Ginsenoside Rh2 Inhibits Cancer Stem-Like Cells in Skin Squamous Cell Carcinoma

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Key Words
Skin squamous cell carcinoma (SCC) • Cancer stem cells (CSCs) • Lgr5 • Ginsenoside Rh2 (GRh2) • β-catenin

Abstract
Background/Aims: Treatments targeting cancer stem cells (CSCs) are most effective cancer therapy, whereas determination of CSCs is challenging. We have recently reported that Lgr5-positive cells are cancer stem cells (CSCs) in human skin squamous cell carcinoma (SCC). Ginsenoside Rh2 (GRh2) has been shown to significantly inhibit growth of some types of cancers, whereas its effects on the SCC have not been examined. Methods: Here, we transduced human SCC cells with lentivirus carrying GFP reporter under Lgr5 promoter. The transduced SCC cells were treated with different doses of GRh2, and then analyzed cell viability by CCK-8 assay and MTT assay. The effects of GRh2 on Lgr5-positive CSCs were determined by flow cytometry and by tumor sphere formation. Autophagy-associated protein and β-catenin were measured by Western blot. Expression of short hairpin small interfering RNA (shRNA) for Atg7 and β-catenin were used to inhibit autophagy and β-catenin signaling pathway, respectively, as loss-of-function experiments. Results: We found that GRh2 dose-dependently reduced SCC viability, possibly through reduced the number of Lgr5-positive CSCs. GRh2 increased autophagy and reduced β-catenin signaling in SCC cells. Inhibition of autophagy abolished the effects of GRh2 on β-catenin and cell viability, while increasing β-catenin abolished the effects of GRh2 on autophagy and cell viability. Conclusion: Taken together, our data suggest that GRh2 inhibited SCC growth, possibly through reduced the number of Lgr5-positive CSCs. This may be conducted through an interaction between autophagy and β-catenin signaling.
Introduction

Carcinoma account for more than 80% of all types of cancer worldwide, and squamous cell carcinoma (SCC) is the most frequent carcinoma. Skin SCC causes a lot of mortality yearly, which requires a better understanding of the molecular carcinogenesis of skin SCC for developing efficient therapy [1, 2]. Ginsenoside Rh2 (GRh2) is a characterized component in red ginseng, and has proven therapeutic effects on inflammation [3] and a number of cancers [4-14], whereas its effects on the skin SCC have not been examined.

Cancer stem cells (CSCs) are cancer cells with great similarity to normal stem cells, e.g., the ability to give rise to various cell types in a particular cancer [15, 16]. CSCs are highly tumorigenic, compared to other non-CSCs. CSCs appear to persist in tumors as a distinct population and CSCs are believed to be responsible for cancer relapse and metastasis after primary tumor resection [15-18]. Recently, the appreciation of the critical roles of CSCs in cancer therapy have been continuously increasing, although the identification of CSCs in a particular cancer is still challenging.

To date, different cell surface proteins have been used to isolate CSCs from a variety of cancers by flow cytometry. Among these markers for identification of CSCs, the most popular ones are prominin-1 (CD133), side population (SP) and increased activity of aldehyde dehydrogenase (ALDH). CD133 is originally detected in hematopoietic stem cells, endothelial progenitor cells and neuronal and glial stem cells. Later on, CD133 has been shown to be expressed in the CSCs from some tumors [19-23], but with exceptions [24]. SP is a sub-population of cells that efflux chemotherapy drugs, which accounts for the resistance of cancer to chemotherapy. Hoechst (HO) has been experimentally used for isolation of SP cells, while the enrichment of CSCs by SP appears to be limited [25]. Increased activity of ALDH, a detoxifying enzyme responsible for the oxidation of intracellular aldehydes [26, 27], has also been used to identify CSCs, using aldefluor assay [28, 29]. However, ALDH has also been detected in other cell types, which creates doubts on the purity of CSCs using ALDH method [30, 31]. Moreover, all these methods appear to be lack of cancer specificity.

The Wnt target gene Lgr5 has been recently identified as a stem cell marker of the intestinal epithelium, and of the hair follicle [32, 33]. Recently, we reported that Lgr5 may be a potential CSC marker for skin SCC [34]. We detected extremely high Lgr5 levels in the resected skin SCC specimen from the patients. In vitro, Lgr5-positive SCC cells grew significantly faster than Lgr5-negative cells, and the fold increase in growth of Lgr5-positive vs Lgr5-negative cells is significantly higher than SP vs non-SP, or ALDH-high vs ALDH-low, or CD133-positive vs CD133-negative cells. Elimination of Lgr5-positive SCC cells completely inhibited cancer cell growth in vitro.

Here, we transduced human SCC cells with lentivirus carrying GFP reporter under Lgr5 promoter. The transduced SCC cells were treated with different doses of GRh2, and then analyzed cell viability by CCK-8 assay and MTT assay. The effects of GRh2 on Lgr5-positive CSCs were determined by flow cytometry and by tumor sphere formation. Autophagy-associated protein and β-catenin were measured by Western blot. Expression of short hairpin small interfering RNA (shRNA) for autophagy-related protein 7 (Atg7) and β-catenin were used to inhibit autophagy and β-catenin signaling pathway, respectively, as loss-of-function experiments. Atg7 was identified based on homology to Pichia pastoris GSA7 and Saccharomyces cerevisiae APG7. In the yeast, the protein appears to be required for fusion of peroxisomal and vacuolar membranes. The protein shows homology to the ATP-binding and catalytic sites of the E1 ubiquitin activating enzymes. Atg7 is a mediator of autophagosomal biogenesis, and is a putative regulator of autophagic function [35-38]. We found that GRh2 dose-dependently reduced SCC viability, possibly through reduced the number of Lgr5-positive CSCs. GRh2 increased autophagy and reduced β-catenin signaling in SCC cells. Inhibition of autophagy abolished the effects of GRh2 on β-catenin and cell viability, while increasing β-catenin abolished the effects of GRh2 on autophagy and cell viability.
Materials and Methods

Cell line and GRh2 in vitro administration

A431 cells are a human skin SCC cell line, which was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO₂ at 37 °C, as has been described before [34]. GRh2 (Weikeqi Bioscience, China) was prepared in a stock of 100mg/ml and applied to cultured GBM cells at 0.01mg/ml, 0.1mg/ml and 1mg/ml, respectively.

Lentivirus preparation, cell transfection and cell transduction

Plasmids carrying GFP reporter under Lgr5 promoter were prepared as has been described before [34]. The coding sequence of human Lgr5 promoter was amplified using human genomic DNA as a template, and cloned into pLVX-ZsGreen1-C1 vector (Clontech, Mountain View, CA, USA). The coding sequence of human β-catenin was amplified using human liver cDNA as a template, and cloned into pLVX-ZsGreen1-C1 vector. The shRNA for autophagy-related protein 7 (Atg7) was purchased from Qiagen (Hilden, Germany). A scramble sequence was used as the mock control (scr). To generate pLgr5-GFP lentiviral particles, NIH HEK293T cells were seeded in a 100mm dish at 50,000 cells/cm² and co-transfected with 10 µg of recombinant DNA plasmids and 5 µg each of packaging plasmids (REV, pMDL and VSV-G) using Lipofectamine-2000 (Invitrogen). The supernatant containing lentiviral particles was collected 48 hours after transfection and filtered through a 0.45 µm syringe filter. The fibroblasts were seeded in 100mm plates at 15,000 cells/cm² one day prior to lentiviral infection. The lentiviral particles were added along with 10µg/ml polybrene (Sigma-Aldrich) to the cell culture for 24 hours. Infected cells were selected by ampicillin resistance. Transfection with either shAtg7 or β-catenin-expressing plasmids was performed with Lipofectamine-2000 (Invitrogen).

Analysis of GFP by flow cytometry

For flow cytometry analysis and sorting, Lenti-pLgr5-GFP-transduced A431 cells were analyzed by GFP direct fluorescence. Flow cytometry was performed using a FACSAria (Becton-Dickinson Biosciences, San Jose, CA, USA) flow cytometer.

MTT assay

For assay of cell viability, cells were seeded into 24 well-plate at 10000 cells per well and subjected to a Cell Proliferation Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm. Experiments were performed 5 times.

Cell counting kit-8 (CCK-8) assay

The CCK-8 detection kit (Sigma-Aldrich) was used to measure cell viability according to the manufacturer’s instructions. Briefly, cells were seeded in a 96-well microplate at a density of 5X10⁴/ml. After 24h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20 ml/well) was added and the plate was incubated at 37 °C for 2 h. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

Primary Tumor Sphere Culture

Purified tumor cells by flow cytometry were washed, acutely dissociated in oxygenated artificial cerebrospinal fluid and subject to enzymatic dissociation. Tumor cells were then resuspended in tumor sphere media (TSM) consisting of a serum-free DMEM, human recombinant EGF (20 ng/ml; Sigma-Aldrich), bFGF (20ng/ml; Sigma-Aldrich), leukemia inhibitory factor (10ng/ml; Sigma-Aldrich) and N-acetylcysteine (60µg/ml; Sigma-Aldrich), and then plated at a density of 2X10⁴ cells/60 mm plate.
Western blot

Protein was extracted from the cultured cells with RIPA lysis buffer (1% NP40, 0.1% Sodium dodecyl sulfate (SDS), 100μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000×g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were rabbit anti-β-catenin, anti-Beclin, anti-Atg7, anti-LC3 and anti-α-tubulin (Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). α-tubulin was used as protein loading controls. The protein levels were first normalized to α-tubulin, and then normalized to control.

Quantitative real-time PCR (RT-qPCR)

Total RNA were extracted from cultured cells with RNeasy kit (Qiagen), for cDNA synthesis. Quantitative real-time PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with 2^\(-\Delta\Delta Ct\) method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to controls.

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferoni correction, followed by Fisher’s exact test. All values are depicted as mean ± standard deviation from 5 individuals and are considered significant if p < 0.05.

Results

Transduction of SCC cells with GFP under Lgr5 promoter

We have recently shown that Lgr5 is CSC marker for skin SCC [34]. In order to examine the role of GRh2 on SCC cells, as well as a possible effect on CSCs, we transduced human skin SCC cells A431 [34] with a lentivirus carrying GFP reporter under Lgr5 promoter (Fig. 1A). The Lgr5-positive cells were green fluorescent in culture (Fig. 1B), and could be analyzed or isolated by flow cytometry, based on GFP (Fig. 1C).

![Fig. 1. Transduction of SCC cells with GFP under Lgr5 promoter. (A) The structure of lentivirus carrying GFP reporter under Lgr5 promoter. (B) The pLgr5-GFP-transduced A431 cells in culture. Lgr5-positive cells were green fluorescent. Nuclear staining was done by DAPI. (C) Representative flow chart for analyzing pLgr5-GFP-transduced A431 cells by flow cytometry based on GFP. Gated cells were Lgr5-positive cells. Scar bar is 20μm.](image-url)
GRh2 dose-dependently inhibits SCC cell growth

Then, we examined the effect of GRh2 on the viability of SCC cells. We gave GRh2 at different doses (0.01mg/ml, 0.1mg/ml and 1mg/ml) to the cultured pLgr5-GFP-transduced A431 cells. We found that from 0.01mg/ml to 1mg/ml, GRh2 dose-dependently deceased the cell viability in either a CCK-8 assay (Fig. 2A), or a MTT assay (Fig. 2B). Next, we questioned whether GRh2 may have a specific effect on CSCs in SCC cells. Thus, we analyzed GFP+ cells after GRh2 treatment were analyzed by flow cytometry, showing that GRh2 dose-dependently decreased the percentage of GFP+ cells, by representative flow charts (C), and by quantification (D). The capability of the GRh2-treated cells to form tumor sphere-like structures was examined, shown by quantification (E), and by representive images (F). *p<0.05. N=5.

GRh2 dose-dependently inhibits SCC cell growth

Fig. 2. GRh2 dose-dependently inhibits SCC cell growth. We gave GRh2 at different doses (0.01mg/ml, 0.1mg/ml and 1mg/ml) to the cultured pLgr5-GFP-transduced A431 cells. (A-B) GRh2 dose-dependently deceased the cell viability in either a CCK-8 assay (A), or a MTT assay (B). (C-D) GFP+ cells after GRh2 treatment were analyzed by flow cytometry, showing that GRh2 dose-dependently decreased the percentage of GFP+ cells, by representative flow charts (C), and by quantification (D). The capability of the GRh2-treated cells to form tumor sphere-like structures was examined, shown by quantification (E), and by representative images (F). *p<0.05. N=5.
representative flow charts (Fig. 2C), and by quantification (Fig. 2D). We also examined the capability of the GRh2-treated cells in the formation of tumor sphere. We found that GRh2 dose-dependently deceased the formation of tumor sphere-like structure, by quantification (Fig. 2E), and by representative images (Fig. 2F). Together, these data suggest that GRh2 dose-dependently inhibited SCC cell growth, possibly through inhibition of CSCs.

**GRh2 treatment decreases β-catenin and increases autophagy in SCC cells**

We analyzed the molecular mechanisms underlying the cancer inhibitory effects of GRh2 on SCC cells. We thus examined the growth-regulatory proteins in SCC. From a variety of proteins, we found that GRh2 treatment dose-dependently decreases β-catenin, and...
dose-dependently upregulated autophagy-related proteins Beclin, Atg7 and increased the ratio of LC3 II to LC3 I, by quantification (Fig. 3A), and by representative Western blots (Fig. 3B). Since β-catenin signaling is a strong cell-growth stimulator and autophagy can usually lead to stop of cell-growth and cell death, we feel that the alteration in these pathways may be responsible for the GRh2-mediated suppression of SCC growth.

**Inhibition of autophagy abolishes the effects of GRh2 on β-catenin**

In order to find out the relationship between β-catenin and autophagy in this model, we inhibited autophagy using a shRNA for Atg7, and examined its effect on the changes of β-catenin by GRh2. First, the inhibition of Atg7 in A431 cells by shAtg7 was confirmed by RT-qPCR (Fig. 4A), and by Western blot (Fig. 4B). Inhibition of Atg7 resulted in abolishment of the effects of GRh2 on other autophagy-associated proteins (Fig. 4B), and resulted in abolishment of the inhibitory effect of GRh2 on β-catenin (Fig. 4B). Moreover, the effects of GRh2 on cell viability were completely inhibited (Fig. C). Together, inhibition of autophagy abolishes the effects of GRh2 on β-catenin. Thus, the regulation of GRh2 on β-catenin needs autophagy-associated proteins.

**Overexpression of β-catenin abolishes the effects of GRh2 on autophagy**

Next, we inhibited the effects of GRh2 on β-catenin by overexpression of β-catenin in A431 cells. First, the overexpression of β-catenin in A431 cells was confirmed by RT-qPCR...
(Fig. 5A), and by Western blot (Fig. 5B). Overexpression of β-catenin resulted in abolishment of the effects of GRh2 on autophagy-associated proteins (Fig. 5B). Moreover, the effects of GRh2 on cell viability were completely inhibited (Fig. 5C). Together, inhibition of β-catenin signaling abolishes the effects of GRh2 on autophagy. Thus, the regulation of GRh2 on autophagy needs β-catenin signaling. This model is thus summarized in a schematic (Fig. 6), suggesting that GRh2 may target both β-catenin signaling and autophagy, which interacts with each other in the regulation of SCC cell viability and growth.

Discussion

Understanding the cancer molecular biology of skin SCC and identification of an effective treatment are both critical for improving the current therapy [1]. Lgr5 has been recently identified as a novel stem cell marker of the intestinal epithelium and the hair follicle, in which Lgr5 is expressed in actively cycling cells [32, 33]. Moreover, we recently showed that Lgr5-positive are CSCs in skin SCC [34]. Thus, specific targeting Lgr5-positive cells may be a promising therapy for skin SCC.

In the current study, we analyzed the effects of GRh2 on the viability of SCC. Importantly, we not only found that GRh2 dose-dependently decreases SCC cell viability, but also dose-dependently decreased the number of Lgr5-positive CSCs in SCC cells. These data suggest that the CSCs in SCC may be more susceptible for the GRh2 treatment, and the decreases in CSCs may result in the decreased viability in total SCC cells. This point was supported by following mechanism studies. Activated β-catenin signaling by WNT/GSK3β prevents degradation of β-catenin and induces its nuclear translocation [39]. Nuclear β-catenin thus activates c-myc, cyclinD1 and c-jun to promote cell proliferation, and activates Bcl-2 to inhibit apoptosis [39]. High β-catenin levels thus are a signature of CSCs. Therefore, it is not surprising that CSCs are more affected than other cells when GRh2 targets β-catenin signaling.

In addition, GRh2 appears to target autophagy. Although altered metabolism may be beneficial to the cancer cells, it can create an increased demand for nutrients to support cell growth and proliferation, which creates metabolic stress and subsequently induces autophagy, a catabolic process leading to degradation of cellular components through the lysosomal system [40]. Cancer cells use autophagy as a survival strategy to provide essential biomolecules that are required for cell viability under metabolic stress [40]. However, autophagy not only results in a starving in cell growth, but also may result in cell death [40]. Increases in autophagy may substantially decrease cancer cell growth. Thus, GRh2 has its inhibitory effect on skin SCC cells through a combined effect on cell proliferation (by decreasing β-catenin) and autophagy [40].

Interestingly, our data suggest an interaction between β-catenin and autophagy. This finding is consistent with previous reports showing that autophagy negatively modulates Wnt/β-catenin signaling by promoting Dvl instability [41, 42], and with other studies showing that β-catenin regulates autophagy [38, 43, 44].

Of note, we have checked other SCC lines and essentially got same results. Together with our previous reports showing that Lgr5-positive cells are CSCs in skin SCC [34], these findings thus highlight a future engagement of Lgr5-directed GRh2 therapy, which could be performed in a sufficiently frequent manner, to substantially improve the current treatment for skin SCC.

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Disclosure Statement

The authors have declared that no competing interests exist.

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