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Rg1 induced anti-cancer effect through a T-cell dependent manner in head and neck squamous cell carcinomas

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Abstract

Purpose: To explore the effect of Ginsenoside Rg1 (Rg1) on the treatment of head and neck squamous cell carcinoma (HNSCC), investigating the involved underlying mechanism.

Methods: Human- and mice-derived cell lines were obtained to confirm the anti-tumor and immunomodulatory effects, the indicators including cell proliferation and cell apoptosis were detected using CCK8 assay, TUNEL staining, respectively. apoptosis protein were detected by western blot. C3H/He mice models were subcutaneously injected with HNSCC cells to establish the tumor model. The tumor growth and apoptosis of the HNSCC model were monitored. Flow cytometry was employed to analyze the percentage of T-cells.

Results: Rg1 was effective to inhibit the HNSCC cell proliferation by inducing the cell apoptosis in a time-dependent way at different time points. Orally Rg1 application in mice inhibited tumor growth compared to control mice, and increased lymphocytes were found in harvested tumors of treated animals. The role of anti-tumor of Rg1 in HNSCC mice is possibly associated with the elevation of T-cell immunomodulatory activities. The suppression of tumor growth by Ginsenoside Rg1 was not reversed by the depletion of NK cells. When the mice were depleted of CD3+ cells, all mNPC01-induced tumor inhibition was abrogated.

Conclusion: Rg1 plays an effective role in the proliferation and induces the cell apoptosis of HNSCC, by elevating the T-lymphocytic infiltration in tumor tissues. This study provided a new therapeutic medicine for patients with HNSCC, identifying that Rg1 may play an effective role in tumor control.

Keywords: Head and neck squamous cell carcinomas; Rg1; proliferation; apoptosis; T-cell.

Introduction

Head and neck squamous cell carcinomas (HNSCC) comprise a group of biologically similar cancers that originate in different structures, starting in the lip, oral cavity (mouth), nasal cavity (inside the nose), paranasal sinuses, pharynx and larynx. HNSCC is the sixth most common malignancy worldwide, it is predicted that the number of HNSCC patients will rise by 30% in 2030 [1]. HNSCC can lead to aesthetic alterations of the face and neck with disturbance of vital functions such as breathing, swallowing, phonation and hearing. Although significant progress has been made in surgical techniques, radiotherapy and chemotherapy, the prognosis is still poor [2]. In recent years, the requirement for a multidisciplinary strategy is essential due to the necessity of combined therapy treatment protocols, it is recommended that HNSCC patients should be treated in high-volume hospitals with experienced multidisciplinary teams [3]. Several novel therapeutic approaches appear on the horizon, such as immune checkpoint therapies and traditional Chinese medicine (TCM), which have achieved optimistic breakthroughs in treatment [4, 5]. With the increasing application of TCM to treat patients with different types of tumors, it is shown several merits including abundant sources, relatively lower side effects, low costs and multiple molecular targets [6]. It is reported that TCM can exert antitumor effects by upregulating immune responses even in the immunosuppressive tumor microenvironment, such as inhibition of M2-type macrophages and Treg cells [7].

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Our previous study identified a prescription named NPC01, a Chinese herbal formula, playing an anti-tumor role in the treatment of HNSCC patients [8]. In latest study, we upgraded the original ingredients and named the new prescription [9-11], the major composition include high dose Rg1. Rg1 was the active ingredient extract from the herb Panax ginseng, exhibited a wide range of pharmacological effects, including anti-cancer, anti-apoptotic and neuroprotective effects [11]. However, the action mechanism of Rg1 in regulating immunomodulatory and the underlying mechanism of the tumor-suppressive effects in HNSCC is currently poorly understood. In this study, we aim to investigate the therapeutic role of Rg1 and its underlying mechanism involved in anti-tumor effect, hoping to provide basic information and clinical guidance for patients with HNSCC.

Materials and Methods

Drugs: Ginsenoside Rg1 (C42H72O14) was purchased from Purifa Technology Development Co. Ltd.

Cell culture: The hypopharyngeal carcinoma (HP) cell line HN30 (human) and SCC7 (mice) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were both cultured in DMEM(Gibco, NY,USA) containing 10% fetal calf serum (FCS, Gibco, USA) , and put in a humidified incubator with 5% CO2 at 37°C.

Rg1-treated mice model: Female C3H/He mice aged 6-8 weeks were purchased from Tianjin Medical Institute (Tianjin, China). All mice were maintained under SPF conditions in the animal facilities of the Nankai Hospital. SCC7 cells (6×105) were subcutaneously injected into the right flank. When tumors became palpable, the tumor-bearing mice were treated with Rg1 (orally administrated with 200 mg/kg/day ginsenoside Rg1 for 14 consecutive days by gavage) and model control (MC, 0.5% sodium carboxymethyl cellulose solution by gavage) groups. The tumor growth and survival analysis were recorded. All the mice were sacrificed, the tumor volume and the weight were calculated. Tumor tissues were extracted and processed for subsequent experiments. Tissue samples including serum, tumors and some major organs were collected for further experiments. Animal experiments were approved by the Animal Ethics Committee. All the experimental procedures were approved by the guidelines of the Ethical Committee Experimental Animal Center of Tianjin Medical University.

H&E, TUNEL and Immunohistochemistry Assay

Collected tissues were fixed in 4%paraformaldehyde (PFA) and cut into 4μm sections. HE staining was performed using an HE staining kit, purchased from Beyotime (Jiangsu, China). Necrosis and apoptosis conditions of cells were assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay with an apoptosis detection kit (Roche, California, USA), according to the manufacturer's instruction. As for immunohistochemical analysis, the tumor or organ tissue sections were incubated with primary antibodies. Then the slides were processed by DAB Substrate Kit (Zhongshan Jinqiao Corp. Beijing, China). These staining slides were captured under the microscope (Olympus,Tokyo, Japan) and quantitatively analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA).

ELISA assay

The serum levels of cytokines were measured by ELISA kits (MultiSciences Biotech Co., Ltd., Hangzhou, China). Besides, the serum levels of aspartate aminotransferases (AST) and alanine aminotransferase (ALT) were detected by commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

CCK8 assay

CCK8 assay was applied to check the splenic lymphocytes, analyzing the lymphocyte proliferation. Briefly, the splenic suspension was put in RPMI-1640 medium, these cells were treated with the lysis buffer (0.747% Tris-NH4Cl buffer, pH7.4) to get rid of red blood cells, then centrifuged at 200×g for 5min. The splenocytes (100 μL/well) were seeded into the 96-well plates with or without Con A (5 μg/mL) or LPS (10 μg/mL). 10 μL CCK8 reagent was added to each well and incubated for 2h, the results were read at an absorbance of 450nm using a microplate reader. Splenic lymphocytes were regarded as effector cells for splenic NK cell activity assay, and the cell was used as the target cell for NK. Briefly, splenic lymphocytes were co-cultured with target cells at a ratio of 50:1 in the 96-well plates. Then the plates were incubated at 37◦C for 22h. The cytotoxic activity of NK cells was measured as the same as CCK-8.

Flow cytometry

The spleen lymphocytes were collected and incubated with anti-CD3+ (FITC), anti-CD4+ (PE-Cy5), anti-CD8+ (PE) and anti-NK1.1 (PE-Cy7) for 30 min at 4◦C in the dark. Then the percentages of NK, CD4+ T and CD8+ T cells were analyzed by flow cytometry (Beckman Coulter FC500, Miami, FL, USA).

Western blot

Total tumor protein was extracted from tumor tissues of mice with RIPA lysis buffer (Beyotime Institute of Biotechnology, Beijing, China), and the concentration was detected by a BCA kit (Beyotime). The protein samples were separated using 10% SDS-PAGE and transferred onto PVDF membrane (Merck Millipore, Billerica, MA, USA). The next day, the PVDF membranes were incubated with corresponding secondary antibodies for 1h. The bands were checked by the enhanced chemiluminescence reagent (ECL) (Millipore), the images were captured by AlphaImager HP system (Cell Biosciences, Inc., Santa Clara, Ca, USA).

Statistical Analysis

Data were represented as mean ± SD. All data were analyzed by Student's t-test (two groups) or one-way ANOVA (three or more groups) using Prism version 5.0 (GraphPad Software, Inc.). $P < 0.05$ was considered statistically significant.

Results

Rg1 induces apoptosis of HN30 cells

To evaluate the potential apoptotic effects of Rg1 on HN30 cells, the CCK8 and TUNEL assays were applied here. The results showed that Rg1 was effective to induce cell apoptosis in a time-dependent way at the time points of 0 h, 12 h, 24h and 48h (**Figure 1a**). Besides, the TUNEL assay indicated that the cell apoptosis was induced by mNPC01 in a dose-dependent manner after 72h interference (**Figure 1b**).

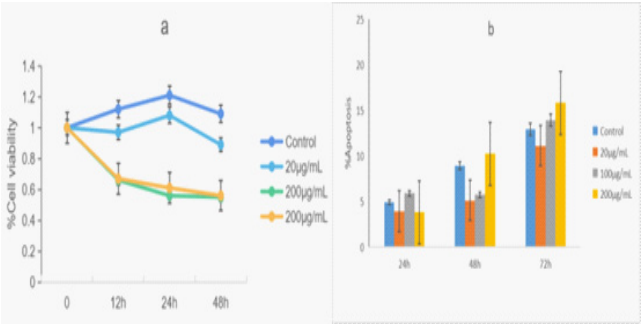

Figure 1: *Cell viability and apoptosis in HN30 treated by Gg1. a. CCK8 assay was applied to evaluate the Cell viability of HN30. HN30 cells cultured in serum-free DMEM served as a control. b. Cell apoptosis was induced by Rg1 with a dose-dependent manner detected by TUNEL assay.*

Rg1 inhibits the proliferation in HP cells

To assess the effects of the Rg1 on the proliferation of HN30 and SCC7 cells, the viable cells were then counted using a hemocytometer after trypan blue staining to exclude dead cells, after being treated with Rg1 (20/100 /200μg/mL) for 48 h. Our data showed a significant reduction in the number of NH30 and SCC7 cell lines after being treated, compared to the control group (**Figure 2a**). Moreover, SCC7 cell line was subcutaneously injected into the female C3H/He mice aged 6-8 weeks, daily oral administration of the Rg1 was then carried out from 3 days after cells injection. The results represented Rg1 plays an important role of anti-tumor growth in vivo (**Figure 2b**).

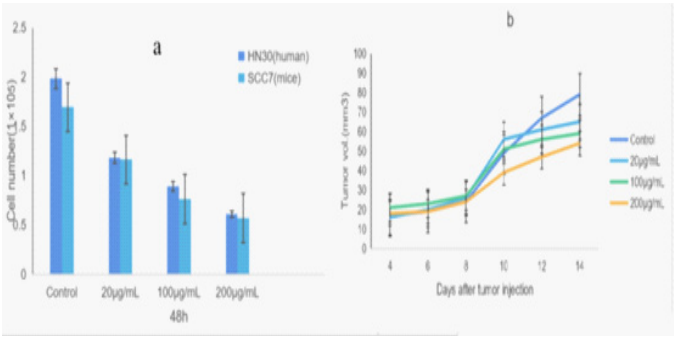

Figure 2: *Rg1 inhibits the proliferation of hypopharyngeal carcinoma cell line HN30 and SCC7. a. Viable cells were counted using hemocytometer after trypan blue staining. b. Tumor growth curve in C3H/He mice subcutaneously injected with SCC7 cell line.*

Rg1 induces cell apoptosis in HP Cells

To determine the underlying mechanism of the anti-tumor growth effect of Rg1, the morphological changes associated with apoptosis indicators were observed after the Rg1 treatment for 48h. HP cells showed obvious morphological changes including cell shrinkage were calculated. As shown in **Figure 3a**, the percent of impaired cells was significantly increased in Rg1 treatment groups in a dose-independent manner. Besides, the levels of expression of cell apoptotic markers (caspase-3, caspase-8, Bax and Bcl-2) were detected using western blot. **Figure 3b** showed that caspase-3, caspase-8and Bax proteins expression were increased after 48h of Rg1 treatment, while the bcl-2 protein expression level was decreased.

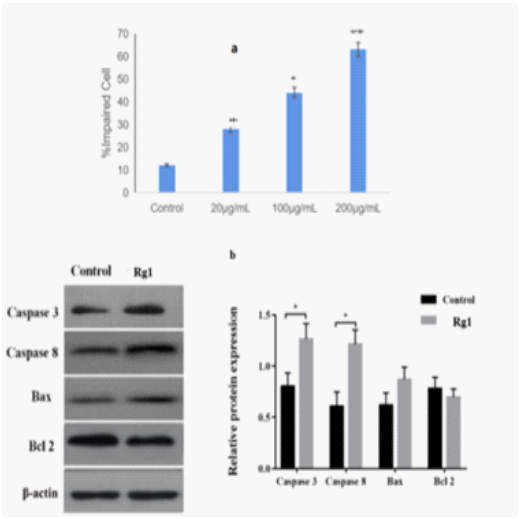

Figure 3: *Rg1 induces cell apoptosis in HP Cells. a. The percentage of impaired HP cells were counted stained by trypan blue. b. The expressions of apoptosis proteins were detected by western blot.*

Rg1 enhances the production of immune cytokines in vivo

The mice tumor was collected, and then flow cytometry was employed to assess the production levels of immune cytokines. Our results showed that Rg1 could significantly promote splenocyte restoration compared to the control group, and T lymphocyte was elevated in the Rg1 group. No difference was found in B lymphocyte proliferation index ($P < 0.05$) (**Figure 4A-C**). Moreover, the infiltration of helper T cells, cytotoxic T cells and NK cells and their relevant cell subtypes were analyzed. It is shown that the percentages of CD4+ and CD8+ T cells were increased in the Rg1 group (**Figure 4D-E**), and the percentage of NK cells was significantly upregulated as well (**Figure 4F**).

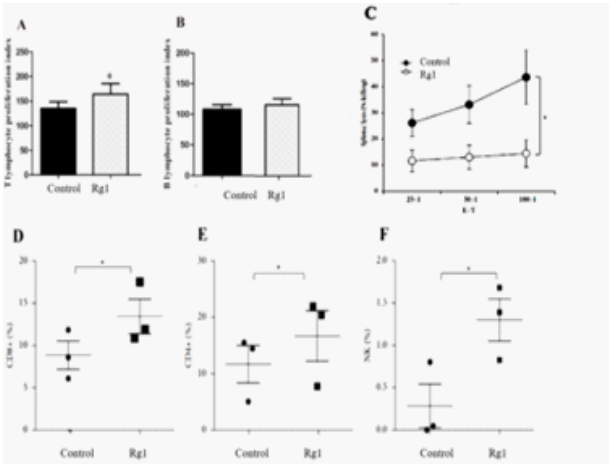

Figure 4: *Rg1 enhances the production of immune cytokines in HP cells. A, B. Effects of Rg1 on proliferation of T and B lymphocytes. Splenic lysis was analyzed as shown in 4C. The percentages of CD4+, CD8+ T and NK cells were represented in 4D-F, respectively.*

Rg1 induces the lymphocytic infiltration without NK Cell depletion in vivo

Since Rg1 could increase NK cell infiltration, we further explored the relationship between tumor inhibition and NK cells. After mice were treated with Rg1, the depletion of NK cells was accomplished by using the anti-NK1.1 antibody

PK136. Tumors were harvested after 10 days and stained for CD4+ and CD8+ lymphocytes within the tumors. The results indicated that the tumor volumes were significantly reduced in mice treated with Rg1, compared to control mice (P<0.01). Intriguingly, the inhibition of tumor growth including tumor volume and weight in the presence of Rg1 was not reversed by PK136 antibody (**Figure 5a, b**), demonstrating the tumor inhibition was not in a NK cell-dependent way. We detected a marked increase in the numbers of both CD4+and CD8+ cells infiltrating the tumor tissues when compared to control (**Figure 5c**). These results demonstrate that Rg1 inhibits tumor growth in vivo and induces lymphocytic infiltration into the tumors of treated mice.

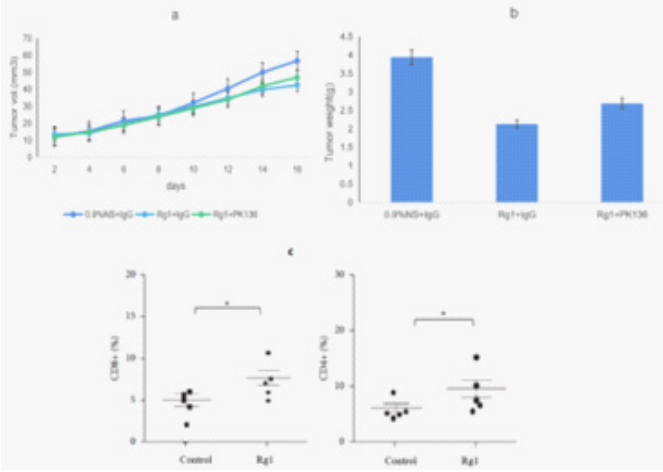

Figure 5: *Rg1 induces the lymphocytic infiltration without NK Cell depletion in vivo. Tumor volume (a) and tumor weight (b) were analyzed. c. The percentage of CD4+and CD8+ cells was detected in tumor tissues.*

Tumor growth inhibition of Rg1 is abrogated by depleting lymphocytes in vivo

C3H/He mice were treated with 3 doses of 200μg anti-CD3 monoclonal antibody given intraperitoneally, and antibody was delivered for two days prior to cells implantation and on day 3 of Rg1. The data showed the tumor volume in Rg1+antiD3 mice was similar to the control mice (**Figure 6a**). We further analyzed the percentage of CD3+ cells in tumor tissues, and no difference was found in Rg1treated and control groups.

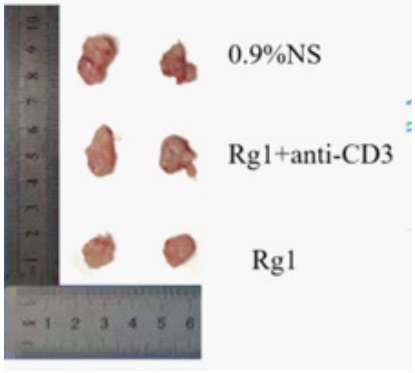

Figure 6: *Tumor growth inhibition of Rg1 is abrogated by depleting lymphocytes in vivo. a. Tumor volume was analyzed in vivo.*

Discussion

HNSCC is widely distributed in southern China with high mortality, leading to a huge socio-economic burden. Although there are several widespread treatments such as optimization of chemotherapy strategies and intensity-modulated radiotherapy, the survival of patients remains poor [12]. Therefore, it is imperative to find novel therapeutic approaches for patients. In the current study, we identified the treatment effects of Rg1, on HNSCC progression. Intriguingly, Rg1 could effectively inhibit cell proliferation through enhancing the T-lymphocytic infiltration. Our study provided a new insight of the treatment strategy for patients with HNSCC, identifying that Rg1 may play an effective role in tumor control.

Burgeoning studies identified that TCM could be regarded as a mainstream form of complementary and alternative therapy with beneficial effects for patients with various malignancies, such as HNSCC, breast cancer, lung cancer, hepatic carcinoma, and colorectal cancer [6, 13, 14]. Besides, it has been proved the relatively safe feature with fewer side effects [6]. For instance, TCM could effectively improve the life quality, prolong survival rate, and improve immediate tumor response with fewer toxic effects [15, 16]. Several typical TCM components such as wogonin, ginseng radix, silibinin, quercetin, radix salviae miltiorrhizae and tanshinone have been deeply explored and they possess the antitumor effects in many cancers through a unique molecular mechanism.

TCM has been confirmed to be able to modulate the tumor immune microenvironment, stimulating the cancer stem cells and alleviating the side effects of antitumor treatment [7, 17]. In colon cancer patients, the frequency of T-helper 1 cells can be enhanced after curcumin therapy [18]; and combining curcumin-polyethylene glycol conjugate with a vaccine can enhance the response of cytotoxic T-lymphocyte and interferon-γ release in vivo [19]. Yuening et al. [6] indicated that TCM may regulate the balance between oncogenes and tumor suppressor genes and epigenetic modification, which plays a crucial role in tumor progression. Here, we first proved that the Rg1 application could effectively inhibit the HNSCC tumor cell proliferation in vivo and in vitro, then the underlying mechanism was explored. The role of Rg1-mediated tumor inhibition might be related to the regulation of cell apoptosis proteins caspase-3 and Bcl-2. Similarly, a previous study has reported that TCM resveratrol upregulated the expressions of caspase-3 and caspase-9, whereas downregulated the expression of Ras, Raf, MEK, and ERK1/2 in a dose-dependent manner in human colon cancer [20]. Another TCM formulation Yanggan Jiedu Sanjie induces apoptosis, and activates caspase activity in human hepatocarcinoma Bel-7402 cells [21].

The adaptive immune system is a highly participated in tumor-related immune responses, involved in the clearance of pathogenic bacteria and tumor cells [22]. Relevant studies have shown that the active components of TCM exert antitumor effects by regulating T cell subset differentiation and cytokine secretion [23, 24]. Consistently, we identified that mNPC01 could significantly enhance the percentages of CD4+ and CD8+ T cells with the upregulation of NK cells in HNSCC. In addition, marked increases in the numbers of both CD4+and CD8+ cells infiltrating the tumor tissues were found, suggesting that mNPC01 inhibits tumor growth in vivo and induces lymphocytic infiltration into the tumors of treated mice. It is well known that peripheral T lymphocytes including CD4+ and CD8+ T cells play a crucial role in anti-tumor immunity. For example, the number of Th1 cells is significantly reduced while the number of Th2 cells is increased in acute lymphoblastic leukemia, confirming the crucial role of Th1/Th2 imbalance

in the occurrence of cancers [25]. It is reported that Ginsenoside promoted the transformation of naive T cells into Th1 cells through acting on DCs, thus increasing the production of a large amount of IFN-γ [26].

Natural killer (NK) cells are the population of innate lymphoid cells, involved in the regulation of host immune responses against infection and tumor growth [27]. NK cells have a powerful cytotoxic activity orchestrated by an intricate network of inhibitory and activating signals. It is proved that the high level of tumor-infiltrating NK cells is related to a better prognosis in different human malignancies [28]. In the current study, we found that Rg1 could enhance the tumor-infiltrating NK cells and subsequently increase the NK cell-related killing activity in HNSCC tumor tissues. Besides, Rg1 also plays a pivotal role in modulating serum cytokines, including the reduction of IL-10 and TGF-β, and the elevation of IL-2, also, Rg1 could upregulate the ratio level of CD4+ /CD8+ T cells, which partly explains the anti-tumor effects of tumor-infiltrating NK cells [29, 30].

Altogether, we elucidated that Rg1 plays an effective role in the proliferation and induces the cell apoptosis of HNSCC, through elevating the T-lymphocytic infiltration in tumor tissues. This study provided a new therapeutic medicine for patients with HNSCC, identifying that Rg1 may play an effective role in tumor control. Further investigation and thorough mechanism of Rg1 treatment in HNSCC are needed for future studies.

Conflict interests

There is no conflict of interest.

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