Significant cytostatic effect of everolimus on a gefitinib-resistant anaplastic thyroid cancer cell line harboring PI3KCA gene mutation

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Abstract. We previously demonstrated the efficacy of gefitinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR), on an anaplastic thyroid cancer (ATC) cell line. We also observed that gefitinib was not effective in regulating cell growth in a different ATC cell line that exhibited an altered EGFR-initiated signal transduction pathway. In the present study, we attempted to regulate the downstream effector of EGFR-Akt-mammalian target of rapamycin (mTOR) pathway by an mTOR inhibitor, everolimus. A total of 8 ATC cell lines were employed, 7 of which were established in our institute. OCUT-2 was known to carry a mutation in the phosphoinositide-3-kinase, catalytic, α polypeptide gene (PI3KCA) and to be gefitinib-resistant, whereas ACT-1 exhibited a remarkable growth arrest by gefitinib. All the cell lines were tested for the cytotoxic effect of everolimus. The mechanisms of cellular toxicity were investigated by EGFR stimulation, cell cycle and concurrent exposure to paclitaxel. In OCUT-2, but not in any of the other cell lines, everolimus achieved a significant growth inhibition (inhibition of 30 and 50% was achieved by concentrations of 0.8 and 5 nM, respectively). The growth in OCUT-2 was inhibited by everolimus, even with concordant EGFR stimulation. This effect was demonstrated by a G2M cell cycle arrest. An additive effect of everolimus onto the cytotoxic effect of paclitaxel was demonstrated at a dose of 1-2 nM. A significant growth inhibitory effect of everolimus on the gefitinib-resistant ATC cell line was demonstrated, suggesting a possible correlation between the efficacy of everolimus and PI3KCA gene mutation and the significance of molecular-targeted therapy in the management of ATC.

Introduction

Anaplastic thyroid cancer (ATC) is a rare entity, accounting for 1-3% of all thyroid cancer cases. ATC is one of the most aggressive malignancies in humans. Multimodal therapies, including surgery, radiation and chemotherapy, are generally used to manage this highly malignant disease. However, the prognosis is poor and the majority of the patients succumb to the disease within a year, with a median survival time of <6 months from the initial diagnosis (1-3). Recently, new approaches to treating ATC by targeted molecular markers have been developed to overcome therapeutic resistance (4-6). We previously demonstrated the efficacy of gefitinib, a tyrosine kinase inhibitor (TKI) of the epidermal growth factor receptor (EGFR), on an ATC cell line, as a potential novel therapeutic strategy. However, we observed that gefitinib was not effective in regulating cell growth in a different cell line that exhibited an altered EGFR-initiated signal transduction pathway (7). A mutation of the phosphoinositide-3-kinase, catalytic, α polypeptide gene (PI3KCA) was later identified in the gefitinib-resistant cell line (8). In the present study, we attempted to regulate the downstream effector of EGFR-Akt-mammalian target of rapamycin (mTOR) pathway by an mTOR inhibitor, everolimus, in a gefitinib-resistant cell line and to demonstrate the mechanism to overcome resistance to EGFR-targeted therapy.

Materials and methods

Chemicals. Everolimus (RAD001) was provided by Novartis (Basel, Switzerland). Paclitaxel was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell lines and cell cultures. We used a panel of 8 authentic human ATC cell lines, including 2 cell lines (OCUT-2 and ACT-1) that were used in our previous experiment (7). OCUT-1-6 were established and characterized in our laboratory. A mutation of B-Raf V600E was found in OCUT-1-5 and a N-Ras mutation was found in the OCUT-6 and ACT-1 cell lines (9). OCUT-2

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Table I. Inhibitory concentrations (30 and 50%) of everolimus in different cell lines.

Cell line	Known gene mutations	IC$_{30}$ (nM)	IC$_{50}$ (nM)
OCUT-1C	B-Raf	14	-[a]
OCUT-1F	B-Raf	8.5	80
OCUT-2	B-Raf, PI3KCA and EGFR	0.8	5
OCUT-3	B-Raf	2.5	19
OCUT-4	B-Raf	23.5	-[a]
OCUT-5	B-Raf	8.4	24
OCUT-6	N-Ras	14	-[a]
ACT-1	N-Ras	17	80

[a]The inhibition rate of the cells did not reach 50% at the highest concentration of 100 nM. IC, inhibitory concentration; EGFR, epidermal growth factor receptor.; PI3KCA, phosphoinositide-3-kinase, catalytic, α polypeptide gene.

was known to carry a mutation of the PI3CA gene (8). ACT-1 was kindly provided by Dr S. Ohata of Tokushima University. Each cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C with 5% CO_2 in a humidified incubator.

MTT assay. The inhibitory effects of everolimus on the viability of these cell lines were measured by the MTT assay (7). Cells (1×10^3) were seeded in each well of a 96-well plastic culture plate and left overnight under the same conditions. The cells were then treated with the intended doses of everolimus for 3 days. After the incubation period, MTT was added to a final concentration of 0.5 mg/ml and the cells were incubated again for 2 h under the same conditions. The culture plate was centrifuged at 200 x g for 5 min and the supernatant was removed. Dimethyl sulfoxide was added for reaction and the absorbency was measured with a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) and calculated using the supplied software. The experiments were performed three times independently, in triplicate each time and the average values of the three independent experiments were calculated.

The effect of everolimus on cell viability following paclitaxel treatment was also measured by the MTT assay. OCUT-2 cells were exposed to 1 nM of everolimus 1 h before, concomitantly, or 1 h after treatment with 1-100 nM of paclitaxel for 72 h.

Effect of everolimus on tumor cell proliferation under EGF stimulation in vitro. Cells (5×10^4) were spread onto a 10-mm plastic dish and left overnight. The cells were then cultured in DMEM without FBS. One nmol of EGF (no. 26190U, Upstate, Lake Placid, NY, USA) was added to a plate to stimulate the EGFR of the cells. The efficacy of everolimus (1 and 10 nM) was investigated by immediate addition following EGF exposure. The cells were counted after 48 h of incubation. The experiments were performed independently in triplicate.

Cell cycle analysis by flow cytometry. Flow cytometry was used to measure the DNA content of individual cells, which allowed us to assess the cell-cycle profiles of the cells treated with everolimus. In preparation for flow cytometry, cells treated with 2 nM of everolimus for 16-72 h were collected
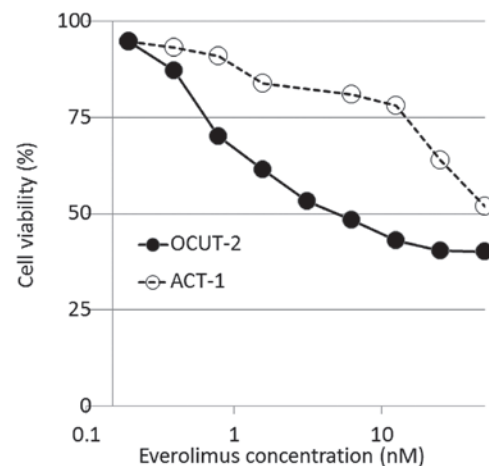

Figure 1. Results of the MTT assay. Similar to the results of the ACT-1 cell line, everolimus exerted a weak growth inhibitory effect on all the cell lines, apart from the gefitinib-resistant OCUT-2 cell line, which harbors a PI3KCA gene mutation and in which everolimus achieved a significant growth inhibition. PI3KCA, phosphoinositide-3-kinase, catalytic, α polypeptide gene.

following brief trypsinization, washed with phosphate-buffered saline and fixed with 70% cold ethanol. The samples were then treated with ribonuclease (R6513; Sigma-Aldrich Corp., St. Louis, MO, USA), stained with 10 mg/ml propidium iodide and analyzed by a FACScan cell sorter (Becton Dickinson, Mountain View, CA, USA). Cell cycle distributions were quantified using CellQuest software (Becton Dickinson).

Results

Growth inhibitory effect of everolimus. A similar growth inhibitory effect of everolimus was observed in all the cell lines, except for OCUT-2. The 30 and 50% inhibitory concentrations of everolimus ranged from 8.4 to 23.5 and from 19 to >100 nM, respectively (Table I). However, in OCUT-2, everolimus achieved a significant growth inhibition compared to that in other cell lines, with 30 and 50% inhibitory concentrations of 0.8 and 5 nM, respectively. The maximal growth inhibitory effect of everolimus on OCUT-2 cells was demonstrated at the concentration of ~20 nM, where ~60% of the cells were growth-inhibited and no further effect was observed
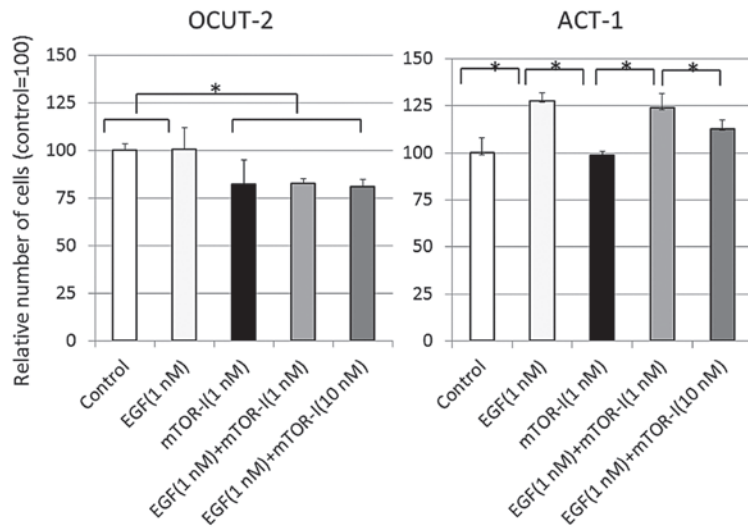
Figure 2. Cancer cell proliferation in reaction to everolimus under epidermal growth factor (EGF) stimulation. OCUT-2 cells did not exhibit any change in proliferation under EGF stimulation and everolimus achieved a significant inhibition of cell proliferation, even under EGF stimulation. By contrast, ATC-1 cell proliferation was significantly stimulated by EGF, but not affected by everolimus. (*P<.01). mTOR, mammalian target of rapamycin.
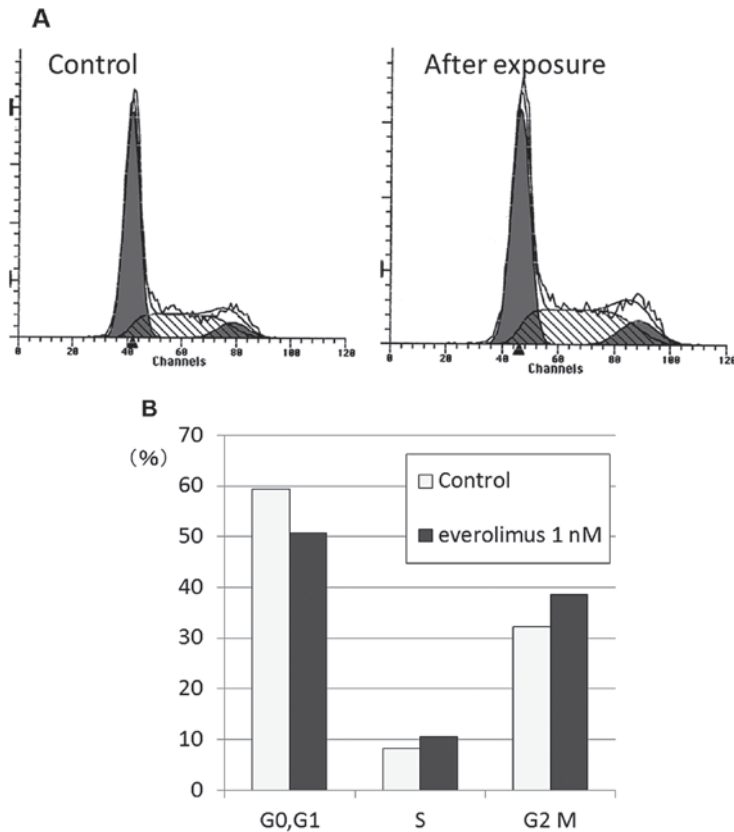

Figure 3. (A) DNA histograms of control cells and cells following exposure to everolimus. (B) An increase in S and G2M phase cell population was observed following exposure, indicating G2M cell cycle arrest.

by increasing the concentration (Fig. 1). There was a significant difference in the sensitivity to everolimus between the ACT-1 and OCUT-1, -3, -4, -5 and -6 cell lines (data not shown).

The proliferation of cancer cells in reaction to EGF clearly differed between the ACT-1 and OCUT-2 cell lines. ACT-1 cells exhibited a significant upregulation of proliferation by stimulation with 1 nM of EGF, as previously reported (7). There was no change in OCUT-2 cell proliferation by EGF stimulation.

Everolimus achieved a significant inhibition of cell proliferation in the OCUT-2 cell line, even under EGF stimulation. A concentration of 1 nM was sufficient to achieve maximal growth inhibition in OCUT-2 cells. By contrast, ATC-1 cell proliferation was significantly stimulated by EGF, but not affected by exposure to 1 nM of everolimus. A high concentration (10 nM) of everolimus was required to achieve a partial inhibition of the upregulated cellular growth of ACT-1 stimulated by EGF (Fig. 2).
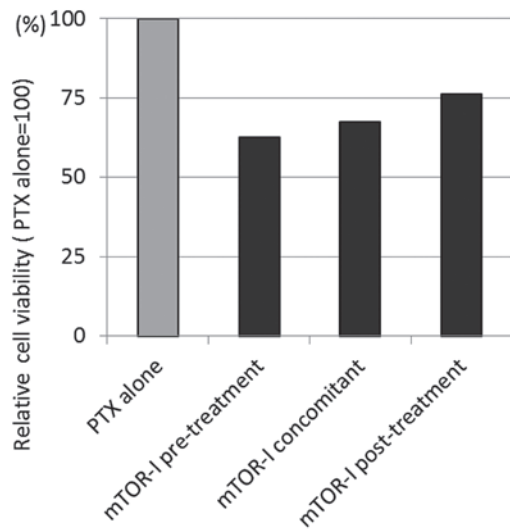
Figure 4. Additive effect of everolimus onto the cytotoxic effect of paclitaxel (PTX). The additive effect of everolimus was more pronounced when it was administered prior to paclitaxel exposure. mTOR, mammalian target of rapamycin.

G2M cell cycle arrest was observed after a 16-h exposure to everolimus (1 nM), with a 19% increased ratio of cells in the G2M phase (32-39%). The G2M arrest continued until 72 h after exposure (Fig. 3).

An additive effect of everolimus onto the cytotoxicity of paclitaxel was demonstrated at a dose of 1 nM. There was a 32% decrease in cell viability following exposure of OCUT-2 cells to 1 nM of paclitaxel in the presence of 1 nM of everolimus compared to paclitaxel alone. The additive effect of everolimus was more pronounced when it was administered prior to paclitaxel exposure (Fig. 4).

Discussion

Conventionally, ATC has been managed with a multimodal therapeutic approach by combining surgery, chemotherapy and external beam radiation. However, the effect of these approaches is limited due to the highly aggressive nature of the tumor and rapid acquisition of resistance to treatment. To overcome this resistance, molecular-targeted approaches have been applied as a possible novel therapeutic strategy. To date, there has been some success in managing this disease (4-6). EGFR is a well known cell membranous receptor and has been reported to be highly expressed in ATC cells (10). Thus, we attempted to manage the disease by inhibiting the kinase activity of EGFR by gefitinib in a previous study and demonstrated the significant efficacy of this TKI in inhibiting cancer cell growth. In the ACT-1 cell line, the cell-proliferating signal through EGFR to mitogen-activated protein kinase kinase (MEK) was clearly inhibited by gefinitib (7). Furthermore, this significant effect was not observed in OCUT-2, a cell line exhibiting a lower level of EGFR expression.

There are two major pathways inducing the proliferation of cancer cells downstream of cell surface EGFR, namely the Raf/Ras/MEK and PI3K/Akt/mTOR pathways. Alterations in both pathways have often been observed in thyroid cancer (11-15). B-Raf, Ras, PI3KCA and phosphatase and tensin

homolog mutations have also been reported in ATC (16-19). Several cell lines harbor one of these gene mutations. By contrast, OCUT-2 is a unique cell line exhibiting alterations in both B-Raf and PI3KCA genes, resulting in completely aberrant proliferation signal generation independent of EGFR-mediated signaling (9). The results of our previous study may, thus, be interpreted as that the efficacy of EGFR-targeted therapy may be determined by the signaling status downstream of EGFR. Therefore, in this study, we attempted to inhibit mutated PI3KCA-generated aberrant growth signaling by an mTOR inhibitor to overcome gefitinib resistance.

A significantly more prominent inhibitory effect on cell proliferation was demonstrated by everolimus in the OCUT-2 cell line, compared to that in other cell lines not carrying alterations in the PI3K/Akt/mTOR pathway, as expected. This observation may indicate the significance of detecting alterations in the PI3K/Akt/mTOR signaling pathway as an indicator of the possible efficacy of everolimus. According to the results of the effects on ACT-1, everolimus was only able to impair part of the proliferation signal generated from EGFR when the signaling pathway was intact. By contrast, a major signal for proliferation was blocked in OCUT-2 cells harboring an aberrantly activated PI3K/Akt/mTOR pathway by a PI3KCA mutation. In addition, OCUT-2 also carrying an active B-Raf mutation, which should cause alternative generation of the proliferating signal in the absence of the PI3K/Akt/mTOR axis, resulted in the incomplete impairment of cell growth.

We observed a marginal increase in the number of cells in the S and G2M phase following exposure to everolimus in the OCUT-2 cell line, suggesting G2M cell cycle arrest. Everolimus is known to cause G0G1 arrest by inhibiting the expression of cyclin D1. However, a very high concentration (5,000-20,000 nM) of everolimus was reportedly required to achieve G0G1 arrest (20,21) compared to the concentration we used in the present experiment. The concentration of everolimus we used in this study was 2 nM, since we were unable to achieve additional inhibition in cell proliferation when increasing the dose to >20 nM in the initial experiments investigating the effect of everolimus on cell viability. The DNA histogram analysis did not identify any cell population with lower or fragmented DNA contents, suggesting apoptosis. These observations may indicate that the effect of everolimus is expressed as a moderate cell cycle arrest in G2M, but not as cell killing within physiological doses.

In the present study, we demonstrated that everolimus inhibited cell growth in cancer cells harboring an altered PI3K/Akt/mTOR signaling pathway. This effect was limited to cell growth arrest and no complete cytotoxicity was observed. As stated earlier, alterations in the PI3K/Akt/mTOR pathway in ATC was not a major genetic abnormality, compared to B-Raf- or Ras-mediated pathway. Farstino *et al* (22) suggested the possible mechanism of LKB1-mediated mTOR pathway upregulation in thyroid carcinoma harboring the B-Raf mutation. Dual inhibition of the Raf/Ras/MEK and PI3K/Akt/mTOR pathways was attempted, with promising preclinical results (23). A combination of a cytotoxic drug with everolimus may be another practical choice to increase the efficacy of treatment. We observed an additive effect of paclitaxel to that of everolimus in the OCUT-2 cell line. Paclitaxel exerts its cytotoxic effect by inhibiting the polymerization of tubulin

in the G2M phase. Further studies are required to maximize the inhibitory effect of everolimus on the mTOR pathway.

In conclusion, a significant growth inhibitory effect of everolimus on a gefitinib-resistant ATC cell line was demonstrated. A possible correlation between the efficacy of everolimus and PI3KCA gene mutation requires further investigation using additional ATC samples with accurate information on genetic alterations. This study indicated the significance of identifying target molecules, or applying target-oriented therapeutic strategies in managing patients with highly malignant ATC.

Acknowledgements

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