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20(S)-Ginsenoside Rh2 Suppresses Oral Cancer Cell Growth by Inhibiting the Src-Raf-ERK Signaling Pathway

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Abstract. *Background: 20(S)-Ginsenoside Rh2 (G-Rh2) has demonstrated therapeutic effects in many types of cancers. We aimed to investigate the potential anticancer activity and underlying mechanisms of G-Rh2 in oral cancer cells. Materials and Methods: The antigrowth effect of G-Rh2 in oral cancer cells was stimulated by cell proliferation, soft agar colony formation, and migration and invasion assay. The cell cycle and apoptosis were detected by flow cytometry. The underlying mechanism of G-Rh2 in oral cancer cells was explored by immunoblotting. Results: G-Rh2 significantly inhibited oral cancer cell growth by inducing apoptosis and cell cycle G_0/G_1-phase arrest. G-Rh2 inhibited oral cancer cell migration and invasion through regulation of epithelial–mesenchymal transition (EMT)-related proteins. G-Rh2 inhibited the Src/Raf/ERK signaling pathway in YD10B and Ca9-22 cells. Conclusion: G-Rh2 exerted anticancer activity in vitro by inhibiting the Src/Raf/ERK signaling pathway in oral cancer. G-Rh2 is a potential therapeutic drug for oral cancer treatment.*

Oral cancer is one of the most common malignancies in the world (1), and approximately 90% of oral and oropharyngeal malignancies are squamous-cell carcinomas (SCC) (2). Despite advances in the diagnosis and treatment of oral cancer, it still remains a global health problem. Surgery is the mainstay of treatment for oral cancer, and patients with advanced-stage cancer or who are inoperable are usually treated with combination therapy (3). Drug resistance and adverse side-effects are complications of oral cancer chemotherapy. Hence, it is urgent that more effective, safer drugs and novel therapies be developed for oral cancer treatment.

Src is a non-receptor tyrosine kinase protein that has been reported to have high expression in late-stage tumor tissues. Thus, Src is considered an indicator of poor clinical prognosis (4). Src was reported to promote chemotherapy drug resistance in various cancer types (5, 6). Src was found to regulate the STAT3/PI3K signaling pathway in cancers (7). Several Src inhibitors, such as dasatinib and bosutinib, have been used clinically for the treatment of chronic myelogenous leukemia.

Phytochemicals have become a promising approach in the management of malignancies (8, 9). G-Rh2 is one of the main active components of Panax ginseng that exerts various pharmacological activities, including antioxidation (10), anti-inflammation (10, 11), anticancer (12, 13), and reverse drug resistance (14-16). Recent studies have demonstrated the anticancer potential of G Rh2 in various human cancer cell lines, including lung cancer (17), colorectal cancer (18, 19), and liver cancer (20). For example, G-Rh2 was found to inhibit NF-κB activation by targeting annexin A2 (ANXA2) in breast cancer cells (21). G-Rh2 was also reported to suppress cancer cell growth by inhibiting the PI3K/Akt/mTOR (22), Wnt/beta-catenin (23), and TNF-α signaling pathways (24). Moreover, G-Rh2 could reverse 5-fluorouracil (5-FU) and oxaliplatin resistance in colorectal cancer cells (15, 16). However, the effects of G-Rh2 in oral cancer cells and its underlying mechanisms have not been well studied.

In this study, we investigated the anticancer effects of G-Rh2 in YD10B and Ca9-22 oral cancer cells and their underlying molecular mechanisms. We found that G-Rh2

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significantly inhibits oral cancer cell proliferation, migration, and invasion, and induces cell cycle G_0/G_1 phase arrest and apoptosis. In addition, G-Rh2 treatment inhibited the Src/Raf/ERK signaling pathway in oral cancer cells.

Materials and Methods

Reagents and antibodies. G-Rh2 (purity: >98% assessed by high-performance liquid chromatography [HPLC]) was obtained from Harvey Biotech Co., Ltd (Beijing, China). The different working concentrations of G-Rh2 were dissolved in dimethyl sulfoxide (DMSO). The primary antibodies, cleaved caspase-3, cleaved PARP, p53, Bax, cyclin E1, E-cadherin, N-cadherin, Src, p-Src (Tyr416), B-Raf, p-B-Raf (Ser445), ERK1/2, p-ERK1/2 (Thr202/Tyr204), and Slug, were purchased from Cell Signaling Technology (Danvers, MA, USA). Vimentin was purchased from Abcam company (Cambridge, MA, USA). β-Actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. YD10B and Ca9-22 human oral cancer cell lines were purchased from the Korean Cell Line Bank (KCLB; Seoul, Republic of Korea). The cells were incubated in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (Gibco) and 1% antibiotics. Cells were maintained at 37˚C in a 5% CO_2 incubator.

Cell viability assay. Cell Counting Kit-8 assays were performed to assess cell viability. Briefly, oral cancer cells were plated on 96-well plates at a density of 1,000 cells/well. After 24 h of culturing, the medium was replaced with a new medium containing specified concentrations of G-Rh2. Then, the cells were cultured for 0, 24, 48, 72, and 96 h, followed by incubation with 10 μl CCK-8 regent per well for an additional 1 h at 37˚C. The optical density of each well at 450 nm was measured using a microplate reader (BioTek Instruments).

Soft agar colony formation assay. YD10B and Ca9-22 cells (8×10^3 cells/well) were suspended in complete growth medium containing 0.3% agar with specified concentrations of G-Rh2, then overlaid into 6-well plates containing 0.6% agar and specified concentrations of G-Rh2. The cultures were incubated for 14 days in a 5% CO_2 incubator at 37˚C. Next, photographs were taken under a microscope (Leica), and the number of colonies was counted using ImageJ software.

Cell cycle and apoptosis analysis. Oral cancer cells were seeded into 60-mm culture dishes (1×10^5 cells/dish) and cultured overnight at 37˚C. The cells were then treated with the indicated concentrations of G-Rh2 for 48 h. To analyze the cell cycle, cells were collected after they were digested by trypsin and centrifuged at 800 rpm for 5 min. Then, the cells were washed with cold phosphate-buffered saline (PBS) twice and fixed in 70% ethanol at −20˚C overnight. Cells were centrifuged at 2,000 rpm for 5 min and washed with PBS followed by incubation with propidium iodide (PI, 20 μg/ml) and RNase (100 μg/ml) in the dark for 30 min. Next, the cell characteristics were detected and measured using flow cytometry. To analyze apoptosis, the cells were stained with Annexin V-FITC (BioLegend, USA) and PI in the dark for 20 min and subsequently analyzed using a FACS Verse flow cytometry (BD Science, CA, USA).

Invasion and migration assays. Migration assay was performed in 24-well Transwell plates (8 μm pore size, Corning) according to the manufacturer's instructions. Cells were seeded in the upper chambers at a density of 8×10^4 cells in 100 μl serum-free DMEM with the indicated concentrations of G-Rh2. The lower chambers were filled with 600 μl culture medium containing 10% FBS to stimulate cell movement. After 48 h of culturing, the cells were fixed using 4% paraformaldehyde for 20 min, the non-invaded cells were removed using a cotton swab, and the invaded cells were stained with 0.05% crystal violet. For invasion assays, the chamber was pre-coated with Matrigel®, before repeating the same steps as for the migration assay. The invaded cell numbers were quantified by counting the stained cells under a microscope.

Western blotting. Oral cancer cells (1×10^6) were plated in 10-cm dishes and incubated with 0, 10, 20, and 40 μM G-Rh2 at 37˚C for 48 h. Subsequently, the cells were collected and lysed using the PRO-PREP™ lysis buffer (Intron Biotechnology, Republic of Korea). The protein concentrations were measured by NanoDrop™ 2000 (Thermo Fisher Scientific). A total of 30 μg protein was loaded, separated *via* SDS-PAGE, and then transferred to a polyvinylidene difluoride (PVDF) membrane (0.22 μm, Merck Millipore). The membranes were blocked in 5% BSA for 1 h, then incubated with the corresponding primary antibodies at 4˚C overnight, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The protein signals were detected with an ECL detection kit (GE Healthcare, Seoul, Republic of Korea) using a Da Vinci Fluorescence Imaging System (Da Vinci-K, Seoul, Republic of Korea). β-Actin was used as the loading control.

Statistical analysis. Results are presented as the mean±SD from three independent experiments. Statistical significance was determined using a Student's *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

Results

G-Rh2 exhibits antiproliferative effects in oral cancer cells. The chemical structure of G-Rh2 is shown in Figure 1A. To evaluate the effect of G-Rh2 on the growth of human oral cancer cells, we treated YD10B and Ca9-22 cells with different concentrations (0, 10, 20, 30, 40, and 50 μM) of G-Rh2 for 48 h. As shown in Figure 1B, the cell morphology changed to round, and the cell density decreased with the G-Rh2 treatment. Most oral cancer cells died with 50 μM G-Rh2. Thus, we selected the concentrations 0, 10, 20 and 40 μM G-Rh2 in the following experiments. Cell proliferation was analyzed using CCK-8 assay. As shown in Figure 1C, G-Rh2 inhibited oral cancer cell proliferation in a dose- and time-dependent manner. The soft agar colony formation assay showed that the number and size of clones in YD10B and Ca9-22 cells were markedly reduced by G-Rh2 treatment in a dose-dependent manner. These results indicate that G-Rh2 effectively inhibits oral cancer cell growth.

G-Rh2 inhibits the migration and invasion of oral cancer cells. Metastasis of cancer cells is a difficult problem in

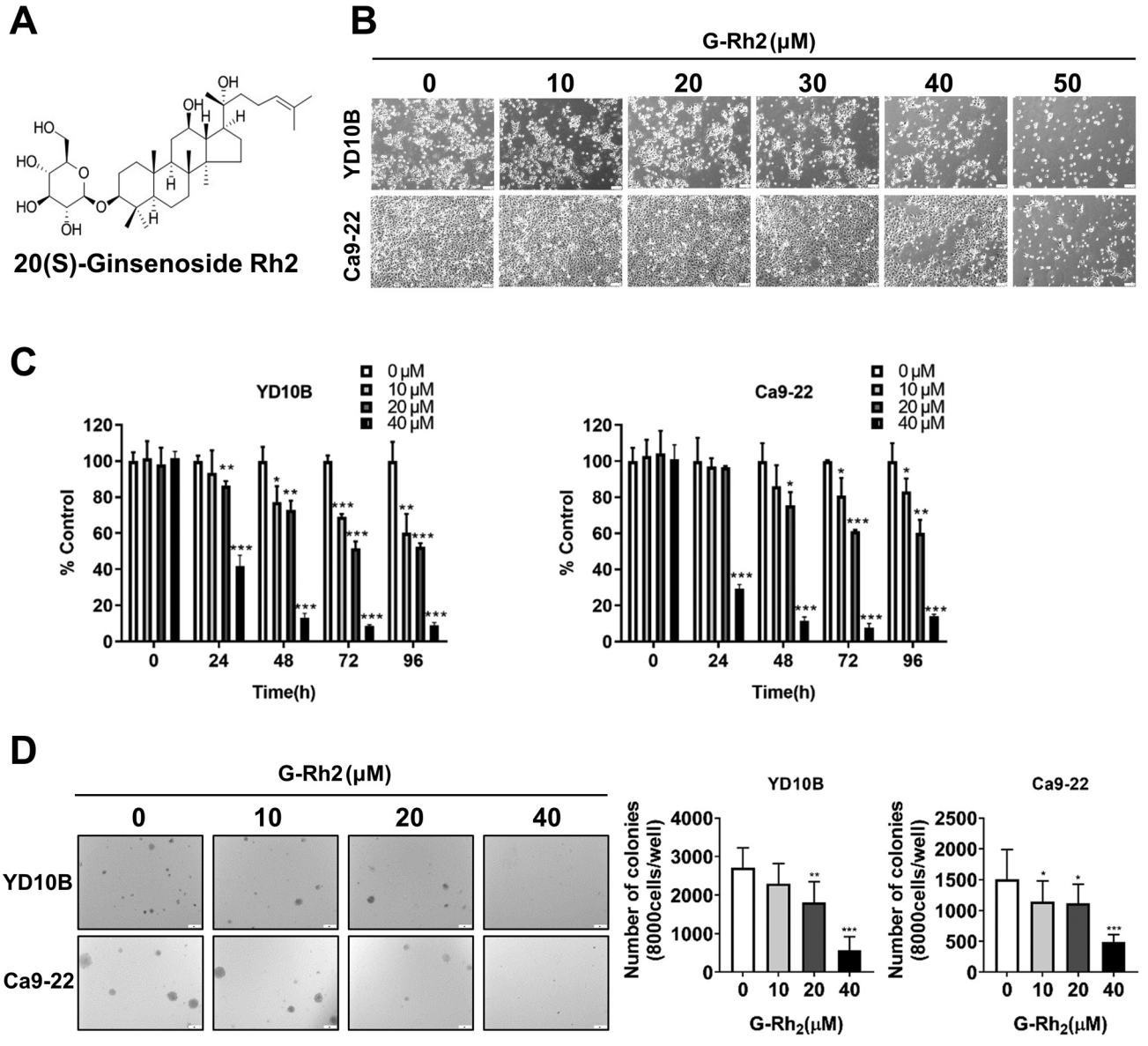

Figure 1. *G-Rh2 exhibits antiproliferative effects in oral cancer cells. (A) Chemical structure of G-Rh2. (B) Cell morphology of YD10B and Ca9-22 cells after treatment with the indicated concentrations of G-Rh2 for 48 h, observed under a light microscope (magnification, 100×). (C) Oral cancer cells were treated with 0, 10, 20, and 40 μM G-Rh2 for 0, 24, 48, 72, and 96 h. Cell viability was measured with CCK-8 assay. (D) Effects of G-Rh2 on colony formation in YD10B and Ca9-22 cells (magnification, 50×). *p<0.05; **p<0.01; ***p<0.001.*

cancer treatment. We wondered whether G-Rh2 could inhibit oral cancer cell metastasis. We then detected the effect of G-Rh2 on the migration and invasion properties of oral cancer cells by using Transwell migration and invasion assays, and the results showed that the migration and invasion capability of oral cancer cells was significantly inhibited by G-Rh2 in a dose-dependent manner (Figure 2A-D). Accumulated evidence showed that the epithelial-mesenchymal transition (EMT) process was associated with cancer cell growth and metastasis (25, 26). Thus, we further explored the effect of G-Rh2 on the expressions of EMT marker proteins, including E-cadherin, N-cadherin, and vimentin. The immunoblot results showed that G-Rh2 down-regulated N-cadherin and vimentin and up-regulated E-cadherin in both YD10B and Ca9-22 cells (Figure 2E). These results indicate that G-Rh2 inhibits the migration and invasion of oral cancer cells by reversing the EMT process.

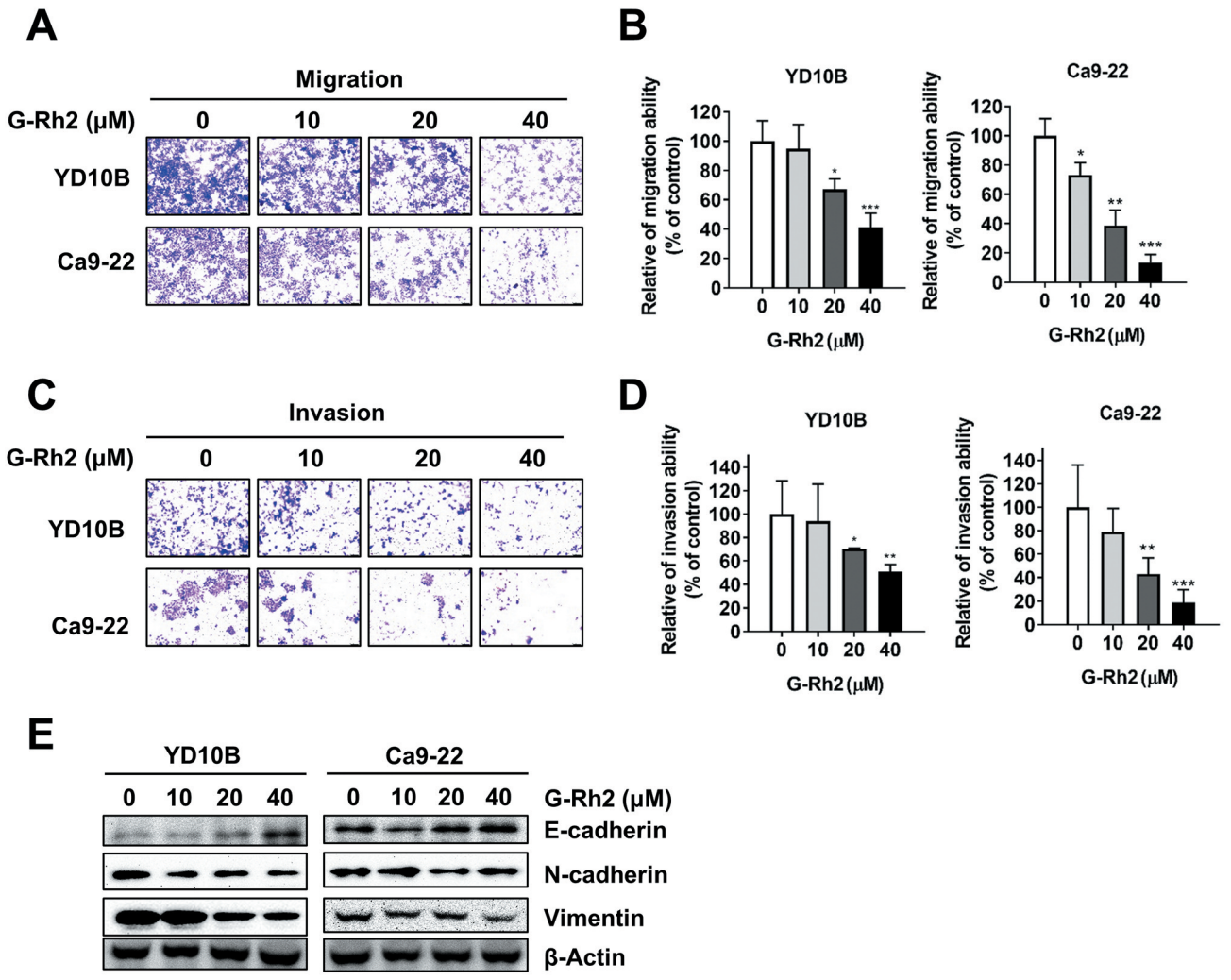

Figure 2. *G-Rh2 inhibits the migration and invasion of oral cancer cells. (A) and (B) Migration abilities of YD10B and Ca9-22 cells determined after treatment with the indicated concentrations of G-Rh2 for 48 h. (C) and (D) The invasion abilities of YD10B and Ca9-22 cells were determined after treatment with the indicated concentrations of G-Rh2 for 48 h. (E) Western blot analysis of expression of vimentin, N-cadherin, and E-cadherin in YD10B and Ca9-22 cells after treatment with the indicated concentrations of G-Rh2 for 48 h. *p<0.05; **p<0.01; ***p<0.001.*

G-Rh2 induces the cell-cycle G0/G1-phase arrest of oral cancer cells. To explore the effect of G-Rh2 on the cell cycle, we evaluated cell-cycle distributions by flow cytometry following a treatment of G-Rh2 (0, 10, 20, 40 μM). The results indicated that G-Rh2 significantly increases the proportion of G_0/G_1-phase cells in YD10B and Ca9-22 cells (Figure 3A and B). Then, we examined the effects of G-Rh2 on the expression of G0/G1-phase-associated proteins, including cyclin D1, cyclin E1, CDK4, and CDK6, by western blot analysis. As shown in Figure 3C, G-Rh2 down-regulated the expression levels of cyclin D1, cyclin E1, CDK4, and CDK6 in YD10B and Ca9-22 cells.

G-Rh2 induces apoptosis of oral cancer cells. To determine the effect of G-Rh2 on apoptosis in oral cancer cells, the cells were treated with 0, 10, 20, and 40 μM G-Rh2 for 48 h, and the results showed that G-Rh2 induced oral cancer cell apoptosis in a dose-dependent manner (Figure 4A and B). The apoptosis induced by G-Rh2 was further evidenced by the increase of apoptotic markers, including p53, cleaved caspase-3, cleaved PARP, and Bax (Figure 4C). Collectively, these results indicated that G-Rh2 can trigger apoptosis to suppress proliferation of oral cancer cells.

G-Rh2 suppresses the Src/Raf/ERK signaling pathway in oral cancer cells. The Src/Raf/ERK signaling pathway plays a crucial role in tumorigenesis (27, 28). We then determined whether G-Rh2 had any effect on the Src/Raf/ERK signaling pathway. As presented in Figure 5A, G-Rh2 treatment

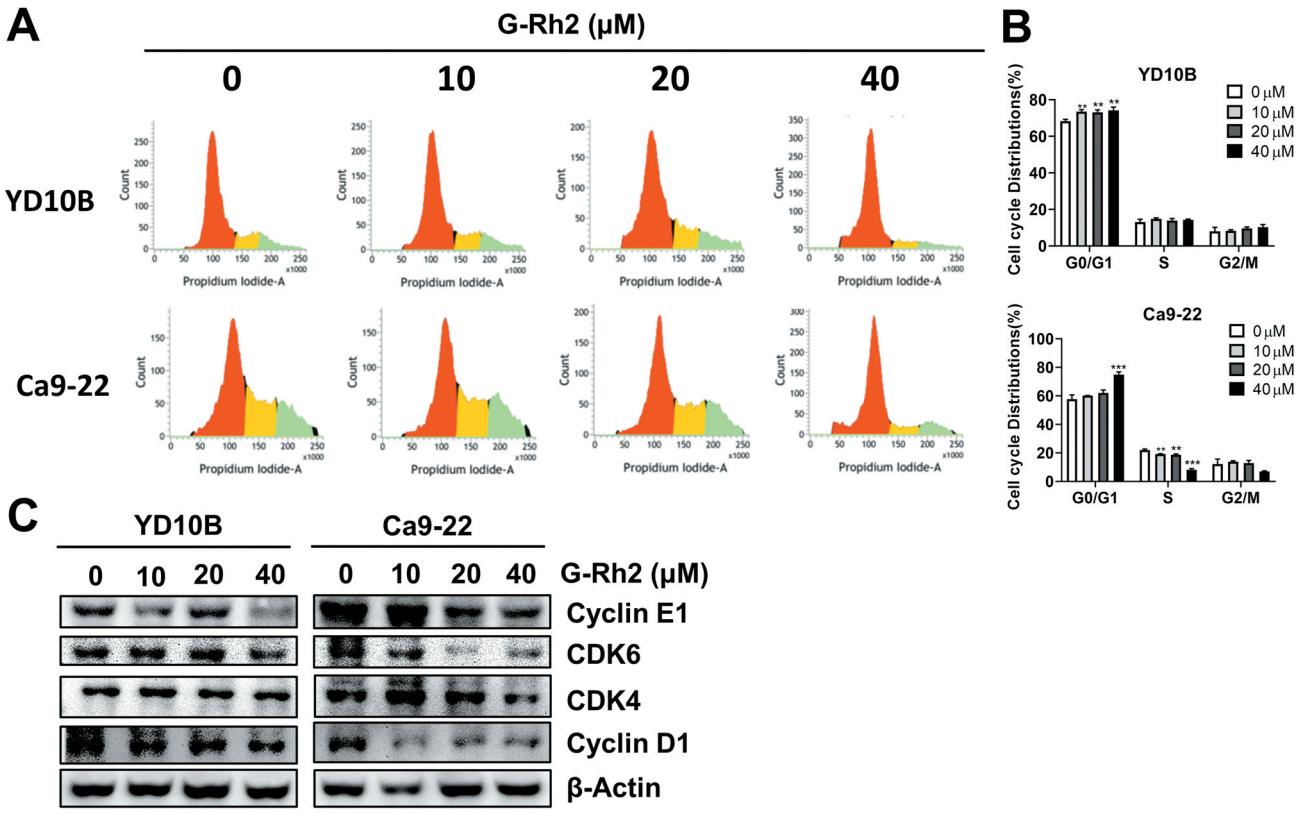

Figure 3. *G-Rh2 induces cell cycle G_0/G_1-phase arrest of oral cancer cells. (A) The effects of G-Rh2 on cell cycle distribution was quantitated by flow cytometry. (B) Bar graph representing the quantified values of cells in different phases. (C)The protein expression of Cyclin D1, Cyclin E1, CDK4, and CDK6 were determined using western blotting. *p<0.05; **p<0.01; ***p<0.001.*

suppressed p-Src, p-B-Raf, p-ERK1/2 expression in YD10B and Ca9-22 cells. Additionally, slug expression was down-regulated by G-Rh2 treatment. The mechanism of G-Rh2 in oral cancer cell growth was shown in Figure 5B. These results suggest that G-Rh2 inhibits oral cancer cell growth by inactivating the Src/Raf/ERK signaling pathway.

Discussion

Oral cancer is still a relatively high-incidence cancer with a poor prognosis. New treatment methods and effective drugs are required to conquer this disease. The present study demonstrated that G-Rh2 significantly inhibits oral cancer cell proliferation, colony formation, migration, and invasion, while inducing apoptosis and cell-cycle G_0/G_1 phase arrest. Mechanism studies showed that G-Rh2 can increase the expression of apoptotic proteins, inhibit the expression of cell cycle G_0/G_1-phase-related proteins, and regulate the level of EMT-related proteins, as well as inhibit the Src/Raf/ERK signaling pathway in oral cancer cells.

Natural products such as paclitaxel, camptothecin, and vinblastine play an important role in the clinical treatment of cancers (29). Many studies have demonstrated that G-Rh2 has a powerful therapeutic effect by inhibiting cancer-related signaling pathways in numerous cancer cells (17, 30, 31). However, there is only one report about the anticancer effects of G-Rh2 on oral cancer cells, which indicated that G-Rh2 inhibited oral cancer cell growth through decrease of MMP-2 and VEGF protein levels (32). In this study, we explored in anticancer activity of G-Rh2 in oral cancer cells through various *in vitro* experiments. Activation of apoptosis pathway is one of the main strategies for cancer treatment. According to our results, G-Rh2 increased cleaved caspase-3 and cleaved PARP expression in oral cancer cells, which indicates that G-Rh2 induced the apoptosis of oral cancer cells. Meanwhile, cell-cycle deregulation is one of the characteristics of cancer cells, whilst blocking the cell cycle of cancer cells is also one of the strategies for cancer treatment. CDK/cyclins have crucial roles in the regulation of cell cycle progression and other major biological processes (33). Cyclin D and CDK4/6 are regulators of the G_0/G_1 phase (34). In the current study, we found that G-Rh2 inhibits oral cancer cell growth by inducing G_0/G_1-phase arrest and that the molecular mechanisms were related to

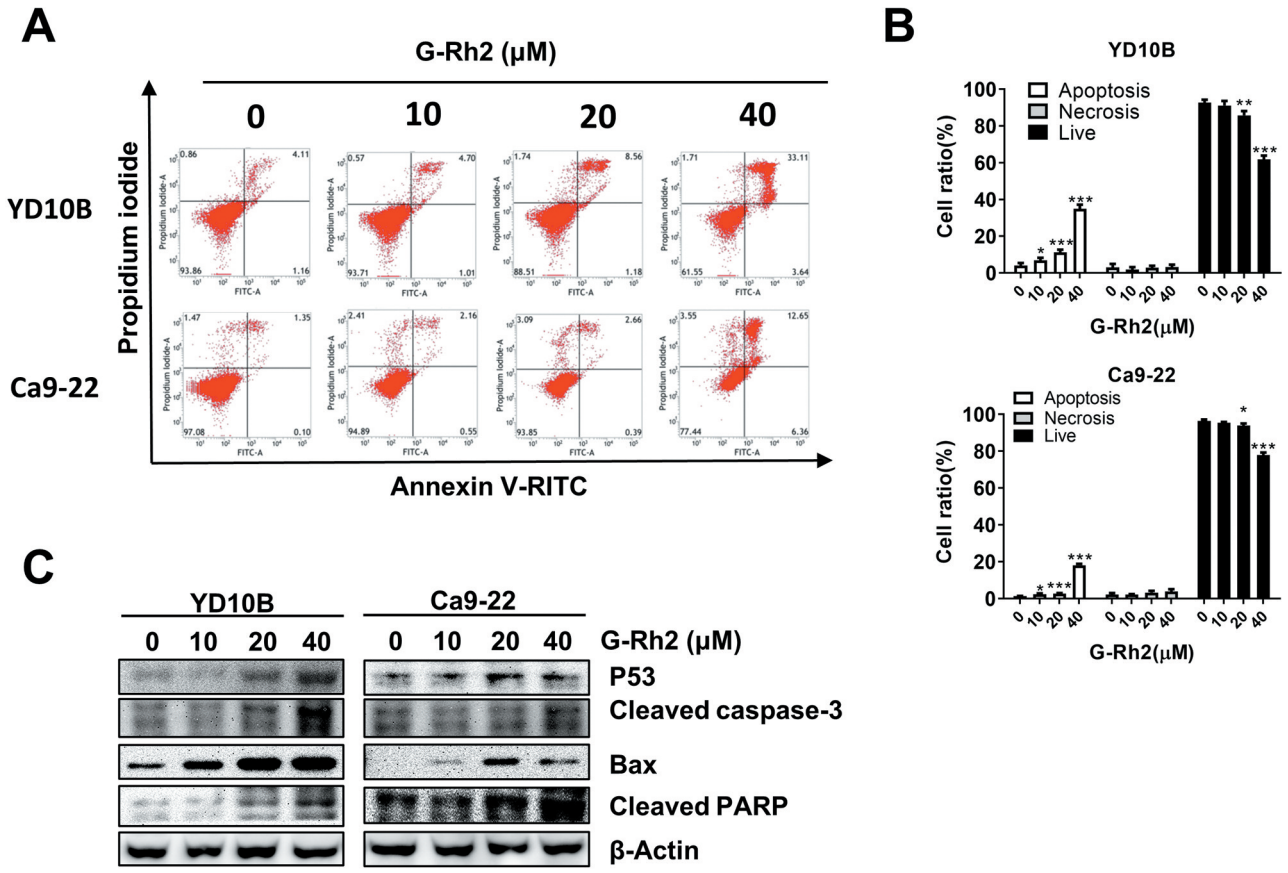

Figure 4. *G-Rh2 induces apoptosis of oral cancer cells. (A) and (B) The effects of G-Rh2 on YD10B and Ca9-22 cell apoptosis were quantitated by flow cytometry. (C) The effects of G-Rh2 on the expression of cleaved PARP, cleaved caspase-3, p53, and Bax are shown. *p<0.05; **p<0.01; ***p<0.001.*

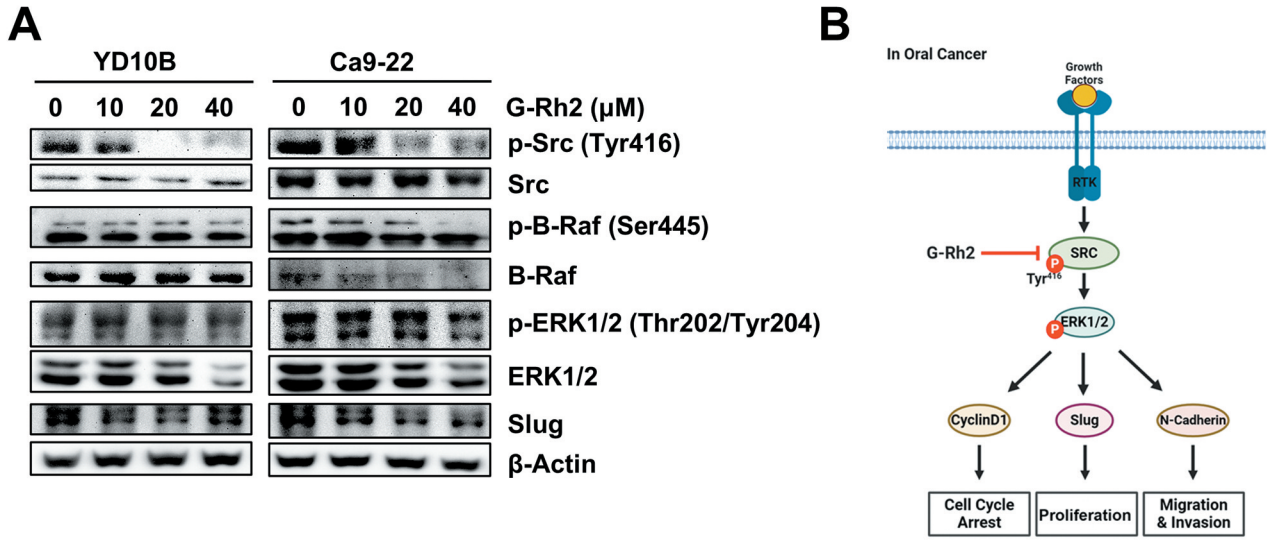

Figure 5. *G-Rh2 suppresses the Src/Raf/ERK signaling pathway in oral cancer cells. (A) The protein expression of p-Src, Src, p-B-Raf, B-Raf, p-ERK1/2, ERK1/2, and Slug were determined by western blotting. (B) The mechanism of G-Rh2 in oral cancer cell growth.*

down-regulation of cyclin E1, cyclin D1, CDK4, and CDK6. These results indicated that G-Rh2 was a potential therapeutic agent for oral cancer treatment through activation apoptosis and blocking cell cycle of oral cancer cells.

Numerous studies have proven that the Src/Raf/ERK pathway can activate a series of processes that are related to tumorigenesis and development, including cell proliferation, differentiation, and metastasis, which makes the Src/Raf/ERK pathway an attractive target for anticancer therapy (35-38). Studies have indicated that the overexpression of Src promotes breast cancer cell resistance to tamoxifen (39), inhibits Src activity through dasatinib to significantly prevent the tumorigenic and metastatic ability of P-cadherin overexpression in breast cancer cells (40). Furthermore, Src inhibition could prevent breast cancer (41) and reverse fulvestrant resistance in ovarian cancer cells (42). All these factors indicate that Src is a potential anticancer target. The current study demonstrates that G-Rh2 treatment can significantly down-regulate the protein levels of p-Src, p-B-Raf and p-ERK1/2, which indicated that G-Rh2 inhibits oral cancer cell proliferation, migration and invasion by down-regulating the Src-Raf-ERK signaling pathway.

Cancer metastasis is the major cause of cancer recurrence and death (43). EMT plays an important role in the physiology of all epithelial tissues, wound healing and tumorigenic process (44). EMT is a key biological mechanisms leading to cancer cell metastasis. Loss of E-cadherin expression and increased expression of mesenchymal markers (such as N-cadherin, vimentin) are one of the characteristics of EMT (45). It was reported that G-Rh2 inhibits cell migration and invasion in endometrium cancer cells by increasing E-cadherin and suppressing TGF-β, Snail and vimentin expression (46). Another study reported that G-Rh2 effectively suppressed Bxpc-3 cell migration and invasion through down-regulation of MMP-2 and MMP-9 (47). In this study, we investigated the effect of G-Rh2 in oral cancer cell migration and invasion ability through Transwell assay, we found that G-Rh2 significantly inhibited oral cancer cell migration and invasion ability, moreover, G-Rh2 markedly down-regulated N-cadherin and vimentin and up-regulated E-cadherin expression in oral cancer cells, indicating that G-Rh2 suppresses EMT process and prevents the metastasis of oral cancer.

Taken together, we confirmed the anticancer activity of G-Rh2 in oral cancer cells through Src/Raf/ERK signaling pathway. These findings highlight the potential of G-Rh2 as a therapeutic agent for the treatment of oral cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

Author's Contributions

HBZ performed experiments and wrote the manuscript. JKY, EYK, HH, KK and EKK designed experiments and analyzed data. YC for funding acquisition and data analysis. MYK and ZYR supervised the experiments and reviewed the manuscript.

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