

ORIGINAL ARTICLE

FTI-277 and GGTI-289 induce apoptosis via inhibition of the Ras/ERK and Ras/mTOR pathway in head and neck carcinoma HEp-2 and HSC-3 cells

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Summary

Purpose: Head and neck squamous cell carcinoma (HNSCC) is a major malignancy worldwide. Ras overexpression in HNSCC is known to promote tumor cell growth; therefore, inhibition of Ras activation could lead to tumor growth suppression in HNSCC patients. Here, we investigated the effect of FTI-277, a farnesyl transferase inhibitor, and GGTI-287, a geranyltransferase 1 inhibitor, on the Ras signaling pathway in HNSCC cell lines-HEp-2 and HSC-3.

Methods: Cell viability was analyzed using the trypan blue staining exclusion assay. The apoptosis of cells was assessed by flow cytometry and caspase activation analysis. The expression levels of proteins were examined using western blot analysis.

Results: FTI-277 and GGTI-287 induced cell death, enhanced caspase 3 activity, and increased the number of an-

nexin V-positive cells in HEp-2 and HSC-3 cells. FTI-277 and GGTI-287 induced apoptosis in HSC-3 cells at much lower concentrations than that in HEp-2 cells. FTI-277 and GGTI-287 decreased the concentration of phosphorylated ERK1/2 and mTOR via membrane localization of Ras and enhanced Bim expression. Furthermore, FTI-277 and GGTI-287 induced cell death in v-H-Ras-transfected NIH3T3 (NW7) cells and not in empty vector-transfected NIH3T3 (NV20) cells.

Conclusion: FTI-277 and GGTI-287 may be useful as potential therapeutic agents for treating HNSCC patients; moreover, farnesyl transferase and geranylgeranyltransferase 1 inhibitors can be further developed as anticancer agents.

Key words: FTI-277, GGTI-287, head and neck squamous cell carcinoma, Ras

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the major cancers worldwide and includes malignancies arising in the mucous membranes of the oral cavity, pharynx, nose, nasal cavity and larynx [1,2]. Remedial strategies for HNSCC include surgery, radiotherapy and chemotherapy; however, the prognosis for patients in the advanced stage of HNSCC is poor [3].

Ras family proteins, such as H-Ras, K-Ras and N-Ras, are activated in many human cancers; activation of Ras leading to prenylation is accelerated via mutation of receptor tyrosine kinases, including epidermal growth factor receptor, c-MET, and platelet-derived growth factor receptor, and via the constitutive activation of these receptors [4-7]. Mutations in the Ras family proteins have

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been implicated in the pathogenesis of various cancers including lung, pancreatic, and colorectal carcinomas [7-9]. Although the rate of Ras mutation is relatively low in HNSCC, overexpression of the wild-type K-Ras protein in HNSCC cells has been reported and is known to contribute to tumor growth in HNSCC [6,8,10]. K-Ras promotes the activation of a downstream kinase cascade, which includes c-Raf/mitogen-activated protein kinase kinase 1/2 (MEK1/2)/extracellular signal regulated-protein kinase 1/2 (ERK1/2) and phosphoinositol-3 kinase/mammalian target of rapamycin (mTOR), leading to cancer cell growth, survival, and metabolism [11,12]. Therefore, it is possible that the suppression of the Ras signaling cascade could prevent Ras-dependent cell growth in HNSCC cells.

Farnesyl transferase and geranylgeranyltransferase 1 are essential enzymes for the prenylation of Ras proteins; they act by binding to farnesyl pyrophosphate and geranylgeranyl pyrophosphate [13-15]. Previous reports indicate that farnesyl transferase inhibitors, such as FTI-277, induce cell death via the inhibition of Ras farnesylation, and geranylgeranyltransferase 1 inhibitors, such as GGTI-287, promote apoptosis through the suppression of the geranylgeranylation of Ras [16,17]. Additionally, Ras prenylation inhibitors, including statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) and nitrogen-containing bisphosphonates, induce apoptosis via the inhibition of Ras signaling pathways [18-22]. However, the mechanisms by which FTI-277 or GGTI-287 induce cell death by modulating the Ras signaling pathways in HNSCC cells remain unclear. Therefore, in the present study, we investigated the mechanism by which FTI-277 and GGTI-287 induce cell death in HNSCC cell lines, such as HEP-2 and HSC-3 cells.

Methods

Materials

FTI-277 and GGTI-287 were purchased from Merck Millipore (Nottingham, UK). A stock solution of 10 mM FTI-277 was prepared by dissolving it in water and 10 mM GGTI-287 stock solution was prepared by dissolving it in dimethyl sulfoxide before use in the experiments described below.

Cell culture

HSC-3 and HEP-2 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and DS Pharma Biomedical Co., Ltd (Osaka, Japan), respectively. These cells were cultured in minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin, 100 U/ml streptomycin (Gibco, Carlsbad, CA, USA), and 25 mM HEPES (pH 7.4; FUJIFILM Wako,

Tokyo, Japan) in a humidified atmosphere containing 5% CO₂ at 37°C. NV20 control (empty vector-transfected NIH3T3 cells) and NW7 (v-H-Ras-transfected NIH3T3 cells) cells were supplied by Dr. Hiwasa (Chiba University, Chiba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 100 µg/ml penicillin, 100 U/ml streptomycin (Gibco, Carlsbad, CA, USA), and 25 mM HEPES (pH 7.4; FUJIFILM Wako, Tokyo, Japan) in a humidified atmosphere containing 5% CO₂ at 37°C.

Trypan blue dye exclusion assay

The effect of FTI-277 and GGTI-287 on cell viability was assessed using the trypan blue dye exclusion assay as described previously [23,24].

Measurement of the proteolytic activity of caspase 3

The activity of caspase 3 was determined using a caspase-3/CPP32 fluorometric assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. The cells were treated with FTI-277 and GGTI-287 for 36 h. After incubation, the cells were washed with PBS and lysed using the lysis buffer included in the caspase-3/CPP32 fluorometric assay kit. The cell lysates were treated with 1 mM Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (AFC) at 37°C for 1 h. The concentration of AFC released from the substrate was measured on a fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at emission and excitation wavelengths of 505 and 400 nm, respectively.

Annexin V apoptosis assay

Apoptosis was measured using an annexin V-FITC apoptosis detection kit (Becton Dickinson, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, the cells were washed twice in PBS, incubated in binding buffer containing annexin V-FITC for 15 min at room temperature, and analyzed using a BD-LSR Fortessa cell analyzer (Becton Dickinson, Bedford, MA, USA).

Western blot analysis

Whole cell lysates were extracted using lysis buffer (100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% NP-40, 1 µM pepstatin, 1 µM leupeptin, 2 mM sodium orthovanadate, 1 µM calpain inhibitor, phosphatase inhibitor cocktail I/II, and 1 mM phenylmethylsulfonyl fluoride). The cytoplasmic and membrane fractions were extracted using a ProteoExtract Subcellular Proteome Extraction Kit (Merck Millipore, USA). The protein content in the lysates was quantitated using a bicinchoninic acid (BCA) protein assay kit (FUJIFILM Wako, Tokyo, Japan). The cell lysates (40 µg of protein) were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked with 3% skimmed milk and incubated overnight at 4°C with the following antibodies: anti-phospho-mTOR (#2971), anti-phospho-ERK1/2 (#9101), anti-mTOR (#2972), anti-ERK1/2 (#9102), anti-Bcl-2 Interacting Mediator of cell death (Bim) (H-191) (Santa Cruz Biotechnology, CA, USA), and anti-β-actin (Sigma, USA). The membranes

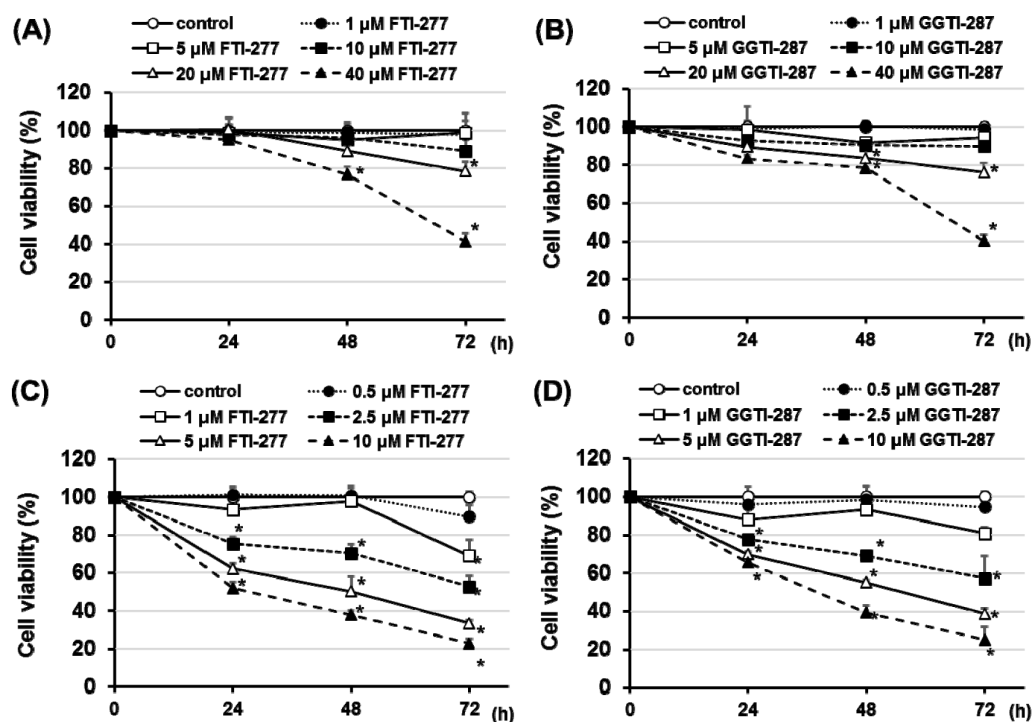


Figure 1. FTI-277 and GGTI-287 decrease the viability of HEP-2 cells and HSC-3 cells. HEP-2 cells were treated with 1–40 μ M concentrations of (A) FTI-277 or (B) GGTI-287, and HSC-3 cells were treated with 0.5–10 μ M concentrations of (C) FTI-277 or (D) GGTI-287. The trypan blue exclusion assay was performed in the HEP-2 and HSC-3 cells after 24, 48, and 72 h of incubation with FTI-277 and GGTI-287. The values are expressed as the mean \pm SD of five independent experiments. *indicates $p < 0.01$ vs. control (ANOVA with Dunnett's test).

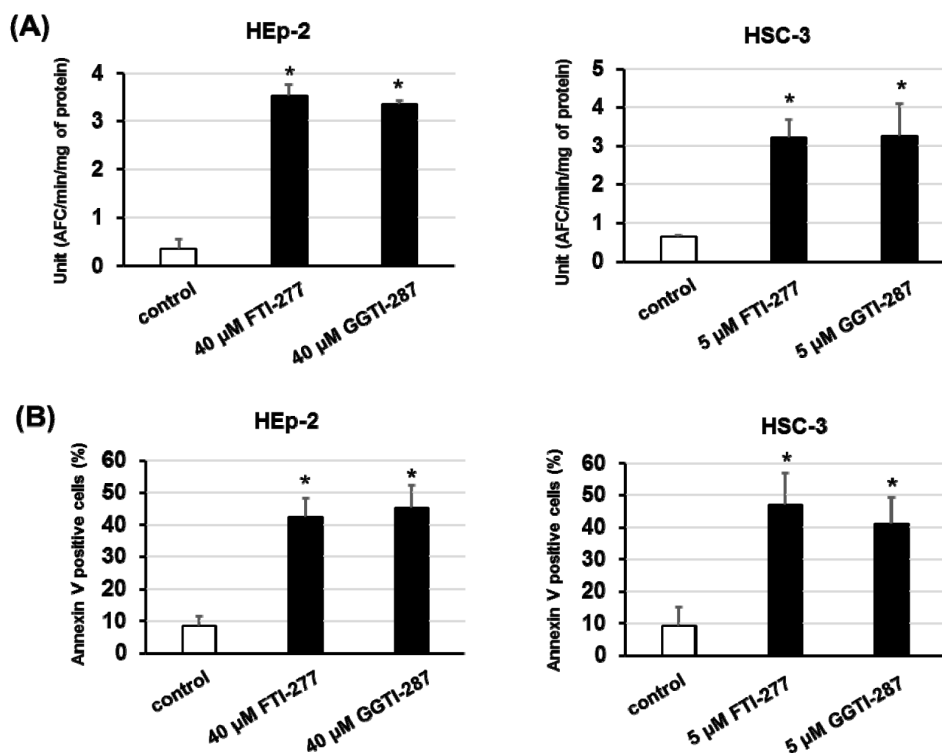


Figure 2. An increase in the percentage of annexin V-positive cells and activity of caspase 3 is associated with FTI-277- or GGTI-287-induced cell death. HEP-2 and HSC-3 cells were exposed to the indicated concentrations of FTI-277 and GGTI-287 for 36 h. (A) The activity of caspase 3 was measured and expressed as picomoles of caspase 3 substrate DEVD-AFC cleaved proteolytically per hour per mg of protein. (B) Apoptosis was measured using an annexin V-FITC apoptosis detection assay kit, and values are expressed as a percentage of annexin V-positive cells. The values are expressed as the mean \pm SD of five independent experiments. *indicates $p < 0.01$ vs. control (ANOVA with Dunnett's test).

were incubated with horseradish peroxidase-coupled sheep anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) for 1 h at room temperature. The reactive proteins were visualized using Luminata Forte Western HRP substrate (Merck Millipore, USA) according to the manufacturer's instructions.

Statistics

All results are expressed as the mean \pm standard deviation (SD) of at least five independent experiments. Multiple comparisons of the data were performed using one-way analysis of variance (ANOVA) with Dunnett's test. P values <0.05 were considered statistically significant.

Results

FTI-277 and GGTI-287 decrease the viability of HEp-2 and HSC-3 cells

The effect of FTI-277 and GGTI-287 on the viability of HEp-2 and HSC-3 cells was assessed using the trypan blue exclusion assay. HEp-2 cells were treated with 1, 5, 10, 20, and 40 μ M concentrations of FTI-277 or GGTI-287 for 24, 48, and 72 h. After treatment with 1, 5, 10, 20, and 40 μ M concentrations of FTI-277 for 72 h, the viability of the HEp-2 cells was 97.9, 98.9, 89.1, 78.3, and 41.5%, respectively (Figure 1A). After treatment with 1, 5, 10, 20, and 40 μ M concentrations of GGTI-287 for 72 h, the viability of the HEp-2 cells was 98.6, 94.3, 89.9, 76.0, and 40.3%, respectively (Figure 1B). HSC-3 cells were incubated with 0.5, 1, 2.5, 5, and 10 μ M concentrations of FTI-277 or GGTI-287 for 24, 48, and 72 h. After treatment with 0.5, 1, 2.5, 5, and 10 μ M FTI-277 for 72 h, the viability of the HSC-3 cells was 89.7, 69.1, 52.9, 33.4, and 23.0%, respectively (Figure 1C). After treatment with 0.5, 1, 2.5, 5, and 10 μ M GGTI-287 for 72 h, the viability of the HSC-3 cells was 94.6, 80.9, 57.4, 38.7, and 25.3%, respectively (Figure 1D). These results indicate that FTI-277 and GGTI-287 decreased the viability of the HEp-2 and HSC-3 cells in a concentration-dependent manner; moreover, FTI-277 and GGTI-287 induced cell death in the HSC-3 cells at much lower concentrations than that in the HEp-2 cells.

FTI-277 and GGTI-287 induce apoptosis by the activation of caspase 3

Apoptosis is induced by an interaction between various initiator and effector caspases. Among these, caspase 3 is considered a crucial effector of the apoptosis pathway [20]. Therefore, we investigated the effect of FTI-277 and GGTI-287 on caspase 3 activation in HEp-2 and HSC-3 cells. Treatment with FTI-277 and GGTI-287 led

to a significant increase in the activity of caspase 3 in the HEp-2 and HSC-3 cells (Figure 2A). Additionally, we observed that treatment with FTI-277 and GGTI-287 significantly increased the percentage of annexin V-positive cells in the HEp-2 and HSC-3 cells (Figure 2B). Thus, these results indicate that FTI-277 and GGTI-287 induced apoptosis in the HEp-2 and HSC-3 cells via caspase 3 activation.

FTI-277 and GGTI-287 suppress the phosphorylation of ERK and mTOR and increase the expression of Bcl-2 Interacting Mediator of cell death (Bim).

FTI-277 is a farnesyl transferase inhibitor, and GGTI-287 is a selective peptidomimetic inhibitor of geranylgeranyltransferase 1. Ras prenylation by farnesyl pyrophosphate and geranylgeranyl pyrophosphate via farnesyl transferase and geranylgeranyltransferase 1, respectively, is required for the membrane localization of Ras [17]. Therefore, we confirmed the effect of FTI-277 and GGTI-287 on the membrane localization of Ras and the phosphorylation of ERK1/2 and mTOR, the downstream signaling molecules of Ras, by west-

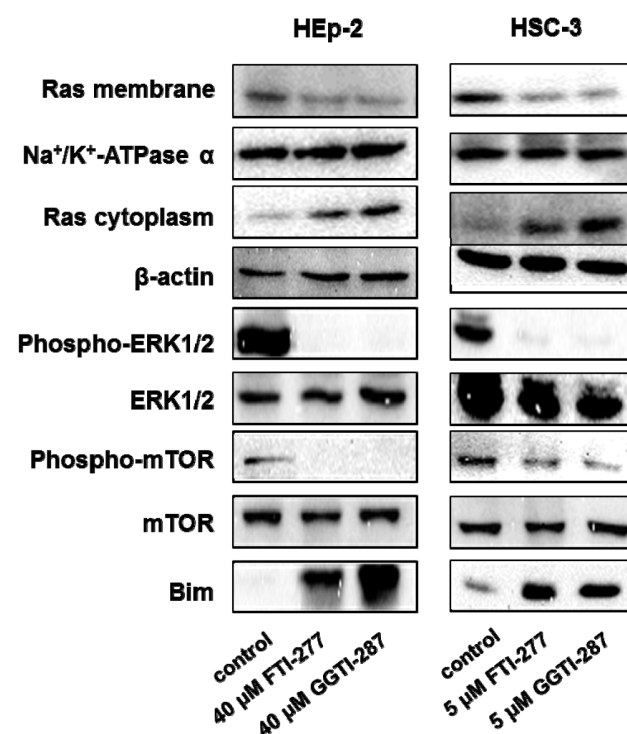


Figure 3. FTI-277 and GGTI-287 suppress Ras membrane localization and phosphorylation of ERK1/2 and mTOR and increase the expression of Bim. HEp-2 and HSC-3 cells were treated with FTI-277 and GGTI-287 for 2 days. The cytoplasmic and membrane fractions were separated on SDS-PAGE; the proteins were transferred on PVDF membranes and probed with antibodies against Ras, phospho-ERK1/2, phospho-mTOR, ERK1/2, mTOR, Bim, Na/K ATPase, and β-actin.

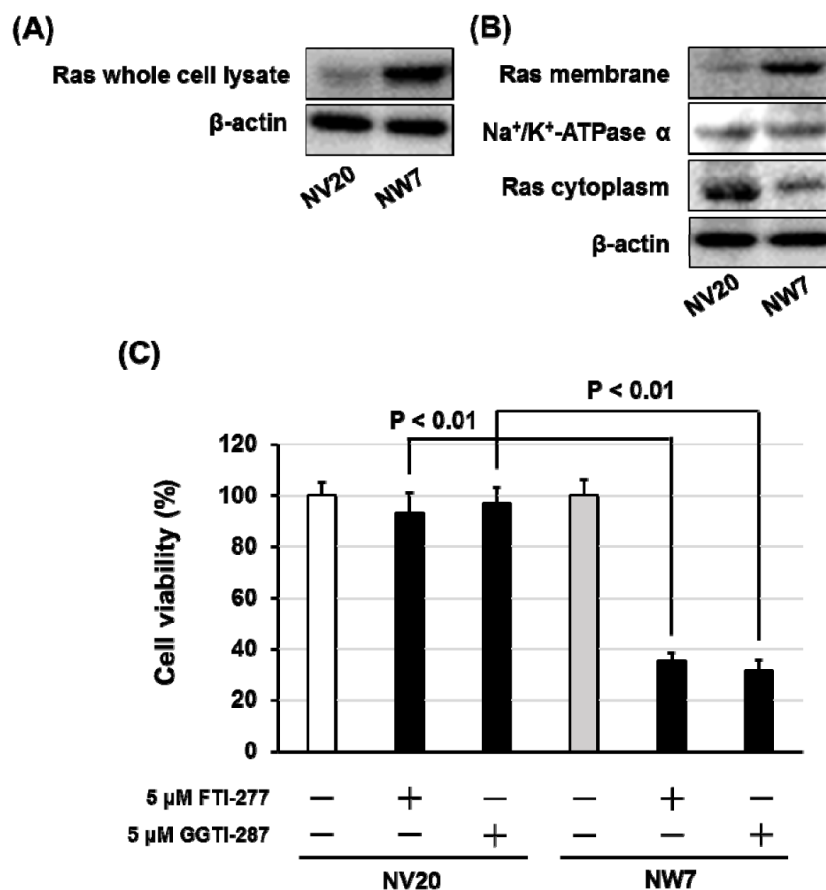


Figure 4. Overexpression of Ras in cells is correlated with their sensitivity toward FTI-277 and GGTI-287. **(A)** NV20 (control, empty vector-transfected) and NW7 cells (v-H-Ras-transfected) were cultured for 2 days. Whole cell lysates were separated on SDS-PAGE; the proteins were transferred on PVDF membranes and probed against Ras and β -actin antibodies. **(B)** The cytoplasmic and membrane fractions were probed against Ras, Na/K ATPase, and β -actin antibodies. **(C)** The viability of the NV20 and NW7 cells treated with 5 μ M FTI-277 and GGTI-287 for 72 h as measured by the trypan blue dye exclusion assay. *indicates $p < 0.01$ vs. control (ANOVA with Dunnett's test).

ern blotting. Treatment with FTI-277 and GGTI-287 led to significant inhibition of the membrane localization of Ras and a decrease in the expression of phosphorylated ERK1/2 and mTOR in the HEP-2 and HSC-3 cells (Figure 3). ERK1/2 and mTOR are known to regulate the expression of Bim [18,20]. We found a significant increase in the concentration of Bim in the HEP-2 and HSC-3 cells treated with FTI-277 and GGTI-287 (Figure 3). Thus, our results indicate that FTI-277 and GGTI-287 increased the expression of Bcl-2 Interacting Mediator of cell death (Bim) via the inhibition of the phosphorylation of ERK1/2 and mTOR in the HEP-2 and HSC-3 cells. We previously reported that the sensitivity of head and neck carcinoma cells to statin-induced apoptosis is related to their Ras expression status [19]. To examine the effect of the overexpression of Ras on the sensitivity of head and neck carcinoma cells toward FTI-277 and GGTI-287, we evaluated the effect of FTI-277 and GGTI-287 on cell death in v-H-Ras-transfected NIH3T3 (NW7) cells and empty vector-transfected

NIH3T3 (NV20) cells. We observed that the expression of Ras was significantly higher in the NW7 cells than in the NV20 cells, and Ras was localized on the membrane of the NW7 cells (Figures 4A and B). Treatment with FTI-277 and GGTI-287 led to a significant decrease in the viability of the NW7 cells compared to that of the NV20 cells (Figure 4C). Overall, our results indicate that FTI-277 and GGTI-287 induced apoptosis in Ras-overexpressing cells.

Discussion

FTI-277 is known to induce cell death via the activation of Ras through farnesyl transferase in SH-SY5Y neuroblastoma cells [25]. IMB-1406, a farnesyl transferase inhibitor, has also been reported to exhibit antitumor activity against lung, liver, colon, and breast cancer cells [26]. GGTI P61A6, a geranylgeranyl transferase inhibitor, suppressed tumor cell growth and induced apoptosis in an MMTV-v-H-Ras transgenic mouse

model and a lung adenocarcinoma A549 xenograft model [27]. In this study, we demonstrate that FTI-277 and GGTI-287 induce apoptosis in HEP-2 and HSC-3 cells via the activation of caspase 3.

Farnesyl transferase and geranylgeranyltransferase 1 are essential for Ras membrane localization following Ras prenylation [28]. We observed that FTI-277 and GGTI-287 suppressed Ras membrane localization and the activation of Ras downstream signal molecules, namely ERK1/2 and mTOR, and increased the expression of Bim in HEP-2 and HSC-3 cells. R115777, a farnesyl transferase inhibitor, suppressed ERK1/2 phosphorylation leading to apoptosis in lung cancer cells [29]. Additionally, FTI-277 has been reported to suppress the mTOR/p70S6kinase pathway in multiple myeloma cells [30]. Geranylgeranyltransferase inhibitors, including GGTI-287, are known to induce apoptosis via the suppression of Ras prenylation [17]. Statins and nitrogen-containing bisphosphonates suppressed the Ras/ERK1/2 and Ras/mTOR pathways via farnesyl pyrophosphate and geranylgeranyl pyrophosphate and enhanced the expression of Bim, leading to the activation of caspase 3 in various cancer cells [18-20]. Furthermore, the sensitivity of HNSCC cells to statins was correlated with their Ras expression status [18]. Our findings suggest that FTI-277 and GGTI-287 induced apoptosis via the suppression of Ras prenylation, the activation of ERK1/2 and mTOR, and an increase in the expression of Bim. We also demonstrated that the

sensitivity of head and neck carcinoma cells to FTI-277 and GGTI-287 was correlated with their Ras expression status.

Conclusion

This study provides evidence that FTI-277 and GGTI-287 induce apoptosis by enhancing the expression of Bim via the suppression of the Ras/ERK and Ras/mTOR pathways. Additionally, the sensitivity of HNSCC cells to treatment with FTI-277 and GGTI-287 was found to be associated with the expression level of Ras, suggesting that FTI-277 and GGTI-287 may act more effectively on tumors with increased expression of Ras. Thus, our findings support the development of farnesyl transferase or geranylgeranyl transferase inhibitors as potential anticancer agents.

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Conflict of interests

The authors declare no conflict of interests.

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