의학박사 학위논문

Anti-proliferative effect of sodium selenite in thyroid cancer via ERK downregulation

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Anti-proliferative effect of sodium selenite in thyroid cancer via ERK downregulation

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Abstract

Anti-proliferative effect of sodium selenite in thyroid cancer cells via ERK downregulation

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Background:

Thyroid cancer is basically an ERK-driven carcinoma, as up to 75% of thyroid cancers are caused by mutations which activate RAS-RAF-MEK-ERK pathway. Although ERK pathway plays major roles

in the progression of thyroid cancer, the use of ERK inhibitors has been limited by its toxicity. I investigated the effect of sodium selenite as an adjunct for ERK inhibitors to avoid the toxicity of ERK inhibitors.

Methods:

Human thyrocyte cell line HTori-3, human papillary thyroid cancer cell line TPC1, and Human anaplastic thyroid cancer cell line 8505C cells were treated with U0126 (ERK inhibitor) and cell viability was counted in the Neubauer chamber. The synergistic effects of sodium selenite and U0126 were also measured. To determine the signaling molecules responsible for sodium selenite treatment, I investigated expression of signaling molecules including *AKT, p-AKT, ERK, p-ERK, ERK, p-JNK, p21* and *p53*. To confirm the effect of sodium selenite on ERK signaling, expression of *ERK, p-ERK*, and $p90^{RSK}$ was determined by western blot.

To validate the effect of sodium selenite *in vivo*, a total of 3628 patients including 114 patients with non-occult papillary thyroid cancer with Graves' disease were reviewed. The primary outcome

measure was recurrence-free survival according to the selenium treatment.

Results:

Treatment with U0126 inhibited proliferation of TPC1 and 8505C cells in a dose-dependent manner. Based on the cell survival analyses, I selected the concentration of 1 μM of U0126 and 5 μM of sodium selenite in the ensuing studies to observe the efficacy of sodium selenite. When 5 μM sodium selenite was added to 1 μM U0126, relative cell survival further decreased compared to the treatment of 1 μM U0126 alone. Signaling pathway analysis results indicated that ERK signaling was the major pathway affected by sodium selenite treatment in thyroid cells. Decreased expression of $p90^{RSK}$ further confirmed that sodium selenite down-regulated ERK signaling.

Of the 114 patients with GD, sodium selenite treatment was performed in 41 patients (36.0%). Recurrence was found in one patient (1.3%) in the GD without selenium treatment group and none (0.0%) in the GD with selenium treatment group, while 67 patients

(1.9%) without GD developed recurrence (p = 0.425). The recurrence-free survival showed no difference among groups (p = 0.452).

Conclusions:

The combination of U0126 and sodium selenite inhibited proliferation of thyroid cancer cells through ERK inhibition. The effect of sodium selenite *in vivo* should be further validated.

Keywords: thyroid cancer, selenium, ERK inhibitor, U0126, ERK pathway

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chamber.

Figure 4. Expression of *AKT, p-AKT, ERK, p-ERK, ERK, p-JNK, p21* and *p53* after sodium selenite treatment for 72 h. A total of 5×10^5 cells of TPC1, 8505C, and HTori-3 cell lines were seeded in DMEM containing 10% fetal bovine serum.

Figure 5. Viability after sodium selenite and MEK-ERK inhibitor (U0126) treatment. Thyroid cells were treated with 0.01% ethanol (CTL), 1μM U0126, 5μM sodium selenite, and 1μM U0126 with 5μM sodium selenite for 72 h. Viable cells were counted in a Neubauer chamber.

Figure 6. Expression of *ERK, p-ERK,* and *p90RSK* after sodium selenite treatment for 72 h. A total of 5×10^5 cells of TPC1, 8505C, and HTori-3 cell lines were seeded in DMEM containing 10% fetal bovine serum.

I. Introduction

Thyroid cancer is one of the most common cancers in Korea and its incidence has rapidly increased over the past 20 years [1]. The incidence of thyroid cancer in Korea increased about 5.8 times from 6.3 cases per 100,000 in 1999 to 36.8 cases per 100,000 in 2017. Papillary thyroid carcinoma (PTC) comprises the vast majority of all thyroid cancers, which is generally associated with favorable outcomes [2,3]. However, some patients present with aggressive disease, including regional or distant metastasis, and experience significant progression [4-6]. Recurrence and cancer-related death can occur more than 20 years after the initial diagnosis of PTC [7]. Furthermore, anaplastic transformation can occur in the untreated or treatment-refractory PTCs [8]. Many therapeutic modalities were thoroughly evaluated to treat refractory PTCs including tyrosine or Aurora kinase inhibitors, gene therapy using oncogenic viruses, apoptosis-inducing agents, and immune therapy [9] .

Of these modalities, researchers paid special attention to the

targeted inhibitors including tyrosine kinase inhibitor. From a molecular biological point of view, thyroid cancer is basically an ERK-driven carcinoma, as up to 75% of thyroid cancers are caused by mutations which activate RAS-RAF-MEK-ERK pathway [10]. Because of high dependency on ERK activation of thyroid cancer, various kinase inhibitors of this pathway have been investigated for the effective treatment of thyroid cancer. US Food and Drug Administration recently approved BRAF and MEK inhibitors for the treatment of radioactive iodine-refractory advanced thyroid cancers [11,12]. These kinase inhibitors such as sorafenib and levatinib showed some improvement of survival in patients with metastatic thyroid cancer [13]. Therapeutic responses of these inhibitors, however, is only temporary and limited by toxicity in highly proliferative tissues such as skin and the intestinal epithelial barrier.

To enhance the efficacy of kinase inhibitors, many drugs and compounds regulating kinases including selenium have been thoroughly investigated [14]. Selenium is an essential trace element

in the human body and required for maintaining optimal health. As selenium derivatives modulated many kinases including MAP, ERK, Akt, JNK, Cdc2, and Cyclin B_1, selenium has been recognized as an effective chemopreventive agent [15]. Furthermore, selenium can stimulate cellular immune system, which enhances anticancer properties [16]. These anticancer activities of selenium compounds can be different according to its chemical form, dose, degree of selenium bioavailability, and cancer type [16]. Selenium compounds are categorized into three groups: inorganic, organic, and selenium-containing nanoparticles. Of these selenium compounds, inorganic selenite is one of the most redox-active forms and exhibits high cytotoxic activity [17].

Recent studies indicated that selenium intake decreased the risk of several cancers including esophagus, liver, pancreas cancers, and thyroid cancer [18]. Epidemiologic studies reported that the serum selenium level was lower in patients with thyroid cancer than in healthy controls [19,20]. Metere et al. indicated that selenoprotein

expression including glutathione peroxidase and thioredoxin reductase decreased to ~50–60% in thyroid cancer tissue compared with healthy thyroid tissue [21]. Zagrodzki further demonstrated that other selenoproteins such as DIO1 (2.49 ± 1.56 pmol I/h/mg vs 6.37 ± 5.44 pmol I/h/mg, $p = 0.016$) and DIO2 (6.57 ± 3.99 fmol I/h/mg vs 31.6 ± 27.2 fmol I/h/mg, $p < 0.001$) showed lower activity in the thyroid tissue of patients with PTC than of those with benign disease [22]. Decreased serum selenium level was also correlated with higher stage of thyroid cancer [23]. On the contrary, selenium supplementation can reduce the inflammatory response of the autoimmune thyroid disease, which may decrease the risk of thyroid cancer [24].

Many studies have suggested that selenium is also related with benign thyroid diseases including autoimmune thyroiditis and Graves' disease (GD). Thyroid glands have a higher concentration of selenium than most other organs, reflecting the importance of selenium to thyroid metabolism. Thyrocytes express a number of selenoproteins,

including deiodinase family (DIO1 and DIO2), glutathione peroxidase isoenzymes, members of thioredoxine reductases, selenoprotein 15, selenoprotein M, selenoprotein P, selenoprotein S. As oxidative stress has been implicated in the pathogenesis of autoimmune thyroiditis and GD, the antioxidant properties of seleoproteins can be inversely associated with those diseases. These selenoproteins further participate in numerous physiologic processes including thyroid hormone regulation, redox homeostasis, inflammatory responses, carbohydrate metabolism, and brain function maintenance.

Among these selenium-related thyroid diseases, GD is an autoimmune thyroid disease whose central mechanism is an activation of thyroid-stimulating autoantibodies (TSAbs) [25,26]. Patients with newly diagnosed GD had lower serum concentration of selenium than controls, and patients achieving remission had higher selenium level than those with relapse [27,28]. A recent meta-analysis also indicated that selenium supplementation enhanced the effect of methimazole for biochemical restoration of euthyroidism, which

indicated a reduced activity of TSAbs [29]. Since TSAbs promotes tumor formation and angiogenesis, GD can be associated with an increased risk of thyroid cancer [30,31]. TSAbs also upregulate various growth factors and enhance tumor invasiveness [32]. Therefore, selenium supplementation in patients with GD may lower the occurrence of thyroid cancer, and improve the prognosis of thyroid cancer.

Contrary to the large number of clinical researches, a few previous studies investigated the mechanism of selenium in thyroid follicular cells. In one experiment, supplementation of sodium selenite enhanced growth and reduced death of normal thyroid cells [33]. Modulation of proapoptotic and antiapoptotic mRNA levels was possible underlying mechanism and high dose of sodium selenite might further prevented the ER-stress apoptosis. Pace et al. indicated that selenium reduced cell oxidative stress and decreased the level of thyroid autoantibodies [34]. In another study, seleno-methionine supplementation induced cell-cycle arrest in S and G2/M phase in

thyroid cancer cells including ARO, NPA, WRO and FRO cell lines [35]. In these cancer cell lines, time-dependent upregulation of GADD gene families inhibited cancer growth. However, there has been few studies about the effect of sodium selenite to the thyroid cancer cells.

Possible mechanism of sodium selenite included stimulation of cellular immune system, activation of natural killer cells, inhibition of angiogenesis, enhancement of damaged DNA fragment repair, and initiation of apoptosis in various cancers [36-40]. Sodium selenite also played an important role at the initial phase of carcinogenesis with its stimulation of the cellular immune system [16]. Some experimental studies further demonstrated that selenite might induce apoptotic process in various cancers [41]. Selenium compounds could produce reactive oxygen species, which had a pro-oxidative effect on apoptosis of cancer cells [42]. On the other hand, recent studies demonstrated that sodium selenite suppressed cell differentiation through inhibiting ERK activation in vascular smooth muscle cells [43,44]. As the activation of RAS-RAF-MEK-ERK axis is the major

driver of thyroid cancer, sodium selenite may also enhance the growth inhibition of thyroid cancer cells.

I hypothesized that sodium selenite could inhibit ERK pathway and exhibit anti-cancer activity against thyroid cancer cells. The role of sodium selenite as an adjunct for ERK inhibitors was also investigated to reduce toxicity of ERK inhibitor. The apoptotic activity of sodium selenite in thyroid cancer cells was further evaluated as a possible anti-cancer mechanism. At last, I tried to validate the effect of selenium supplement in patients PTC with GD.

The primary objective of this study is to investigate the effect of sodium selenite for thyroid cancer cells with combination of MEK-ERK inhibitor.

II. Materials and Methods

Cell culture

Human thyrocyte cell line HTori-3, human papillary thyroid cancer cell line TPC1, and Human anaplastic thyroid cancer cell line 8505C cells (Korean Cell Line Bank, Seoul, Republic of Korea) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco Thermo Fisher Scientific). The cells were seeded at 5×10^5 cells in Falcon™ standard tissue culture dishes (BD Biosciences, San Jose, CA, USA), and were incubated at 37 °C in a humidified, 5% CO_2 atmosphere.

Cell viability assay after treatment

HTori-3, TPC1, and 8505C cells ($\times 5 \times 10^5$) were seeded in DMEM supplemented with 10% FBS. Following 24 h of culturing, the cells were washed twice with phosphate-buffered saline (PBS), and fresh

medium was added. The cells were treated with 0.01% Ethanol (CTL) or with U0126 (Target Molecule, MA, USA) at 1nM, 10nM, 100nM, 1 μM, or 10 μM for 72 h. The cells were also treated with distilled water (CTL) or with sodium selenite (Sigma-Aldrich, St. Louis, MO, USA) at 1 μM, 5 μM, or 10 μM for 72 h. To observe co-treatment effect of U0126 and sodium selenite, cells were treated with U0126 at 1 μM or 5 μM sodium selenite and/or with 1 μM U0126 plus 5 μM sodium selenite for 72 h. Viable cells were counted in a Neubauer chamber. Relative cell survival was shown as the percentages of control viable cells.

Flow cytometry for cell death analysis.

For cell death analysis, cells treated with distilled water (control), 1 μM of U0126, 5 μM of sodium selenite and 1 μM of U0126 plus sodium 5 μM of sodium selenite for 72 h and then collected by centrifugation at 300 x g for 3 min at 25 ° C. Annexin V staining was performed according to the manufacturer' s protocol (BD Biosciences,

San Jose, CA, USA). After centrifugation, cells were washed with PBS, and resuspended in binding buffer at 1×10^6 cells/mL. An aliquot (100μL) of the solution containing 1×10^5 cells was transferred to tube, and 5μL each of Annexin V-FITC and 1 μg/mL PI were added. After vortexing, cells were incubated for 1 h at 4 °C in the dark, and 400μL of binding buffer was added to each tube. Flow cytometry was performed on a FACSCalibur system (BD Biosciences, San Jose, CA, USA) within 1 h.

Protein expression assay by immunoblotting

Total cell lysates were prepared in 200 μL of lysis buffer according to the manufacturer's protocol (Cell Signaling Technology, Beverly, MA, USA). Protein concentrations were measured using the Bradford assay with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's directions. Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad

Laboratories) and electrotransferred onto Hybond™-ECL nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK). Blots of proteins were immuno-labeled with anti-phospho-Akt1/2/3 rabbit polyclonal antibodies (1:1000, Santa Cruz Biotechnology), anti-P21 rabbit monoclonal antibody (1:1000, Cell Signaling Technologies, Beverly, MA, USA), anti-P53 mouse monoclonal antibody (1:1000, Cell Signaling Technologies, Beverly, MA, USA), anti-phospho-ERK mouse monoclonal antibody (1:1000, Cell Signaling Technologies, Beverley, MA, USA), anti-phospho-JNK mouse monoclonal antibody (1:1000, Cell Signaling Technologies, Beverley, MA, USA), and anti-β-actin mouse monoclonal antibody (1:1000, Cell Signaling Technologies, Beverley, MA, USA). Blots were washed with TBST buffer (Tris-buffered saline; 0.2% Tween 20; Sigma-Aldrich) and incubated for 1 h at room temperature with peroxidase-conjugated AffiniPure rabbit anti-mouse IgG (1:2500; Cat No: 315-005-045, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or peroxidase-conjugated AffiniPure mouse anti-rabbit IgG (1:2500; Cat No: 211-005-109, Jackson ImmunoResearch

Laboratories). Labeled proteins were detected using an enhanced chemiluminescence detection system (Amersham Bioscience). The phosphorylated forms of Akt, ERK, JNK, and 90^{RSK} were calculated in relation to their total forms. The quantitative measurement was performed by Image J program ver. 1.80 (National Health Institute, USA).

Patients

The research protocol was approved by the institutional review board (IRB No. 2019-10-039-001), and the need for written informed consent was waived. A total of 3628 patients with PTC who underwent total thyroidectomy were reviewed. The cases of occult thyroid cancer, which was occasionally found at histologic exam after surgery were excluded. Of those 3628 patients, 114 non-occult cases with concomitant GD were identified and included in the present study. All patients underwent neck ultrasonography and computed tomography preoperatively to evaluate tumor location and cervical

lymph node (LN) metastasis. Patients with suspicious LN enlargement performed therapeutic LN dissection in addition to total thyroidectomy. Data pertaining to patient age, sex, body mass index, and pathological features, including tumor size, extrathyroidal extension (ETE), resection margin involvement, and LN metastasis were collected. Follow-up duration and recurrence status were also recorded.

Postoperative management and follow-up

Sodium selenite treatment was performed in accordance with patient or surgeon's preference. In case of selenium supplementation, 1000 μg of sodium selenite was used at both 1 day before and the day of surgery. After total thyroidectomy, all patients underwent thyroid-stimulating hormone suppression therapy. Radioactive iodine treatment was considered in patients with high risk for recurrence (incomplete tumor resection, gross ETE, or distant metastases). Follow-up evaluations, including physical examination, neck

ultrasound, serum thyroglobulin, and thyroglobulin antibodies, were performed at intervals of 6–12 months.

Statistical analysis

The statistical significance of the differences between groups was assessed using Student's t-test with R 3.4.0 (R Development Core Team, Vienna, Austria). Data are expressed as the mean±standard error of the mean (S.E.M.) of at least three independent experiments. Categorical variables were compared using the chi-squared test. Data derived from continuous variables of different groups were compared by Student T test. Statistical significance was determined using a p value of < 0.05 as the threshold.

III. Results

The effect of MEK-ERK inhibitor (U0126) on cell proliferation.

To determine the optimal concentration for ERK signaling inhibition, I treated TPC1 cells and 8505C cells with 1nM, 10nM, 100nM, 1 μM, and 10 μM of U0126. As shown in Fig. 1, U0126 reduced viability of both TPC1 and 8505C cells in a dose-dependent manner. Treatment with 1 μM U0126 decreased survival of thyroid cancer TPC1 cells to 47% and that of 8505C cells to 55%, respectively (Fig. 2). However, treatment of normal thyroid HTori-3 cells with 10 μM U0126 did not lead to the same reduction in viability as observed in the other two cell lines. These results indicated that ERK inhibition is not crucial for normal thyroid cells, but for thyroid cancer cells. I selected the concentration of 1 μM of U0126 in the ensuing studies to observe the efficacy of sodium selenite.

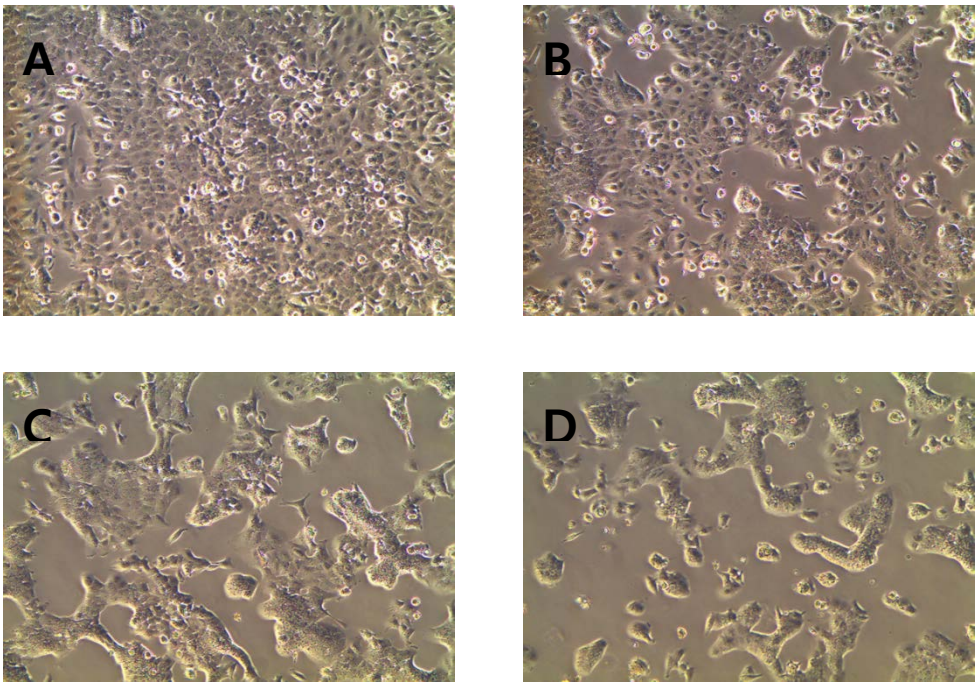

Figure 1. Morphologic change of 8505C thyroid cancer cells after U0126 treatment of A) 10nM, B) 100nM, C) 1 μM, and D) 10 μM for 72 h.

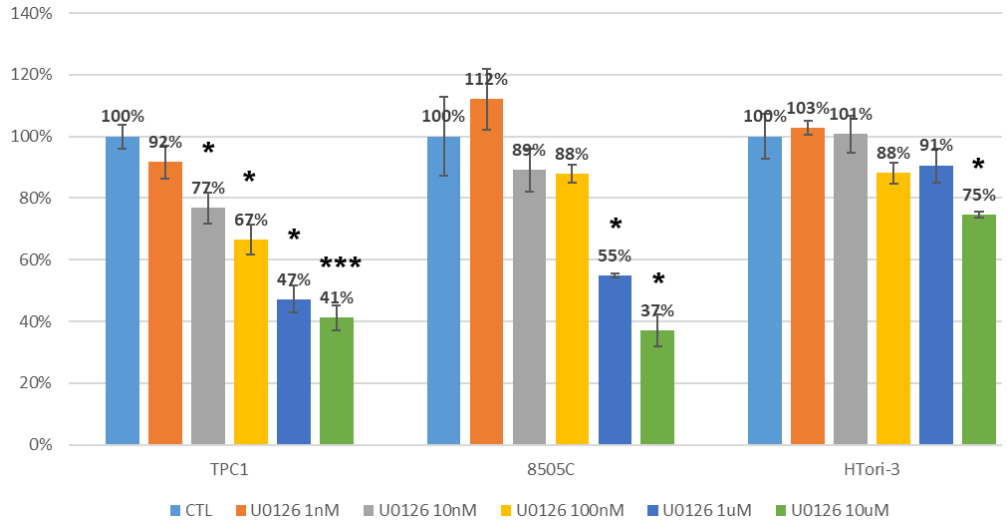

Figure 2. Effect of U0126 treatment on cell proliferation in human thyroid cells. Cells with the RET-PTC1 rearrangement (TPC1), and cells with BRAF mutation (8505C), and normal human thyroid cells (HTori-3) were treated with 1nM, 10nM, 100nM, 1uM, and 10uM of U0126 for 72 hours. Cells treated with 0.01% ethanol was defined as control. Results are represented as mean ± S.E.M. The results are representative of four independent cultures with each conditions in quadruplet. * and *** represent a significant effect of U0126 as compared to the control at $p < 0.05$ and $p < 0.001$, respectively.

The effect of sodium selenite on cell proliferation.

To investigate the anti-proliferative effects of sodium selenite on thyroid cells, I treated HTori-3, TPC1, and 8505C cells with 1 μM, 5 μM, and 10 μM of sodium selenite. Treatment with 5 μM and 10 μM of sodium selenite significantly decreased the viability of HTori-3, TPC1, and 8505C cells (Fig. 3). I selected the concentration of 5 μM of sodium selenite in the ensuing studies to observe the co-treatment effect of sodium selenite.

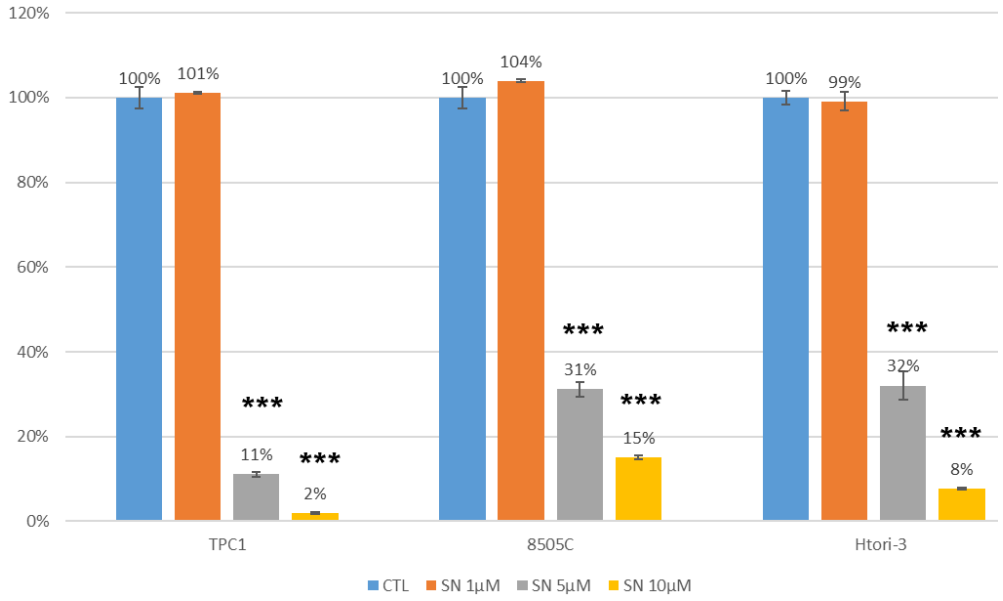

Figure 3. Effect of sodium selenite treatment on cell viability in human thyroid cells. Cells were treated with distilled water (CTL) or with 1 μM, 5 μM, or 10 μM of sodium selenite for 72 hours. Viable cells were counted in a Neubauer chamber. Results are represented as mean ± SEM. The results are representative of four independent cultures with each conditions in quadruplet. * and *** represent a significant effect of U0126 as compared to the control at $p < 0.05$ and $p < 0.001$, respectively.

Signaling pathway analysis by immunoblotting after sodium selenite treatment.

To determine the signaling molecules responsible for sodium selenite treatment, I investigated expression of some important signaling molecules including *AKT, p-AKT, ERK, p-ERK, ERK, p-JNK, p21* and *p53* (Table 1). Expression of *p-ERK* was significantly downregulated among them in HTori-3, TPC1, and 8505C cells (Fig. 4). Other signaling molecules did not show consistent changes. These results indicated that ERK signaling was the major pathway affected by sodium selenite treatment in thyroid cells.

Table 1 Expression of *AKT, p-AKT, ERK, p-ERK, ERK, p-JNK, p21* and *p53* after sodium selenite treatment for 72 h

Protein expression	HTori-3		TPC1		8505C	
	Control	5uM Selenium	Control	5uM Selenium	Control	5uM Selenium
Akt	73.5 ± 7.9%	54.9 ± 8.1%	89.1 ± 3.3%	56.4 ± 5.9%	86.5 ± 0.7%	67.7 ± 3.7%
Phosphorylated-Akt	91.8 ± 6.5%	88.0 ± 9.4%	88.5 ± 5.6%	67.9 ± 2.1%	97.8 ± 0.6%	55.9 ± 0.2%
ERK	76.9 ± 1.7%	63.9 ± 5.9%	74.1 ± 0.8%	70.2 ± 5.7%	65.3 ± 3.5%	78.5 ± 0.1%
Phosphorylated-ERK	88.4 ± 3.3%	27.8 ± 3.5%	84.9 ± 6.0%	32.4 ± 7.6%	96.3 ± 7.1%	36.5 ± 7.9%
Phosphorylated-JNK	99.4 ± 5.9%	101 ± 7.1%	70.0 ± 1.7%	5.6 ± 0.4%	99.0 ± 6.7%	93.7 ± 9.9%
p21	74.8 ± 2.1%	67.5 ± 5.4%	15.2 ± 0.6%	73.9 ± 4.8%	78.1 ± 1.1%	66.3 ± 7.3%
p53	99.0 ± 0.1%	94.1 ± 5.2%	16.0 ± 8.3%	90.8 ± 2.0%	101 ± 4.1%	90.6 ± 7.2%

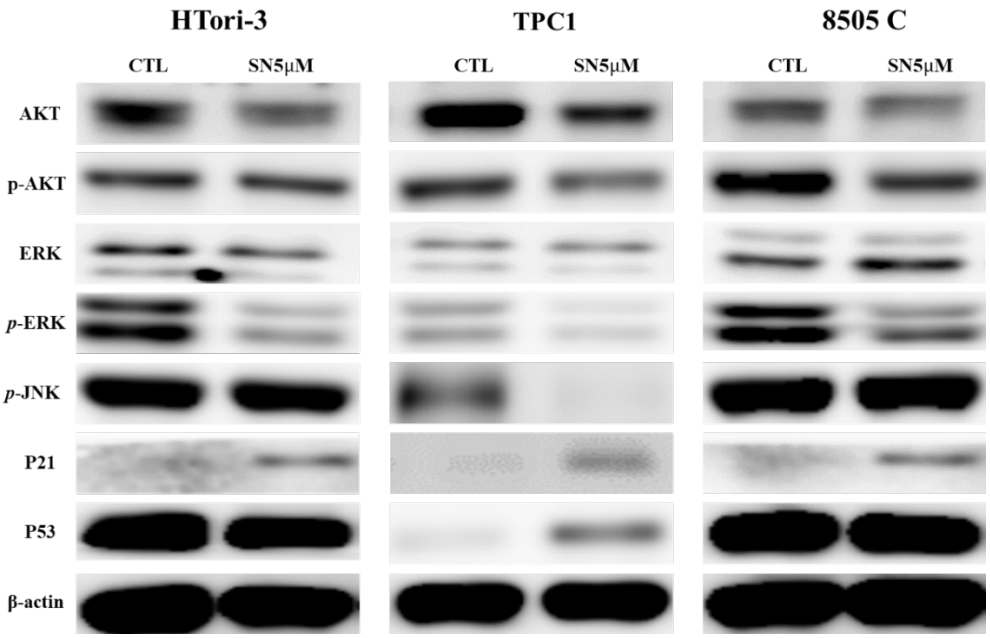

Figure 4. Expression of *AKT, p-AKT, ERK, p-ERK, ERK, p-JNK, p21* and *p53* after sodium selenite treatment for 72 h. A total of 5 × 10^5 cells of TPC1, 8505C, and HTori-3 cell lines were seeded in DMEM containing 10% fetal bovine serum. Cell extracts were analyzed by western bolt to detect the proteins indicated on the right.

The combination effect of sodium selenite and MEK-ERK inhibitor (U0126).

To observe the combination effect of U0126 and sodium selenite on thyroid cancer cells, TPC1 and 8505C cells were co-treated with 5 μM of sodium selenite and 1 μM of U0126. The combination treatment led to a reduction in the survival of cells (Fig. 5). These results showed that the combination of U0126 and sodium selenite enhanced anti-cancer effect of U0126 in thyroid cancer cells.

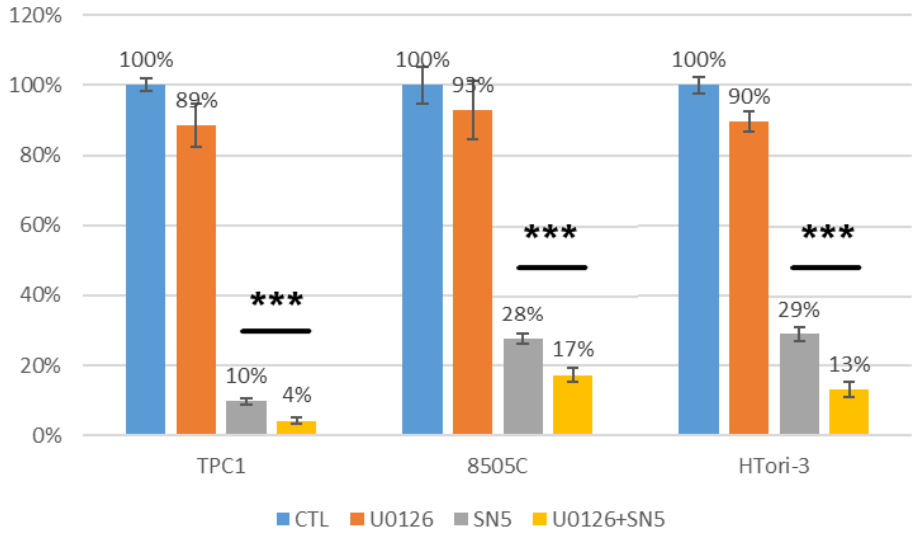

Figure 5. Viability after sodium selenite and MEK–ERK inhibitor (U0126) treatment. Thyroid cells were treated with 0.01% ethanol (CTL), 1 μM U0126, 5 μM sodium selenite, and 1 μM U0126 with 5 μM sodium selenite for 72 h. Viable cells were counted in a Neubauer chamber. Results are represented as mean ± S.E.M. The results are representative of four independent cultures with each conditions in quadruplet. *** represent a significant effect of U0126 with 5 μM sodium selenite as compared to the 5 μM sodium selenite alone at $p <$ 0.001.

Downregulation of p-ERK expression after sodium selenite treatment.

To confirm the effect of sodium selenite on ERK signaling, I investigated its effect on the expression of *ERK*, *p-ERK*, and $p90^{RSK}$(Table 2). Expression of *p-ERK* was the most significantly down-regulated in both of TPC1 and 8505C cancer cells after sodium selenite treatment (Fig. 6). Decreased expression of $p90^{RSK}$ further indicated that sodium selenite down-regulated ERK signaling in thyroid cancer cells. These results showed that ERK signaling is involved in anti-cancer effect of sodium selenite on the growth of thyroid cancer cells.

Table 2. Expression of *ERK*, *p-ERK*, and *p90^{RSK}* after U0126 and sodium selenite treatment for 72 h

Protein expression	HTori-3	TPC1	8505C
Control			
ERK	68.2 ± 4.8%	60.6 ± 4.5%	71.0 ± 6.0%
Phosphorylated-ERK	57.7 ± 5.5%	96.6 ± 3.9%	44.5 ± 1.4%
p90^{RSK}	86.3 ± 1.5%	77.0 ± 5.5%	86.5 ± 1.1%
After U0126 treatment			
ERK	84.9 ± 5.7%	63.8 ± 1.2%	78.2 ± 4.4%
Phosphorylated-ERK	56.7 ± 1.6%	63.1 ± 0.5%	87.2 ± 2.7%
p90^{RSK}	89.0 ± 3.5%	87.9 ± 0.2%	73.2 ± 3.3%
After U0126 and selenite treatment			
ERK	84.5 ± 3.8%	102.3± 4.2%	94.6 ± 0.5%
Phosphorylated-ERK	29.3 ± 2.4%	17.9 ± 2.0%	66.5 ± 4.8%
p90^{RSK}	50.0 ± 2.1%	51.2 ± 3.6%	59.6 ± 1.1%

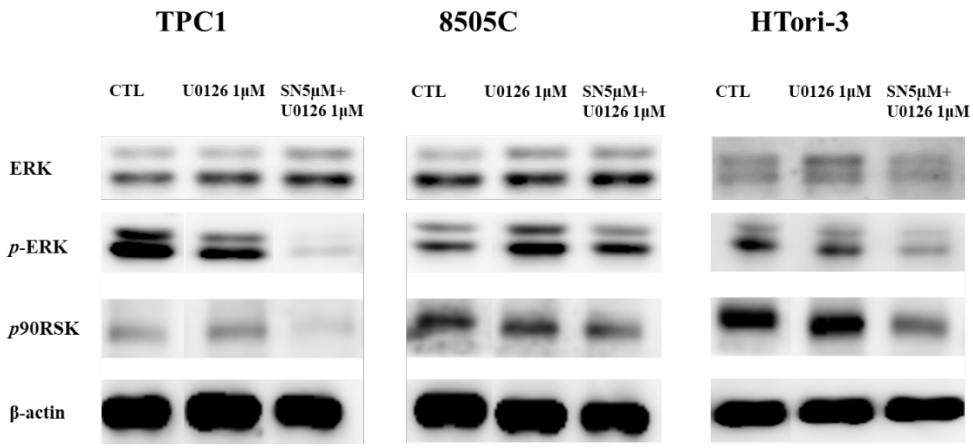

Figure 6. Expression of *ERK, p-ERK,* and $p90^{RSK}$ after sodium selenite treatment for 72 h. A total of 5 × 10^5 cells of TPC1, 8505C, and HTori-3 cell lines were seeded in DMEM containing 10% fetal bovine serum. Cell extracts were analyzed by western bolt to detect the proteins indicated on the right.

Characteristics of the patients with GD

Mean age of the patients at the time of surgery was 46.3 ± 12.9 years and 99 (86.8%) were women. The average tumor size was 0.8 ± 0.5 cm. There were 4 (3.5%) patients with gross ETE and 51 (44.7%) patients with microscopic ETE, while 59 (51.8%) patients had no ETE. Lymphatic invasion was found in 2 (1.8%) patients. One (0.9%) patient showed microscopic margin involvement. Central LN metastasis was identified in 34 (29.8) patients, whereas 80 (70.2%) patients had no LN involvement. Distant metastasis was not found in all patients. median follow-up period of patients with GD was 94.1 months [interquartile range (IQR), 69.3-118.8 months].

Comparison of the recurrences rates

The baseline characteristics of the patients with or without selenium supplementation are summarized in Table 3. Of the 114 patients with GD, sodium selenite treatment was performed in 41 patients (36.0%).

Patients with selenium supplementation group showed higher rate of ETE than those without supplementation ($p = 0.019$). There was no significant difference in sex, age, tumor size, and AJCC 7th TNM classification. Recurrence was found in one patient (1.3%) in the GD without selenium treatment group and none (0.0%) in the GD with selenium treatment group. The recurrence-free survival (RFS) showed no difference among groups; the 5-year RFS were 100% in the GD with selenium treatment group, 100% in the GD without selenium treatment group.

Table 3. Comparison of clinicopathological features between patients with and without selenium supplementation

Characteristics	GD with selenium (n=41)	GD without selenium (n=73)	*p* value
Age (years)	47.3 ± 12.4	45.8 ± 13.3	0.551
Female sex	33 (80.5%)	66 (90.4%)	0.133
Body mass index (kg/m^2)	23.9 ± 3.5	23.7 ± 2.7	0.717
Pathologic features			
Tumor size (cm)	0.8 ± 0.4	0.8 ± 0.6	0.747
Extrathyroidal extension			0.019
No	14 (34.1%)	45 (61.6%)	
Microscopic	25 (61.0%)	26 (35.6%)	
Gross	2 (4.9%)	2 (2.7%)	
Lymphatic invasion	2 (4.9%)	0 (0.0%)	0.057
Vascular invasion	0 (0.0%)	0 (0.0%)	NA
Margin involvement	1 (2.4%)	0 (0.0%)	0.180
LN metastasis			0.922

N0	29 (70.7%)	51 (69.9%)	
N1a	12 (29.3%)	22 (30.1%)	
N1b	0 (0.0%)	0 (0.0%)	
^{131}I remnant ablation	18 (43.9%)	25 (34.2%)	0.307
Follow up (months)	101.4 ± 14.0	94.3 ± 35.2	0.129
Recurrence	0 (0.0%)	1 (1.4%)	0.452

GD Graves' disease, *LN* lymph node, *NA* not applicable.

Data presented as mean and standard deviation if not noted otherwise. Categorical data were compared using the chi-squared test. Data derived from continuous variables of different groups were compared by Student T test

Discussion

The Cancer Genome Atlas (TCGA) has extended the understanding about the genetic basis of thyroid cancers [45]. The TCGA analysis and subsequent studies identified various genetic alterations and molecular markers, which played a significant role in the tumorigenesis of PTCs. Those markers can also serve as potential targets for treatment and predictors of aggressive biology [46]. Genetic alterations identified in the TCGA demonstrated two distinct patterns of signaling pathways involved in the growth and proliferation of thyroid tumor: the mitogen-activating protein kinase (MAPK) and the phosphatidylinositol-3-kinase/AKT (PI3K/AKT) pathways [47]. Mutations in the MAPK-signaling pathway are responsible in about 75% of all PTCs and constitutive MAPK activation may lead to dedifferentiation of PTC [48,49].

The MAPK pathway is important in translating extracellular stimuli to a broad range of cellular response. The MAPK pathways can be categorized into three key pathways: the ERK1/ERK2, the Jun

N-terminal kinase (JNK), and p38 MAPK pathways [50]. The ERK1/ERK2 can be up-regulated in various human cancers, whereas JNKs and p83 MAPKs have major roles in inflammation and homeostasis [51]. Although JNKs and p38 MAPKs also have some roles in cancer development including cell differentiation, proliferation, migration, and survival, their functions are complex and not yet fully understood [52,53]. Furthermore, in thyroid cancer, BRAF mutation that alters a key serine/threonine kinase in the MAPK signaling pathway, mostly leads to increased ERK1/ERK2 activation [54]. BRAF V600E mutation increased the activity of BRAF kinase about 500-fold more than the wild type BRAF kinase [55]. Therefore, the downstream molecules in the MAPK-signaling cascade including ERK1/ERK2 can be the main target for patients with thyroid cancer, especially harboring BRAF mutation.

ERK1 and ERK2 have been intensively studied more than 30 years, as the ERK signaling is associated with cancer, neurodegeneration, developmental disorder, and aging [56]. The ERK

signaling pathway is in a pivotal position in the cell signal-transduction network, and any mistakes in activation can profoundly affect cellular process [57]. Therefore, dysregulation in the ERK pathway is one of the major triggers for the development of cancers [58]. ERK signaling pathway also plays a crucial role in cancer invasion and metastasis [59]. Elevated activation of the ERK signaling pathway can be closely associated with the aggressiveness of cancer [56]. Increased ERK expression has been observed in various human cancers including colon, breast, ovarian, and lung cancer [60,61]. ERK signaling cascade can also be activated in thyroid cancer [62].

U0126 is a specific and noncompetitive inhibitor of both MEK1 and MEK2, and suppresses ERK phosphorylation and activation [63]. Henderson et al. demonstrated that over 10 μM of U0126 completely blocked ERK phosphorylation and inhibited thyroid cancer cell growth, while phosphorylated ERK was detectable with treatment of 1 μM to 5 μM of U0126 [64]. Specht et al. further indicated that the growth inhibition of thyroid cancer cells was significant at >10 μM of U0126,

when PTC cells were treated 1 to 25 μM of U0126 for 3 days [65]. In the present study, after treatment with 1 μM U0126 for 72h, relative cell survivals of both TPC1 and 8505C were 47% and 55%, respectively. Western blot analysis also indicated that 1 μM U0126 did not fully suppress ERK expression as expected. Therefore, I selected 1 μM U0126 as the treatment dose to observe the synergistic effects of the sodium selenite.

Previous studies indicated that sodium selenite exhibited anti-proliferative activity by modulation of various pathways including ERK activation [36-40]. Sodium selenite could up-regulate ERK expression at lower doses, while decrease ERK activation at higher doses [63]. Liu et al. further demonstrated that sodium selenite suppressed oxidative stress, which induced activation of PI3K/AKT and ERK signaling pathways [66]. My results were also compatible with those previous studies, and I selected the optimal dose of 5 μM sodium selenite for ERK down-regulation. When 5 μM sodium selenite was added to 1 μM U0126, ERK cascade was further

downregulated in thyroid cancer cells. Decreased expression of p90RSK confirmed that sodium selenite additionally inhibited ERK signaling pathway. These results supported my notion that selenium inhibited thyroid cancer growth and could be used as adjuvant therapy for thyroid cancer.

Some researchers suggested that sodium selenite induced apoptosis and inhibited cell proliferation in many tumors including breast and colorectal cancers [67–69]. High dose of selenium generated oxygen free radicals and led to an apoptosis of cancer cell, which was provoked by oxidation and crosslinking of sulfhydryl groups [16]. Reactive oxygen species promoted cellular senescence and apoptosis, and thus eliminated cancer cells [70,71]. Selenium also enhanced the *p*53 activity toward DNA repair or apoptosis [72]. In the present study, *p*53 expression showed non–significant changes after treatment of sodium selenite, which suggested that apoptosis might not be essential for the anti–proliferative effect of sodium selenite and U0126 on thyroid cancer cells.

There is no consensus or recommended protocol for the use of sodium selenite in patients with GD-associated PTC, although GD is associated with higher risk of PTC. Despite selenium has been assumed to exert anti-cancer properties, there are a few evidences that selenium supplementation can prevent thyroid cancer [73,74]. Moreover, Wang et al. suggested that selenium treatment did not change the total antioxidant capacity and TSAbs level in mouse model, respectively [75]. A recent review also indicated that data about the association between selenium and thyroid cancer were still inconclusive [76]. In the present study, although selenium treatment did not significantly alter the prognosis of the patients with GD, GD with selenium treatment group showed no recurrence. When I calculated the sample size was calculated using Table 3, over 1000 patients would be required to show the positive results with 5% of significance level and 80% of power. Larger multicenter trial may give us the answer.

This study had several limitations. First, the response to

U0126 can differ in thyroid cancer cell lines harboring mutations [64]. Most thyroid cancers have mutually exclusive single driver mutations. BRAF mutation is found in 60% of PTC, followed by mutations in the RAS gene (13%) with predominant NRAS mutation [45]. In the present study, I used cell lines with the most common mutations including BRAF and RET/PTC1 rearrangement. Additional studies with cell lines harboring other mutations including RAS mutations are needed. Second, although I demonstrated the additive effect of sodium selenite on MEK inhibitors, these results should be validated in *in vivo* studies. Moreover, the anti-proliferative effect of selenium was not replicated in patients with GD. Individual determination of selenium status and personalized treatment would be needed to minimized biases. Last, in the present study, I focused on ERK inhibition by sodium selenite. However, sodium selenite can affect various pathways simultaneously. Further studies for other pathways are warranted.

In conclusion, sodium selenite had an anticancer effect in

thyroid cancer cells through downregulation of ERK. Sodium selenite enhanced anti-proliferative effect of ERK inhibitor. These results suggested that selenium would be a therapeutic agent for treatment-refractory thyroid cancer.

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국문 초록

갑상선암에서 ERK 경로 억제를 통한 아셀레늄산나트륨의 증식억제효과

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배경 및 목적: 갑상선암은 분자유전학적으로 ERK 경로의 돌연변이에 의해 주로 발생하며, RAS-RAF-MEK-ERK 경로를 활성화시키는 돌연변이가 갑상선암 환자의 약 75%에서 발견된다. ERK 경로가 갑상선암의 발생 및 진행에 중요한 역할을 담당하고 있지만, ERK 억제제의 독성 때문에 ERK 억제제는 제한적으로 사용되고 있다. 이번 연구에서는 ERK 억제제의 독성을 피하기 위해, 아셀레늄산나트륨을

사용하여 ERK 억제제의 효과를 증진시킬 수 있는지 확인하였다.

대상 및 방법: 정상 갑상선 세포주로 HTori-3를 사용하였고, 갑상선 유두암 세포주 TPC1과 갑상선 역형성암 세포주 8505C를 갑상선암 세포주로 사용하여 실험을 시행하였다. ERK 억제제인 U0126을 각각의 세포주에 투여하여 세포 생존률을 확인하였다. 아셀레늄산나트륨과 U0126를 병용 투여하였을 때, 세포 생존률을 추가로 감소시키는지 확인하였다. 아셀레늄산나트륨의 작용에 관계하는 분자경로를 확인하기 위하여 *AKT, p-AKT, ERK, p-ERK, ERK, p-JNK, p21* 및 *p53* 단백질 발현을 웨스턴 블롯으로 확인하였다. 아셀레늄산나트륨의 ERK 신호전달경로에 대한 효과를 확인하기 위하여 *ERK, p-ERK, $p90^{RSK}$* 단백질의 변화를 추가로 확인하였다.

아셀레늄산나트륨의 인체 내에서의 효과를 확인하기 위하여, 114명의 그레이브스병이 동반된 갑상선암 환자를 포함하여 총 3628명의 환자 자료를 검토하였다. 일차적 목표로 셀레늄 투약 여부에 따른 갑상선암의 무병 생존률의 차이를 분석하였다.

결과: U0126을 처리하였을 때, TPC1과 8505C 세포주에서는 용량-의존적으로 세포 생존률의 감소가 관찰되었다. 50% 세포가 생존할 수 있는

IC50 값을 기준으로 U0126은 1㎛M, 아셀레늄산나트륨은 5㎛M의 농도로 향후 실험을 진행하기로 결정하였다.

갑상선암 세포주에 1㎛M U0126과 5㎛M 아셀레늄산나트륨을 동시에 투여하였을 때, 1㎛M U0126만 투여하였을 때 보다 추가적인 세포 생존률의 감소가 관찰되었다. 아셀레늄산나트륨의 작용에 관계하는 신호전달경로를 확인하였을 때 ERK 경로가 주요 경로로 확인되었다. 또한 추가 실험에서 $p90^{RSK}$의 감소를 확인하여 아셀레늄산나트륨의 작용이 ERK 경로의 억제에 의한 것임을 입증하였다.

114명의 그레이브스병이 동반된 갑상선암 환자 중, 41명 (36.0%)의 사람이 아셀레늄산나트륨을 투여받았다. 아셀레늄산셀레늄을 투여받지 않은 그레이브스병 동반 환자군 중 1명 (1.3%)에서 재발이 있었으며, 아셀레늄산나트륨을 투여받은 그레이브스병 환자군에서는 재발이 없었다. 각 환자군에서 무병 생존률은 차이가 없었다.

결론: 갑상선암 세포에서 아셀레늄산나트륨은 ERK 경로의 억제를 통해 U0126의 효과를 증가시킬 수 있음을 확인하였다. 아셀레늄산나트륨의 인체 내에서의 효과에 대해서는 추가적인 검증이 이루어져야 한다.

주요어: 갑상선암, 셀레늄, ERK 억제제, U0126, ERK 경로

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