGFR1) is significantly induced while TGF-β mediated EMT and plays a crucial role of the metastatic cancer. These previous researchs are support to our hypothesis, at the same time lead to drug resistance of poorly differentiated cancer-cancer stem cell through EMT-mediated by FGFR signaling pathway. The molecules and mechanisms that are closely associated with the poor clinical results of the advanced thyroid cancer. Among of them, we concentrated to the epithelial-mesenchymal transition (EMT) and drug resistance on the cancer stem cell (CSC) properties that are caused by EMT as one of the potential bring about of the poor clinical result. Evidence of the lately research was proved that EMT of cancer cells not only causes induced metastasis, but also contributed drug resistance.

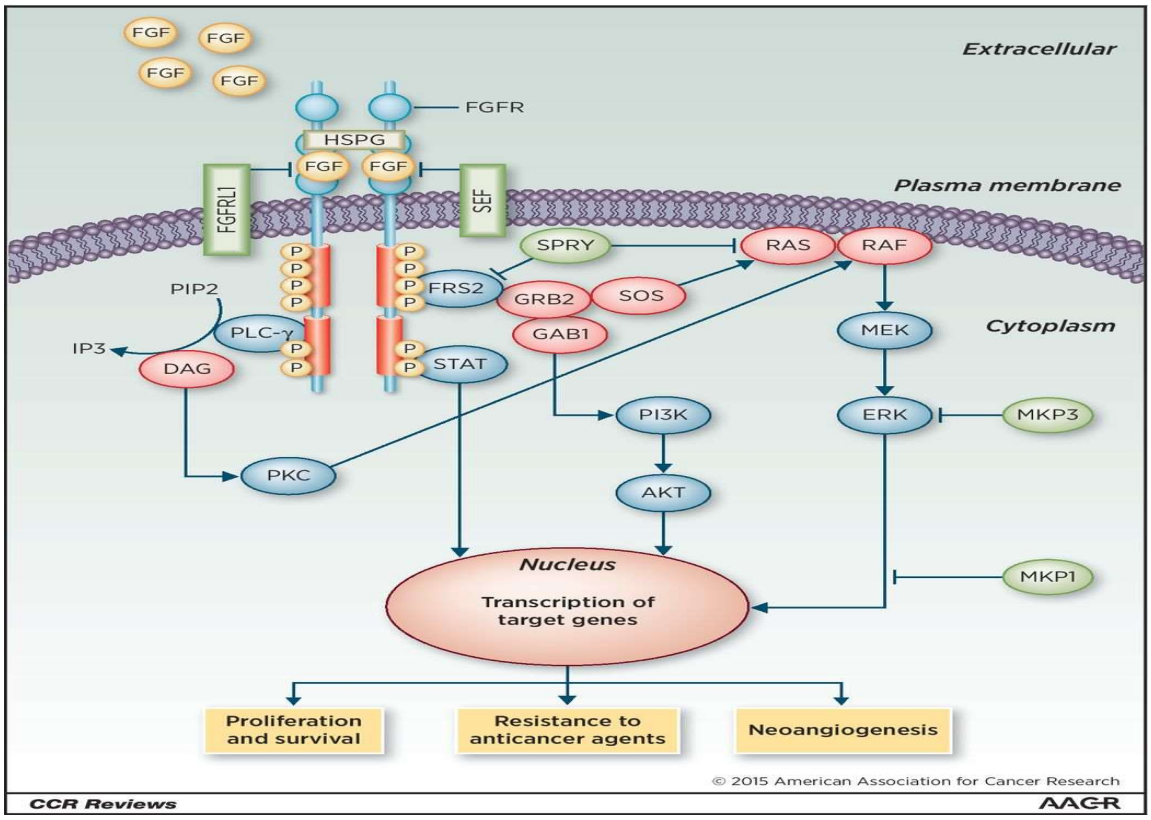

Figure 8. FGFR signaling pathway mediated target gene expression.

In this research, we based on gene expression profiles between PDTC and DTC, patient derived thyroid cancer cell, how inhibited to drug resistance *via* FGFR signaling and the epithelial-mesenchymal transition (EMT) develops in cancer in response to current treatments and how these problems are being addressed.

II. MATERIALS AND METHODS

Patients/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. For goals of this research, we chose one patient with thyroid cancer. The research protocol was approved by 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

On the morrow of resection, tumors were kept into normal saline with antifungal and antibiotics, and moved to the laboratory. Normal tissue and fat were removed and the tissues were rinsed with 1X HBSS. Tumors were minced into tube with dissociation medium. Dissociation medium contained DMEM/F12 with 20% FBS supplemented with 1 mg/ml of collagenase type IV (Sigma, USA). Minced and suspended tumor cells were filtered through sterile 70-micron pores nylon cell strainers (BD Falcon) rinsed with 50 ml 1X HBSS and centrifuged at 1400 rpm for 5 min. Cells were resuspended with RPMI-1640 (Hyclone, South Logan, UT, USA) medium, with 10 % fetal bovine serum (Hyclone) and 2% penicillin/streptomycin solution (Gibco, Grand Island, NY, USA). Cell viability was decided by the trypan blue dye exclusion method used.

Cell culture

The patient-derived PTC and ATC cells were isolation from the patient and grown in RPMI-

1640 medium with 10% fetal bovine serum (Authentication by short tandem repeat profiling / karyotyping / 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 the incubated for the indicated times prior to determination of cell viability using the MTT reagent according to the manufacturer's protocol, and measurement of absorbance at 490 nm. Viable cells were counted with trypan blue exclusion. Data were expressed as a percentage of the signal observed in vehicle-treated cells and shown as the mean ± standard error of the mean (SEM) of triplicate experiments.

Microarray experiment and data analysis

Gene expression data from the cancer cell lines were generated by hybridizing labeled RNAs to Human-6 v2. Expression BeadChips (Illumina). Total RNA was isolated from cells harvested after HNHA or Sorafenib or Lenvatinib alone or in combination with HNHA for 24 h, for the indicated time busing the mirVana miRNA Isolation Kit (Ambion Inc. AM1560) according to manufacturer's protocol. Biotin-labeled cRNA was prepared using the Illumina Total Prep RNA Amplification Kit (Ambion Inc.). Total RNA (500 ng) was used for the synthesis of cDNA followed by amplification and biotin labeling. Biotinylated cRNA (1.5 μg) per sample was hybridized to Illumina Human-6 BeadChip v.2 microarray and signals were developed by Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences). All statistical analysis was performed using R 2.3.0 and BRB Arraytools Version 3.5 (https://brb.nci.nih.gov/BRB-ArrayTools/) with quantile normalization[26].

Immunofluorescence analysis and confocal imaging

Expression analysis of β-catenin was performed with immunofluorescent staining. Cells grown on glass-bottomed dishes glass bottom dishes (MatTek, Ashland, MA) were fixed with 4% formaldehyde solution (R&D systems, UK) for 10 min. and permeabilised with 0.5% TritonX-100 in PBS for 10 min. Slides were air-dried and washed with PBS, and incubated with anti-β-catenin (1:25, abcam) in 3% bovine serum albumin (BSA) in PBS. After washing with PBS, slides were incubated with Alexa 488 (1:200, Molecular Probes, Eugene, U.S.A.) Nucleus was stained with Hoechst 33342 (Life Technologies, Grand Island, NY, USA) to visualize nuclei. Images were observed under a confocal microscope (LSM Meta 700) and were analyzed with the Zeiss LSM Image Browser software, version 4.2.0121.

Immunoblot analysis

Cells were washed twice with cold phosphate-buffered saline and lysed on ice with protein extraction buffer (Pro-Prep, iNtRON Biotechnology, Korea) following the manufacturer's protocol. Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (20 μg) were separated in 8-10% SDS-polyacrylamide gels; the resolved proteins were then electro-transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were subsequently blocked with 5% nonfat milk in TBST for 1 h at room temperature and incubated with appropriate concentrations of primary antibodies for Ki-67 (Abcam), Cyclin D1 (Santa Cruz Biotechnology), CDK4 (Santa Cruz Biotechnology), p21 (Santa Cruz Biotechnology), p53 (Santa Cruz Biotechnology), p-ERK 1/2 (Santa Cruz Biotechnology), ERK 1/2 (Santa Cruz Biotechnology), Apaf-1 (Abcam), p-NFκB (Santa Cruz Biotechnology), Bcl-2 (Santa Cruz Biotechnology), Caspase 3 (Santa Cruz Biotechnology), Vimentin (Abcam), E-cadherin (Abcam), Snail (Abcam), Zeb1 (Abcam) and β-actin (Santa Cruz Biotechnology) were overnight at 4°C. The membranes were then rinsed 3 ~ 5 times with TBS-T and probed with the corresponding secondary antibodies conjugated to HRP (Santa Cruz)

at room temperature for 1 h. After rinsing, blots were developed with ECL reagents (Pierce, Rockford, US) and exposed to Kodak X-OMAT AR Film (Eastman Kodak, Rochester, US) for 3 ~ 5 min.

Flow cytometry analysis of the cell cycle

Cells were treated with Sorafenib and Lenvatinib alone or in switching in RPMI-1640 medium containing 10% FBS for 40 h, 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 min at 37°C. Cell cycle distribution was then analyzed in the FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). The proportions of cells in the sub-G0/G1, G0/G1, S, and G2/M phases were analyzed by FlowJo v8 software for MacOSX (Tree Star, Ashland, OR, USA). This experiment was repeated thrice, and the results were averaged.

Human thyroid cancer cell xenograft

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 grouped randomly (n = 10/group) and with 10 mg/kg Lenvatinib (given p.o.) and 40 mg/kg Sorafenib (given p.o.), Lenvatinib or Sorafenib once every 2 days for injections. Tumor size was measured every other day using calipers. Tumor volume was estimated using the following formula: L × S2/2 (where L, longest diameter; S, shortest diameter). Animals were maintained under specific pathogen-free (SPF) 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 by 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 min. Sections were incubated for 5 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Immunohistochemistry results were subjected to ANOVA followed by a Bonferroni *post hoc* test. Values are expressed as means ± SD. P values < 0.05 indicated statistical significance.

III. RESULTS

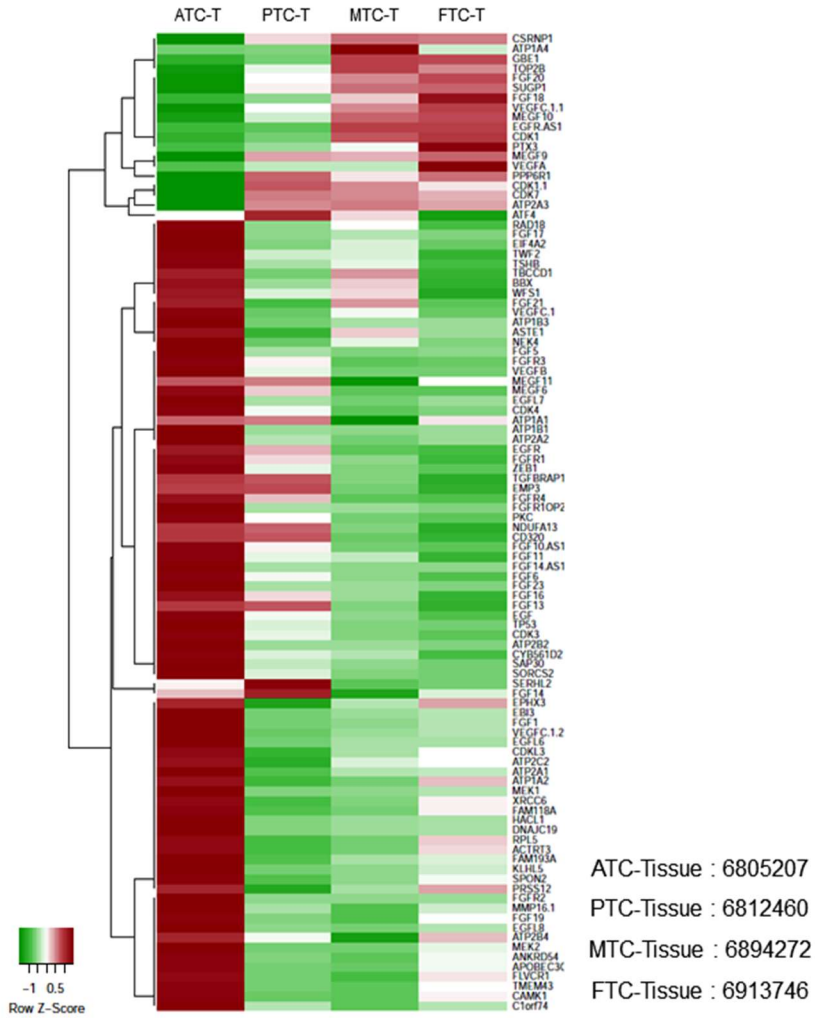

Figure 9. Gene expression profiles measured by microarrays. Hierarchical clustering analysis in between differentiated-, poorly differentiated-and dedifferentiated thyroid cancer of patient tissue.

We used expression microarrays to assess changes in gene expression of FGFR signalling cascades between differentiated- and poorly differentiated thyroid cancer of patient tissue. FGFR, EGFR and EMT markers were high expressed in poorly differentiated thyroid cancer.

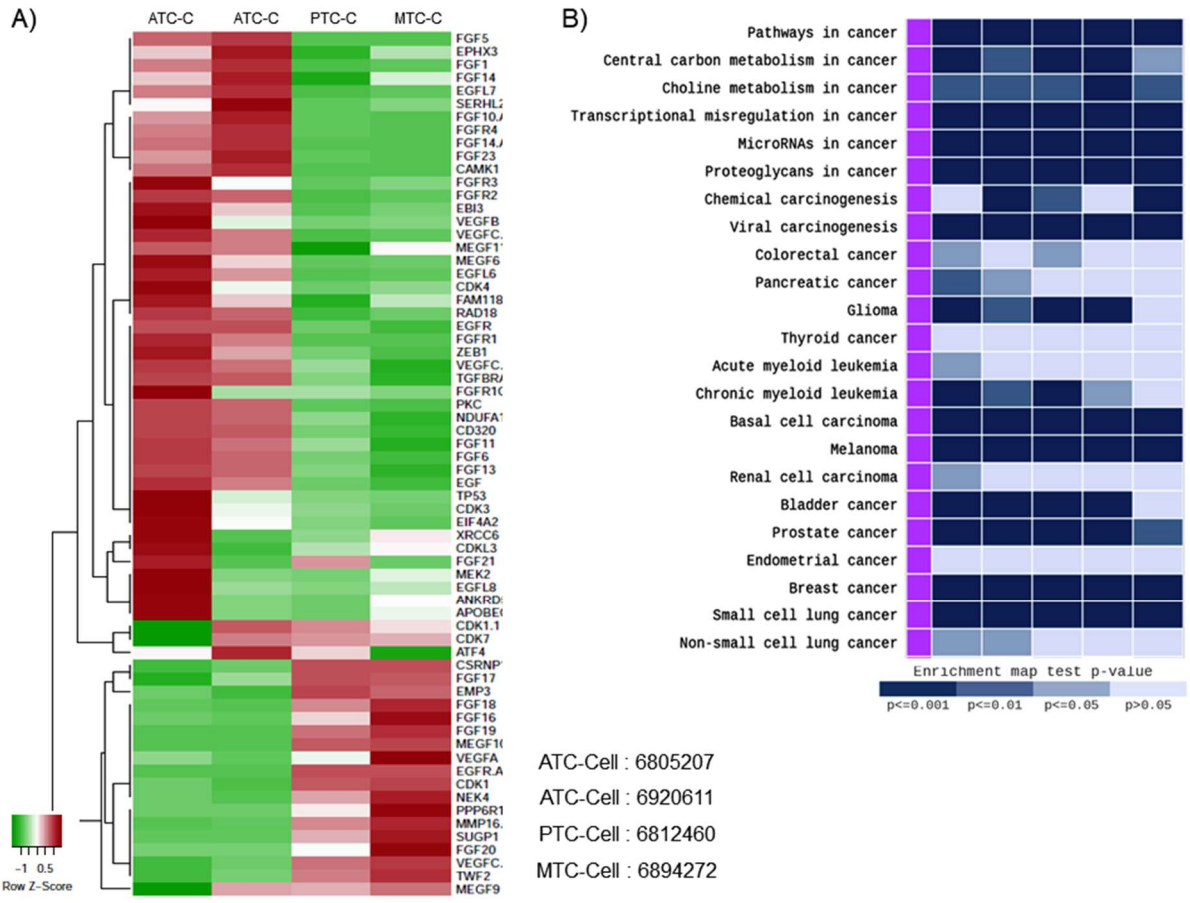

Figure 10. A, Gene expression profiles measured by microarrays. B, Gene expression pattern of the cancer subtype. Hierarchical clustering analysis in between differentiated-, poorly differentiated-and dedifferentiated patient-derived thyroid cancer cell.

The patient-derived ATC, PTC and MTC cells were isolation from the patient tissue. A, there is no significantly difference of gene expression compare microarrays of patient tissue. B, patient-derived ATC, PTC and MTC cells were confirmed by gene express pattern analysis of cancer subtype. The patient-derived ATC, PTC and MTC cells were showed coterminous gene express pattern compared with thyroid cancer FGFR, EGFR and EMT markers were high expressed in poorly differentiated thyroid cancer.

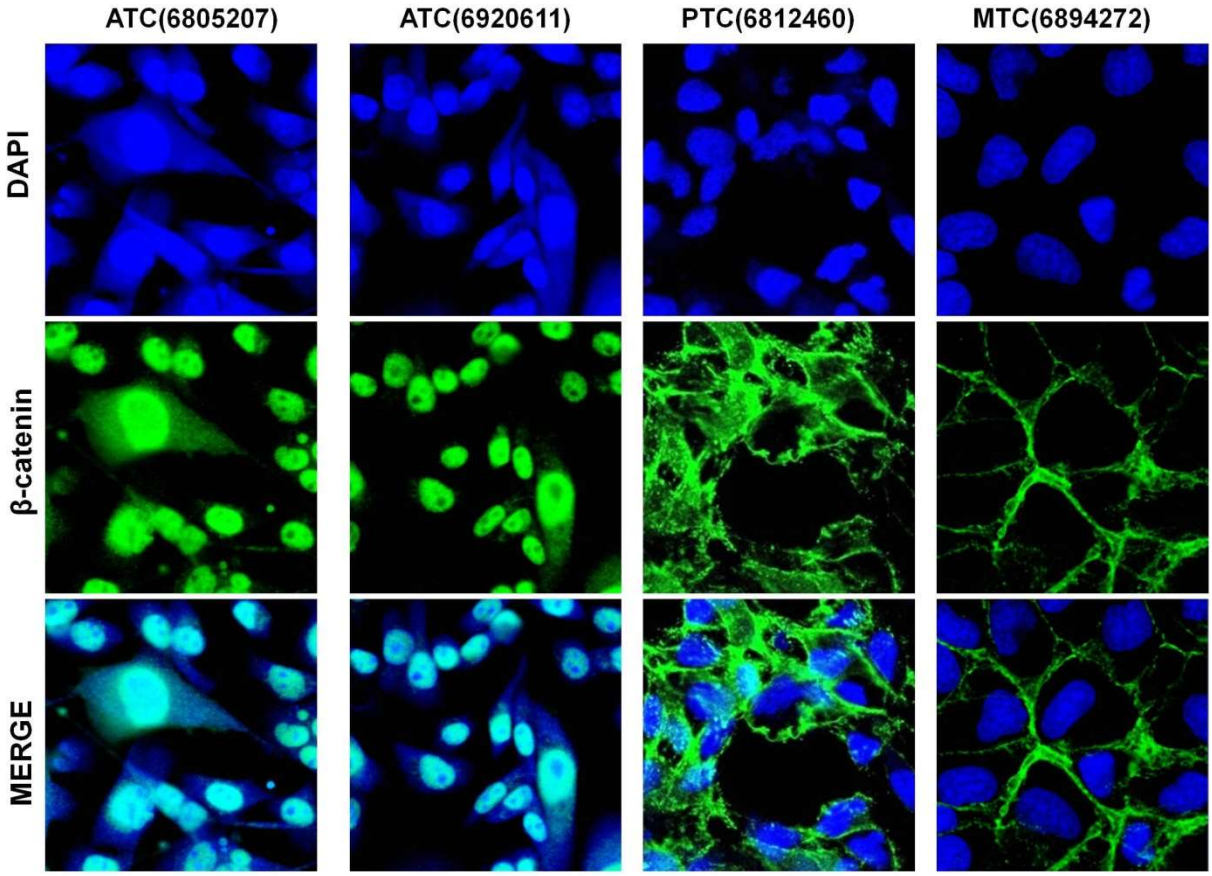

Figure 11. Present study was to investigate the nuclear localization of β-catenin in patient-derived-advanced thyroid cancer cells.

β-catenin, EMT marker, plays a key role in the induction of EMT, patient-derived advanced thyroid cancer cells were acquired target gene expression *via* EMT activation mediated β-catenin nuclear localization more than non-advanced thyroid cancer cells (Figure 8). β-catenin nuclear localization act synergistically to promote target gene expression related drug resistant.

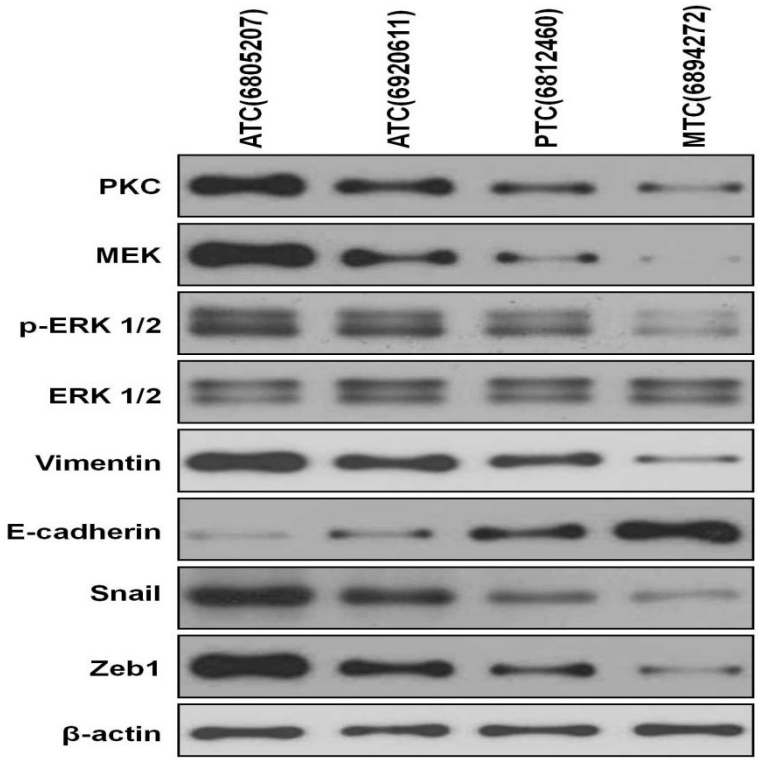

Figure 12. Profiling of the FGFR and EMT signaling pathway between patient-derived, advanced- and non-advanced thyroid cancer cells.

FGFR signaling pathway was well known that an evolutionary conserved signaling cascade for contribute a few biological procedures, target gene expression, including for drug resistance. This signaling pathway was more activated by EMT induction mediated FGFR signaling pathway in advanced cancer cells for target gene expression (Figure 9).

Table 1. Cell line characteristics, viability after drug treatment of all thyroid cancer cell lines examined

	GSP1	GSA1	GSA2
Age at diagnosis	31	74	56
Gender	Female	Female	Male
Primary disease site	Thyroid	Thyroid	Thyroid
Stage	IVc	IVc	IVc
Primary pathology	Papillary thyroid cancer	Anaplastic thyroid cancer	Anaplastic thyroid cancer
Classification of specimen used for culture	Fresh tumor	Fresh tumor	Fresh tumor
Obtained from	Gangnam Severance Hospital, Seoul, Korea	Gangnam Severance Hospital, Seoul, Korea	Gangnam Severance Hospital, Seoul, Korea

Table 2. IC50 (half maximal inhibitory concentration) determination using a cell proliferation assay. HNHA and Lenvatinib combination treatment is a lower IC50 than HNHA and Sorafenib combination or Sorafenib, Lenvatinib and HNHA alone. Each data point represents the mean of 3 independent MTS assays for IC50 performed in triplicate. SD, standard deviation.

Cell line	Cell proliferation IC50 (μM)				
	Sorafenib	Lenvatinib	HNHA	HNHA+S	HNHA+L
GSP1	9.42 ± 0.2	10.51 ± 0.1	5.15 ± 0.4	4.95 ± 0.5	3.84 ± 0.3*
GSA1	23.51 ± 0.2	41.54 ± 0.5	20.05 ± 0.4	9.63 ± 0.1	6.49 ± 0.2*
GSA2	21.11 ± 0.3	35.13 ± 0.2	18.33 ± 0.3	11.47 ± 0.5	7.32 ± 0.5*

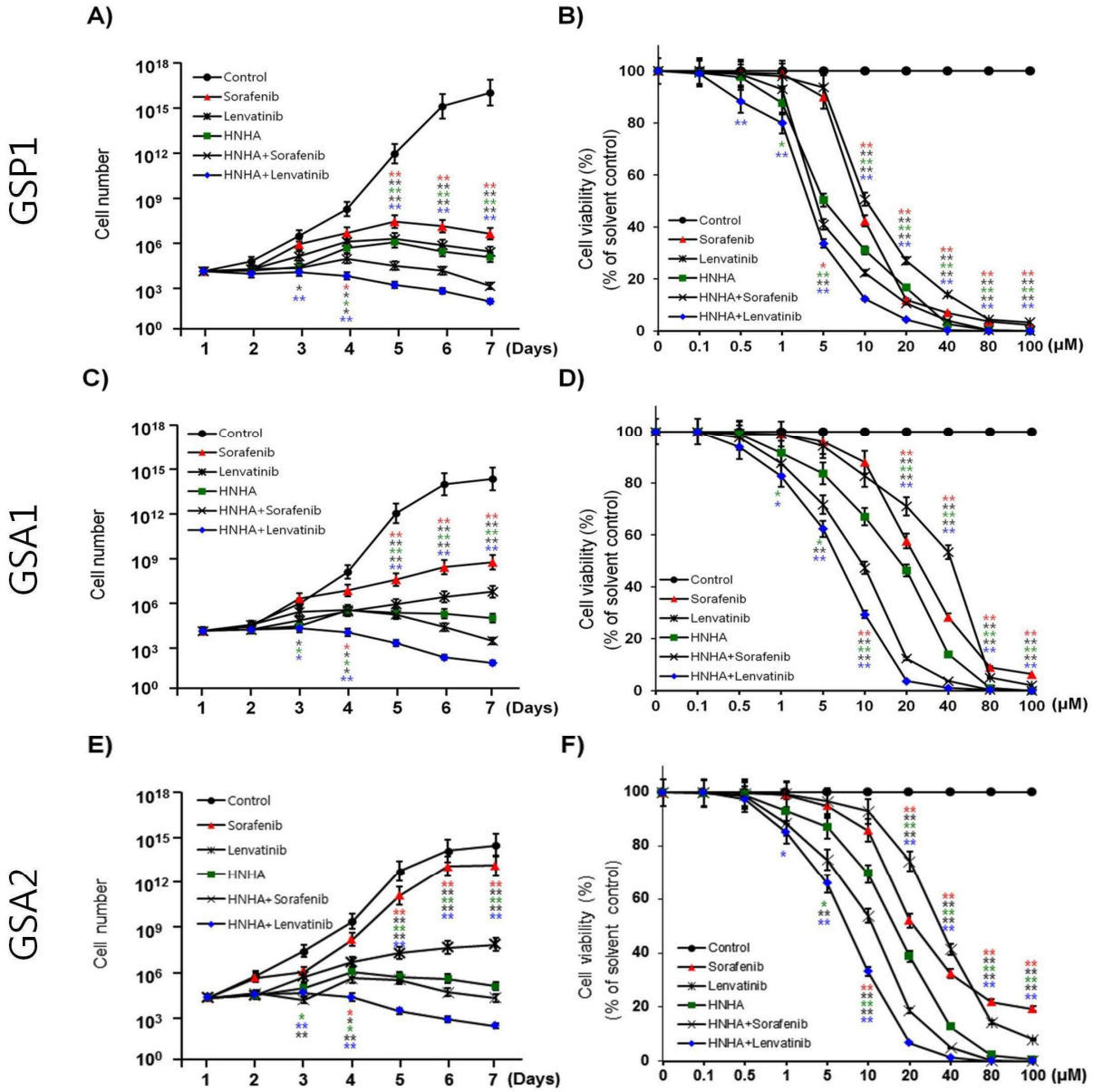

Figure 13. Synergistic suppression of cancer cell proliferation by HNHA and Lenvatinib was stronger than any other groups in patient –derived thyroid cancer cells.

To investigate of 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 of established a PTC and ATC on Gangnam Severance hospital) cell proliferation in the presence and absence of these

compounds by MTT assay (Figure 11A, C and E). IC_{50} of the combination of HNHA and Lenvatib had the lowest IC_{50} any other groups in 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 by either agent alone or combination of HNHA and Sorafenib. The combination of HNHA and Lenvatinib suppressed cell proliferation better than either agent used singly or combination of HNHA and Sorafenib (Figure 11A, C, and E); moreover, this effect was concentration-dependent (Figure 11 B, D, and F).

Table 3. Flow cytometry analysis of the cell cycle of the GSP1, GSA1 and GSA2.

A) GSP1

Status	Sub-G_0G_1	G_0G_1	S	G_2/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

B) GSA1

Status	Sub-G_0G_1	G_0G_1	S	G_2/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

C) **GSA2**

Status	Sub-G_0G_1	G_0G_1	S	G_2/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

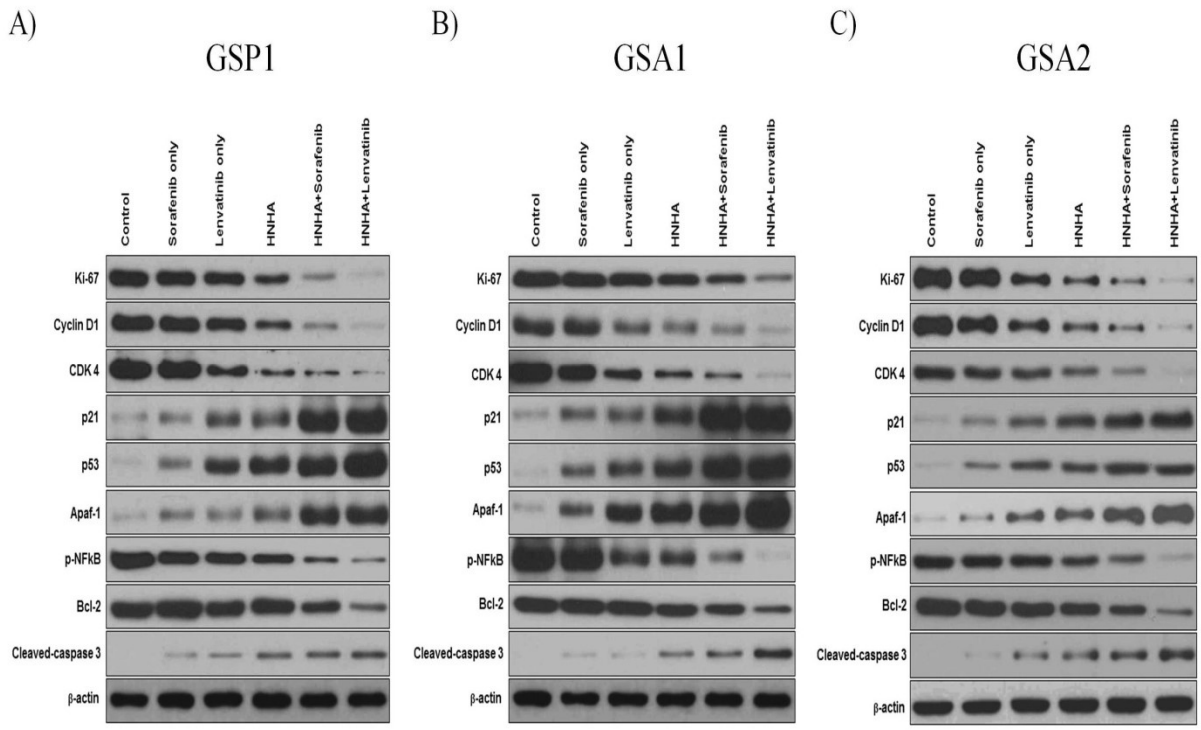

Figure 14. The HNHA and Lenvatinib combination significantly induced apoptosis and cell cycle arrest in patient–derived thyroid cancer cells

The combination treatment of HNHA and Lenvatinib showed the most significant induction of the sub-G_0G_1 population, showing the induction of cell death in GSP1, GSA1 and GSA2 cells (Table

3). The synergistic effect of HNHA and Lenvatinib most potently induced sub-G_0G_1 population, leading to apoptosis, cell cycle arrest, and strong inhibition of GSP1, GSA1 and GSA2 cells viability.

Immunoblot analyses of protein levels in GSP1, GSA1 and GSA2 cell lines indicated that the HNHA and Lenvatinib combination induced most marked increases in the levels of p53 and p21-well-known arrestors of the cell cycle-and decreases in the levels of cyclin D1, CDK 4-positive regulators of the cell cycle-as compared with either agent alone or combination of HNHA and Sorafenib (Figure. 12A, B and C). It is a noteworthy fact that proliferation marker (Ki-67) and anti-apoptotic (phosphorylated NF-κB p65 and Bcl-2) markers were most suppressed to HNHA and Lenvatinib combination treated group compared with either agent alone or combination of HNHA and Sorafenib (Figure. 12A, B and C). Whereas the expression of apoptotic markers (Apaf- and cleaved-caspase 3) were most induced to HNHA and Lenvatinib combination treated group compared with either agent alone or combination of HNHA and Sorafenib (Figure. 12A, B and C).

These results suggest that HNHA and Lenvatinib combination was a potent agent for effectively suppressor on advanced cancer.

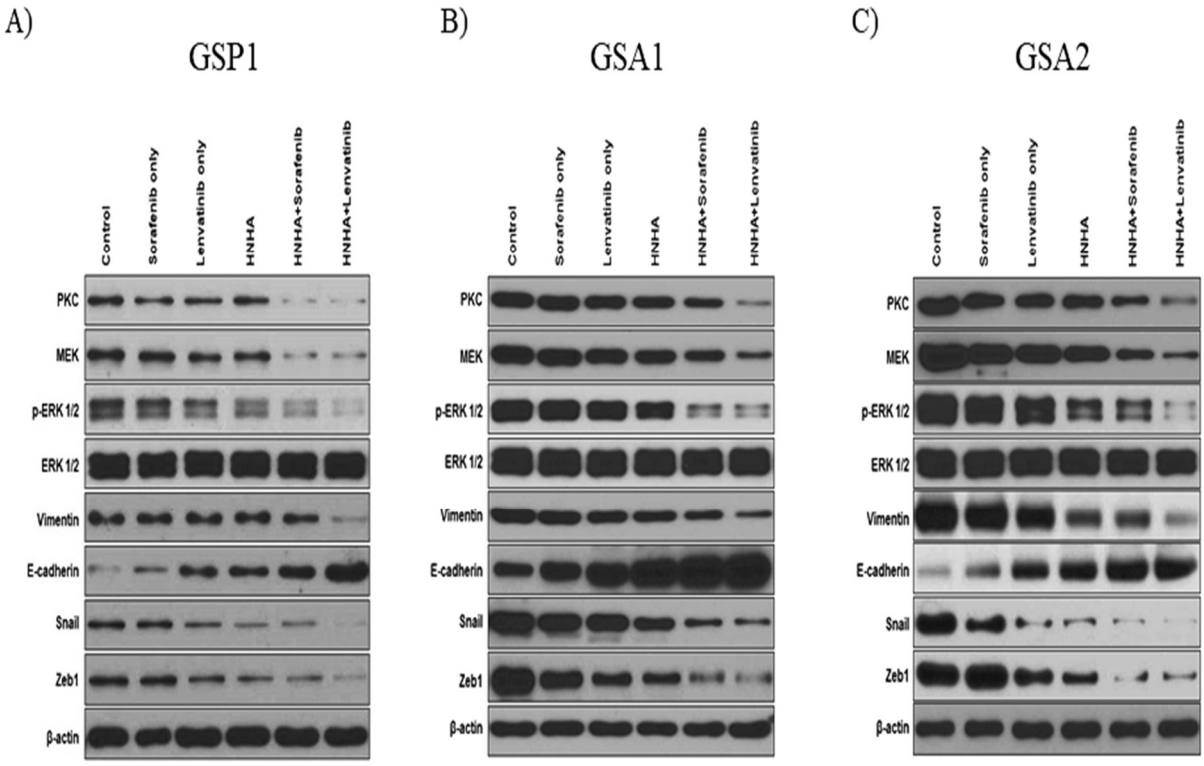

Figure 15. More advanced cancer cells, cancer stem cells, were more resistant to drug by EMT induction mediated FGFR signaling pathway activation in GSP1, GSA1 and GSA2.

We also investigated whether these combinations were restrained epithelial-mesenchymal transition (EMT) activation through prevention of fibroblast growth factor (FGFR) signal transduction. FGFR signaling pathway was well known that an evolutionary conserved signaling cascade for contribute a few biological procedures, target gene expression, including for drug resistance. HNHA and Lenvatinib combination was most suppressed FGFR signaling pathway (PKC, MEK and p-ERK1/2) which led to the inhibition of EMT (vimentin, E-cadherin, snail, and zeb1) (Fig. 13A, B and C) in GSP1, GSA1, and GSA2. On ligand binding, FGFR bring about a cascade of downstream signaling pathways, containing mitogen-activated protein kinase enzyme MEK and ERK (a component of the MAPK pathway) and PKC through PLCγ activation (Fig. 13A, B and C).

This evidence suggests that more advanced cancer cells, cancer stem cells, were more resistant to drug by EMT induction mediated FGFR signaling pathway activation, while synergistic effect of the

HNHA and Lenvatinib combination was an effective deterrent by suppression of EMT induction mediated inhibition of FGFR signalling pathway in GSP1, GSA1 and GSA2.

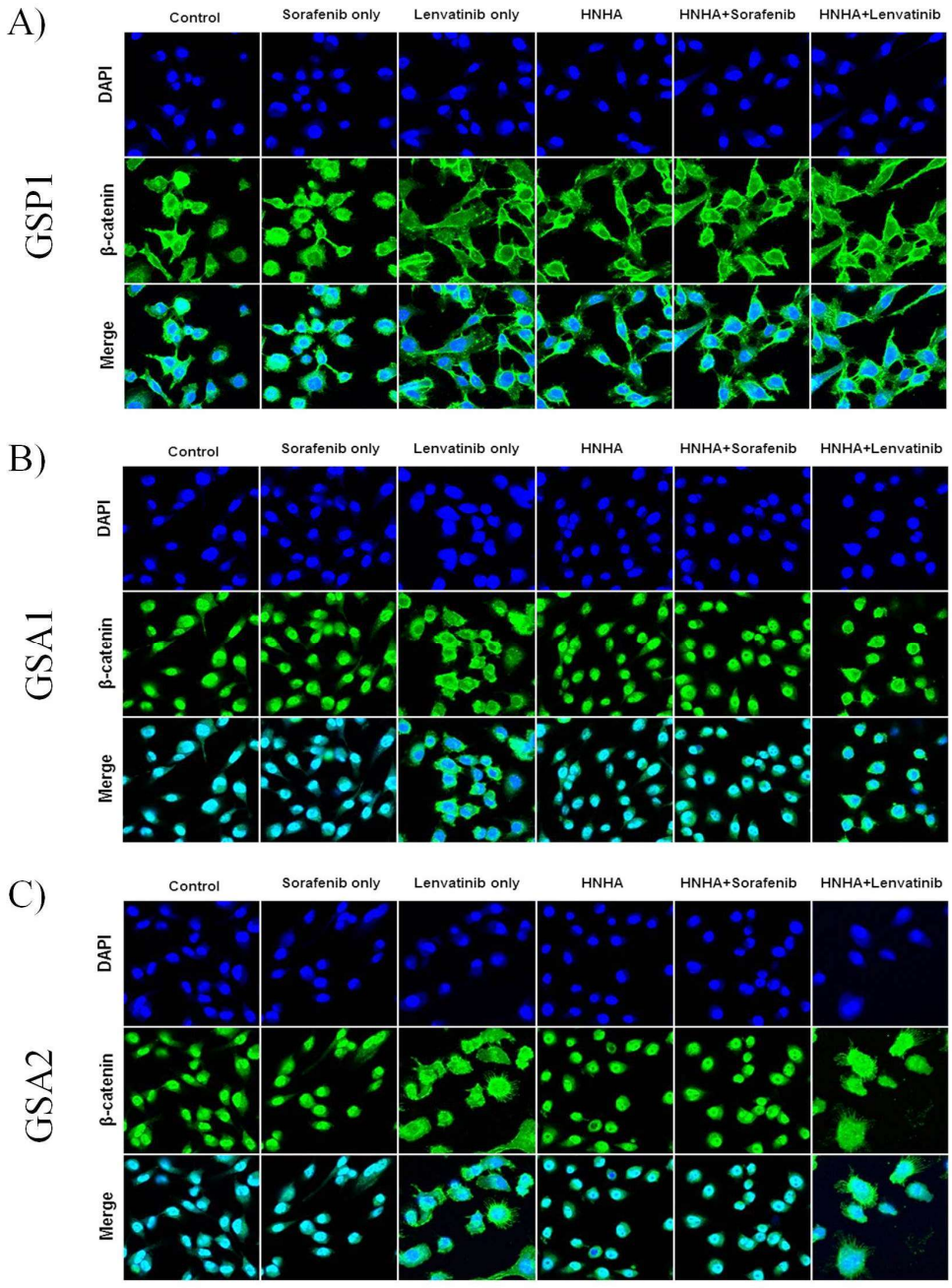

Figure 16. β-catenin, EMT marker, plays a key role in the induction of EMT by nuclear localization on advanced thyroid cancer cell.

β-catenin is a well-known multi-regulator and evolutionary conserved molecule that a crucial role in a tumorigenesis and correlates with poor prognosis. β-catenin nuclear localization act synergistically to promote target gene expression related drug resistant. Advanced thyroid cancer cells were acquired drug resistant *via* EMT activation mediated β-catenin nuclear localization. In GSA1 and 2, patient-derived anaplastic thyroid cancer 1 and 2, β-catenin nuclear localization is more increase than patient-derived papillary thyroid cancer 1, GSP1 (Fig. 14A, B and C, each control panel). β-catenin nuclear localization on GSA1 and 2 was significantly inhibited by FGFR inhibitor, Lenvatinib compare than non FGFR inhibitor, HNHA or Sorafenib (Fig. 14A, B and C).

This result implied many different things, one of them is disturbed EMT activation *via* inhibition of FGFR signaling pathway was contributed to drug resistant by Lenvatinib

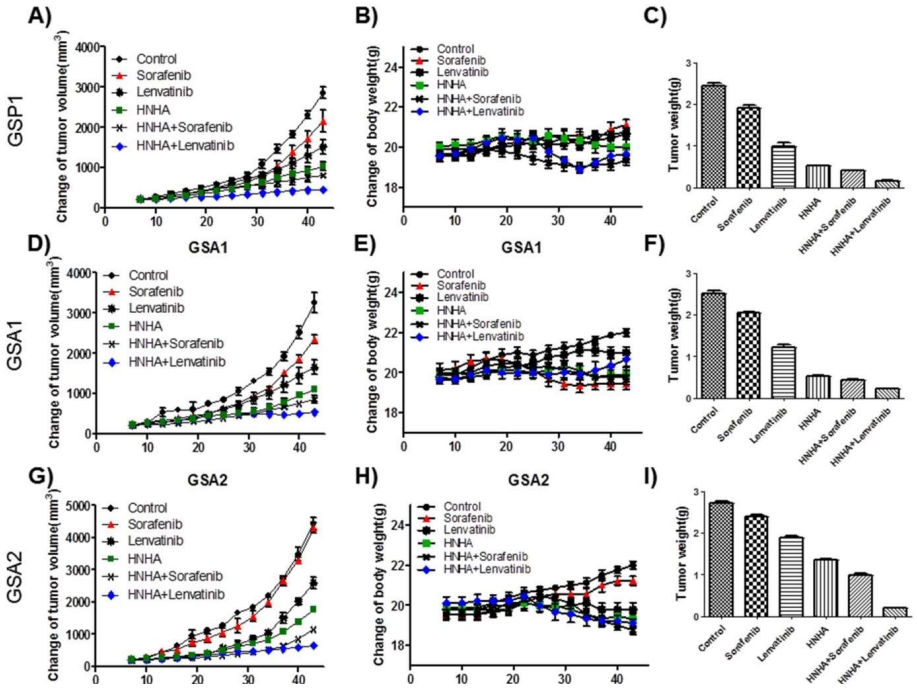

Figure 17. Tumor shrinkage was significantly induced by the combination treatment of the HNHA and Lenvatinib in xenograft model.

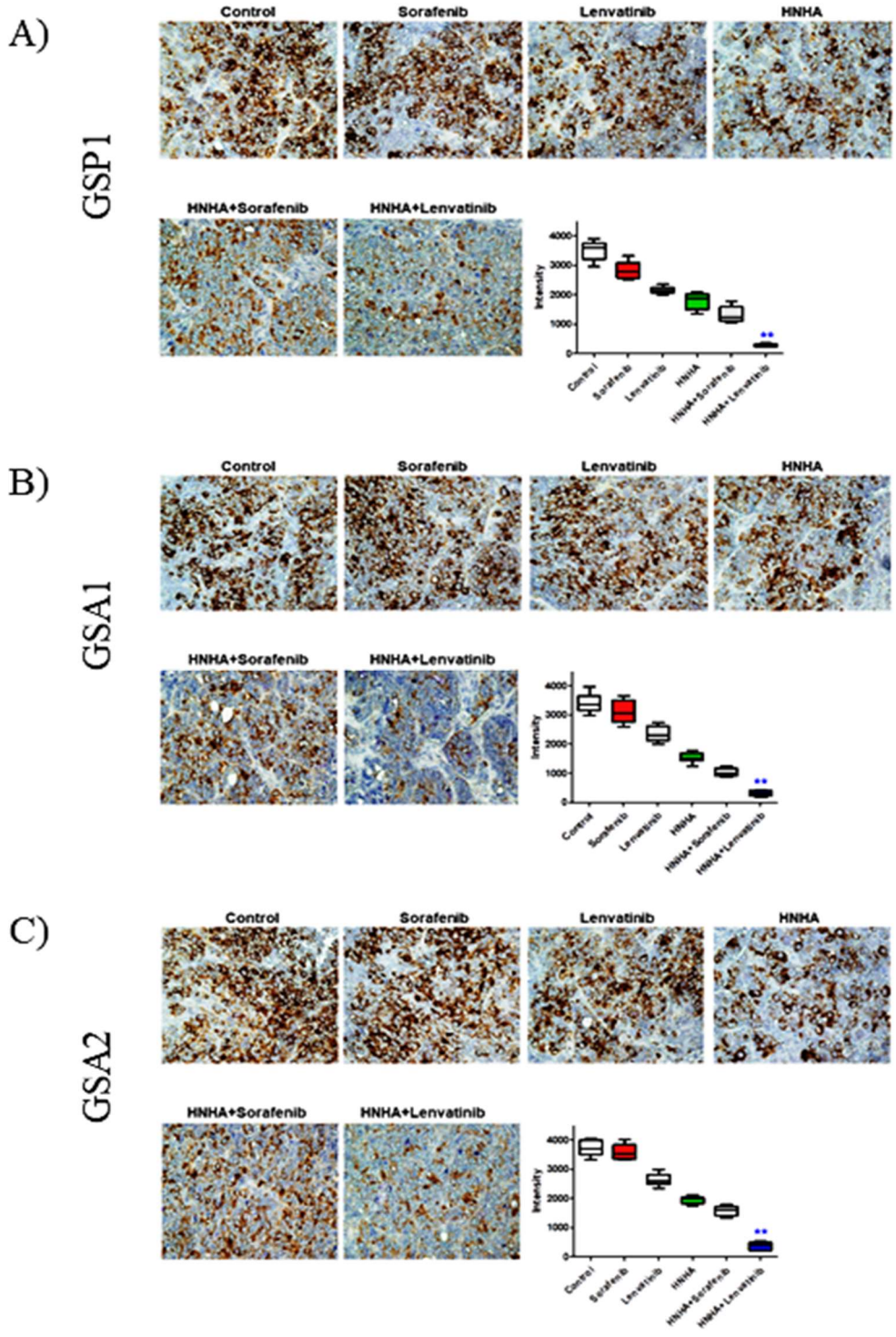

Figure 18. Immunohistochemistry analysis of Bcl2, anti-apoptotic marker GSP1, GSA1 and GSA2 cell xenograft tumors.

To investigate the synergistic anti-cancer effect of combination treatment of the HNHA and Lenvatinib *in vivo*, we developed a mouse xenograft tumor model using GSP1 (patient-derived papillary thyroid cancer 1), GSA1 and GSA2 (patient-derived anaplastic thyroid cancer 1 and 2).

Each agent used alone or combination of HNHA and Sorafenib were not markedly suppressed GSP1, GSA1 and GSA2 cell xenograft tumors; however, combination treatment of the HNHA and Lenvatinib was exhibited a greater suppression of these tumors (Fig. 15A, D and G). Moreover, there is no proof of systemic toxicity or treatment-related death was found in any group. There was no significant effect on the body weight of mice treated with Sorafenib or Lenvatinib or HNHA (Fig. 15A, D and G). The HNHA and Lenvatinib combination treatment group indicated significantly smaller tumor volumes compared to each agent used alone or combination of HNHA and Sorafenib (Fig. 15C, F and I).

Anti-apoptotic activity is a fundamental factor in the evaluation of the biological conduct of tumorigenesis. Bcl-2 is the most important marker of anti-apoptosis. We identified this marker by immunohistochemistry analysis of GSP1, GSA1 and GSA2 cell xenograft tumors and found that the HNHA and Lenvatinib combination treatment group showed the strongest decrease in Bcl-2 (Fig. 7A, B and C).

When all the results were combined, the HNHA and Lenvatinib combination treatment has potent anti-cancer activity in the cancer stem cell, advanced cancer cell xenograft model.

IV. DISCUSSION

According to a recent study, EMT is an extremely connected cellular procedure that involved to cancer growth, including metastasis, therapeutic resistance and recurrent. Cancer stem cells (CSCs) delegate a portion of poorly differentiated cancer cells that prove stem cell-like features[27]. CSC has a capability to self-renewal, metastases and accounts for therapeutic resistance[28]. Recent studies have concentrated a link EMT as well as drug resistance on CSC[29]. A well-known study of the CSCs, CSCs and EMT-type cells, which portion of molecular characteristics with CSCs, have been believed to performing crucial roles in drug resistance and cancer metastasis as proved on some human malignant cancer[29]. EMT is appropriate to gaining of stem cell-like properties and is adequate to provide differentiated normal and cancer cells with stem cell properties. Furthermore, CSCs frequently show EMT properties. For a decade, there has been many studies on the relationship between EMT and drug resistance in CSCs. In this paper, we investigate to EMT-mediated drug resistance *via* FGFR signaling pathway of the patient-derived anaplastic thyroid cancer cell, cancer stem-like cell. These cancer stem-like cells were highly induced marker of the EMT and FGFR signaling pathway. Some studies show that FGF2 and β3 integrin are part of an EMT signature that contribute to FGFR1-mediated drug resistance and metastatic progression[30]. One thing to look for is that drug resistance of the CSCs was dependent to EMT mediated FGFR signaling pathway. Consequently, we focused to inhibition of FGFR signaling pathway by TKI (tyrosine kinase inhibitor) on the CSCs.

TKIs are suggested in Radioiodine (RAI)-refractory DTC patients with metastatic, rapidly progressive, symptomatic, and/or imminently threatening disease not otherwise amenable to local control with other approaches. Advantage of systemic therapeutics has been demonstrated in the form of improved progression-free survival in three randomized, double-blinded, placebo-controlled clinical trials: vandetanib, sorafenib and lenvatinib[3,31,32]. Sorafenib is known to inhibit RAF-1 a member of the RAF/MEK/ERK signaling pathway, BRAF activity as well as VEGFR-2, VEGFR-3, PDGFR-β and c-

KIT[33]. Lenvatinib has a potent inhibitory effect on VEGFR-2, VEGFR-3, PDGFRα/β, KIT, RET and other than sorafenib, FGFR 1-4. The most important difference of lenvatinib to other drugs are the ability to inhibit FGFR 1, representing an effective drug in those cases in which a resistance to VEGFR inhibitors is developed[34-36]. Although both lenvatinib and sorafenib show good results in phase III trials and although they are the first line treatment in RAI refractory DTCs, most patients eventually stop responding to them and many are not able to continue medication because of its toxicity. In patients who have disease progression during initial kinase inhibitor therapy without prohibitive adverse effects, second-line kinase inhibitor therapy should be considered, whereas only lenvatinib may be used as second-line treatment[3]. There are several mechanisms for TKI resistance such as receptor autophosphorylation, autophagy, hypoxia-inducing factor, epigenetic regulation and epithelial-mesenchymal transition[37,38]. Yet, there are several EMT inducing cytokines known such as TGF-β, FGF, HGF, insulin-like growth factor and IL-6[39,40].

EMT in thyroid cancer is known to be induced in more aggressive forms, with increased expression of ZEB1, which can promote drug resistance through EMT-dependent and EMT-independent mechanisms[41-43]. Studies showed that downregulation of ZEB1 expression could restore drug sensitivity[44,45]. Sorafenib was able to inhibit EMT in hepatocellular carcinoma, to attenuate HGF secretion in polarized macrophages and to decrease plasma HGF. Sorafenib abolished polarized-macrophage-induced activation of the HGF receptor Met[46]. Reversion of EMT resulted in overcoming drug resistance in lung adenocarcinoma[39].

This study proved that drug resistance of the CSC-like cancer cell- Sorafenib resistant-patient derived thyroid cancer cell- was inhibited by combination therapy of HNHA and Lenvatinib, through inhibition of EMT mediated FGFR signaling pathway. Synergistic effect of HNHA and Lenvatinib was more efficient than either agent used singly or combination of HNHA and Sorafenib. Which could induce markers of cell cycle arrest and apoptosis while reduce markers of anti-apoptosis, EMT and FGFR signaling pathway more efficient. Not only in cell culture studies, but also in in vivo studies of

xenograft model, significantly tumor shrinkage induced in HNHA and Lenvatinib combined treatment group. We propose that these effects might be due to reduced EMT-mediated drug resistance in CSCs model. HNHA and Lenvatinib combined treatment blocked FGFR signaling pathway. FGFR signaling pathway is a major signaling pathway for EMT and metastasis, and inhibition of this pathway showed significant reduction of EMT[47].

The most important things of this study, the combination of targeted therapy with HNHA and Lenvatinib has possible new clinical approach of care in patients with CSCs, drug resistant properties.

V. CONCLUSION

These results propose that HNHA in combination with Lenvatinib has significant anti-cancer activity in preclinical models, potentially suggesting a new clinical approach for patients of advanced thyroid cancer type.

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ASTRACT (IN KOREAN)

분화갑상선암 및 미분화갑상선암 환자 유래 갑상선암 세포 사이에서 유전자 발현의 차이에 따른 치료 저항성 차이에 대한 연구

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이 용 상

갑상선은 일반적으로 좋은 예후를 보이는 분화갑상선암, 그리고 임상적으로 예후가 매우 좋지 않으며 불완전하게 분화 된 미분화갑상선암에 이르기까지 난포 세포로부터 유래된 다양한 종양이 발생한다.

미분화갑상선암은 클론성 확장과 암으로의 진행을 일으키는 유전적 및 후생유전학적 변화의 다양한 과정을 통해 기존의 잘 분화 된 암으로부터의 진행으로 인해 발생하는 것으로 알려져 있다. 저분화갑상선암과 미분화갑상선안 간 유전 변이와 후성적 변화는 아직 명확하게 밝혀진 바가 없는 실정이며, 다만 저분화갑상선암과 미분화갑상선암이 분화갑상선암에서 유래될 수 있다고 추정할 뿐이고, 미분화갑상선암이 유두갑상선암과 여포갑상선암의 일반적인 유전적 변화와는 차이가 있을 것이라고 생각한다.

분화갑상선암과 미분화갑상선암에 있어 BRAF, NRAS와 같은 유전 인자들의 변이

를 근거로 삼는데 p53 은 일반적으로 저분화갑상선암이나 미분화갑상선암에서 많이 발현되며 진단 마커로도 사용된다. 특히 미분화갑상선암은 예후가 매우 좋지 않아 생존율이 매우 낮은데 이러한 이유는 항암제에 대한 저항성을 가지기 때문에 표준항암치료법의 적용을 할 수가 없는 실정이다.

본 연구는 분화갑상선암과 미분화갑상선암 간 유전적 발현 차이를 근거로 하여 항암제 저항성 관련 유전자 발현 차이를 이용한 항암제 조합을 발굴하고자 한다.

중심어: 유두갑상선암, 여포갑상선암, 분화갑상선암, 미분화암, 유전자 발현 프로파일, 약물 저항성