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ORIGINAL ARTICLE

Involvement of RhoA and RalB in geranylgeranyltransferase I inhibitor-mediated inhibition of proliferation and migration of human oral squamous cell carcinoma cells

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Abstract
Purpose Geranylgeranyltransferase I is required for the prenylation of the small GTPases. The effect of GGTase I inhibitors (GGTIs) on oral squamous cell carcinoma (SCC) cells was examined.
Methods The GGTI-treated cells were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, flow cytometric analysis, transwell chamber assays, and immunofluorescent staining. Small GTPases were detected by immunoblot analysis, and siRNA were used for silencing RalA and RalB.
Results GGTI suppressed the proliferation of oral SCC cells and induced cell cycle arrest at G_1, but the sub-G_1 fraction was small. The expression of the cyclin-dependent kinase (CDK) inhibitor $p21^{Waf1/Cip1}$, but not $p27^{Kip1}$, was markedly increased by GGTI. There was an apparent increase in the expression and reduction in the membrane localization of RhoA and RalB, but not Ras and RalA. Assays with transwell chambers and wound healing and invasion revealed the migrative and invasive capabilities of SAS cells to be inhibited by GGTI. Actin filaments were rearranged and stress fibers and peripheral cell processes were lost, accompanying cell rounding. siRNA for RalB, but not RalA, significantly suppressed the migration of SAS cells.
Conclusion These results suggest that GGTI inhibits the geranylgeranylation of RhoA and increases the $p21^{Waf1/Cip1}$ level, resulting in cell cycle arrest at G_1 to decrease cell proliferation, and that of RalB to suppress the migration and invasion by oral SCC cells. GGTIs may be useful as inhibitors of invasion and metastasis in cases of oral SCC.

Keywords Geranylgeranyltransferase I inhibitor · Oral squamous cell carcinoma · GGTase · Rho · Ral · Migration

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Introduction

Oral squamous cell carcinoma (SCC) accounts for about 80% of all oral malignant tumors. Oral SCC still has a very unfavorable prognosis, in spite of progress in diagnosis, surgery, and chemotherapy, with a 5-year survival rate in the past 20 years of approximately 56% [1, 2]. This relatively high mortality is predominantly due to widespread lymphatic and distant metastasis. Hence, there is an urgent need for a safe and effective chemotherapy to control invasion and metastasis in cases of oral SCC.

The small GTPase Ras superfamily including the Ras, Rho, Arf, Rab, Ran, and Rad/Gem families are regulatory proteins whose activity is controlled by a GDP/GTP cycle [3]. The Ras family, particularly H-Ras, N-Ras, and K-Ras, is known to contribute widely to human tumorigenesis through both the activation of mutations and overexpression [4, 5]. Rho family members such as RhoA, Rho C, Rac1, and cdc42 are also involved in many cancers [6, 7]. Mutated forms of the small G protein Ras are found in about 30% of all human cancers, including 95% of pancreatic cancers and 50% of colon cancers [4, 8]. In oral cancer, Ras mutations have been reported in 20–30% of cases from India, whereas lower frequencies (4%) were reported in the United Kingdom and no mutation has been reported in the United States [9–11]. The Ras signals were transduced into downstream signaling branches including

the Raf/MEK/ERK pathway, phosphatidylinositol 3-kinases (PI3K) signals, and the Ral guanine nucleotide exchange factor (GEF)/Ral pathway [12, 13]. While the Raf and PI3K pathways are well-validated effectors in cancers, recent studies have shown that RalA and RalB in the RalGEF/Ral pathway contribute to tumorigenesis and metastasis [14–17]. It has been also shown that the RalGEF/Ral and not the Raf or PI3K pathway is sufficient for Ras' transformation of a variety of human cell types [18, 19]. These findings suggest that small GTPases in the RalGEF/Ral branch of the Ras pathway play important roles in tumorigenesis, even if the tumors have no mutations in Ras genes [20].

Small GTPases are synthesized through the mevalonate pathway. After their translation, they are modified by prenylation [21, 22]. The two enzymes responsible for the prenylation of Ras family proteins are farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) [7, 22], which covalently attach 15-carbon farnesyl and 20-carbon geranylgeranyl lipids, respectively, to the cysteine of the carboxyl-terminal motif CAAX (C is cysteine, A is any aliphatic amino acid, and X is any carboxyl-terminal amino acid). The prenylated small G proteins are then transported to the plasma membrane and act as transmitters of external signaling [23]. Several geranylgeranylated CAAX proteins are critical for tumorigenesis downstream of Ras [12]. Therefore, FTase and GGTase I are considered potential molecular targets, and GGTase I inhibitors (GGTIs) have been developed. GGTIs can inhibit cell proliferation and induce apoptosis in pancreatic cancer, breast cancer, and bladder cancer [24–27]. However, the antineoplastic targets of GGTIs in most cancers remain unclear.

In the present study, we investigated the effect of GGTIs on the prenylation of Ras, RalA, RalB, and RhoA as possible targets of these inhibitors in oral SCC. The results suggest that the Ral and Rho pathways are involved in the inhibitory effect of GGTI on cell proliferation, migration, and invasion.

Materials and methods

Cell culture

The human oral SCC cell lines SAS, Ca9-22, and HSC-3 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin and grown in an incubator at 37°C in a humidified atmosphere with 5% CO_2.

Reagents

GGTI-298, GGTI-2133, and GGTI-2147 were obtained from Calbiochem-Novabiochem (San Diego, CA), and a stock solution was made in dimethyl sulphoxide.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Cells (1×10^3) in 96-well culture plates were cultured overnight prior to experiments. After treatment with GGTI, 10 μl of a 5 mg/ml MTT (Sigma, St. Louis, MO) solution was added to each well with 100 μl of medium and cells were incubated at 37°C for 4 h. Next, 100 μl of 0.04 N HCl in isopropanol was mixed thoroughly to dissolve the dark blue crystal, and the plates were kept at room temperature overnight. Readings were made on a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA) with a reference wavelength of 630 nm and a test wavelength of 570 nm. Background absorbance at 630 nm was subtracted from the 570-nm reading.

Flow cytometric analysis

Cells were harvested and washed twice with PBS, and the pellets were fixed in cold 70% ethanol at −20°C overnight. The fixed cells were washed twice with ice-cold PBS and treated with 1 mg/ml RNase at 37°C for 30 min. Cellular DNA was stained with 50 μg/ml propidium iodide in PBS and analyzed with a fluorescence-activated cell sorter (FACSort; Becton–Dickinson, Mountain View, CA). DNA histograms were used to determine the percentages of cells in the sub-G_1, G_0/G_1, S, and G_2/M phases. Cells with less DNA content than in the G_0/G_1 phase, the sub-G_1 fraction, were considered apoptotic [28].

Migration assay

Cell migration was assessed using transwell chambers and a wound healing assay. For the transwell chamber assay, BD BioCoat Cell Culture Inserts (8-μm pore size; Becton–Dickinson Labware, Bedford, MA) were used. A total 2.5×10^4 cells in 500 μl of serum-free DMEM were transferred to the upper chamber. An equal volume of DMEM containing 5% FBS was added to the lower chamber. After 12 h at 37°C in 5% CO_2, GGTI was added at 5 μM in the upper chamber and the incubation continued for a further 24 h. Cells remaining on the upper surface of the filters were removed with cotton swabs, and cells on the lower surface were fixed with 100% methanol, stained with hematoxylin–eosin (H–E), and counted.

For the wound healing assay, cells were grown to a confluent monolayer in 6-well plates for 24 h. The

monolayers were wounded by scraping with a P200 micropipette tip. After two washes with PBS, cells were incubated in the presence or absence of 5 μM GGTI and observed by phase-contrast microscopy. At specified time points after the scraping, the cell monolayers were photographed using a Canon EOS 40D digital camera (Canon, Lake Success, NY), and the area denuded was measured using NIH image software.

Invasion assay

A cell invasion assay was carried out using BioCoat Matrigel Invasion Chamber (Becton–Dickinson Labware, Bedford, MA) consisting of transwell filter inserts in a 24-well tissue culture plate. A total of 5×10^4 cells in 500 μl of serum-free DMEM were added to the upper chamber, and DMEM medium containing 5% FBS was placed in the lower well. After 12 h at 37°C, GGTI was added at 5 μM in the upper chamber and the incubation continued for 24 h. Cells on the lower surface were counted as in the migration assay.

Cell fractionation

The membranous and cytosolic fractions were prepared using ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions. A total of 4×10^6 cells were washed in cold PBS, suspended in 150 μl of ice-cold Extraction I containing 0.75 μl of a protease inhibitor cocktail, and incubated at 4°C for 10 min. The suspension was centrifuged at $1{,}000 \times g$ for 10 min at 4°C. The supernatant was removed and kept as the cytosolic fraction. The pellet was suspended in 150 μl of ice-cold Extraction II containing 0.75 μl of protease inhibitor cocktail and incubated at 4°C for 30 min. The mixture was centrifuged at $6{,}000 \times g$ for 10 min at 4°C. The supernatant was used as the membranous fraction.

Immunoblot analysis

Cells were washed in PBS and lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate, and the protease inhibitor cocktail. After sonication on ice and subsequent centrifugation at $15{,}000 \times g$ for 10 min at 4°C, the supernatant was collected and the protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA). Sample protein (20 μg) was separated through polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a PVDF membrane (Millipore, Bedford, MA) by electroblotting. The membrane was probed with antibodies, and antibody binding was detected using an enhanced chemiluminescence kit (GE Healthcare, Amersham, Buckinghamshire, England) according to the manufacturer's instructions. The antibodies used were rabbit polyclonal antibodies against Ras, RalA, RalB, RhoA, and $p27^{Kip1}$ (Cell Signaling Technology, Beverly, MA), mouse monoclonal antibodies against $p21^{Waf1/Cip1}$ (Cell Signaling), Hsp-90 and Calnexin (Novagen, Madison, WI) and β-actin (Sigma, St. Louis, MO), and horseradish peroxidase–conjugated secondary antibodies (Cell Signaling).

Small interfering RNA transfection

Chemically synthesized siRNA against RalA, RalB, and nonsense oligonucleotides (nonsense siRNA) were purchased from Dharmacon (Lafayette, CO) with the following target sequences: RalA, 5′-GACAGGUUUCUGUAGAAGA-3′; RalB, 5′-AAGCUGACAGUUAUAGAAA-3′; negative control GL2, 5′-CGTACGCGGAATACTTCGA-3′. Cells were plated in 6-well plates at a density of 2×10^5/well, cultured for 24 h, and transfected with 20 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The medium was replaced with DMEM at 3 h after transfection. For the immunoblot analysis, cells were incubated for a further 45 h. Prior to the MTT assay and transwell chamber assays, si-RNA-transfected cells were cultured for 24 h.

Visualization of the actin cytoskeleton

Cells were plated in 6-well plates containing coverslips and treated with GGTI. Forty-eight hours later, they were fixed with 4% paraformaldehyde for 15 min and permealized with 0.1% Triton X-100 in PBS for 3 min. After being washed, the cells were stained with Alexa Flour 546 phalloidin (Invitrogen, CA) diluted 1:40 for 20 min and washed with PBS. Coverslips were mounted onto microscope slides using VECTASHIELD Hard Set Mounting Medium with 4′,6′-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA). The slides were observed under a confocal laser-scanning microscope, TCS SP5 (Leica Instruments, Germany).

Statistical analysis

The statistical analysis was performed using a standard Student's *t* test with Microsoft Excel (Microsoft, Redmond, WA).

Results

Inhibition of cell growth by GGTIs

The effect of GGTIs on the viability of oral SCC cells was examined by MTT assay. SAS cells were cultured in the

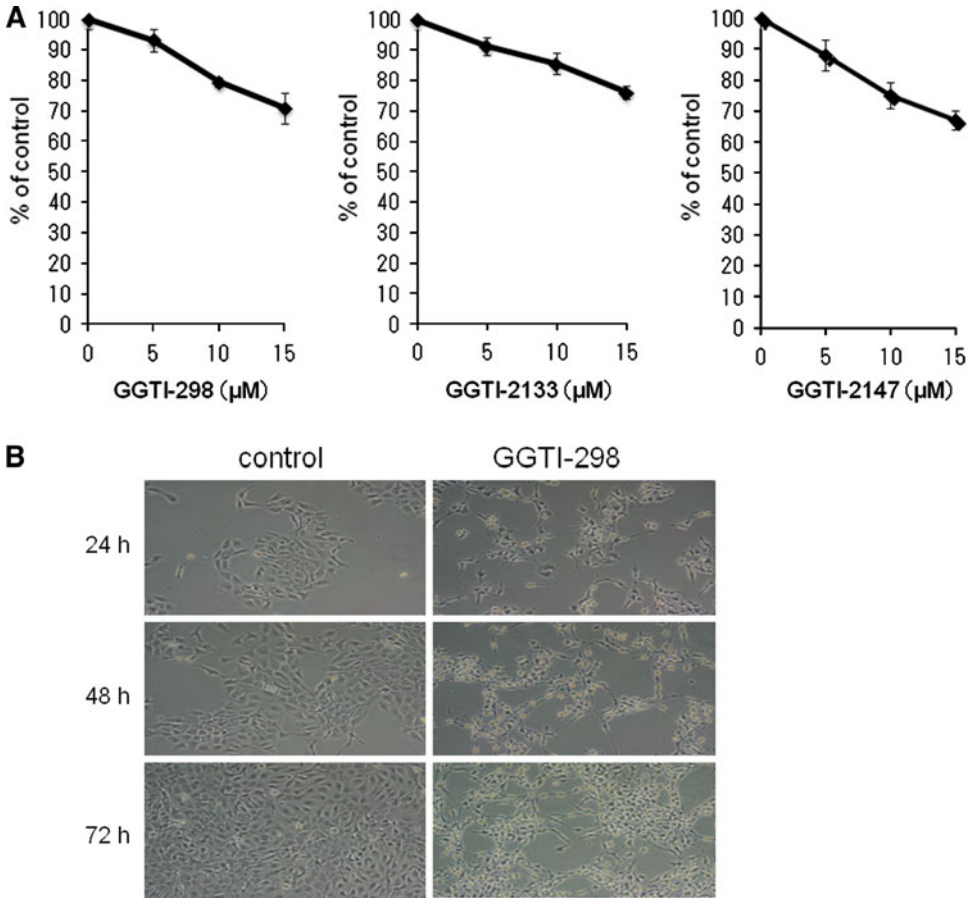

Fig. 1 Inhibition of cell growth and changes in cell morphology caused by GGTIs. **a** SAS cells were treated with GGTI-298, GGTI-2133, and GGTI2147 at 5, 10, and 15 μM for 48 h, and cell viability was determined by MTT assay. Data are the mean ± SD of six determinations. **b** SAS cells were treated with GGTI-298 at 15 μM for the period indicated, and cell morphology was photographed under a phase-contrast microscope

presence of GGTIs at a concentration of 5, 10, or 15 μM for 48 h (Fig. 1a). Cell growth was inhibited by all GGTIs and the proportion of viable cells in cultures treated with 15 μM GGTI-298, GGTI-2133, and GGTI-2147 was 71, 75, and 67% of the control, respectively. When other oral SCC cells, Ca9-22 and HSC-3 cells, were treated with 15 μM GGTI-298 for 48 h, the proportion of viable cells decreased to 64 and 70% of the control, respectively. The morphology of SAS cells was affected by 15 μM GGTI-298. Cells became rounded and were connected to neighboring cells with fine intercellular cell processes at 24 h, and the morphological change was maintained until the 72-h mark (Fig. 1b).

Cell cycle arrest by GGTI and induction of the CDK inhibitor $p21^{Waf1/Cip1}$

To determine whether GGTI could induce cell cycle arrest, SAS cells were treated with 15 μM GGTI-298 and subjected to a flow cytometric analysis. In the untreated control, the proportion of cells in the G_0/G_1, S, and G_2/M phases was 41, 28, and 29%, whereas for the cells treated with 15 μM GGTI-298 for 72 h, it was 55, 24, and 16%, respectively (Fig. 2a), indicating an increase in the number of cells at G_0/G_1. Initially, the sub-G_1 fraction accounted for 1.9% of cells, but 72 h after the treatment with GGTI-298, this had increased to 4.6% (Fig. 2b).

The involvement of cyclin-dependent kinase (CDK) inhibitors in the GGTI-induced cell cycle arrest at G_1 was examined by immunoblotting. When SAS cells were treated with 15 μM GGTI-298 for 48 h, the expression of $p21^{Waf1/Cip1}$ was markedly increased, whereas $p27^{Kip1}$ remained at an undetectable level (Fig. 2c)

Expression of Ras, RalA, RalB, and RhoA and inhibition of translocation of small GTPases in GGTI-treated SAS cells

SAS cells were treated with GGTI-298, and the expression of Ras, RalA, RalB, and RhoA was examined by immunoblotting. Although RalA and Ras were not specifically altered by GGTI-298 for 48 h, there was a slight increase in RalA at 72 h. The expression of RalB increased markedly from 48 h and that of RhoA from 12 h (Fig. 3a). Unprenylated GTPases whose geranylgeranylation is inhibited by GGTI migrate slower than prenylated GTPases in SDS–PAGE gels [25–27]. Two bands representing unprenylated

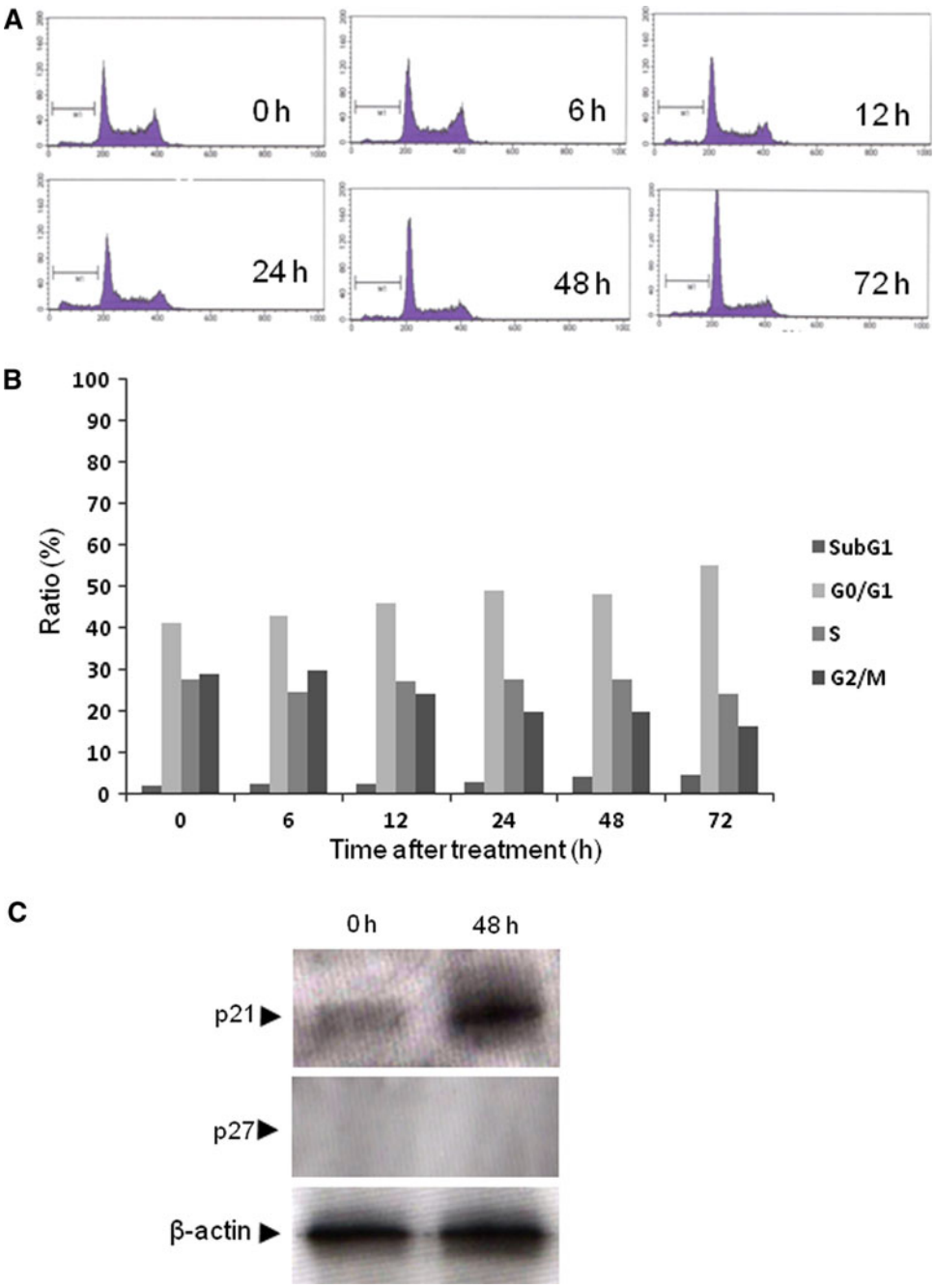

Fig. 2 Cell cycle arrest and induction of the CDK inhibitor $p21^{Waf1/Cip1}$ by GGTI. **a** SAS cells were treated with 15 μM GGTI-298 for the period indicated and subjected to a flow cytometric analysis. **b** From an analysis of DNA histograms, the percentages of cells in the sub-G_1, G_0/G_1, S, and G_2/M phases were evaluated. **c** SAS cells were treated with GGTI-298 for 48 h. Cells were subjected to an immunoblot analysis for the expression of the CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. A representative result is shown

and prenylated GTPases were not clearly separated under the conditions.

Prenylated small GTPases are transported to the plasma membrane and act as transmitters of external signaling, whereas unprenylated GTPases accumulate in the cytoplasm [22, 23]. To determine whether GGTI could inhibit the translocation of these small GTPases, membranous and cytosolic fractions were prepared from cells treated with GGTI-298 for 48 h and subjected to an immunoblot analysis. Hsp90 and Calnexin were used as markers for the cytosolic fraction and membranous fraction, respectively. It was found that the amounts of RalB and RhoA in the membranous fraction were decreased and those in the cytosolic fraction were increased (Fig. 3b). In contrast, the amount of Ras was not specifically altered. The change in RalA was unremarkable, but the cytosolic level was slightly increased.

Rearrangement of actin fiber by GGTI

RhoA regulates the actin cytoskeleton [29, 30]. RalA and RalB also have interactions with the actin cytoskeleton [31,

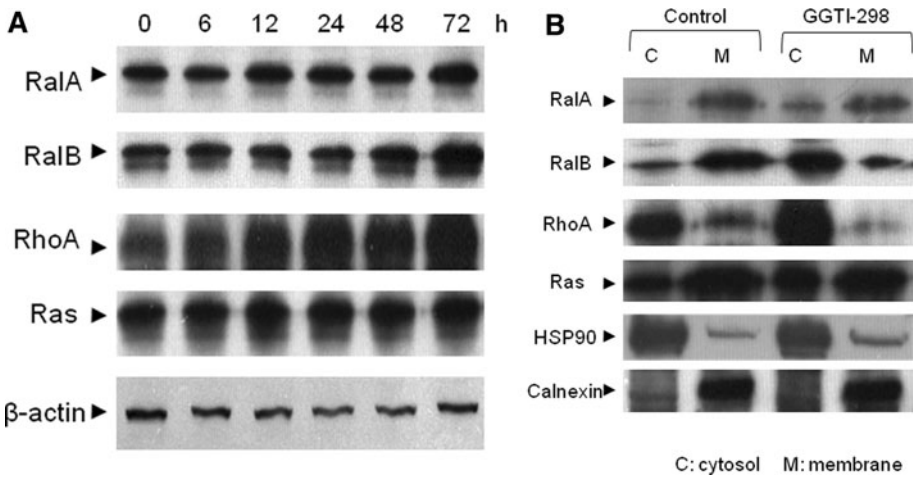

Fig. 3 Expression of Ras, RalA, RalB, and RhoA and inhibition of the translocation of small G proteins in GGTI-treated SAS cells. **a** SAS cells were treated with GGTI-298 for the period indicated. They were then subjected to an immunoblot analysis for the expression of Ras, RalA, RalB, and RhoA. **b** SAS cells were treated with 15 μM GGTI-298 for 48 h. Membranous and cytosolic fractions were prepared and subjected to immunoblotting to determine the expression of Ras, RalA, RalB, RhoA, Hsp90, and Calnexin. Hsp90 and Calnexin were used as a control for protein in the cytosol and membrane, respectively. A representative result is shown

32]. After treatment with GGTIs, SAS cells became rounded and the translocation of RhoA and RalB was markedly inhibited. Thus, the structure of actin fibers was examined by staining using Alexa Fluor 546 phalloidin. Untreated control cells had many prominent actin stress fibers running most of the length of the cell. After treatment with 5 μM GGTI-298 for 24 h, there were markedly fewer and less prominent stress fibers and peripheral processes (Fig. 4).

Inhibition by GGTIs of migration and invasion by oral SCC cells

Small G proteins, Ral and Rho, are involved in cell motility [17, 19, 33]. The effect of GGTIs on the migration of SAS cells was examined with a transwell chamber assay. Twenty-four hours after incubation in the presence of 5 μM GGTI-298, the migration of SAS cells was inhibited, and the numbers of migrating cells decreased to 26% of the control value (Fig. 5a). In the presence of GGTI-2133 and GGTI-2147, the numbers of migrating SAS cells were decreased to 45 and 11% of the control value, respectively. The difference between control and GGTI-treated cells was significant ($P < 0.001$).

Cell migration was also assessed with the wound healing assay. The denuded surface was significantly ($P < 0.001$) smaller in the cultures treated with GGTI-298 or GGTI-2147 than in control cultures (Fig. 5b, c). In the case of GGTI-2133, there was no decrease in the width of the denuded surface, although cell density was low in GGTI-treated cultures when compared with the control (Fig. 5b).

Cell invasion was assayed with matrigel chambers. In the presence of GGTIs, invasion under the membrane was inhibited. The numbers of invading cells in cell cultures treated with GGTI-298, GGTI-2133, and GGTI-2147 were 23, 27, and 8% of the control value, respectively (Fig. 5d). The difference between control and GGTI-treated cells was significant ($P < 0.001$).

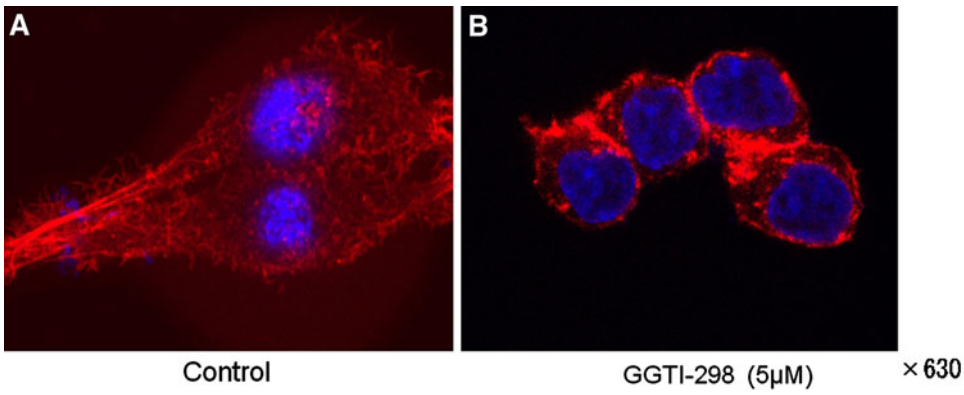

Fig. 4 Rearrangement of actin fibers by GGTI. Cells were plated in 6-well plates containing coverslips and incubated in the absence **a** or presence **b** of 5 μM GGTI-298. Twenty-four hours later, they were fixed with 4% paraformaldehyde for 15 min, stained with Alexa Flour 546 phalloidin and DAPI, and observed under a confocal laser-scanning microscope

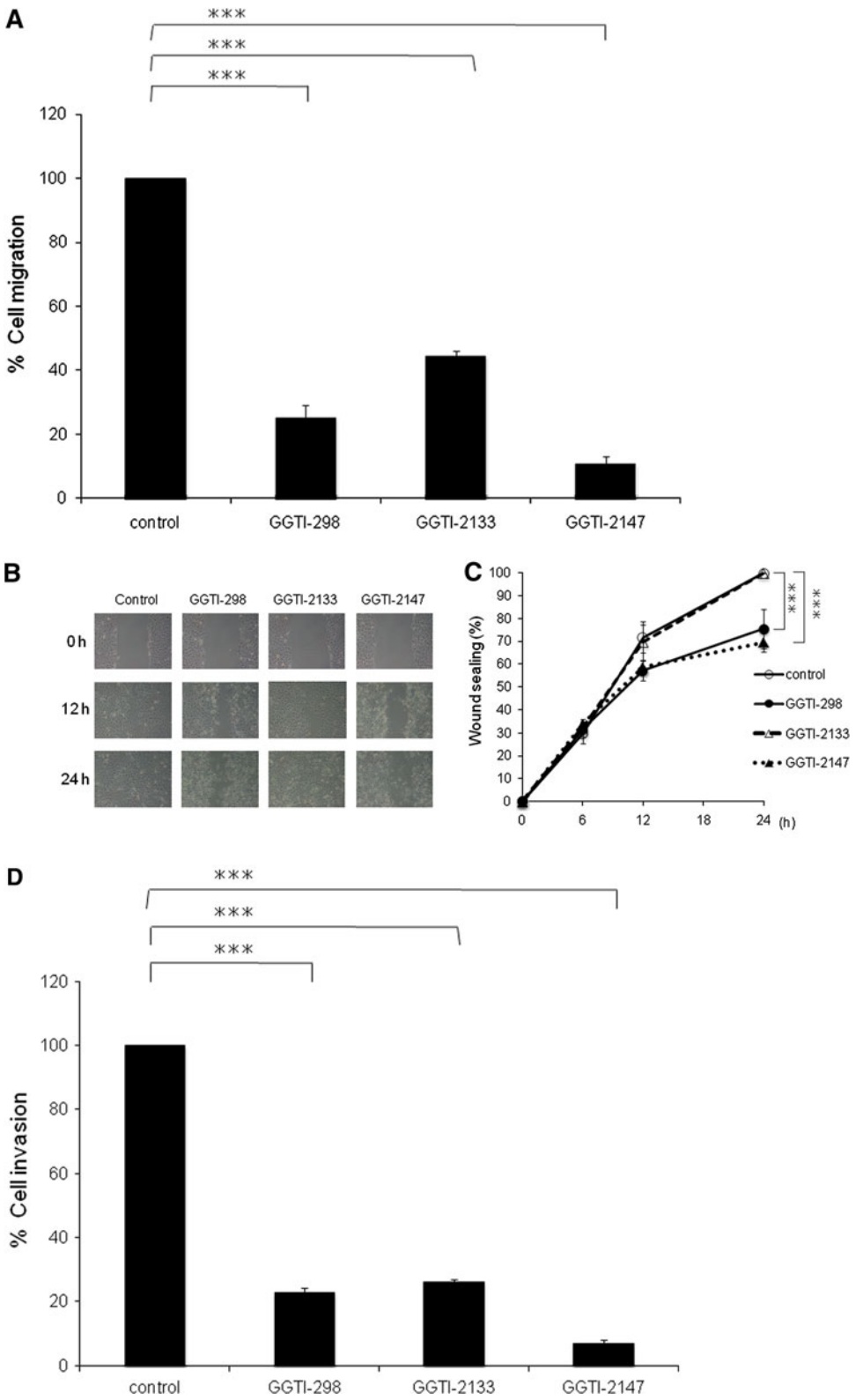

Fig. 5 Inhibition of cell migration and invasion by GGTIs. **a** A transwell chamber assay was performed with BD BioCoat Cell Culture Inserts. Cells were added to the upper chambers of transwell filters in a 24-well culture plate. The lower chambers contained medium with 5% FBS. From 12 h after plating, cells were treated with 5 μM of GGTI-298, GGTI-2133, or GGTI-2147. After incubation for a further 24 h, cells on the lower surface of filters were stained by H–E and counted under a light microscope at 200× magnification in each of 10 fields. **b** For the wound healing assay, cells were grown to a confluent monolayer in 6-well petri dishes for 24 h. The medium was replaced with one containing GGTIs, and cells in monolayers were wounded with a micropipette tip. At the indicated time points after scraping, the cell monolayers were photographed under a phase-contrast microscope. **c** In the wound healing assay, the denuded areas after incubation in the presence or absence of GGTIs were measured using NIH image software. **d** A cell invasion assay was carried out using BioCoat Matrigel Invasion Chamber. Cells were added to the upper chambers of transwell filters in a 24-well culture plate. The lower chambers contained medium with 5% FBS. From 12 h after plating, cells were treated with 5 μM of GGTI-298, GGTI-2133, or GGTI-2147. After incubation for a further 24 h, cells on the lower surface of filters were stained by H–E and counted. Data are the mean ± SD of three determinations. ***$P < 0.001$

Suppression of RalA and RalB by siRNA

To further determine the role of RalA and RalB in the proliferation and migration of oral SCC cells, the effects of downregulating the expression of Ral GTPases were examined. SAS cells were transfected with siRNA for RalA or RalB and cultured for 48 h. The expression of each small G protein was suppressed (Fig. 6a). In the cultures

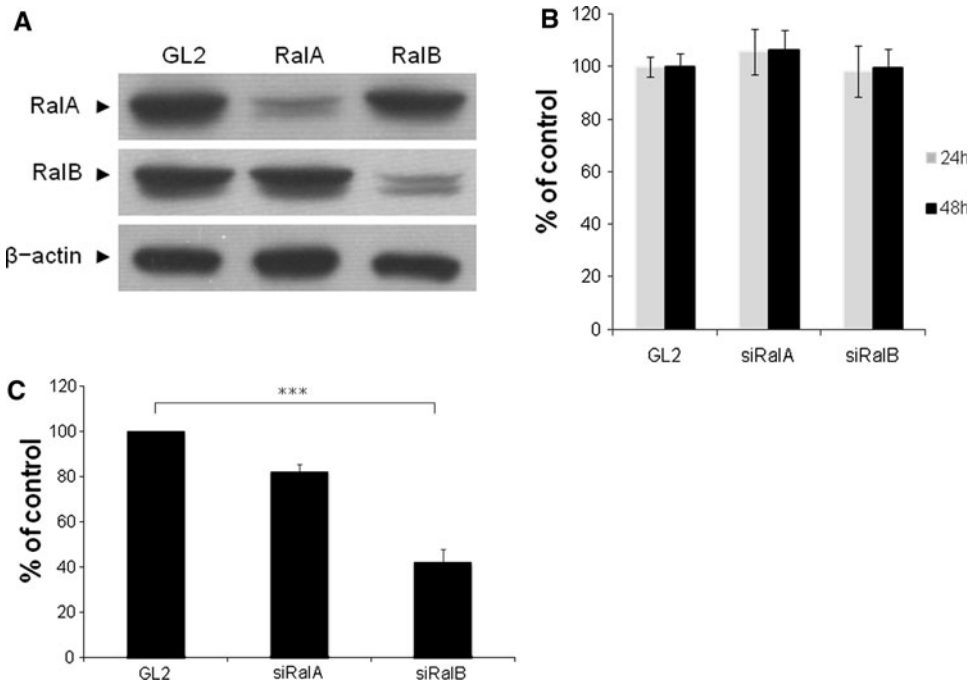

Fig. 6 Suppression of RalA and RalB by siRNA. **a** SAS cells were transfected with siRNA for RalA or RalB or nonsense siRNA and cultured for 48 h. The expression of RalA and RalB in transfected cells was examined by immunoblotting. **b** Transfected cells were incubated for 24 and then used for a MTT assay. Data are the mean ± SD of four determinations. **c** A transwell chamber assay was performed with transfected SAS cells. Twenty-four hours after transfection, cells were added to the upper chambers of transwell membranes and cultured for a further 24 h. Cells on the lower surface of filters were stained by H–E and counted. Data are the mean ± SD of three determinations. ***$P < 0.001$

transfected with the siRNA for RalA or RalB, no significant decrease in cell viability was observed when compared with the control (cells transfected with nonsense siRNA) (Fig. 6b). However, transfection of the siRNA for RalB resulted in a significant ($P < 0.001$) decrease in the number of migrating cells to 42% of the control value (Fig. 6c). The effect of the siRNA for RalA was less prominent, and the number of migrating cells was 83% of the control (Fig. 6c).

Discussion

A number of GGTIs have been developed, but studies of their inhibitory effects on cell proliferation are limited. Falsetti et al. [26] reported that the IC_{50} of GGTI-2147 for pancreatic cancer cells, incubated for 72 h, was approximately 3 μM. When assayed by anchorage-independent colony formation, the IC_{50} was 30 μM. Consistent with these results, three GGTIs, GGTI-298, GGTI-2133, and GTTI-2147, inhibited the proliferation of SAS cells. At a concentration of 15 μM, the viability of the treated cells decreased from 65% to 75% of the control value. The proportion of viable cells was reduced by GGTI-298 in other oral SCC cell lines, Ca9-22 and HSC-3. We also found that GGTIs affected cell morphology and induced rapid cell rounding. It can be concluded that GGTIs suppress the proliferation of oral SCC cells in a dose-dependent manner.

The cell cycle analysis of various human cells lines revealed an increase in cells at G_1 due to GGTI. An antiproliferative effect of GGTI-298 was demonstrated in fibroblasts, epithelial cells, and smooth muscle cells, and the cell growth inhibition appeared to be mediated through G_1 arrest and apoptosis [34]. In the case of pancreatic MiaPaCa2 cells, the rate of apoptosis was 4%. In MDA-MB-468 breast cancer cells, the IC_{50} of GGTI-2147 was reported to be 3 μM and the proportion of dead cells was 21% after treatment with 50 μM GGTI-2147 [27]. We also found an accumulation of G_0/G_1 cells after treatment with 15 μM GGTI-298 for 48 h. However, the sub-G_1 fraction accounted for just 4.6% of cells. Thus, GGTIs seem to decrease the proliferation of oral SCC cells, but the effect is not accompanied by extensive cell death.

Cell cycle progression is driven by CDKs in association with cyclins. The relative levels of cyclins, CDKs, and CDK inhibitors determine whether cell-cycle progression will occur [35]. One possible mechanism that may involve a GGTI-298-mediated G_1 phase block in cultured human tumor cells is the induction of CDK inhibitors [36]. Indeed, Vogt et al. [24] have shown that GGTIs arrested tumor cells in the G_0/G_1 phase and upregulated $p21^{Waf1/Cip1}$ expression at the transcriptional level. In breast cancer cells, the ability of GGTI to reduce the proliferation of cancer cells was ascribed, in part, to an accumulation of nuclear $p27^{Kip1}$ [27]. In the present study, $p27^{Kip1}$ was undetectable in SAS cells and not induced to express by GGTI-298, whereas the expression of $p21^{Waf1/Cip1}$ was markedly increased. Thus, it is likely that the G_1 arrest observed in GGTI-298-treated SAS cells is due to an

elevation of the $p21^{Waf1/Cip1}$ level, resulting in the inhibition of CDKs.

The antineoplastic targets of GGTIs remain unknown. Rho family proteins were initially shown to regulate the actin cytoskeleton and motility [29], but they also influence gene expression, cell cycle progression, and survival [7, 30, 37, 38]. Higher vertebrates have 3Rho GTPases, RhoA, RhoB, and RhoC, which share 85% amino acid sequence identity. RhoA plays a key role in the regulation of actomyosin contractility. RhoB, which is localized primarily on endosome, has been shown to regulate cytokine trafficking and cell survival, while RhoC may be important in cell locomotion. This suggests that although the 3 isoforms of Rho are structurally highly homologous, they have different cellular functions [39]. Adnane et al. [40] indicated that one mechanism by which GGTI-298 upregulates $p21^{Waf1/Cip1}$ transcription is by preventing RhoA from repressing $p21^{Waf1/Cip1}$ expression in human pancreatic carcinoma cells. Thus, we examined the geranylgeranylation of RhoA and found the increased expression and decreased membrane translocation of RhoA. It is considered that GGTI-298 suppressed the geranylgeranylation and translocation of RhoA, thereby suppressing its function, and increased $p21^{Waf1/Cip1}$ levels, leading to arrest at G_1. Geranylgeranylation is required for V14RhoA to induce actin stress fibers and focal adhesion [38]. RhoA may also contribute to the rearrangement of the actin cytoskeleton in GGTI-treated oral SCC cells. The roles of RhoB and RhoC in this circumstance remain to be clarified.

In the absence of Ras mutations, RalA and RalB appear to play a role in the growth of several cancer cell lines [20]. In the present study, the expression of RalB was increased, and the membranous fraction of RalB was markedly decreased, and as a consequence, the protein level of the cytosolic fraction became much higher than that in the untreated control. By contrast, there was no apparent decrease in Ras or RalA in the membranous fraction, suggesting that Ras mutations do not play a major role in the tumorigenesis of SAS cells. It is likely that RalB is the target of GGTI-298 in oral SCC cells. Increased expression of Rho and Ral caused by GGTI, as observed in the present study, had been reported in pancreatic cancer and lung cancer cells [25, 26]. In this regard, Delarue et al. [41] indicated that promoter acetylation is a mechanism by which RhoB expression is regulated following treatment with FTIs and GGTIs. A similar mechanism may act to increase levels of RhoA and RalB in oral SCC cells. Nevertheless, the role of the cytoplasmic accumulation of small GTPases triggered by GGTI remains undetermined.

Despite sharing 85% amino acid identity, with 100% identity in the sequence important for effector binding, RalA and RalB participate in different cellular functions [42]. Lim et al. [19] reported that RalA, but not RalB, is critical for the anchorage-independent and tumorigenic growth of pancreatic carcinoma cells, whereas RalB, and to a lesser degree RalA, is critical for cell invasion and metastasis. Oxford et al. [32] indicated that RalA and RalB have opposing roles in the regulation of cell migration, with RalB-stimulating and RalA-inhibiting motility. The distinct functions of RalA and RalB may be due, in part, to the different downstream effectors utilized. In transwell chamber and wound healing assays, all GGTIs inhibited the migration of SAS cells in a growth factor–dependent manner. Cell invasion was also remarkably suppressed, consistent with the results of a previous study [43]. Furthermore, we found that siRNA for RalA and RalB did not affect the proliferation of oral SCC cells, but RalB significantly reduced the numbers of migrating cells. The effect of RalA was much weaker than that of RalB. It is concluded that the inhibition of RalB's geranylgeranylation by GGTI is an important step in the suppression by GGTIs of cell motility. RalB has a role in the organization and/or maintenance of actin fibers [29]. There were markedly fewer and less prominent stress fibers in SAS cells that had been treated with GGTI-298. It is possible that RalB as well as RhoA is involved in the rearrangement of the actin cytoskeleton in oral SCC cells.

Several previous findings suggested RalA to have a positive role in tumorigenesis. RalA expression was upregulated in breast, bladder, brain, and prostate cancer [20, 44, 45]. However, more recently, Sowalsky et al. [46] indicated that RalA expression is specifically reduced in head and neck SCCs and the decrease is strongly associated with poorly differentiating tumors. They also proposed an important factor in the early stages of SCC to be a modest decrease in RalA gene expression that magnifies the effects of decreased E-cadherin expression by promoting its degradation. In this case, knockdown of RalA by GGTI may reduce E-cadherin levels and promote the malignant progression of head and neck cancer. However, this is unlikely, because the effect of GGTI-298 on the membranous localization of RalA in SAS cells was marginal (Fig. 3b).

In conclusion, GGTI-298 inhibited the geranylgeranylation of RhoA and increased the $p21^{Waf1/Cip1}$ level, resulting in cell cycle arrest at G_1 to decrease cell proliferation, and that of RalB to suppress the migration and invasion by SAS cells. GGTIs may be useful as inhibitors for invasion and metastasis in cases of oral SCC.

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Conflicts of interest No potential conflicts of interest were disclosed.

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