Growth arrest by activated BRAF and MEK inhibition in human anaplastic thyroid cancer cells

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Abstract. Anaplastic thyroid cancer (ATC) is a rare malignancy that progresses extremely aggressively and often results in dismal prognosis. We investigated the efficacy of inhibiting the activated RAS/RAF/MEK pathway in ATC cells aiming to clarify the mechanism of effect and resistance. Four human ATC cell lines (ACT-1, OCUT-2, OCUT-4 and OCUT-6) were used. OCUT-4 had a BRAF mutation. OCUT-2 had both BRAF and PI3KCA mutations. ACT-1 and OCUT-6 had wild-type BRAF and NRAS mutations. The effects of dabrafenib, a selective inhibitor of the BRAF^{V600E} kinase, and trametinib, a reversible inhibitor of MEK activity, were investigated. Dabrafenib strongly inhibited the viability in BRAF mutated cells by demonstrating G0/G1-arrest via the downregulation of MEK/ERK phosphorylation. Upregulated phosphorylation of MEK was observed in RAS mutated cells after dabrafenib treatment and caused VEGF upregulation, but was not related to the cellular proliferation. Trametinib inhibited the cellular viability to variable degrees in every cell by downregulating ERK phosphorylation. Dual blockade by both inhibitors demonstrated clear cytostatic effect in all the cells. OCUT-4 showed the weakest sensitivity to trametinib, no additional effect of either inhibitor in combination with the other, and an increase of SNAI1 mRNA expression after treatment with inhibitors, suggesting a mechanism for resistance. Our findings demonstrated the efficacy of a mutation-selective BRAF inhibitor and a MEK inhibitor in human ATC cells in a genetic alteration-specific manner.

Introduction

Anaplastic thyroid cancer (ATC) is a rare orphan disease that accounts for 1-3% of thyroid cancers. ATCs progress rapidly

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and are extremely aggressive toward both adjacent organs by invasion and distant organs by hematological dissemination. Because of the highly malignant potential, ATC cases often become lethal within 6 months from the initial diagnosis, despite intensive therapeutic efforts (1-3). No standardized therapeutic strategy has been documented to manage ATC, and experimental multimodal therapies with surgery, chemotherapy and/or radiation therapy have been attempted practically. Regrettably, no effective therapeutic method for ATC has been established to date (4,5).

However, several molecular targeted therapies have achieved successful results against ATCs (6-8). Rosove *et al* (9) reported an impressive case of an ATC patient successfully treated with a selective BRAF^{V600E} inhibitor, vemurafenib. Possible clinical application of this inhibitor has been demonstrated recently in a phase 2 trial in BRAF^{V600E} mutation-positive ATC patients, demonstrating an overall response rate of 29% (2/7) (10). In a study by Kim *et al* (11), BRAF mutation in papillary thyroid cancer (PTC) was found more frequently in East Asian countries compared to the Western countries, and the proportion of PTCs among differentiated thyroid cancers (DTCs) was higher in Japan than Western countries. A considerable proportion of ATCs is thought to be derived from long-lasting DTC, and BRAF mutation was found to be maintained during the phenotypical change from DTC to ATC (12). Although the incidence of BRAF mutation was reported to be relatively less common in ATC (15-24%) than that found in PTC (13-15), a preliminary finding indicated that the rate of BRAF mutation in a population of Japanese ATC patients was high (6 of 14 patients) [Uchino, *et al*, Proceedings of the 20th Annual Meeting of Japan Association of Endocrine Surgeons, O-11 65, 2008 (In Japanese)]. In addition, six of seven thyroid cancer cell lines in our series have a BRAF mutation (16). These observations suggest that the frequency of BRAF mutation in ATC is much higher in Japan compared to Western countries.

A previous study of our group demonstrated a possible effect of molecular therapies targeting epidermal growth factor receptor (EGFR), although the effect was limited to the cells with a preserved RAS/RAF/MEK pathway (17). Our more recent study demonstrated that part of this EGFR-targeted therapy resistance could be overcome with an mTOR inhibitor, although we again observed that the efficacy was limited to the cells with an altered PI3K/AKT/mTOR pathway (18). These
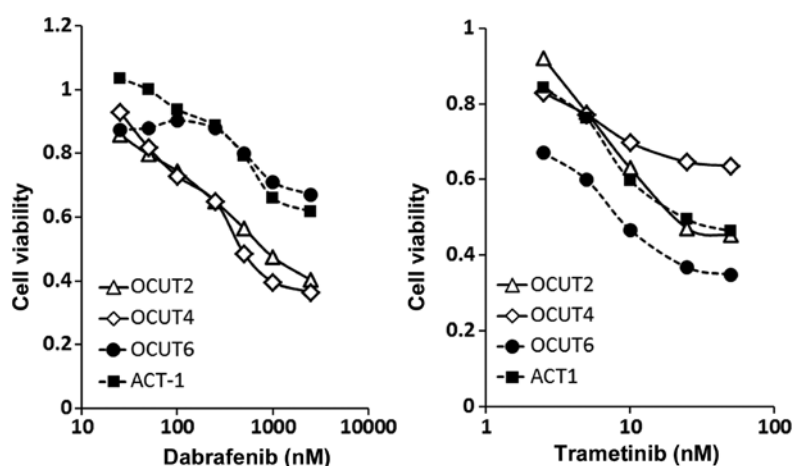
Figure 1. The cell viability assay results demonstrate dose-dependent effects of the inhibitors. Dabrafenib had different effects according to the mutational status of BRAF (left). The trametinib effect was not influenced by the differences in genetic alterations (right).

observations indicated the importance of direct targeting to the RAS/RAF/MEK pathway to manage ATC.

Another research group also demonstrated the importance of BRAF mutation in the aggressive characteristics of thyroid cancer and the efficacy of its inhibition on the management of the disease (19). Several studies described important roles of BRAF gene alteration in genome-wide aberrant methylation (20), vascular endothelial growth factor (VEGF) expression (21), and the induction of epithelial-mesenchymal transformation (EMT) (22).

We conducted a preclinical investigation of the efficacy of inhibiting the RAS/RAF/MEK pathway in a series of authentic ATC cell lines harboring a genetic alteration in either BRAF or NRAS (16). Our specific aims were to determine the efficacies of BRAF/MEK inhibitors in ATC cells and to identify possible differences in the mechanism of blockade between the cell lines according to the differences in the genetic alterations of the cell lines, the levels of VEGF secretion, and/or the expression of EMT markers.

Materials and methods

Cell lines. Four human ATC cell lines, ACT-1, OCUT-2, OCUT-4 and OCUT-6 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin at 37°C with 5% CO_2 in a humidified condition. The ACT-1 cell line was kindly provided by Dr S. Ohata of Tokushima University. The other three cell lines were established in our institute (16). The OCUT-4 cell line had a BRAF (1799T>A; V600E) gene mutation. The OCUT-2 cell line had both BRAF (1799T>A; V600E) and PI3KCA (3140A>G; H1047R) gene mutations. The ACT-1 line harbored the wild-type BRAF gene and an NRAS (181C>A; Q61K) mutation. The OCUT-6 cells had the wild-type BRAF gene and an NRAS (182A>G; Q61R) mutation.

Inhibitors and drugs. Dabrafenib and trametinib were provided by Novartis (Basel, Switzerland).

Cell viability after exposure to the inhibitors. Cells (1x10³) were seeded in each well of a 96-well plastic culture plate

and left overnight. They were then treated with the intended doses of inhibitors for 72 h. After the incubation period, MTT reagent (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, Dojindo Laboratories, Kumamoto, Japan) was added to the final concentration of 0.5 mg/ml, and the cells were incubated again for 2 h under the same condition. The culture plate was centrifuged at 200 g for 5 min, and the supernatant was removed. Dimethyl sulfoxide was added for reaction, and the absorbency at 570 nm was measured with a microplate reader (Infinite F50, Tecan Trading, Männedorf, Switzerland) and calculated using the supplied software. The experiments were carried out three times independently, in triplicate each time, and the average values of the three independent experiments were calculated (17).

Western blotting. Cells were incubated in 10 ml of DMEM containing 1,000 nM dabrafenib or 500 nM trametinib for 1 h. The cells were then rinsed with phosphate-buffered saline (PBS) and lysed with Pro-Prep (iNtRON Biotechnology, Kyungki-Do, Korea). After the protein concentration of each sample was adjusted, the lysates were electrophoretically separated using 4-12% Tris/Gly gels (Novex, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad, Hercules, CA, USA). Membranes were blocked with skim milk and incubated either with anti-human p44/42 MAPK antibody (#4695S; Cell Signaling Technology, Beverly, MA, USA), anti-human phospho-p44/42 MAPK antibody (T202/Y204) (#9101S; Cell Signaling Technology), anti-human MEK1/2 antibody (#8727S; Cell Signaling Technology), anti-human phospho-MEK1/2 (S217/221) (#9154S; Cell Signaling Technology) and anti-human β-actin antibody (#4963; Cell Signaling Technology) using SNAP i.d. (Merck, Darmstadt, Germany). The bands were detected using an enhanced chemiluminescence system (ImageQuant LAS 4000mini, General Electric, Fairfield, CA, USA).

Cell cycle analysis by flow cytometry. The cells treated with 100 nM of dabrafenib or 5 nM of trametinib for 24 h were collected after brief trypsinization, washed with PBS and fixed with 70% cold ethanol. The samples were then treated with
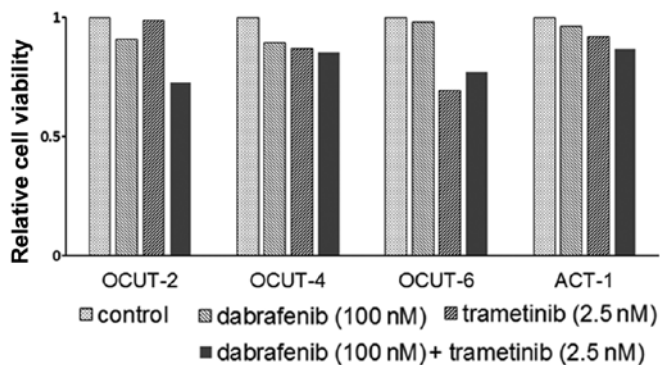
Figure 2. Effects of the dual inhibition of dabrafenib and trametinib on cellular viability. Cells were incubated with the indicated dose of dabrafenib and/or trametinib for 72 h. Cells without inhibitor(s) were set as the control, and the relative viabilities were calculated.

ribonuclease (R6513: Sigma-Aldrich, St. Louis, MO, USA), stained with 10 mg/ml propidium iodine and analyzed by a cell sorter (FACScan, Becton-Dickinson, Mountain View, CA, USA). The cell cycle distributions were quantified using CellQuest software (17).

Measurement of VEGF secretion. Approximately 1×10^5 cells were seeded on a 10-mm plastic culture plate in 5 ml of culture medium, and treated with either or both 100 nM of dabrafenib or 5 nM of trametinib for 24 h. The conditioned medium was then sampled, and the concentrations of VEGF were measured by an enzyme-linked immunosorbent assay (ELISA; Mitsubishi, Tokyo, Japan). Culture medium without cells was used to measure the baseline concentrations (16).

Reverse transcription-polymerase chain reaction (RT-PCR). The cells were treated with 1,000 nM of dabrafenib or 500 nM of trametinib for 1 h. After incubation, total cellular RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) and was reverse transcribed into cDNA with the use of ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a

StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), with TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) for GAPDH (Hs02758991), SNAI1 (Hs00195591), SNAI2 (Hs00950344) and TWIST1 (Hs01675818). The threshold cycle (CT) values were used to calculate the relative expression ratios between control and treated cells. We performed the relative quantification of gene expression by the $2^{-\Delta\Delta CT}$ method (23).

Results

Cell viability after exposure to the inhibitors. The dabrafenib treatment resulted in dose-dependent inhibitions of cell viability. The cellular viability was significantly more strongly inhibited in the OCUT-2 and OCUT-4 cells, which harbor a BRAF V600E mutation, compared to the ACT-1 and OCUT-6 cells, which have the wild-type BRAF gene (Fig. 1, left). The efficacy of trametinib was found in all four cell lines, with no relationship to the gene mutation status. The OCUT-6 line (the NRAS mutant) showed the weakest sensitivity to dabrafenib and the highest sensitivity to trametinib among all of the cell lines. The OCUT-4 line (the BRAF mutant) showed the weakest sensitivity to trametinib (Fig. 1, right). Significant impairment of the cellular viability by trametinib in addition to that by dabrafenib was observed in all cell lines tested (Fig. 2).

Alteration of the phosphorylation status of ERK and MEK after exposure to inhibitors. There was a clear downregulation in the phosphorylation of ERK and MEK after exposure to dabrafenib in the BRAF mutant cell lines, OCUT-2 and -4. The phosphorylation of ERK was also significantly decreased after trametinib exposure in these cell lines, but the phosphorylation of MEK was increased at the same time. The combination treatment with dabrafenib and trametinib resulted in the additional shut-down of ERK phosphorylation. In contrast, an upregulation of the phosphorylation of ERK was observed in the two NRAS mutant cell lines ACT-1 and OCUT-6 after exposure to dabrafenib. Trametinib clearly inhibited the phosphorylation of MEK in these NRAS mutant cells, and significant shut-down of ERK phosphorylation was observed after the dual blockade with dabrafenib and trametinib (Fig. 3).
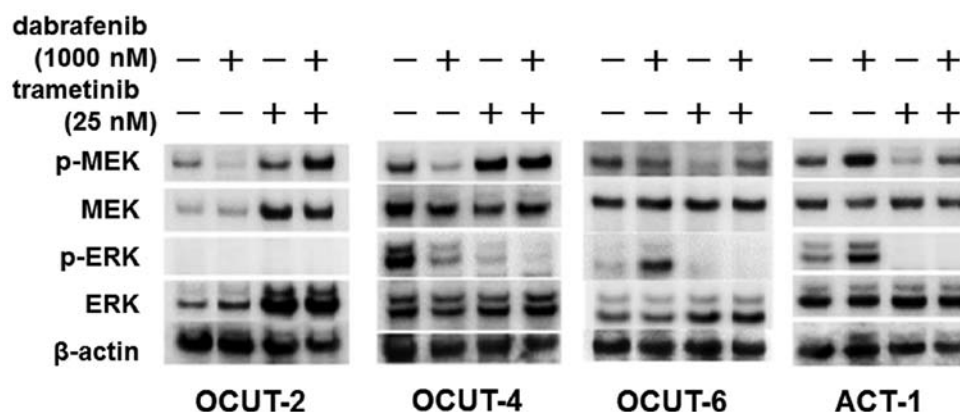

Figure 3. Effects of the inhibitors on the phosphorylation of MEK and ERK protein as shown by western blotting. The phosphorylation of ERK was clearly downregulated with dabrafenib treatment in the OCUT-2 and -4 cells lines with BRAF mutation. In contrast, a marked upregulation of phospho-ERK was observed in the ACT-1 and OCUT-6 cell lines with NRAS mutation. Trametinib decreased the level of phospho-ERK in all four cell lines.
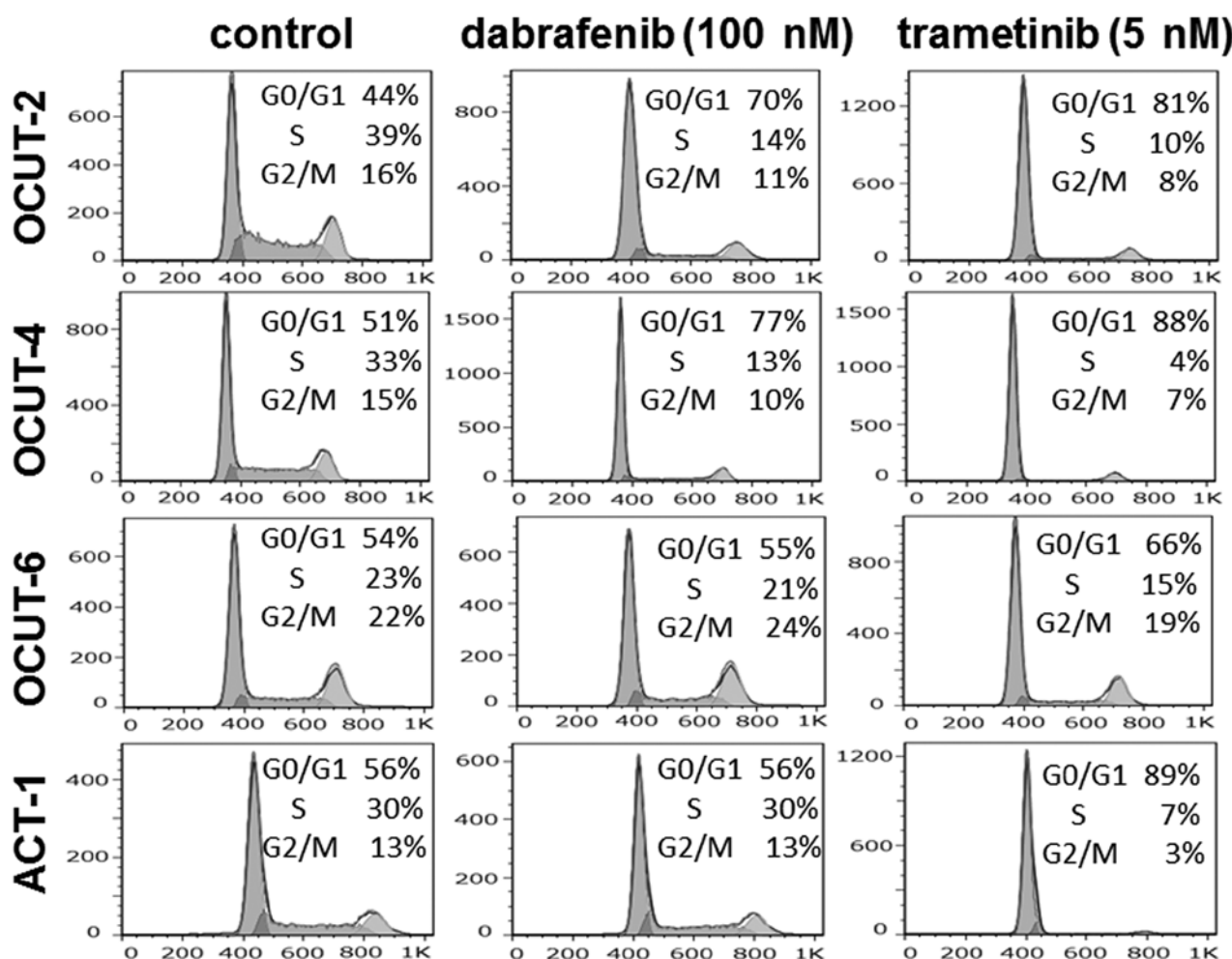
Figure 4. Cell cycle alterations after exposure to the inhibitors. Dabrafenib treatment resulted in cellular arrest in the G0/G1 phase in the OCUT-2 and -4 cells (with BRAF mutations). Trametinib treatment resulted in G0/G1 cell cycle arrest in each cell line.

The effects of the inhibitors on cell cycle progression.
Significant increases in the proportion of cells in the G0/G1 phase were observed after exposure to dabrafenib in both the OCUT-2 and -4 lines. The G0/G1 arrest was not observed in the RAS mutant ACT-1 or OCUT-6 cells after dabrafenib treatment. Trametinib induced G0/G1 arrest in all four cell lines. Sub-G1 population cells were scarcely observed (0.7-4.4%) after treatment (Fig. 4).

The effects of the inhibitors on VEGF secretion of the cell lines. The VEGF concentration in the conditioned medium varied among the cell lines. The OCUT-2 cells demonstrated the highest concentration at 13,500 pg/ml and the OCUT-4 cells showed the lowest concentration at 384 pg/ml in the stable condition. The concentration of VEGF decreased after dabrafenib treatment in the OCUT-2 and -4 cells, whereas it increased after dabrafenib treatment in the two cell lines with wild-type BRAF, i.e., the OCUT-6 and ACT-1 cells. A decrease in the VEGF concentration in the conditioned medium was observed in all four cell lines after trametinib treatment (Fig. 5).

The effects of the inhibitors on the expression of EMT markers.
A significant decrease in the mRNA expression of the EMT markers snail (SNAI1), slug (SNAI2) and twist (TWIST) was
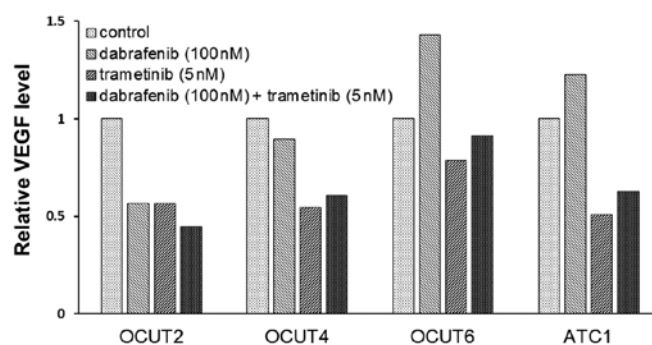

Figure 5. The VEGF concentrations in the conditioned medium after treatment with the two inhibitors relative to the non-treated control. The VEGF concentrations in the conditioned media of the ACT-1 and OCUT-6 cells (with NRAS mutation) were increased after dabrafenib exposure.

observed in the OCUT-2 cells after exposure to dabrafenib and trametinib, alone and in combination. Increased expression of SNAI1 mRNA was seen in the OCUT-4 cell line after exposure to either and both inhibitors. The changes of SNAI2 and TWIST expressions after treatment were not significant in the OCUT-4 line (Fig. 6).
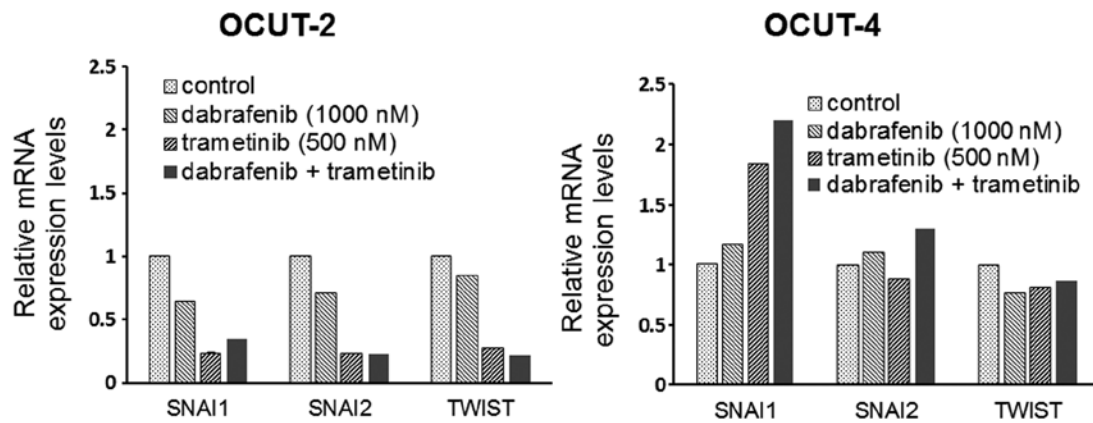
Figure 6. The changes in the mRNA expression of EMT markers after exposure to the inhibitors differed among the cell lines. Downregulations of the markers were clear in the cell lines OCUT-2 (left) and ACT-1 (data not shown). An increase in SNAI1 mRNA was observed after exposure to the inhibitors in the OCUT-4 cells (right).

Discussion

Dabrafenib is a reversible and potent ATP-competitive inhibitor that selectively inhibits the BRAF[V600E] kinase (24). In the OCUT-2 and -4 cell lines, which harbor a BRAF[V600E] mutation, dabrafenib clearly inhibited cellular growth by demonstrating G0/G1 arrest. The strongest inhibitory effect was in the OCUT-4 cells, in which the marked activation of a downstream pathway from BRAF gene was observed in the stable culturing condition. The phosphorylations of MEK and ERK were strongly downregulated by exposure to dabrafenib in OCUT-4 cells, causing a significant G0/G1 arrest. In the OCUT-2 line, the mutation in PI3KCA gene in addition to BRAF[V600E] mutation (16) and signaling through the PI3K/AKT/mTOR pathway can also be expected to contribute to aberrant cell proliferation to some extent. Nevertheless, the dabrafenib treatment resulted in a degree of growth inhibition by G0/G1 arrest in the OCUT-2 cells that was similar to that observed in the OCUT-4 cells. This observation suggested that the activated MAPK/ERK pathway, and not the PI3K/AKT/mTOR pathway, was the main driver for aggressive cell proliferation in OCUT-2 cell line. The results indicate that the inhibition of BRAF[V600E] by dabrafenib might be effective against cancer cells harboring active alterations in both the MAPK/ERK and PI3K/AKT/mTOR pathways.

We observed an upregulation of phosphorylated ERK after dabrafenib exposure in ACT-1 and OCUT-6 cells, which have an NRAS mutation. The mechanism of the upregulation of p-ERK in RAS mutant cells after treatment with a selective BRAF[V600E] kinase inhibitor has been investigated. Dimeric complexes with wild-type BRAF, CRAF or kinase-dead BRAF is able to generate excessive downstream signaling under stimulation by mutant RAS enzyme (25). This mechanism resulted in paradoxical phosphorylation in ERK after BRAF inhibition, but did not contribute to the cell cycle progression or cell growth in the present study. However, the VEGF secretion was clearly stimulated in the NRAS mutant cells after dabrafenib treatment in our study. VEGF is well known as a strong inducer of cancer neo-vasculature that contributes to the arrangement of the cancer microenvironment for aggressive growth. Our present findings indicated one of the potential

mechanisms of tumor growth in dabrafenib-resistant NRAS mutant cancer cells.

The treatment with trametinib, a reversible allosteric inhibitor of MEK1 and MEK2 activation and kinase activity, resulted in universal growth suppression in all four cell lines independent of the mutational status of BRAF or NRAS. A weak growth-inhibitory effect was observed in the BRAF mutant OCUT-4 cells. After trametinib exposure, inhibition of the phosphorylation of ERK was clear, and cell cycle arrest was obviously identified. Nevertheless, the phosphorylation of MEK was strongly induced in OCUT-4 cells by trametinib treatment, suggesting that resistance to trametinib could be caused by a mechanism other than one downstream of the MAPK/ERK pathway. This hypothesis was also suggested by the result of our dual blockade by dabrafenib and trametinib. There was no additional effect of either inhibitor in combination with the other in the OCUT-4 cell line, suggesting a limited effect of inhibiting the MAPK/MEK pathway. In addition, the OCUT-4 cells showed a different EMT marker expression profile after exposure to the inhibitors. Only this cell line showed an upregulation in the expression of the mRNA of SNAI1.

The expression of EMT markers is thought to have a role in the acquisition of resistance to a cytotoxic drug (26). Our present findings indicated that the phenotypical change through the EMT also contributed to the mechanism of resistance to these inhibitors. Several mechanisms have been confirmed to trigger resistance to BRAF inhibition (27-30). Additional investigations are needed to clarify the involvement of the EMT in the effect of BRAF inhibition.

Dabrafenib and trametinib, as monotherapy or in combination, were approved for the treatment of melanomas by the US Food and Drug Administration. Dabrafenib as a treatment for advanced thyroid cancer resulted in durable responses in BRAF-mutant DTC patients (31). A recent report suggested the re-differentiation of iodine-refractory thyroid cancer after dabrafenib treatment (32). These observations clearly indicate the usefulness of BRAF/MEK inhibitors for the management of advanced and inoperable thyroid cancer. The results of the present study suggest the importance of selecting ATC patients in accord with the mutation status of BRAF and RAS when applying inhibitors (33).

Our present findings demonstrated the efficacy of a mutation-selective BRAF inhibitor and a MEK inhibitor in human ATC cell lines. Our observations indicated the existence of a unique driver gene for the aggressive proliferation of ATC cancer cells, and we observed that a cellular growth inhibitory effect can be expected when appropriate inhibitor(s) are selected.

Acknowledgements

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