Ginsenoside Rg3 combined with oxaliplatin inhibits the proliferation and promotes apoptosis of hepatocellular carcinoma cells via downregulating PCNA and cyclin D1

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Summary

The present study aims to investigate the effects of ginsenoside Rg3 combined with oxaliplatin on the proliferation and apoptosis of hepatocellular carcinoma cells and the related mechanism. In this study, MTT assay was applied to examine the proliferation rate of hepatocellular carcinoma cell SMMC-7721 with different treatment. Flow cytometry was performed to examine apoptosis rate of hepatocellular carcinoma cells with different treatment. Immunofluorescence and western blot methods were used to evaluate the expressions of PCNA and cyclin D1 in different groups. We found that ginsenoside Rg3, oxaliplatin or ginsenoside Rg3+oxaliplatin significantly suppressed the proliferation and promoted the apoptosis of SMMC-7721. Meanwhile, ginsenoside Rg3, oxaliplatin or ginsenoside Rg3+oxaliplatin also significantly inhibited the expressions of PCNA and cyclin D1. Moreover, compared with ginsenoside Rg3 group and oxaliplatin group, the effect of ginsenoside Rg3+oxaliplatin was more remarkable. Taken together, cells treated with oxaliplatin+ ginsenoside enhanced the anti-tumor effect and may inhibit the proliferation and promoted apoptosis of hepatocellular carcinoma via regulating the expression of PCNA and cyclin D1.

**Keywords:** Ginsenoside Rg3, oxaliplatin, hepatocellular carcinoma
Introduction

Primary liver cancer (PLC) is a common malignancy worldwide, characterized with high incidence and mortality \(^1\). PLC can be divided into three subtypes, of which hepatocellular carcinoma (HCC) accounts for 90%, and cholangiocellular carcinoma and mixed hepatocellular 10% of the total cases \(^2\). Nowadays, the prevalent therapeutic strategies for hepatocellular carcinoma are traditional surgery, chemotherapy, and radiotherapy \(^3\). However, 80% of the patients were diagnosed with local advanced or/and metastatic hepatocellular carcinoma due to the unclear mechanisms of HCC and lack of specific biomarker and therapeutic targets \(^4\). Meanwhile, the overall survival rate of the patients with hepatocellular carcinoma is poor \(^5, 6\). Therefore, there is an urgent need to inaugurate a novel therapy to improve the efficiency in treating hepatocellular carcinoma.

Presently, the clinical outcomes of the anti-HCC therapy with monotherapy is unsatisfactory. Hence, for the treatment of HCC, combination therapies were often used to improve the therapeutic efficacy. Oxaliplatin, as the third generation of platinum antitumor drugs, has many advantages, such as high efficiency and few side effects \(^7\). Many studies showed that oxaliplatin or the combination of oxaliplatin and other cytotoxic agents and/or targeted drugs gained satisfactory effects \(^8, 9\). Ginsenoside Rg3 is a monomer that extracted from a traditional Chinese medication ginseng\(^10\), and it has been proved that ginsenoside Rg3 inhibits the metastasis and invasion as well as progression of multi-type of cancers \(^10-12\). However, the effects of ginsenoside Rg3 on hepatocellular carcinoma has not been fully elucidated, and the therapeutic effects of ginsenoside Rg3 combined oxaliplatin have not been reported.
In the present study, we investigated the anti-cancer effect of ginsenoside Rg3 combined oxaliplatin on hepatocellular carcinoma cells. We found that the combination treatment of RG3 and oxaliplatin was more remarkable in inhibiting the proliferation and promoting the apoptosis of hepatocellular carcinoma cells. This may provide a theoretical basis for the use of combined RG3 and oxaliplatin as a novel therapy for hepatocellular carcinoma.

**Materials and methods**

**Cell culture**

Hepatocellular carcinoma cell lines SMMC-7721 (ATCC, US) cells were incubated at 37°C in RPMI-1640 culture medium (Thermo Fisher Scientific, US) containing 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin in the air humidified with 5% CO₂. Cells in logarithmic phase were applied for the following experiments.

**MTT assay**

Cells were planted in 96-well plates (3×10^3 cells/well) added with 100μl cell suspension and incubated at 37°C in 5% CO₂ for 24 h. Cells were divided into three groups: blank group, ginsenoside Rg3 group, and oxaliplatin group. Then cells were treated for 24h, 48h, and 72h with ginsenoside Rg3 (314197-60-5, Y-S Biotechnology, China) at the concentration of 7.5 μg/ml, 15 μg/ml, 30 μg/ml, 60 μg/ml, and 100 μg/ml) or oxaliplatin (Med Chem Express, US) at the density of 0.0625μg/ml, 0.125 μg/ml, 0.25 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 8μg/ml) respectively. After this, cells were stained with 20μl MTT solutions, and incubated for 4h. Then cells were dissolved with 100ul isopropanol hydrochloride and shaken for 10 min. Subsequently, the absorbance values at the wavelength values of 570nm were determined with automatic microplate spectrophotometer (SpectraMax 190, Sunnyvale, USA).
at 24, 48 and 72 h. Each experiment was conducted in triplicate.

**MTT for ginsenoside Rg3+oxaliplatin**

The appropriate concentration of ginsenoside Rg3 was 15 µg/ml and oxaliplatin 0.25 µg/ml. Then cells were treated with 15 µg/ml ginsenoside, 0.25 µg/ml oxaliplatin, and 15 µg/ml ginsenoside+0.25 µg/ml oxaliplatin for 48 h. The experiment was performed therebefore.

**Flow cytometry**

Cells were divided into four groups: control group, Ginsenoside Rg3 group, oxaliplatin group, and ginsenoside Rg3+oxaliplatin group. After treated with Ginsenoside Rg3 or/and oxaliplatin for 48 h, cells were trypsinized and collected. Then cells were washed with PBS twice. After this, cells were centrifuged at 2000rpm for 5 min and resuspended to 5×10⁵ cells/ml. Then cells were stained with 5µl of Annexin V-FITC and PI and incubated in shade for 10 min. Subsequently, the apoptosis rate were calculated with flow cytometry (BD, USA) using FlowJo software 7.6.2 (FlowJo LLC, USA).

**Western blot**

Western blot was performed to determine the protein level of cyclin D1. Cells were harvested and lysed with 200 µl of precooled RIPA buffer (Sigma-Aldrich; Merck KGaA). Later, the compounds were centrifuged at 13000rpm for 10 min. The total protein was determined with BCA Protein Assay kit (Beyotime Institute of Biotechnology). After isolated with 10% odium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100v, the protein was transferred to nitrocellulose (NC) membranes (Abcam, US). Then the cells were blocked with 5% non-fat milk at room temperature for 1 h and washed with PBS. The membranes were incubated with anti-cyclin D1 (ab134175, 1: 10000, Abcam, US) at 4°C in shade overnight.
and then with horseradish peroxidase-conjugated secondary antibodies (ab6721, 1: 2000, Abcam, US). Subsequently, the membranes were captured and analyzed with Image Lab v3.0 software (Bio-Rad Laboratories, Inc.). β-actin was used as loading control. Each independent experiment was performed in triplicate.

**Immunofluorescence assay**

Cells were incubated at 37°C with 5% CO₂ for 72h. Then cells on cover glass slides were fixed with 5% formaldehyde. After permeabilization with 0.5% Triton X-100 (Thermo Fisher Scientific, US) for 15 min, cells were incubated with anti-PCNA (ab92552, 1: 1000, Abcam, US) at 37°C for 2 h. Then cells were washed with FBS and incubated with second antibody (ab97051, 1: 2000, Abcam, US) stained with FITC at 37°C for 30 min. Later, the slides were supplemented with DAPI and pictured with fluorescence microscopy (BX53, Olympus, Japan).

**Statistical analysis**

Data were analyzed with the software SPSS 17.0. The comparison among multi-groups was analyzed with Analysis of Variance (ANOVA). Two-sided P<0.05 was deemed as statistical significance.

**Results**

*The proliferation of SMMC-7721 cells was decreased after the treatment of Ginsenoside Rg3 + oxaliplatin*

Firstly, MTT assay was conducted to examine the proliferation of SMMC-7721 cells treated with Ginsenoside Rg3 or oxaliplatin at different concentrations. The result suggested that treatment of either ginsenoside Rg3 or oxaliplatin had no significant effect on the
proliferation of SMMC-7721 cells at 24-hour post treatment, especially for the cells treated with low concentration of Ginsenoside Rg3 or oxaliplatin (p>0.05); while on the other hand, the proliferation of the SMMC-7721 cells was significantly decreased at 48- and 72-hour post treatment of ginsenoside Rg3 or oxaliplatin (Table 1, p<0.05). Moreover, the optimal concentrations of ginsenoside Rg3 and oxaliplatin were 15 µg/ml and 0.25 µg/ml, respectively, and then the effects of combination of 15 µg/ml of ginsenoside Rg3 + 0.25 µg/ml of oxaliplatin on the proliferation of SMMC-7721 cells were examined. The result suggested that the proliferation of the cells treated with ginsenoside Rg3 + oxaliplatin was significantly decreased compared with ginsenoside Rg3 group and oxaliplatin group (Figure 1, p<0.05).

**Ginsenoside Rg3 + oxaliplatin significantly promoted the apoptosis of SMMC-7721 cells.**

Flow cytometry assay was applied to examine the effect of ginsenoside Rg3 and oxaliplatin on the apoptosis of SMMC-7721 cells. It was shown in Figure 2A and B, the apoptosis rate of SMMC-7721 cells in ginsenoside Rg3 or oxaliplatin was significantly increased compared with the control group. Moreover, ginsenoside Rg3 + oxaliplatin has shown more significant pro-apoptotic effects on SMMC-7721 cells than either ginsenoside Rg3 or oxaliplatin treatment (p<0.01).

**Ginsenoside Rg3 + oxaliplatin downregulated the protein level of cyclin D1 and PCNA in SMMC-7721 cells.**

Finally, to further explore the underlying mechanism of ginsenoside Rg3 + oxaliplatin induced anti-tumor effects, western blot was used to determine the expression level of anti-apoptotic protein cyclin D1 in SMMC-7721 cells of different treatment. The results
showed that the expression of cyclin D1 in ginsenoside Rg3 group and oxaliplatin group was significantly lower than that in the blank group. Furthermore, the level of cyclin D1 in ginsenoside Rg3 + oxaliplatin group was significantly lower than that in ginsenoside Rg3 group and oxaliplatin group (Figure 3A and B, p<0.001). Moreover, immunofluorescence assay was performed to determine the expression of proliferation-related protein PCNA. The expression of PCNA was significantly decreased after the treatment of ginsenoside Rg3 or oxaliplatin compared with the blank group. The expression of PCNA was significantly lower in the ginsenoside Rg3 + oxaliplatin group compared with ginsenoside Rg3 and oxaliplatin group (Figure 4A and B).

**Discussion**

In the present study, we explored the effects of ginsenoside Rg3 + oxaliplatin on the proliferation and apoptosis of hepatocellular carcinoma cell line SMMC-7721. The results suggested that ginsenoside Rg3 + oxaliplatin might promote apoptosis and repress proliferation of SMMC-7721 cells through downregulating the expression of PCNA and cyclin D1. Moreover, the treatment of ginsenoside Rg3 + oxaliplatin was more efficient than that of ginsenoside Rg3 or oxaliplatin alone. The present study primarily verified the feasibility of the combination of ginsenoside Rg3 + oxaliplatin in treating hepatocellular carcinoma.

Recent studies witnessed the unsatisfactory result of single treatment for hepatocellular carcinoma 13-15). The supplementary and combined therapies can be the potential and potent way to improve the treatment of hepatocellular carcinoma. Oxaliplatin is a new cytotoxic agent widely used in the patients with advanced gastric cancer 16, 17). The results of EACH
study showed that oxaliplatin induced local control and prolonged the survival of the patients with advanced HCC \(^{18}\). Meanwhile, progresses have been made in the study on the combination of oxaliplatin and other drugs for the treatment of HCC. Liao et al reported that the combination of oxaliplatin and histone deacetylase (HDAC) inhibitor vorinostat is of high efficacy in suppressing the growth of hepatocellular carcinoma cells including SMMC7721, BEL7402, and HepG2 in vivo and in vitro \(^{19}\). Zhang et al found that oxaliplatin combined with lentinan induces cytotoxicity against hepatocellular carcinoma cells in vivo and vitro \(^{20}\). Li et al verified that oxaliplatin + β-Elemene enhances the sensitivity of hepatocellular carcinoma cell to oxaliplatin via suppressing the degradation of Copper Transporter 1 \(^{21}\).

Ginsenoside Rg3, a tetracyclic triterpenoids saponin in natural herb ginseng, was reported to inhibit the development of lung carcinoma, gastric carcinoma, intestinal carcinoma, hepatocellular carcinoma, and breast carcinoma \(^{22-25}\). Many studies showed that ginsenoside Rg3 combined with chemotherapeutics have addictive effects \(^{10, 26}\). However, the potential mechanisms of oxaliplatin combined with ginsenoside Rg3 regulating the apoptosis and proliferation of hepatocellular carcinoma cells has not been elucidated.

To our knowledge, this is the first study to investigate the possible effects of ginsenoside Rg3 + oxaliplatin on the growth of hepatocellular carcinoma cell SMMC-7721. We found that ginsenoside Rg3 + oxaliplatin are more efficient in promoting apoptosis and suppressing proliferation of SMMC-7721 compared with ginsenoside Rg3 group and oxaliplatin group, which suggested that ginsenoside Rg3 + oxaliplatin may be a potential and novel strategy for hepatocellular carcinoma.

Moreover, ginsenoside Rg3 strengthens the cytotoxicity of 5-Fluorouracil and oxaliplatin.
and further inhibited the progression of colorectal cancer via regulating the angiogenesis-related genes\(^{27}\). Proliferating cell nuclear antigen (PCNA) is closely correlated with DNA synthesis and plays an important role in regulating cell proliferation\(^{28}\). Cyclin D1 is involved in the proliferation of hepatocellular carcinoma\(^{29}\). Previous studies revealed that ginsenoside Rg3 regulates the adherent property of prostate cancer cells via suppressing the expression of PCNA, prostate specific antigen (PSA), androgen receptor (AR) and 5alpha-reductase (5alphaR) and inhibits the progression of colorectal cancer via regulating CCAT1/cyclin D1 axis\(^{30,31}\). Meanwhile, HOXC8 involved in the proliferation and drug resistance via regulating CyclinD1 and PCNA pathways\(^{32}\). Then we explored the possible effects of ginsenoside Rg3 and oxaliplatin on PCNA and cyclin D1. We found that the expression of PCNA and cyclin D1 in ginsenoside Rg3 + oxaliplatin group was significantly lower than that in ginsenoside Rg3 group and oxaliplatin group. This might work through the underlying mechanism that ginsenoside Rg3 + oxaliplatin downregulates the expression of cyclin D1, and weakens the combination of cyclin D1 and CDK4, fails to form cyclin D1-CDK4 compounds\(^{33,34,35}\), which further inhibits cell proliferation, suppresses the expression of PCNA, and promotes the apoptosis of SMMC-7721.

The present study investigated the potential effects of ginsenoside Rg3 + oxaliplatin on the proliferation and apoptosis of hepatocellular carcinoma cell SMMC-7721. This study is limited to cells. Further study should verify these results in animals and clinical trials and evaluate the possible toxic side effects of ginsenoside Rg3 + oxaliplatin.

To sum up, ginsenoside Rg3 + oxaliplatin repressed proliferation and promoted apoptosis of hepatocellular carcinoma cell SMMC-7721 via downregulating the expression of PCNA
and cyclin D1. The present study integrated ginsenoside Rg3 and oxaliplatin for the treatment of hepatocellular carcinoma, which may lay a basis for further study.

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Conflict of interest:

The authors declare no conflict of interest.
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Figure 1. The combination of ginsenoside Rg3 and oxaliplatin inhibited the proliferation of hepatocellular carcinoma cell SMMC-7721.

*MTT assay* was performed to examine the proliferation of SMMC-7721. Cells were treated with 15 µg/ml of ginsenoside Rg3 and/or 0.25 µg/ml of oxaliplatin. The proliferation was significantly suppressed after the treatment of 15 µg/ml of ginsenoside Rg3 and 0.25 µg/ml of oxaliplatin, while there was no significant difference between ginsenoside Rg3 group and oxaliplatin group. **p<0.01.
Figure 2. Ginsenoside Rg3+oxaliplatin promoted the apoptosis of SMMC-7721.

Cells were treated with ginsenoside Rg3, oxaliplatin, and ginsenoside Rg3+oxaliplatin respectively. Compared with blank group, the cell apoptosis rate of SMMC-7721 in ginsenoside Rg3 group and oxaliplatin group was significantly increased, while ginsenoside Rg3+oxaliplatin was more potent in promoting the apoptosis of SMMC-7721. **p<0.01, ***p<0.001, ****p<0.0001.
**Figure 3 Ginsenoside Rg3+oxaliplatin downregulated the expression of Cyclin D1.**

Western blot was conducted to determine the protein levels of Cyclin D1. The expression of Cyclin D1 in ginsenoside Rg3 group and oxaliplatin group was significantly lower than that in blank group and the expression of Cyclin D1 was significantly lower decreased in comparison with ginsenoside Rg3 group and oxaliplatin group. **p<0.01, ***p<0.001, ****p<0.0001.
Figure 4. Ginsenoside Rg3 combined with oxaliplatin decreased the expression level of PCNA.

A: The results of immunofluorescence assay showed that the protein level of PCNA treated with Ginsenoside Rg3 and/or oxaliplatin was decreased, which was more efficient in ginsenoside Rg3+oxaliplatin group. B: Quantitative analysis of A. **p<0.01.
Table 1 the effects of Ginsenoside Rg3 and oxaliplatin on the proliferation of SMMC-7721

| Group  | 24h (Inhibition rate %) | 48h (Inhibition rate %) | 72h (Inhibition rate %) |
|--------|-------------------------|-------------------------|-------------------------|
| Ginsenoside Rg3 (µg/ml) | 7.5  | 1.23±4.1 | 1.03±3.1 | 10.73±3.16** |
|        | 15 | -4.92±9.02 | 5.34±1.9 | 24.98±1.17** |
|        | 30 | 1.23±7.38 | 5.52±4.48 | 37.96±1.44** |
|        | 60 | 10.25±6.56 | 29.31±2.41** | 59.87±2.16** |
|        | 100 | 38.11±4.1** | 65.52±1.21** | 76.56±1.35** |
| oxaliplatin (µg/ml) | 0.0625 | -1.16±12.36 | 5.17±2.51* | 5.63±1.71** |
|        | 0.125 | 0.77±6.18 | 7.68±3.29* | 15.10±1.39** |
|        | 0.25 | 4.25±6.56 | 8.31±1.41** | 20.08±1.63** |
|        | 1 | 1.93±7.72 | 16.61±2.98** | 41.47±4.65** |
|        | 2 | 12.74±6.95* | 30.56±2.82** | 46.94±1.96** |
|        | 4 | 14.29±4.63** | 39.03±2.51** | 57.71±1.63** |
|        | 8 | 16.22±6.18** | 45.61±2.82** | 61.31±0.24** |