〈Regular Article〉

Anti-cancer stem cell activity of the Src inhibitor dasatinib in thyroid cancer cells

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ABSTRACT Although the prognosis of differentiated thyroid cancer (DTC) is good, those of poorly-differentiated and undifferentiated thyroid cancers (PDTC and UDTC) are poor. Recent preclinical studies have suggested that the Src inhibitor dasatinib is active in thyroid cancer cell lines. We conducted the present study in an attempt to clarify the antitumor activity of dasatinib in PDTC and UDTC.

The expression levels of c-Src, phosphorylated Srcs (p-SrcY416 and p-SrcY527), focal adhesion kinase (FAK), and phosphorylated FAK (p-FAKY861) were immunohistochemically investigated in a case-control series (15 cases of PDTC or UDTC vs. 29 control cases of DTC). The PDTC cell line KTC-1 and UDTC cell line KTC-2 were used to investigate the anti-cell growth and anti-cancer stem cell (CSC) activities of dasatinib. The combined effects of dasatinib and the taxane paclitaxel on anti-cell growth and anti-CSC activities were also tested.

c-Src and p-FAKY861 expression levels were significantly higher, while those of p-SrcY416 were slightly higher in PDTC and UDTC than in DTC. Dasatinib inhibited cell growth in association with G1-S cell cycle retardation and increased apoptosis in both cell lines. Dasatinib significantly decreased the proportion of CSCs and more than additively enhanced the anti-cell growth activity of paclitaxel.

The results of this study suggest that the Src signaling pathway is activated more in PDTC and UDTC than in DTC. The Src inhibitor dasatinib exhibited anti-cell growth and anti-CSC activities. Furthermore, it more than additively enhanced the anti-cell growth activity of paclitaxel.

doi:10.11482/KMJ-E43(2)63 *(Accepted on June 19, 2017)*

Key words: Dasatinib, Src inhibitor, Thyroid cancer, Anaplastic thyroid cancer, Cancer stem cells, Paclitaxel

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INTRODUCTION

Differentiated thyroid cancer (DTC) is the most common type of thyroid cancer, and surgical therapy with or without radioactive iodine (RAI) provides patients with an excellent prognosis. However, poorly-differentiated and undifferentiated thyroid cancers (PDTC and UDTC) have very aggressive biological and clinical behaviors and are resistant to radioactive iodine. Patients with these aggressive tumors have poor outcomes. Therefore, the development of new molecular targeting agents is urgently needed for the control of such incurable thyroid cancers[1].

In recent years, several tyrosine kinase inhibitors (TKIs) have been investigated for the treatment of advanced RAI-resistant thyroid cancers, and some have recently been approved for clinical use: sorafenib and lenvatinib for DTC and PDTC and vandetanib and cabozantinib for medullary thyroid cancers[2]. Lenvatinib has also been used in the treatment of patients with UDTC in Japan due to its favorable efficacy in UDTC patients in a clinical trial conducted in Japan[3]. However, the clinical usefulness of the single administration of these TKIs is limited. More potent TKIs or more active combination therapies with chemotherapeutic agents are needed in order to achieve a more favorable impact in patients with aggressive thyroid cancers.

Recent preclinical studies have suggested that Src family kinases (SKFs) are activated in thyroid cancer cells and the Src inhibitor dasatinib has been shown to suppress their growth and metastasis in various thyroid cancer cell lines including UDTC[4-9]. Therefore, we conducted the present study in an attempt to clarify these findings, particularly the antitumor activity of dasatinib in PDTC and UDTC.

Tumor-initiating cells or cancer stem cells (CSCs) have been suggested to exist in solid tumors including thyroid cancers. CSCs may also play important roles in cancer initiation, progression, recurrence, metastasis, and resistance to anticancer agents and radiotherapy. If an anticancer agent exhibits anti-CSC activity as well as anti-cell growth activity, it may provide cancer patients with longer progression-free survival or a cure[10]. Therefore, we also investigated the anti-CSC effects of dasatinib in PDTC and UDTC cells.

Recent clinical studies have suggested that the taxane paclitaxel is active in patients with advanced DTC or UDTC[11, 12]. In order to examine the hypothesis that dasatinib may enhance the antitumor activity of paclitaxel, the combined effects of dasatinib and paclitaxel on anti-cell growth and anti-CSC activities were also tested in this study.

MATERIALS AND METHODS

Immunohistochemical analysis of Src and focal adhesion kinase (FAK) pathways

A case-control study using formalin-fixed paraffin-embedded thyroid cancer samples was conducted in order to compare the expression levels of molecules related to the Src and FAK pathways in PDTC and UDTC with those in DTC. Fifteen patients with PDTC or UDTC were randomly selected as cases from thyroid cancer patients treated at the Department of Breast and Thyroid Surgery, Kawasaki Medical School between 1983 and 2003, and 29 patients with DTC were selected as gender- and age-matched controls. This study protocol was approved by the Ethical Committee of Kawasaki Medical School (approval number: 2310).

Five-micrometer-thick paraffin sections were dewaxed with xylene and hydrated. Antigen retrieval was performed in a 95℃ water bath for 40 min in Target Retrieval Solution (pH 9.0; Dako, Glostrup, Denmark) for c-Src, p-SrcY416, p-SrcY527, and FAK or in citrate buffer at pH 6.0 for p-FAKY861. Endogenous peroxidase activity was blocked by an incubation in 3% hydrogen peroxide for 5 min. The primary antibodies used were: c-Src (Src antibody, polyclonal, 1:800, Cell Signaling Technology,

Danvers, MA, USA), p-SrcY416 (phosphorylated Src family Tyr416 antibody, polyclonal, 1:100; Cell Signaling Technology), p-SrcY527 (phosphorylated Src family Tyr527 antibody; polyclonal, 1:50; Cell Signaling Technology), FAK (FAK antibody, polyclonal, 1:400, Cell Signaling Technology), and p-FAKY861 (phosphorylated FAK Tyr861 antibody, polyclonal, 1:400; Invitrogen, Camarillo, CA, USA). After an overnight or one-hour incubation, slides were washed in Tris-buffered saline, and EnVision+ (Dako) was then applied. The chromogen used was diaminobenzidine. Sections were counterstained with hematoxylin and mounted. Samples in which the primary antibody was omitted served as negative controls[13, 14].

Protein expression levels in thyroid cancer cells were assessed over the full tissue specimen using the histoscore method. Staining intensity was graded as negative (0), weak (1), moderate (2), and strong (3). The histoscore was calculated by multiplying the percentage of positively-stained tumor cells by the intensity. One pathologist (NK) scored all tissue sections in a blinded manner.

In order to investigate the expression levels of c-Src and phosphorylated Srcs in thyroid cancer cell lines, harvested cells were washed once with cold phosphate-buffered saline (PBS) and centrifuged at room temperature. The cell pellet was fixed with 10% phosphate-buffered formalin overnight and embedded in paraffin[15]. An immunohistochemical analysis was performed as described above.

Western blot analysis

Cells were lysed for protein extraction using Pierce RIPA Buffer with protease inhibitor and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The total protein concentration was measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Isolated proteins were separated by 5-20% SDS-PAGE and transferred to an Immobilon-FL (Merck Millipore Corporation, Billerica, MA, USA). Membranes were blocked with blocking buffer (5% BSA in 1 x Tris buffer saline with 0.1% Tween 20) at room temperature for one hour, subjected to immunoblots using primary antibodies at 4℃ overnight, and then incubated with secondary antibodies at room temperature for one hour. Labeled protein was visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare Japan) with the expression of β-actin as the internal standard. Rabbit monoclonal antibodies against Src, β-actin, p-SrcY416, and FAK were from Cell Signaling Technologies. A polyclonal antibody to p-FAKY861 was purchased from Thermo Fisher Scientific. Secondary antibodies, goat anti-rabbit lgG-HRP, and goat anti-mouse lgG-HRP were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Specific bands were visualized using LAS-1000 UVmini (GE Healthcare, Tokyo, Japan) and analyzed for density using Quantity One 1-D analysis software, ver. 4.5 (BIORAD, Tokyo, Japan).

Reagents

Dasatinib was purchased from CHEMSCENE, LLC (Monmouth Junction, NJ, USA). Paclitaxel was purchased from Sigma Co. (St Louis, MI, USA).

Thyroid cancer cell lines and culture conditions

The poorly-differentiated papillary thyroid cancer cell line KTC-1 and UDTC cell line KTC-2 were established in our laboratory[16, 17]. Both cell lines were routinely maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS).

According to the findings of a comprehensive study conducted by Schweppe RE *et al.*, KTC-1 and KTC-2 were both proven to be unique thyroid cancer cell lines[18]. In all *in vitro* experiments performed in the present study, earlier passages (around the 30th passage) of either KTC-1 or KTC-2 cells were used.

Cell growth analysis

In order to investigate the effects of dasatinib and/or paclitaxel on cell growth, KTC-1 or KTC-2 thyroid cancer cells (1 - 5 × 10^4 cells per well) were seeded on 24-well plates (SB Medical, Tokyo, Japan) and grown in RPMI1640 medium supplemented with 10% FBS at 37℃ in a 5% CO_2 atmosphere for two days. After washing with PBS, cells were incubated with RPMI1640 medium supplemented with 10% FBS plus the indicated concentrations of dasatinib and/or paclitaxel. In the paclitaxel treatment, cells were exposed to paclitaxel for 4 hours, washed with PBS, and cultured with fresh medium without paclitaxel thereafter for three days. In the dasatinib treatment, cells were continuously exposed to dasatinib for three days. After the incubation, cells were harvested and counted with a Coulter counter (Coulter Electronics, Harpenden, UK). Reproducibility was confirmed in at least two separate experiments.

In order to evaluate the antitumor effects of the combined treatments, a combination index based on the 50% inhibitory concentration (IC_{50}) was calculated according to the following formula: combination index = IC_{50} with the combined treatment/IC_{50} with the single treatment. A combination index < 0.5 was considered to be evidence of a more than additive interaction[19].

Cell cycle and apoptosis analyses

In order to investigate the effects of agents on the cell cycle and apoptosis, approximately 5 × 10^5 cells per well were plated into T-25 flasks (Corning Japan, Tokyo, Japan) and cultured in RPMI1640 medium supplemented with 10% FBS for two days. Cells were then washed twice with PBS and cultured for two days in RPMI1640 medium supplemented with 10% FBS plus the indicated concentrations of dasatinib and/or paclitaxel. Duplicate flasks were trypsinized and harvested.

In order to investigate the effects of agents on cell cycle progression, harvested cells were stained with propidium iodide using a CycleTest Plus DNA Reagent kit (Becton Dickinson, San Jose, CA, USA). Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson), and the DNA histogram obtained was analyzed using CELLQuest version 1.2.2 (Becton Dickinson). The percentages of apoptotic cells were measured by FACSCalibur flow cytometry (Becton Dickinson) using an Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's recommendations.

Aldefluor assay

In order to investigate the effects of agents on putative thyroid CSCs, the ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) was used to isolate the population exhibiting strong aldehyde dehydrogenase (ALDH) enzymatic activity. Harvested cells were suspended in Aldefluor assay buffer containing ALDH substrate (BODIP-aminoacetaldehyde, 1 μmol/l per 1 × 10^6 cells) and incubated at room temperature for 40 minutes. As a negative control, cells were treated with 50 mmol/l diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor[20].

Thyrosphere assay

In order to investigate the effects of agents on putative thyroid CSCs, KTC-1 or KTC-2 thyroid cancer cells (approximately 1.5 x 10^5 cells per well) were seeded on 35-mm dishes (SB Medical) and grown in RPMI1640 medium supplemented with 10 % FBS at 37℃ in a 5 % CO_2 atmosphere for two days. After washing with PBS, cells were treated with RPMI1640 medium supplemented with the indicated concentrations of dasatinib and/or paclitaxel. After washing with PBS, cells were incubated with RPMI1640 medium supplemented with 10% FBS plus the indicated concentrations

of dasatinib and/or paclitaxel. In the paclitaxel treatment, cells were exposed to paclitaxel for 4 hours, washed with PBS, and cultured with fresh medium without paclitaxel thereafter for three days. In the dasatinib treatment, cells were continuously exposed to dasatinib for three days. These cells were then dispersed, and single-cell suspensions (5 x 10^3 cells/well) were incubated in MammoCultTM basal medium (STEMCELL Technologies Co., Vancouver, Canada) supplemented with 10% MammoCultTM proliferation supplements (STEMCELL Technologies Co.) in non-adhesive 6-well plates (CORNING Co., NY, USA) for seven days. Thyrospheres larger than 60 μm were counted with an Olympus phase-contrast microscope[20].

Statistical analysis

All values are expressed as the mean ± SE. An ANOVA analysis with StatView computer software (ATMS Co., Tokyo, Japan) was used to compare differences between two groups. A two-sided P value less than 0.05 was considered significant.

RESULTS

Immunohistochemical analysis

No significant differences were observed in gender or age between the PDTC/UDTC group and DTC group (Numbers of male and female patients: 7 and 8, 12 and 17, median age of patients: 67 years old, 66 years old, respectively). Only two cases were diagnosed as PDTC in the PDTC/UDTC group. No variant types of DTCs were included in the DTC group.

The expression levels of molecules related to the Src and FAK pathways in thyroid cancer cells were immunohistochemically evaluated in these thyroid cancer samples using the histoscore system. The expression levels of c-Src and p-FAKY861 were significantly higher in the PDTC/UDTC group than in the DTC group (P = 0.001 and P = 0.0008, respectively, Fig. 1A, E). Similarly, the expression levels of p-SrcY416 and p-SrcY527 tended to be

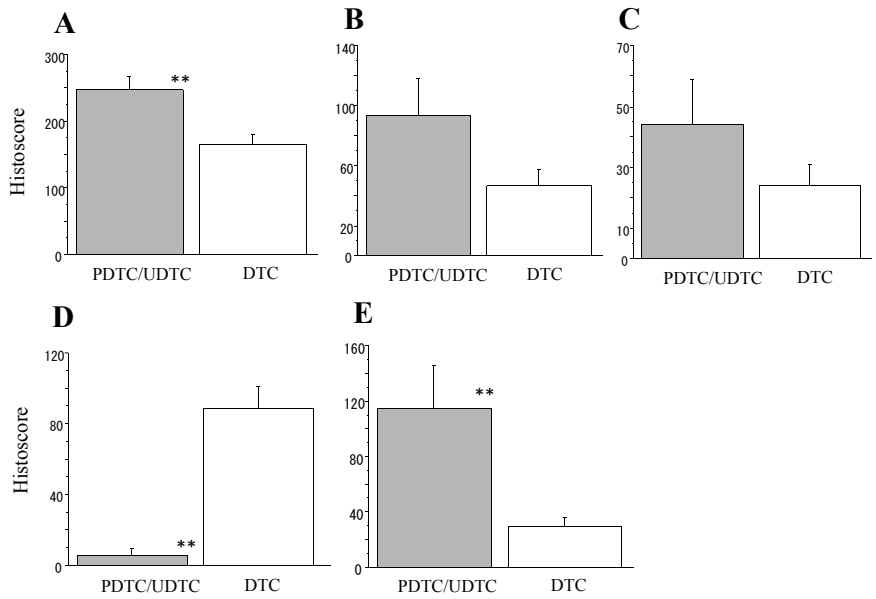

Fig. 1 Immunohistochemical analysis of c-Src, p-SrcY416, p-SrcY527, FAK, and p-FAKY861 expression levels in thyroid cancer samples. Samples were divided into two groups, the PDTC/UDTC group and DTC group. They were immunostained and evaluated using the histoscore system as described in the Materials and Methods. Values are expressed as means ± SEs. *, P < 0.05; **, P < 0.01 significant difference between the two groups.

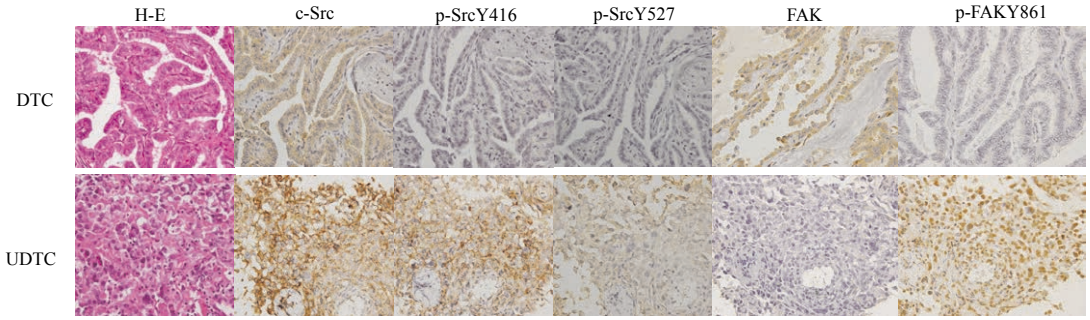

Fig. 2 Representative immunostaining for c-Src, p-SrcY416, p-SrcY527, FAK, and p-FAKY861 as well as H-E staining as controls in DTC and UDTC tissue samples. The original magnification was x 20.

higher in the PDTC/UDTC group ($P = 0.0506$ and $P = 0.1702$, respectively, Fig. 1B, C). In contrast, the expression levels of FAK were markedly lower in the PDTC/UDTC group than in the DTC group ($P < 0.0001$, Fig. 1D). These results suggest that the baseline expression level of c-Src and also the expression level of an activated form of c-Src, p-SrcY416 appeared to be higher in PDTC and UDTC than in DTC. Additionally, a downstream molecule for Src signaling, FAK was strongly activated in PDTC and UDTC. Representative microphotographs for the immunohistochemical analysis are shown in Fig. 2.

Inhibitory activity of dasatinib on Src and FAK signaling

Based on the results of immunocytochemical and Western blot analyses, dasatinib (10 or 100 nM) dose-dependently decreased the expression of p-SrcY416 and p-FAKY861, but not that of c-Src or FAK in the KTC-1 and KTC-2 cell lines (Figs. 3A, B, and 4A-F).

Anti-cell growth activity of dasatinib

Dasatinib dose-dependently inhibited cell growth in the KTC-1 and KTC-2 cell lines (Fig. 5A). IC_{50}s were 11.64 ± 4.85 nM and 18.91 ± 4.94 nM, respectively. Based on our previous study using breast cancer cell lines, IC_{50}s ranged between 100 nM and 5,000 nM under similar culture conditions[13]. These findings suggest that the two thyroid cancer cell lines used in the present study are very sensitive to dasatinib.

In order to elucidate the mechanisms responsible for the anti-cell growth activity of dasatinib, its effects on cell cycle progression and apoptosis were investigated. Dasatinib dose-dependently increased the cell population at the G1 phase and decreased that at the S phase, i.e., induced G1-S cell cycle retardation in both cell lines (Fig. 5B, D). Furthermore, dasatinib dose-dependently increased the cell population at the sub-G1 phase, and also increased apoptotic fractions measured by the Annexin V assay in both cell lines (Fig. 5B-E).

Anti-CSC activity of dasatinib

Dasatinib dose-dependently decreased putative CSC populations, ALDH-positive cell populations measured by the Aldefluor assay, and cell populations building thyrospheres measured by the thyrosphere assay in KTC-1 cells (Fig. 6A, C). Similarly, dasatinib dose-dependently decreased the proportion of ALDH-positive cells and number of thyrospheres in KTC-2 cells (Fig. 6E, F). In addition, representative results of the Aldefluor assay (Fig. 6B) and thyrosphere assay (Fig. 6D) in KTC-1 cells treated with dasatinib are shown.

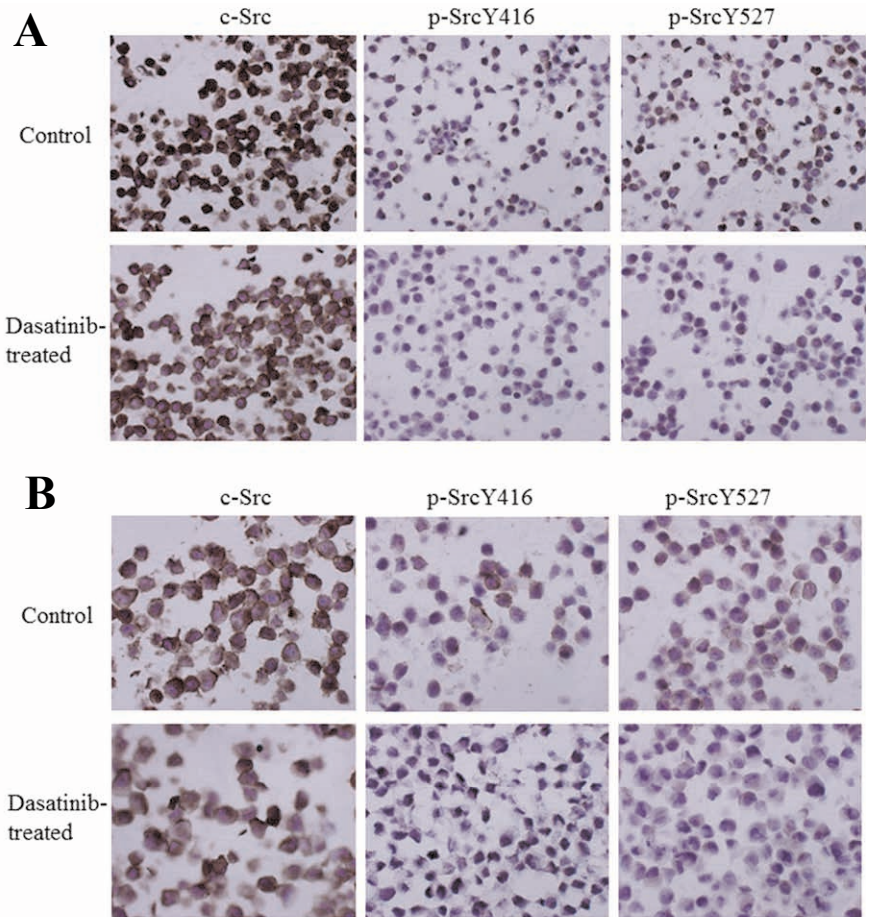

Fig. 3 Immunocytochemical analysis of the effect of dasatinib on c-Src, p-SrcY416, and p-SrcY527 expression levels in KTC-1 cells (A) and KTC-2 cells (B).

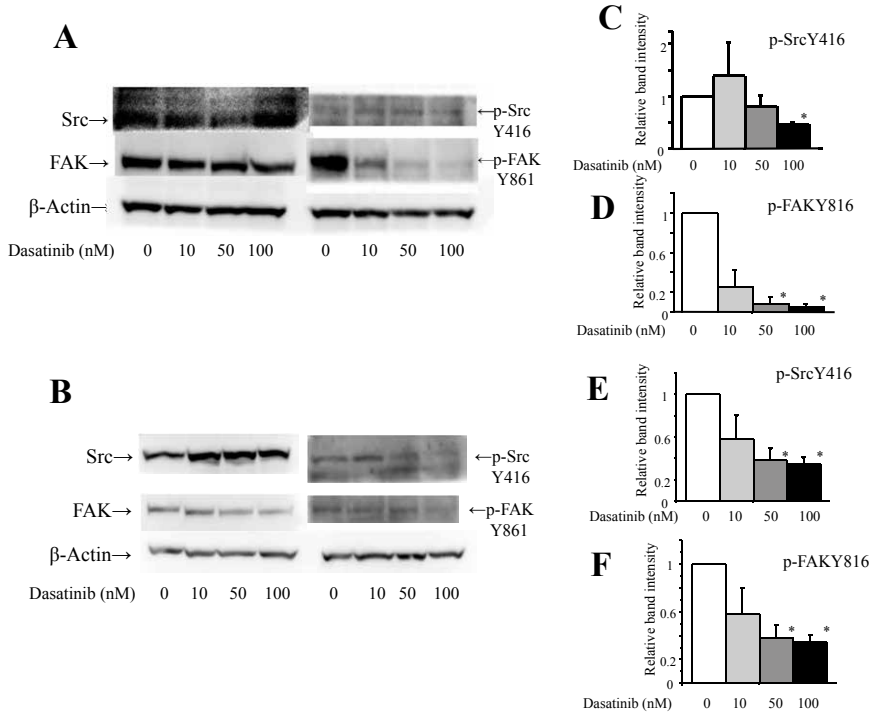

Fig. 4 Western blot analysis of effects of dasatinib (10 or 100nM) on c-Src, p-SrcY416, FAK and p-FAKY861 expression levels in KTC-1 cells (A) and KTC-2 cells (C). Band intensities for p-SrcY416 (B, D) and p-FAKY861 (C, E) were analyzed in KTC-1 and KTC-2 cells, respectively. and of dasatinib (10, 50 or 100nM) on pan-FAK and p-FAKY861 expression levels in KTC-1 cells (B) and KTC-2 cells (D). Values are expressed as means ± SEs of control. *, $P < 0.05$ significantly different from the control.

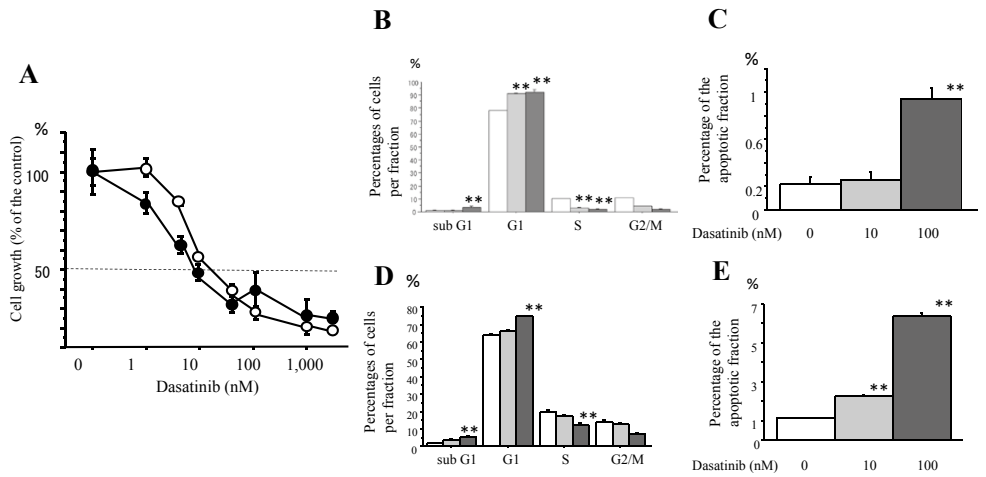

Fig. 5 Effects of dasatinib on growth (A: closed circles for KTC-1 and open circles for KTC-2), cell cycle progression (B for KTC-1 and D for KTC-2), and apoptosis (C for KTC-1 and E for KTC-2) in thyroid cancer cells. Both cell lines were treated with 10 or 100 nM dasatinib for three days in the cell growth assay or two days in the cell cycle and apoptosis assays as described in the Materials and Methods. Values are expressed as means ± SEs of % control. The white bars denote as control values, grey bars as 10 nM dasatinib-treated, and black bars as 100 nM dasatinib-treated. *, $P < 0.05$; **, $P < 0.01$ significantly different from the control.

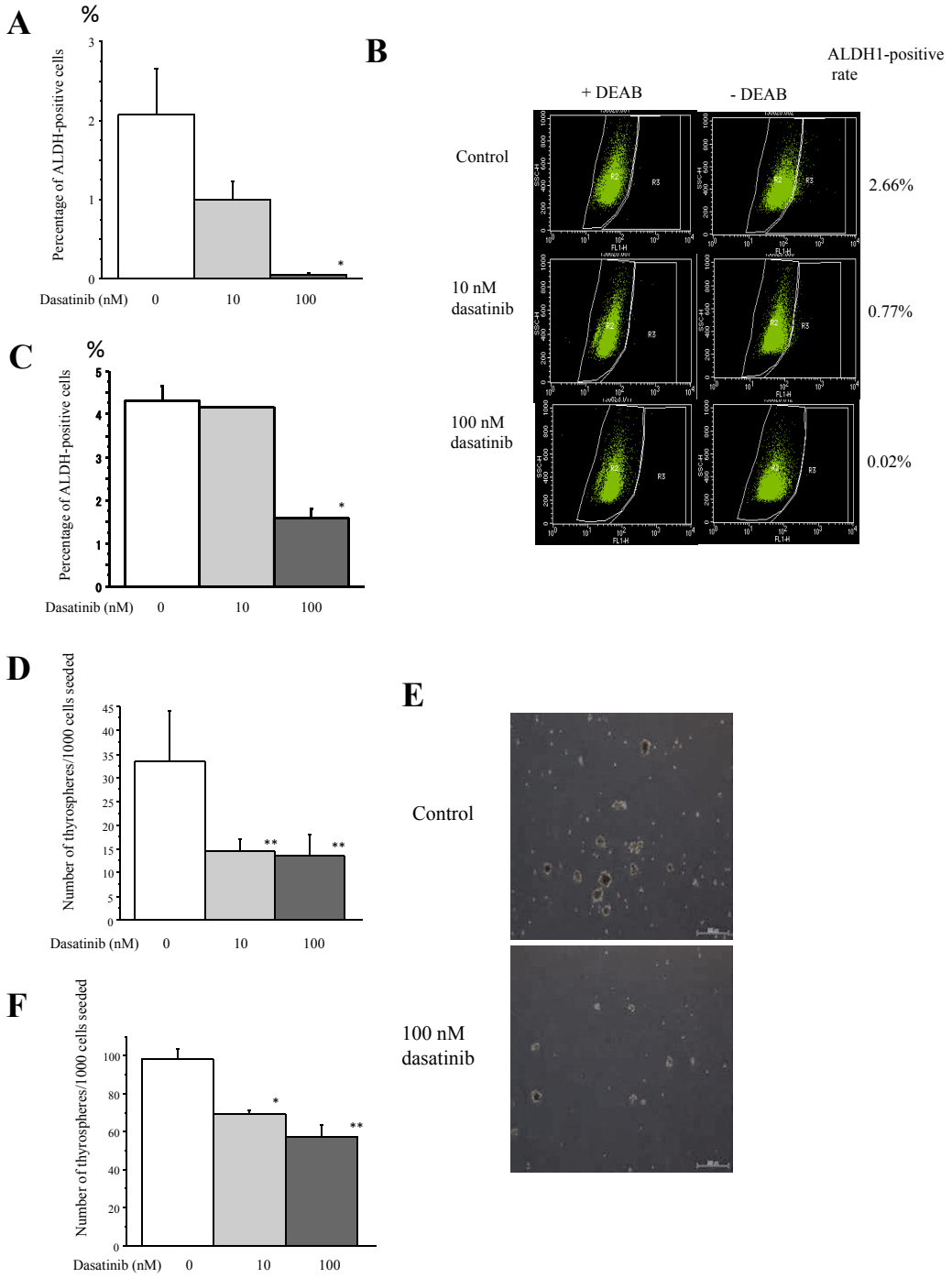

Fig. 6 Effects of dasatinib on the proportion of ALDH-positive cells and number of thyrospheres/1,000 cells seeded in KTC-1 cells (A, C) and KTC-2 cells (D, F). Cells were treated with dasatinib for three days. The percentage of ALDH-positive cells and number of thyrospheres were measured as described in the Materials and Methods. Values are expressed as means ± SEs. White bars, control; light grey bars, 10 nM dasatinib-treated; dark grey bars, 100 nM dasatinib-treated. * $P < 0.05$; ** $P < 0.01$ significantly different from control cells. Representative results of the Aldefluor assay (B) and thyrosphere assay (E) in KTC-1 cells treated with dasatinib are also shown. The dots in the R3 area were ALDH-positive cells (B).

Combined antitumor effects of dasatinib and paclitaxel

Combined treatments of dasatinib and paclitaxel more than additively inhibited cell growth in both cell lines (Fig. 7A, B). Combination indexes at IC_{50} for paclitaxel were 0.03 ± 0.00 (n = 2) for KTC-1 cells and 0.10 ± 0.03 (n = 2) for KTC-2 cells.

In order to elucidate the mechanisms responsible for the effects of the combination treatments, the combined effects of the agents on cell cycle progression and apoptosis were investigated. Dasatinib significantly enhanced increases in apoptotic fractions induced by paclitaxel, but not G1-S cell cycle retardation in both cell lines (Fig. 8A-D).

Combined anti-CSC effects of dasatinib and paclitaxel

Paclitaxel alone modestly decreased the putative CSC population and number of thyrospheres/1000 cells seeded measured by the thyrosphere assay in KTC-1 cells, but not in KTC-2 cells (Fig. 9A-D). The combined treatment of dasatinib and paclitaxel enhanced the decrease in the CSC population induced by paclitaxel in KTC-1 cells (Fig. 9B).

DISCUSSION

SFKs play important roles in cell survival, proliferation, adhesion, invasion, and angiogenesis during cancer development. SFKs comprise at least nine family members that share similar structures and functions. The overexpression and/or activation of SFKs are frequently observed in cancer tissues. These findings suggest that SFKs are promising targets for cancer therapeutics. A number of preclinical studies revealed that small-molecule SFK inhibitors exert potent antitumor and anti-metastatic effects in various cancer models, some of which are under early phase clinical trials[21].

Dasatinib is a potent inhibitor of multiple tyrosine kinases including BCR-ABL, c-KIT, platelet-derived growth factor receptor, and SFKs. It has been mainly used in the treatment of patients with chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. However, the antitumor effects of dasatinib have also been examined in a wide variety of solid tumors in clinical trials as a potent inhibitor of SFKs[22, 23]. Positive findings have been reported for patients with breast or ovarian cancer[24–27].

Regarding thyroid cancer, a few studies have

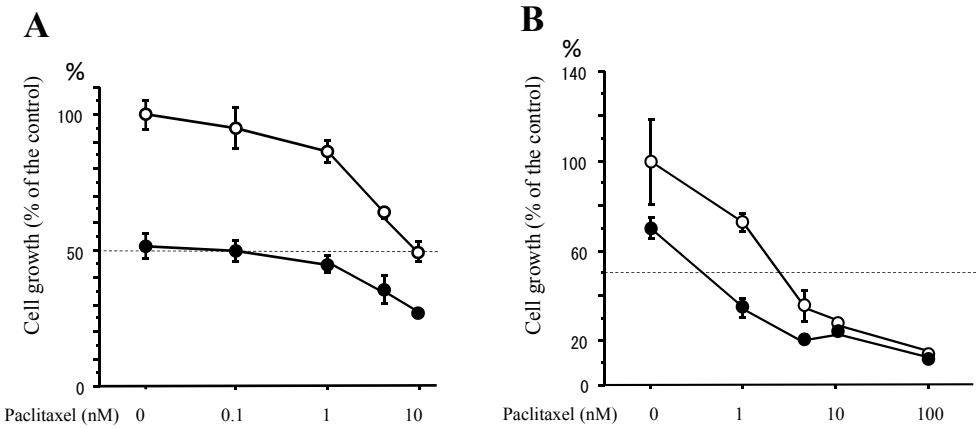

Fig. 7 Growth inhibitory curves of combined treatments of dasatinib and paclitaxel in KTC-1 cells (A) and KTC-2 cells (B). Cells were treated with or without 10 nM dasatinib plus the indicated concentrations of paclitaxel for three days. Values are expressed as means ± SEs. Open circles, paclitaxel alone-treated; closed circles, paclitaxel plus 10 nM dasatinib-treated.

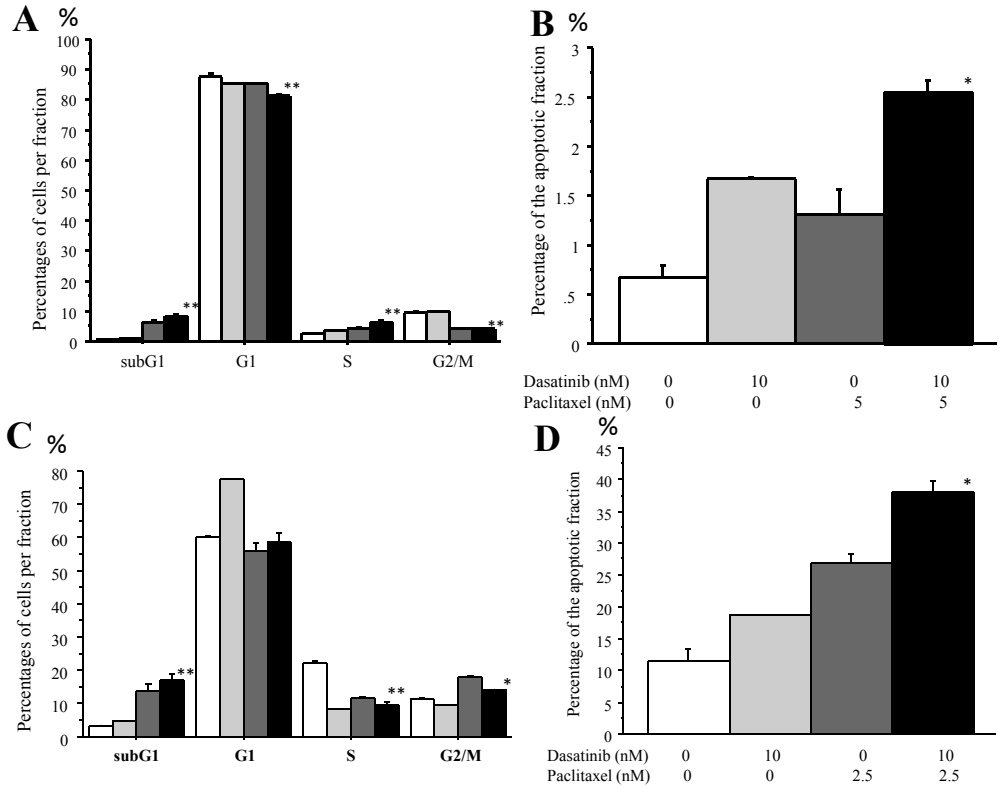

Fig. 8 Effects of combined treatments of dasatinib and paclitaxel on cell cycle progression and apoptosis in KTC-1 cells (A, B) and KTC-2 cells (C, D). Cells were treated with or without 10 nM dasatinib and paclitaxel (2.5 or 5 nM) for two days. The percentages of cells at each cell cycle phase were analyzed as described in the Materials and Methods. The percentage of apoptotic cells was analyzed as described in the Materials and Methods. Values are expressed as means ± SEs. White bars, control; the lightest gray bars, dasatinib alone; the second lightest gray bars, paclitaxel alone; and the darkest gray bars, dasatinib plus paclitaxel. * $P < 0.05$; ** $P < 0.01$ significantly different from control cells.

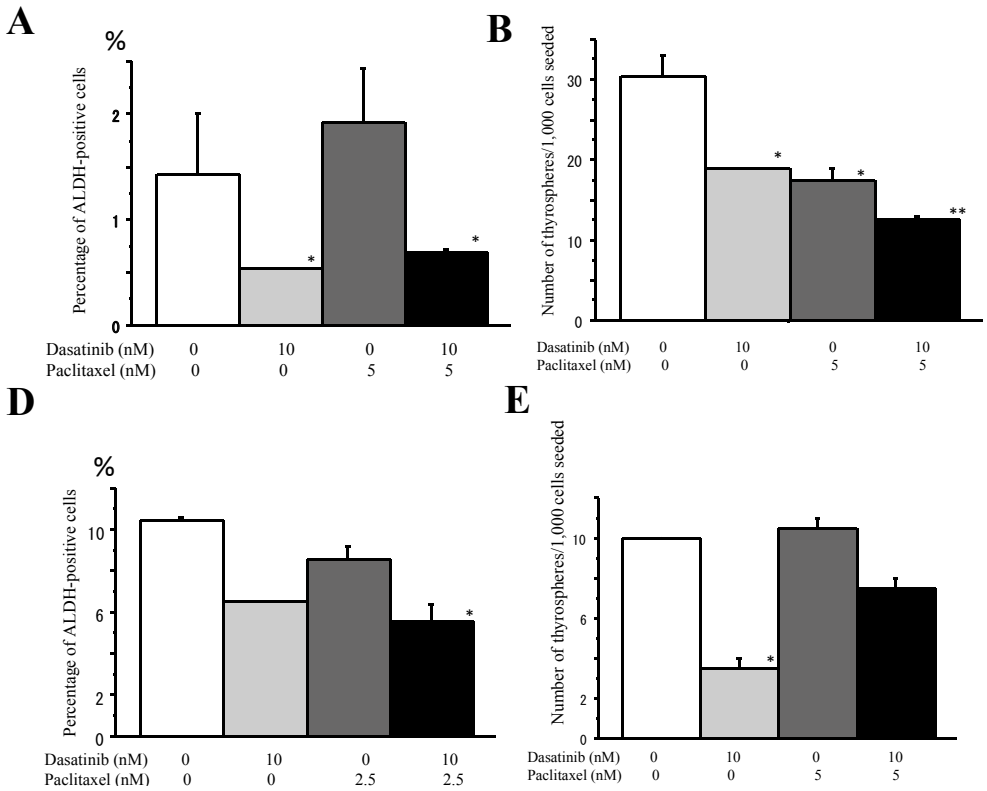

Fig. 9 Effects of dasatinib and paclitaxel on the proportion of ALDH-positive cells and number of thyrospheres/1,000 cells seeded in KTC-1 cells (A, B) and KTC-2 cells (C, D). Cells were treated with or without 10 nM dasatinib and paclitaxel (2.5 or 5 nM) for two days. The percentage of ALDH-positive cells and number of thyrospheres were measured as described in the Materials and Methods. Values are expressed as means ± SEs. White bars, control; the lightest gray bars, dasatinib alone; the second lightest gray bars, paclitaxel alone; and the darkest gray bars, dasatinib plus paclitaxel. * $P < 0.05$; ** $P < 0.01$ significantly different from control cells.

measured the expression levels of SFKs in thyroid cancer tissues. Michailidi C *et al.* reported that the expression levels of total Src and FAK were significantly higher in papillary thyroid cancer than in benign thyroid nodules in an immunohistochemical study. They also suggested that total Src expression levels correlated with tumor size, while total FAK expression levels correlated with capsular and lymphatic invasion in thyroid cancers[28]. Cho NL *et al.* conducted global tyrosine kinome profiling for papillary thyroid cancer tissues and showed that phosphorylated Src expression was up-regulated and correlated with invasion[29]. Schweppe RE *et al.* reported that FAK, a critical substrate and effector of Src, was phosphorylated at tyrosine residue 861 in most papillary thyroid cancers in an immunohistochemical study[14]. These findings prompted us to investigate the expression levels of c-Src, phosphorylated Src, FAK, and phosphorylated FAK using immunohistochemistry in the present study.

Since PDTC and UDTC are relatively rare diseases, an immunohistochemical study using a case-control method (PDTC/UDTC as the case and DTC as the control) with gender and age matching was conducted in this study. The results of the present study suggest for the first time that the expression levels of c-Src, phosphorylated Src, and phosphorylated FAK were significantly higher in PDTC/UDTC than in DTC. In contrast, FAK expression was significantly lower in PDTC/UDTC than in DTC (Figs. 1, 2). FAK expression has been suggested to correlate with malignant progression in thyroid tumors[28]. De-differentiation from DTC to PDTC/UDTC may decrease FAK expression levels in spite of the accelerated activation of FAK. Further immunohistochemical analyses using a larger number of thyroid cancer samples are needed in order to clarify these results.

The anti-cell growth activity of dasatinib has been studied in various solid tumor cell lines. Based on our previous study using six breast cancer cell lines consisting of four different breast cancer subtypes measured by a similar growth assay to that in the present study, the IC_{50} of dasatinib ranged between 0.10 μM and 7.5 μM[13]. According to a study using eight thyroid cancer cell lines including DTC and UDTC cell lines reported by Chan CM *et al.*, the IC_{50} of dasatinib ranged between 0.03 μM and more than 5 μM[5]. These findings suggest that the PDTC cell line KTC-1 and UDTC cell line KTC-2 are more sensitive to dasatinib than the other breast cancer and thyroid cancer cell lines.

The antitumor mechanisms of dasatinib were investigated in the present study. In line with previous findings from several studies, dasatinib dose-dependently induced G1-S cell cycle retardation in association with an increase in the apoptotic fraction in PDTC and UDTC cell lines (Fig. 5). In contrast, paclitaxel induced G2-M accumulation in association with increases in apoptosis in KTC-2 cells (Fig. 8C, D). The combined treatments of dasatinib and paclitaxel additively increased the apoptotic fraction measured by the cell cycle analysis or Annexin V assay in these cell lines (Fig. 8B, D).

Our previous study and other findings suggest that dasatinib exhibits strong anti-CSC activity in breast cancer cells[15, 30]. CSCs have been suggested to play a pivotal role in cancer progression, invasion, metastasis, and resistance to anticancer agents and radiotherapy. Reductions induced in the proportion of CSCs in cancer tissues by certain anticancer agents may be beneficial for cancer patients to achieve the total cell kill of cancer cells or a longer remission time. The results of this study clearly showed for the first time that dasatinib dose-dependently decreased the proportion of CSCs measured by the Aldefluor assay or thyrosphere assay in PDTC KTC-1 cells and UDTC KTC-2 cells (Fig. 8A-D). In addition, dasatinib constantly decreased the proportion of CSCs regardless of the

effects of paclitaxel in the combined treatments of dasatinib and paclitaxel (Fig. 9A-D). Furthermore, combined treatments of dasatinib enhanced the antitumor activity of paclitaxel in a more than additive manner (Fig. 7A, B). These results suggest for the first time that the combined treatment of dasatinib and paclitaxel is more beneficial for controlling PDTC/UDTC cells than that of paclitaxel alone in terms of antitumor activity and anti-CSC activity.

There are some unresolved questions regarding the mechanisms responsible for the anti-CSC effects of dasatinib in PDTC and UDTC cells. It currently remains unclear whether the inhibitory effects of dasatinib on the Src signaling pathway are the main player regulating the CSC population in these thyroid cancer cells. Dasatinib is a multiple tyrosine kinase inhibitor that inhibits the following kinases: BCR-ABL, SFKs, c-KIT, ephrin type-A receptor, and platelet-derived growth factor receptor β. Although the results of our preliminary immunohistochemical analysis in this study suggest that the Src signaling pathway and its effector molecule FAK were up-regulated in PDTC/UDTC cells in clinical thyroid cancer samples (Figs. 1, 2), it is impossible to simply conclude that the Src signaling pathway is a main regulator of the CSC population in these thyroid cancer cells. In addition, a recent study by Thakur R *et al.* indicated that the combined inhibition of STAT3 with Src or FAK reduced CSC properties and tumor sphere formation more significantly than individual inhibitors in breast cancer cells[30]. Another study by Luo M *et al.* using a FAK knockout transgenic mouse model suggested that FAK plays an important role in the maintenance of breast cancer stem/progenitor cells and tumorigenicity[31]. These findings indicate that the inactivation of the signaling pathways of Src and FAK plus others by dasatinib regulates the proportion of CSCs in thyroid cancer cells.

In conclusion, the results of the present study suggest that the Src signaling pathway is activated more in PDTC and UDTC than in DTC. The Src inhibitor dasatinib exhibited not only anti-cell growth activity, but also anti-CSC activity in the PDTC and UDTC cell lines. Furthermore, dasatinib more than additively enhanced the anti-cell growth activity of paclitaxel in these cell lines. These results suggest for the first time that combination therapy with dasatinib and paclitaxel has potential in the treatment of patients with PDTC and UDTC.

Declaration of interest

J. Kurebayashi received advisory/consultation fees and research funding from Takeda Pharmaceutical Co., Limited. J. Kurebayashi received research funding from Takeda Pharmaceutical Co., Eisai Co., Chugai Co., and AstraZeneca Pharmaceuticals. The other authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr. Yasumitsu Nishimura of the Department of Hygiene, Kawasaki Medical School for his technical advice. We also thank Mrs. Kaoru Tsuboi and Ms. Megumi Ogo for their technical assistance. This work was supported by Research Project Grants from Kawasaki Medical School (26-5 and 27-1) and the Ministry of Education, Culture, Sports, Science, and Technology, Japan (26461964).

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