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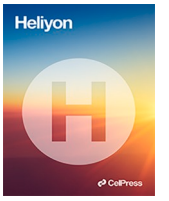

Research article

Ginsenoside Rd reduces cell proliferation of non-small cell lung cancer cells by p53-mitochondrial apoptotic pathway

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ABSTRACT

Ginsenoside Rd is a tetracyclic triterpenoid derivative, widely existing in *Panax ginseng*, *Panax notoginseng* and other traditional Chinese medicines. Many studies have proved that ginsenoside Rd have a variety of significant biological activities on certain types of cancer. However, the mechanism of ginsenoside Rd remains unclear in lung cancer. The findings of this study reveal that GS-Rd inhibits the proliferation of NSCLC cells, induces apoptosis, and suppresses migration and invasion. The results showed Ginsenoside Rd inhibited the cell proliferation (~99.52 %) by S phase arrest in cell cycle and promoted the apoptosis (~54.85 %) of NSCLC cells. It also inhibited the migration and invasion of cells ($p < 0.001$). The expression levels of related mitochondrial apoptosis proteins (Bax/Bcl-2/Cytochrome C) and matrix metalloproteinases (MMP-2/-9) were significantly changed. The results showed that ginsenoside Rd inhibited the proliferation of tumor cells by activating p53/bax-mediated mitochondrial apoptosis and the expression of key enzymes for cell apoptosis caspase-3/cleaved-caspase-3 were significantly increased. This research contributes to a better understanding of the anti-tumor effects and molecular mechanisms of GS-Rd, paving the way for its potential development and clinical application in NSCLC therapy.

1. Introduction

Non-small cell lung cancer (NSCLC) poses a significant threat to human health [1,2], and current treatment methods, including surgical excision, immunotherapy, radiotherapy, and chemotherapy, have limitations [3–5]. In recent years, research has found that Natural compounds have played a crucial role in the treatment and prevention of lung cancer. Novel supplementary therapies for cancer treatment are also being investigated, including those from natural sources. Meanwhile, natural compounds play an extremely

Abbreviations: GS-Rd, Ginsenoside Rd; FBS, fetal bovine serum; NSCLC, Non-small cell lung cancer; CCK8, Cell counting kit 8; DOX, Doxorubicin hydrochloride; MMPs, Matrix Metalloproteinases.

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important role in the treatment and prevention of lung cancer [6].

Ginsenoside Rd (GS-Rd), a tetracyclic triterpenoid compound found in Panax ginseng, has shown promising anti-tumor effects in various cancers. While previous studies have demonstrated the anti-tumor potential of GS-Rd in different cancer types, its specific anti-tumor effects and mechanisms in NSCLC remain unclear [7–9]. In recent years, studies have found that GS-Rd has an excellent anti-tumor effect [10,11]. GS-Rd inhibits the cell proliferation of gastric cancer by regulating the cycle and inducing cell apoptosis [12]. GS-Rd also can inhibit the proliferation of malignant glioma cells by regulating the tumor suppressor gene miR-144-5p and down-regulating toll-like receptor 2 [13]. It also regulates the Akt/mTOR/p70S6K signaling cascade and suppresses angiogenesis and breast tumor growth, by decreasing the volume and the weight of solid tumors in a dose-dependent manner [14]. However, in research on lung cancer, GS-Rd has also been studied for anti-tumor effects. Chian et al. found that NRF2 plays an important role in acquired drug resistance in NSCLC, and GS-Rd may ameliorate this chemoresistance by downregulating the NRF2 pathway [15]. Thus, GS-Rd has a certain potential in the antitumor research of natural products. However, the specific anti-tumor effect and mechanism of GS-Rd on NSCLC are not yet clear.

This study aims to evaluate the anti-NSCLC effect and molecular mechanism of GS-Rd, using NCI–H460 and 95-D cell lines. The research seeks to provide a theoretical basis for the development and clinical application of GS-Rd as an adjuvant therapy for lung cancer.

2. Materials and methods

2.1. Materials and reagents

GS-Rd was purchased from Yuanye Bio-Technology (B21054, Analytical standard, UV $\geq$ 98 %, Shanghai, China). The Cell culture-related consumables were purchased from Sangon Biotech (Shanghai, China). The cell lines 95-D (CL-0011) and NCI–H460 (CL-0299) were obtained from Procell Life Science & Technology (Wuhan, China). All antibodies were purchased from ABclonal (Wuhan, China). Other chemicals and reagents were of analytical grade and supplied by Sigma-Aldrich (St. Louis, USA).

2.2. Cell culture

Two kinds of NSCLC cells (NCI–H460 and 95-D) were cultured in RPMI-1640 (with 10 % FBS and 100 μg/mL of Penicillin-Streptomycine, the concentration of penicillin G sodium salt is 10 kU/L, the concentration of streptomycin sulfate is 10 mg/mL), and incubated at a constant temperature of 37 °C and 5 % carbon dioxide. Continue passaging when cell proliferation covers the culture plate to 90 %.

2.3. Cell proliferation

GS-Rd at a concentration of 50~200 μg/mL were used to treat the NSCLC cells. After filtration with a 0.22 μm membrane, the NCI–H460 cells were treated as a medium for drug administration, and the proliferation ability of the cells was evaluated by cell proliferation detection ($n \geq 3$). 4×10^3 cells were inoculated into each well of the 96-well-plate, with 5 parallel wells set for each concentration. On the second day of cell inoculation, the culture medium of GS-Rd was replaced. After 24–72 h of treatment, the cell proliferation was detected and the proliferation inhibition rate was calculated. The positive control drug used in the study was doxorubicin hydrochloride (DOX, 5 μM). All experimental procedures were performed in accordance with the CCK8 detection kit (MCE, China), and calculations were based on the following formula [16]:

$$\text{Inhibition rate (\%)} = ([A_{\text{without drugs}} - A_{\text{with drugs}}] / [A_{\text{without drugs}} - A_{\text{blank control}}]) \times 100\%$$

2.4. Cell colony formation

Cell colony formation assays were used to assess the cells' ability to form colonies. Cells were seeded into each well of a plate and incubated with various concentrations of GS-Rd for 21 consecutive days, with fresh GS-Rd medium replaced every 3 days [17]. Afterward, the culture medium was removed, and the cells were washed with PBS three times, stained with crystal violet for 15 min, and then observed under an inverted microscope. The number of cell colonies in each well was counted for statistical analysis of GS-Rd's inhibitory effect on cell colony formation. Images were captured using a microscope and camera ($n \geq 3$).

2.5. Cell cycle

For cell cycle analysis, cells were seeded in wells (4×10^5 cells), and 24 h post-seeding, the culture medium was replaced with GS-Rd at concentrations of 50, 100, and 200 μg/mL ($n \geq 3$) [18]. The minimum number of events detected was 10^4 cells. Cells were washed with PBS three times, trypsinized, collected by centrifugation, fixed with 70 % ethanol for 2 h, stained with propidium iodide solution, and then incubated in the dark at 37 °C for 30 min. The samples were analyzed using flow cytometry as per the instructions of the Cell Cycle and Apoptosis Analysis Kit (KeyGEN BioTECH, China).

2.6. Cell apoptosis analysis

Subsequently, the impact of GS-Rd on NSCLC cell apoptosis was preliminarily explored using cell fluorescence staining and morphology. Cells were seeded in wells (4×10^5 cells), and 24 h post-seeding, the culture medium was replaced with GS-Rd at concentrations of 50, 100, and 200 μg/mL ($n \geq 3$). Nuclear morphology was assessed using 4',6-diamidino-2-phenylindole (DAPI) nuclear fluorescence staining, while the situation of cells at different apoptotic stages was evaluated using AO/EB fluorescence staining. Cells were observed under an inverted fluorescence microscope, and images were captured. Additionally, Annexin V-FITC/PI cell Apoptosis Analysis Kits (KeyGEN BioTECH, China) were used to measure the cell proportions at different apoptotic stages by flow cytometry to conduct cluster quantitative analysis of early apoptotic, late apoptotic, and dead cells [19]. The minimum number of events detected was 10^4 cells.

2.7. Cell migration analysis

The effect of GS-Rd on cell migration ability was assessed by scratching the cell surface and treating the cells with GS-Rd at a concentration of 200 μg/mL when the cell coverage reached 85 %. Images of the cells were captured at 0 and 72 h under a microscope to observe their migration behavior. The healing area of the scratch in the treated group was compared with that of the control group to analyze the effect of GS-Rd on cell migration ability ($n \geq 3$) [20].

2.8. Cell invasion analysis

For cell invasion analysis, a layer of Matrigel was applied to the chamber, and then 105 cells were seeded into the chamber. The medium used in the chamber contained 200 μg/mL GS-Rd, while the lower chamber contained a normal medium with 10 % FBS as the inducer to detect cell migration across the membrane ($n \geq 3$). Images of cells crossing the membrane were captured using an inverted microscope, and invasive cells were counted [21].

2.9. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from each sample and used to prepare cDNA samples for measuring gene expression by qPCR with primers listed in Table 1. The reaction conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

2.10. Western blotting assay

To analyze the protein expression of NSCLC cells, Western blot analysis was performed using radioimmunoprecipitation assay (RIPA) buffer. Approximately 60 μg of protein was loaded into each lane, and primary antibodies were added for 2 h. To block nonspecific binding, 5 % BSA was used for 1.5 h. The primary antibodies used in this study were purchased from ABclonal and included anti-β-actin (AC038), anti-Bax (A19684), Cytochrome C (A13430, ABclonal), anti-Bcl-2 (A19693), anti-p53 (A3185), anti-caspase-3 (A19654), anti-MMP-9 (A11147), and anti-MMP-2 (A19080), which were diluted at a concentration of 1:1000. After washing with TBST, horseradish peroxidase (HRP)-conjugated secondary antibodies were added and incubated for 1 h at room temperature. Finally, the target bands were detected using enhanced chemiluminescence detection reagents and quantified using Image J software. β-actin was used as the control. Protein expression was then detected using an ECL kit.

2.11. Statistical analysis

Each group of experiments was conducted at least 3 times. All data are represented by mean ± standard error of the mean (SEM) and analyzed using Microsoft Excel and SPSS 17.0 software. T-test and one-way analysis of variance were mainly used for analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the negative control.

3. Results

3.1. GS-Rd inhibits the proliferation of NSCLC cells

To better assess the activity of GS-Rd on the two types of NSCLC cells, 95-D and NCI–H460, GS-Rd with varying concentrations was co-incubated with NSCLC cells to evaluate cell proliferation activity. Previous reports have indicated that ginsenoside Rd has no lethal

Table 1
IC_{50} of NSCLC cells affected by GS-Rd (μg/mL).

Cells	24 h	48 h	72 h
95-D	867.10 ± 298.00	145.33 ± 65.54	80.60 ± 0.31
NCI–H460	101.00 ± 2.61	68.19 ± 1.14	62.57 ± 1.25

effect on normal cells but can promote the proliferation of nerve cells (PC12) and ameliorate vascular damage [22,23]. Meanwhile, preliminary experiments were conducted at GS-Rd concentrations of 0, 12.5, 25, 50, 100, and 200 μg/mL, and the results showed that the inhibition rate begins to appear at a concentration of 200 μg/mL [24]. The inhibitory rate of GS-Rd on NCI–H460 cells is close to 100 %. Therefore, the following studies will use concentrations of 50, 100, and 200 μg/mL. As the results showed, GS-Rd effectively inhibited the viability of NSCLC cells and significantly suppressed cell proliferation (Fig. 1A–B). Additionally, cell morphology observations revealed that compared with the control group, the cell density in the GS-Rd groups was significantly reduced, and the cell morphology exhibited significant changes (Fig. 1C). We observed changes in cell morphology, noting that compared with the full and elongated tumor cells in the control group, the number and density of cells in the GS-Rd group decreased, and the cell morphology

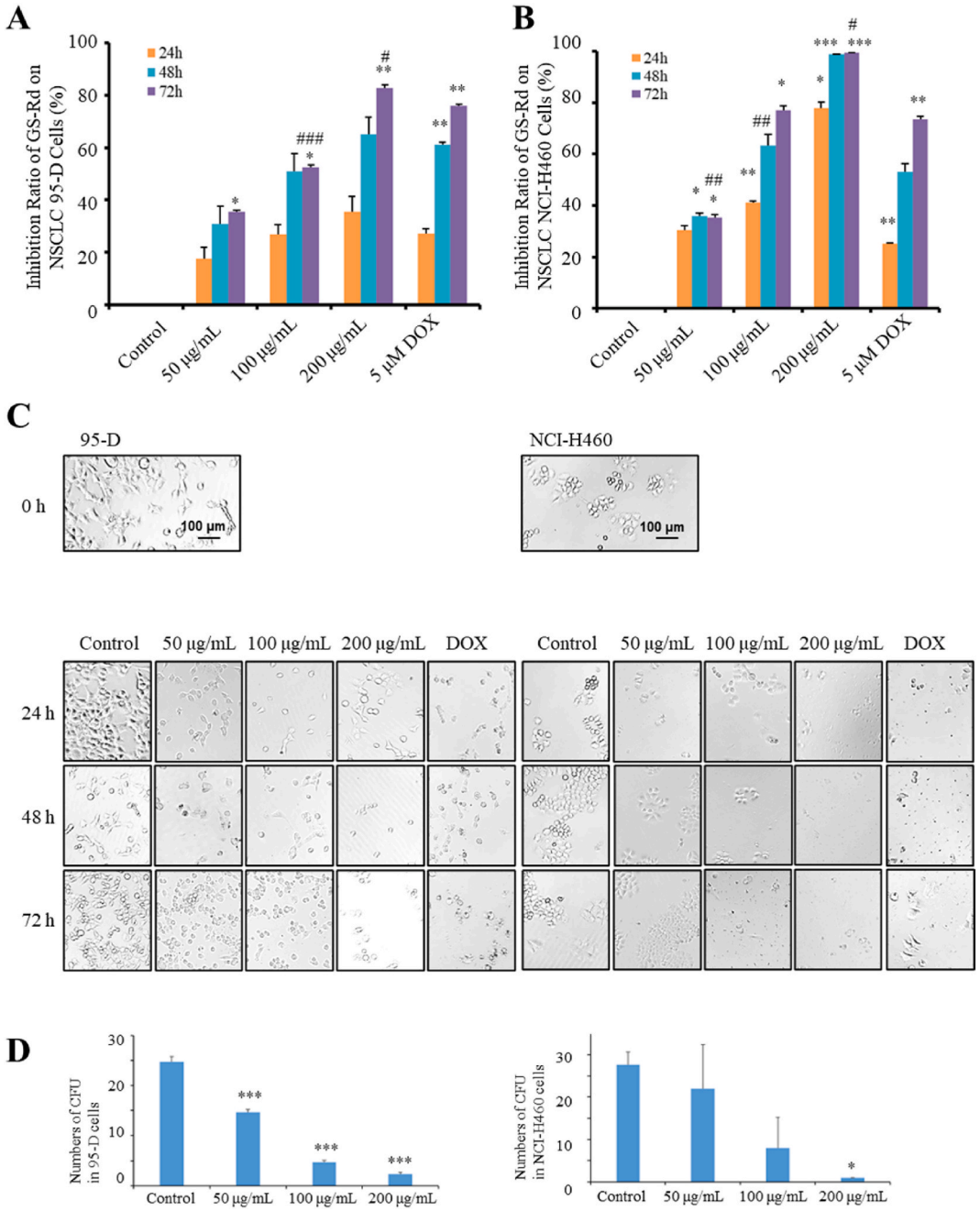

Fig. 1. Effect of GS-Rd on the proliferation of NSCLC cells. (A) Inhibition rate of the cell proliferation on 95-D; (B) Cell proliferation inhibition rate on NCI–H460; (C) Cell morphological changes on NSCLC cells; (D) CFU assay on NSCLC cells. Means ± SD, n = 5, *P < 0.05, **P < 0.01, ***P < 0.001 vs. control, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. Dox.

changed. The edges of the cells gradually contracted and became rough. The culture medium appeared cloudy, with dead cells and cell debris suspended in the medium. These changes were concentration- and dose-dependent.

Colony Forming Units (CFU) Assay is an effective method for detecting the proliferation ability of cultured cells. It can not only detect the ability of cells to proliferate microcellular communities from individual cells, but also reflect the migration and invasion of cells. Due to its ability to test the proliferation of live cells, it avoids experimental result errors caused by counting dead or non-dividing cells in the growth curve. Cell colony formation experiments can be used for the study of cells and tumors. For anti-cancer drugs, colony inhibition rate or colony survival score are important indicators. To investigate the proliferation ability of GS-Rd on cell-independent lesion formation, we performed another proliferative capacity test — CFU ability analysis was conducted (Fig. 1D), and the results showed that 200 μg/mL GS-Rd significantly inhibited in NCI–H460 cells ($P < 0.05$).

In conclusion, the inhibitory effect of GS-Rd on the proliferation of the two cell lines is obvious. Therefore, we calculated the half inhibitory rate (IC_{50}) of GS-Rd (Table 1). The smaller the calculation result, the stronger the effect of GS-Rd on the cells. As the results showed, GS-Rd had a stronger inhibitory effect on NCI–H460 cells, with IC_{50} values of 101.00 ± 2.61 μg/mL, 68.19 ± 1.14 μg/mL and 62.57 ± 1.25 μg/mL, respectively, during 24~72 h.

3.2. GS-Rd regulates the cell cycle of NSCLC in vitro

The cell cycle is divided into four stages, namely G1 phase (pre-DNA synthesis), S phase (DNA synthesis phase), G2 phase (post-DNA synthesis phase), and M phase (division phase). Through proliferation experiments, we found that the cell proliferation ability was significantly inhibited by GS-Rd. First, we examined the intervention effect of GS-Rd on the cell cycle. The results showed that GS-Rd had an obvious blocking effect on the S-phase of two NSCLC cell lines, and the proportion of G2/M-phase was significantly reduced (Fig. 2A). With the increase of GS-Rd concentration, the proportion of S-phase cells increased from 25.16 ± 0.37 % to 38.42 ± 0.29 %

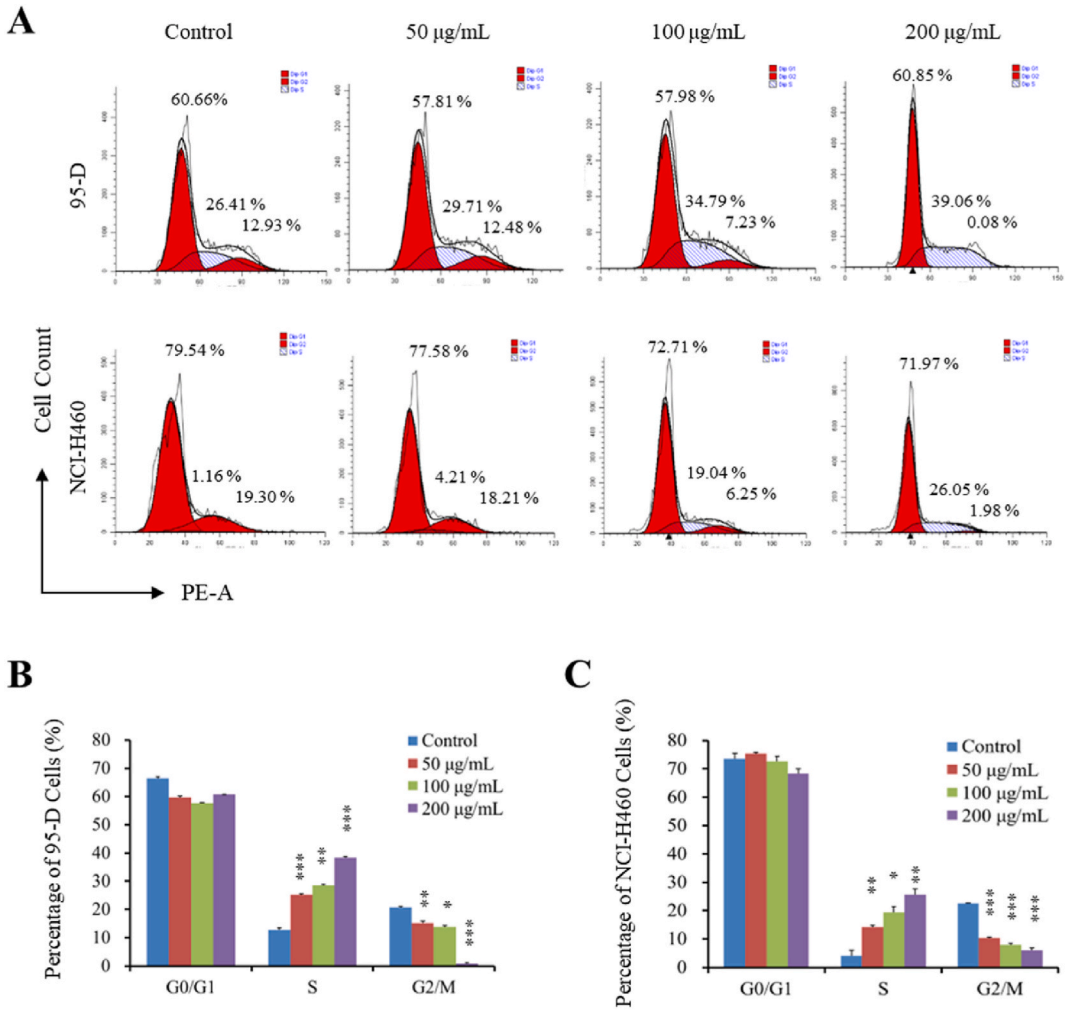

Fig. 2. Effect of GS-Rd on the cell cycle of NSCLC cells. (A) Flow cytometry detection of the effect of GS-Rd on the cell cycle of two NSCLC cell lines; (B,C) Cell cycle assay on NSCLC cells. Means ± SD, n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

in 95-D cells (Fig. 2B), and from 4.03 ± 0.14 % to 19.3 ± 0.48 % in NCI–H460 cells. Then, G2/M phase reduced from 10.76 ± 0.31 % to 0.83 ± 0.29 % in 95-D cells, and from 22.48 ± 1.90 % to 7.00 ± 2.05 % in NCI–H460 cells (Fig. 2C). In the S phase of the cell cycle (DNA synthesis phase), the main characteristic of cells is the synthesis of DNA, which lasts for about 8 h. In the initial stage, DNA polymerase and four types of deoxyribonucleotides are rapidly formed within the cells, while also synthesizing histones. The synthesis of DNA first occurs in loose euchromatin, then replicates in heterochromatin, and by the end stage, the DNA content doubles. These results indicate that GS-Rd affects cell proliferation by regulating cell cycle changes of NSCLC. GS-Rd inhibits the S phase of the NSCLC cell cycle, ultimately leading to impaired DNA synthesis and an inability to maintain cell division and proliferation.

3.3. GS-Rd induced apoptosis of NSCLC cells in vitro

During the co-incubation of GS-Rd and cells, it was found that a large number of dead cells and cell fragments were suspended above the cell medium, and the cell morphology was abnormal. Therefore, we predicted that GS-Rd might promote the apoptosis of cells. Furthermore, we conducted nuclear fluorescence staining to observe the effect of GS-Rd on the nuclear morphology of NSCLC (Fig. 3A–B). The results of fluorescence staining showed that with the increase of GS-Rd concentration and treatment time, the nucleus underwent shrinking, emitting strong blue fluorescence, and apoptotic bodies appeared in the nucleus. These results indicated that GS-Rd induced cell apoptosis in NSCLC.

Next, we used double fluorescence staining AO/EB to detect the cell conditions at different apoptotic stages. The results showed that cell apoptosis increased with the increase of co-incubation time and concentration of GS-Rd. The number of nuclear chromatin green and pinched or beaded indicated early apoptotic cells, and nuclear chromatin orange and pinched or beaded indicated late apoptotic cells, both of which increased significantly (Fig. 4A–B).

Therefore, to quantify the number of apoptotic cells, the cell cycle distribution was measured by flow cytometry at 24 h (Fig. 5). The results showed that with the increase of GS-Rd concentration, the proportion of early apoptosis and late apoptosis increased significantly. In 95-D cells, early apoptosis increased from 2.83 ± 0.33 % to 11.4 ± 0.25 %, late apoptosis increased from 5.73 ± 1.23 % to 24.07 ± 0.85 %, and total apoptosis increased from 8.57 ± 1.52 % to 26.6 ± 1.03 %. In NCI–H460 cells, late apoptosis increased from 4.53 ± 0.08 % to 49.37 ± 2.04 %, and total apoptosis increased from 10.67 ± 0.17 % to 52.93 ± 1.92 %. In conclusion, GS-Rd induced apoptosis of NSCLC cells (Fig. 5A–C).

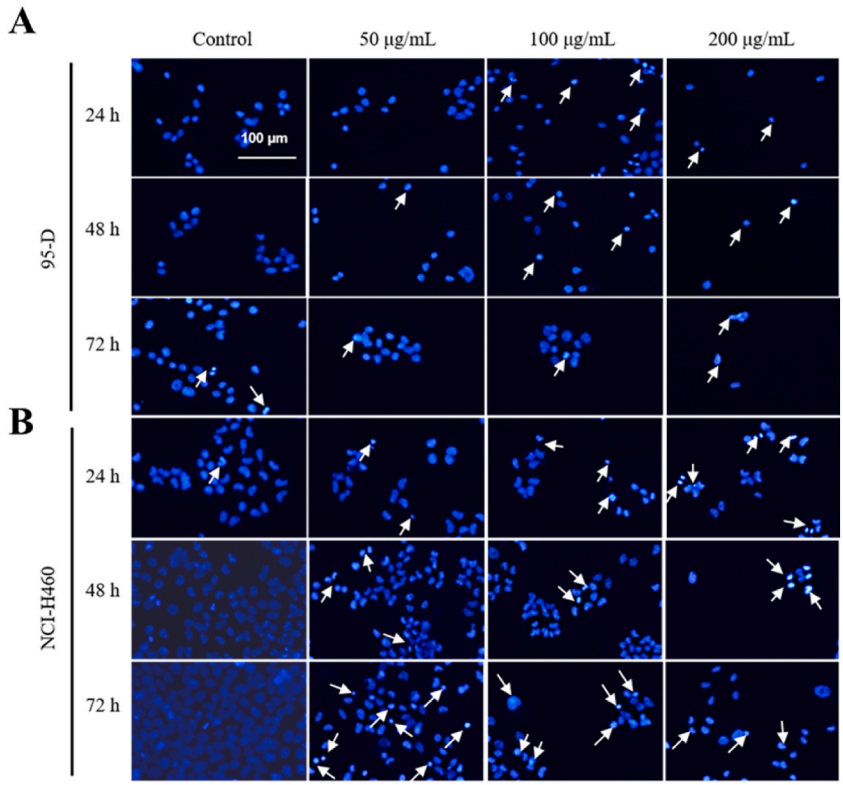

Fig. 3. DAPI fluorescence staining of GS-Rd co-incubated with NSCLC. Arrows pointing towards cells with dense and densely stained nuclei. (200 ×). (A) 95-D cells; (B) NCI–H460 cells.

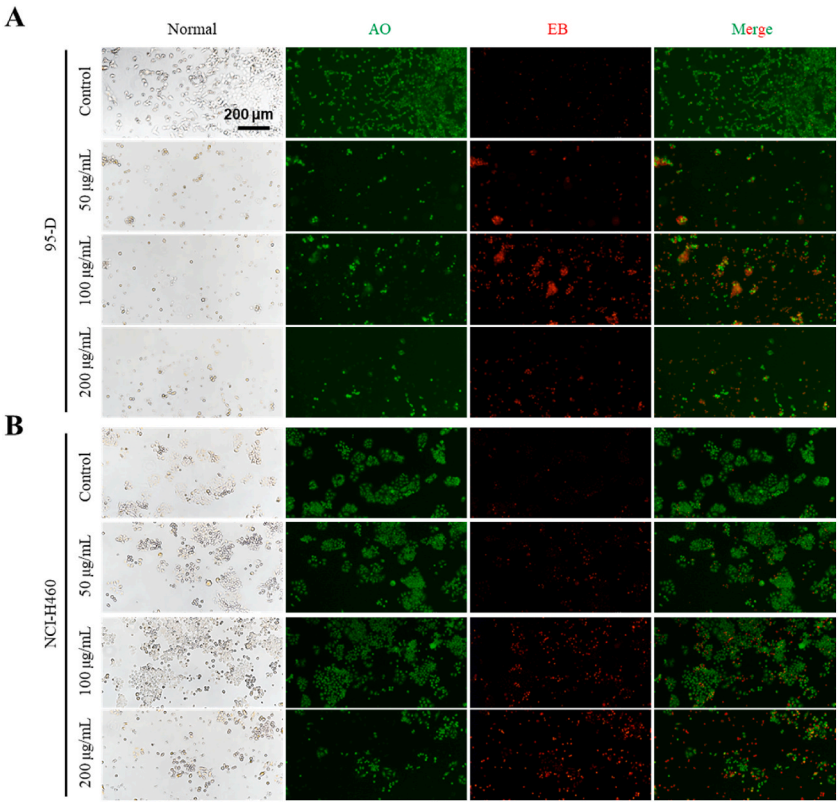

Fig. 4. AO/EB double fluorescence staining of GS-Rd co-incubated with NSCLC. Green chromatin and normal structure, living cells; Nuclear chromatin is green and compact or beaded, early apoptotic cells; Chromatin is orange-red and has a normal structure, non-apoptotic dead cells; Chromatin is orange-red and pinched or beaded, late apoptotic cells. (A) 95-D cells; (B) NCI–H460 cells.

3.4. GS-Rd inhibited the migration and invasion of NSCLC cells

The cell migration and invasion are important features of tumors. We conducted scratch healing and migration chamber tests to assess the migration and invasion ability of the cells. The healing of the scratched area was also measured at 72 h (Fig. 6). The results indicate that GS-Rd effectively inhibits the migration of NSCLC cells.

In the scratch healing test, the width of the scratched area of the control group cells gradually migrated and grew towards the center with the extension of culture time, showing a trend of healing. Conversely, the healing of the scratch area in the GS-Rd group was slower, indicating that under the action of GS-Rd, the speed of cell migration slowed down and was dose-dependent. The higher the concentration of GS-Rd, the poorer the ability of cells to migrate and grow inward, and even had a tendency to widen. Additionally, the culture medium was accompanied by a large number of dead cells, further indicating that GS-Rd significantly inhibits the migration ability of NSCLC cells. For specific statistical analysis, we will use transwell experiments for quantitative analysis.

Subsequently, the transwell assay was employed to quantitatively detect the inhibition of GS-Rd on the migration and invasion of NSCLC cells (Fig. 7A–B, Table 2). The statistical results showed that when the concentration of GS-Rd was 200 μg/mL, the migration and invasion ability of NSCLC cells was significantly inhibited (Fig. 7B). In both 95-D and NCI–H460 cells, the number of migrating and invasive cells decreased significantly, confirming the regulatory effect of GS-Rd on the migration and invasion ability of NSCLC cells. In 95-D cells, the number of migrating cells decreased from 162.8 ± 4.43 to 93.2 ± 2.22, invasive cells decreased from 32.6 ± 1.27 to 19.6 ± 0.59. In NCI–H460 cells, the number of migrating cells decreased from 92.6 ± 2.75 to 49.2 ± 5.15, invasive cells decreased from 48.2 ± 2.67 to 35.2 ± 3.13. These results confirm the regulation of GS-Rd on the migration and invasion ability of NSCLC cells. These results confirm the regulation of GS-Rd on the migration and invasion ability of NSCLC cells.

3.5. Effect of GS-Rd on gene and protein expression in NSCLC cells

Based on the above experimental results, we further investigated the effects of GS-Rd on gene and protein expression in NSCLC cell lines (Figs. 8–9, Table 3). The Q-PCR experiment verified the effect of GS-Rd on the expression of key genes in NSCLC cells, showing significant intervention in the key genes of apoptosis and the matrix metalloproteinase family MMP-9. Furthermore, the results of western blotting indicated significant effects on the expression of Bax, Bcl-2, Cytochrome C, P53, and caspases, confirming that GS-Rd induces S-phase division of NSCLC cells, inhibits cell proliferation, and induces mitochondrial dysfunction. Additionally, it regulates

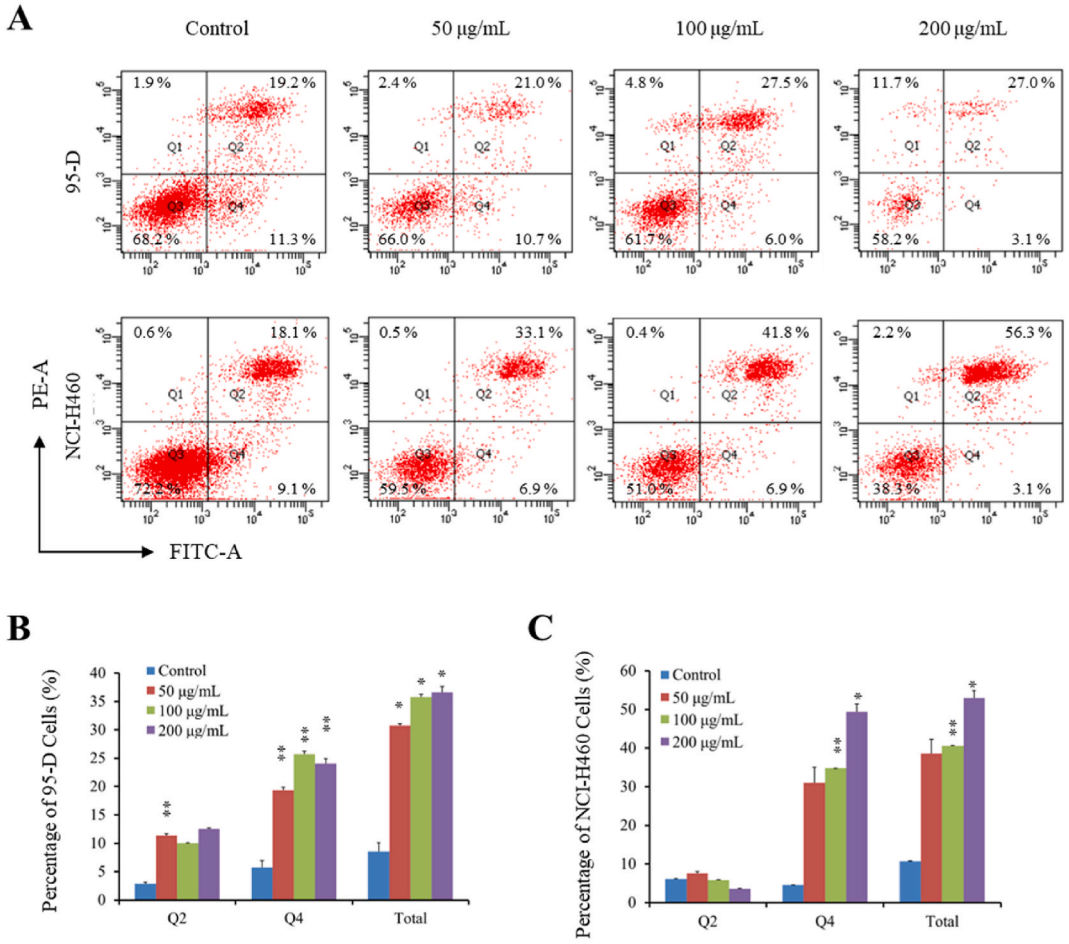

Fig. 5. The effect of GS-Rd on apoptosis of NSCLC cells. (A) Flow cytometry detection of the effect of GS-Rd on the cell apoptosis of two NSCLC cell lines; (B,C) Cell apoptosis assay on NSCLC cells. Means ± SD, n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

the expression of MMP-2 and MMP-9, weakening the migration and matrix degradation ability of NSCLC cells, ultimately limiting cell migration and invasion (Fig. 9A–B). In summary, GS-Rd may regulate tumor occurrence and development from multiple perspectives and pathways.

4. Discussion

Panax ginseng C. A. Meyer, a traditional Chinese medicine, has a variety of biological activities and pharmacological functions, such as antioxidation, enhancing immunity, anticancer, regulating metabolism, and modulating mood disorders [25–29]. Ginsenosides, as the most important active substances in ginseng, are involved in the regulation and improvement of various diseases [30]. More and more studies have found that ginsenosides have high nutritional value and can be widely used as food and drugs in disease improvement and treatment. At the same time, it has been found that it has a significant anti-tumor effect, which is worthy of further study in improving cancer [31]. It is well known that ginsenosides, major bioactive constituents of *Panax ginseng*, are attracting more attention due to their beneficial pharmacological activities. According to literature reports, ginsenoside Rg3, Rh2, CK, Rh1, Re, and other ginsenosides have been found to have excellent anti-tumor effects [17,32–35].

Ginsenoside Rd is the active compound in *P. ginseng* known to have broad-spectrum pharmacological effects to reduce neurological damage that can lead to neurological diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, depression, cognitive impairment, and cerebral ischemia [36]. Ginsenoside Rd, belonging to protopanaxadiol (PPD)-type ginsenosides, exhibits diverse and powerful pharmacological activities, including anti-cancer, anti-diabetic, anti-inflammatory, neuroprotective, cardioprotective, ischemic stroke, immunoregulation, and other pharmacological effects [37].

The antitumor effects of GS-Rd monomers were first reported in 2006. It has been reported that GS-Rd can inhibit the proliferation of human cervical cancer cell HeLa cells by regulating the expression of Bcl-2/Bax, regulating mitochondrial transmembrane potential, activating the expression of caspase family members, and inhibiting the proliferation of human cervical cancer cell HeLa cells through apoptosis [38]. In recent years, it has been reported that GS-Rd can effectively inhibit the activity of various tumor cells such as tongue

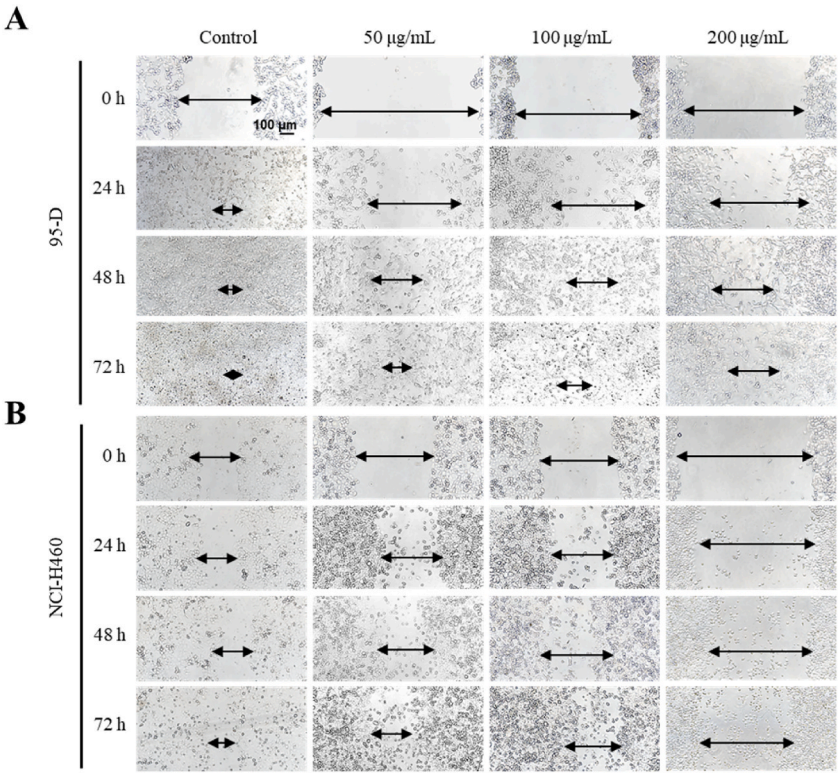

Fig. 6. Effect of GS-Rd on cell migration. (A) 95-D cells; (B) NCI–H460 cells.

cancer, gastric cancer, breast cancer, and colorectal cancer [12,39–41]. Research on tongue cancer has also found that Ginsenoside Rd inhibits migration and invasion of tongue cancer cells through the H19/miR-675-5p/CDH1 axis [10]. However, in the process of inhibiting tumor occurrence and development, GS-Rd exerts anti-tumor effects mainly through cell cycle regulation, inducing apoptosis of tumor cells, inhibiting proteasome activity, and inhibiting cell migration and invasion [42–44].

However, the bioactive effect of GS-Rd on lung cancer is rarely reported, and there is only one study on NSCLC cell A549. It was found that GS-Rd inhibited the proliferation of A549 cells by inducing G0/G1 phase arrest. At the same time, it was found that GS-Rd could inhibit the Nrf2 signaling pathway and significantly increase the sensitivity of drug-resistant cell line A549/DDP to drugs. This study suggests that the Nrf2 signaling pathway may be a potential therapeutic target for NSCLC [15].

Infinite cell proliferation is a critical characteristic of tumor cells, making the induction of tumor cell apoptosis and the inhibition of tumor cell proliferation key challenges in traditional Chinese medicine's approach to tumor research. Currently, many antitumor drugs achieve inhibition of tumor cell proliferation by inducing tumor cell apoptosis [45]. Subsequently, this affects phenotypes such as cell migration and invasion.

Our study initially discovered that GS-Rd can inhibit the proliferation of two types of NSCLC cells, indicating its potential as an inhibitor of NSCLC. Further investigation revealed significant changes in the G2/M-phase and S-phase of the cells, indicating an impact on the cell division process. Additionally, programmed apoptosis was observed, with apoptotic bodies appearing in the nucleus. The proportion of early/late apoptosis and dead cells increased significantly, further indicating the occurrence of the apoptosis process. Moreover, the ability of cell migration/invasion was significantly inhibited. Our study also found that the effect of GS-Rd on NSCLC is both time- and dose-dependent, likely due to the time required for the action and metabolism of GS-Rd. The longer the duration of action on cells and the higher the drug concentration, the stronger the cytotoxicity of cells.

Protein level detection revealed that GS-Rd achieves its inhibitory effect on NSCLC cells by inducing apoptosis, as evidenced by the upregulation of proteins P53, Bax, Cytochrome C, and members of the caspase family Caspase-3 and Cleaved-Caspase-3, while reducing the expression of Bcl-2, MMP-2, and MMP-9.

Current research indicates that cell apoptosis is primarily mediated through two pathways: the mitochondrial apoptosis pathway and the death receptor pathway (Fas-FasL). P53, a human tumor suppressor gene, plays a crucial role in tumor formation due to its inactivation [46]. Our study confirms that the expression of P53 in NSCLC cells is significantly upregulated after the action of GS-Rd. Furthermore, GS-Rd is instrumental in inhibiting NSCLC, with previous research suggesting that GS-Rd mainly induces cell apoptosis through the mitochondrial apoptosis pathway.

As a pre-apoptotic protein, Bax interacts with Bcl-2, influencing the permeability transition stomata, further regulating the mitochondrial membrane potential of cells, promoting the release of Cytochrome C, and mediating the occurrence of mitochondrial apoptosis pathways in tumor cells [47]. During the process of apoptosis, Caspase-3 is cleaved into two fragments with molecular

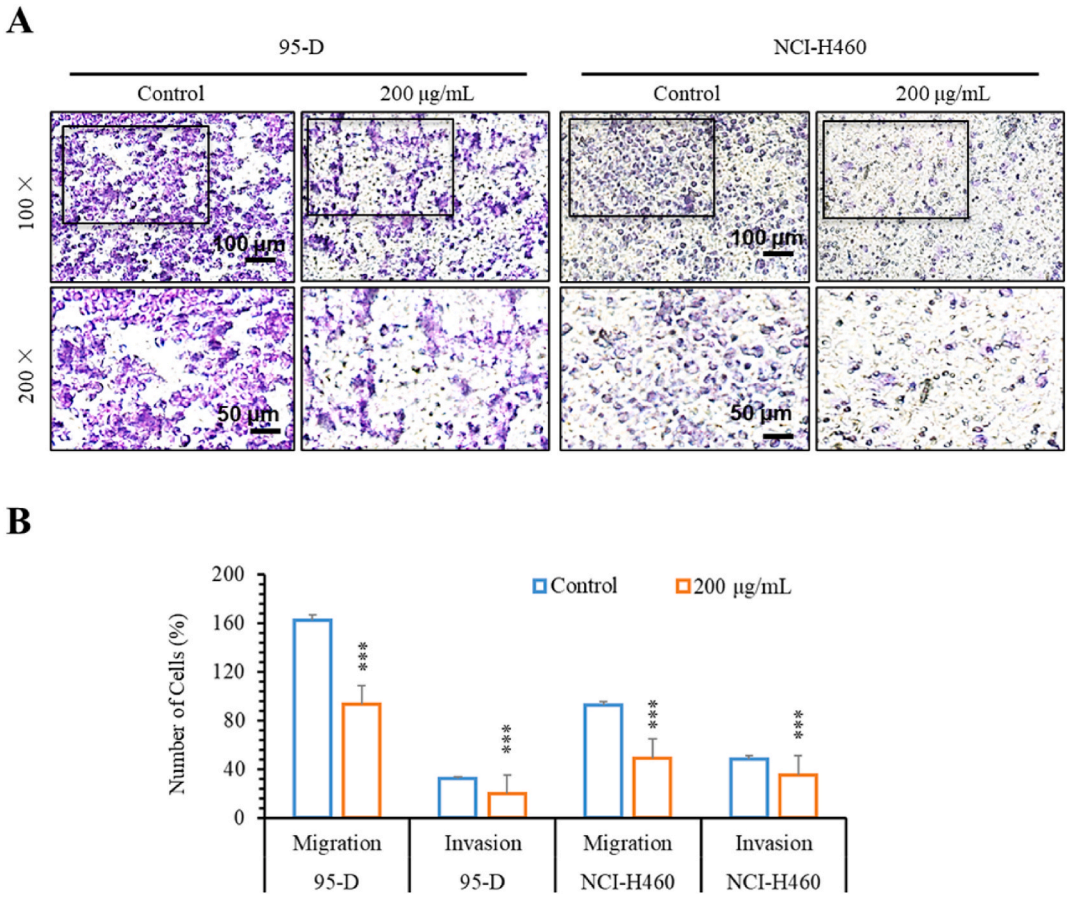

Fig. 7. Effect of GS-Rd on cell migration and invasion in NSCLC cells. (A) Changes in cell invasion; (B) Analysis of cell migration and invasion (48 h). Mean ± SEM, one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

Table 2
Effect of GS-Rd on cell migration and invasion of NSCLC cells after 48 h treatment (%).

Cells	Type	Control	200 μg/mL
95-D	Migration	162.8 ± 4.43	93.2 ± 2.22***
	Invasion	32.6 ± 1.27	19.6 ± 0.59***
NCI–H460	Migration	92.6 ± 2.75	49.2 ± 5.15***
	Invasion	48.2 ± 2.67	35.2 ± 3.13***

Mean ± SEM, *one-way ANOVA*. ***P < 0.001 vs. control.

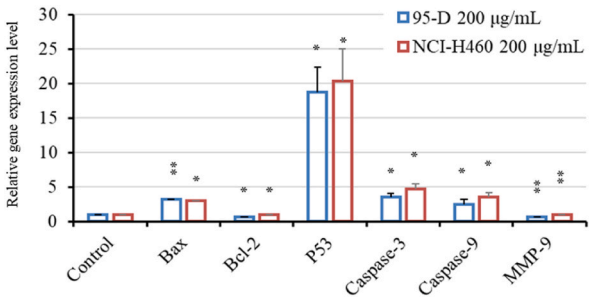

Fig. 8. Effect of GS-Rd on key gene expression levels in NSCLC cells (48h). Mean ± SEM, one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

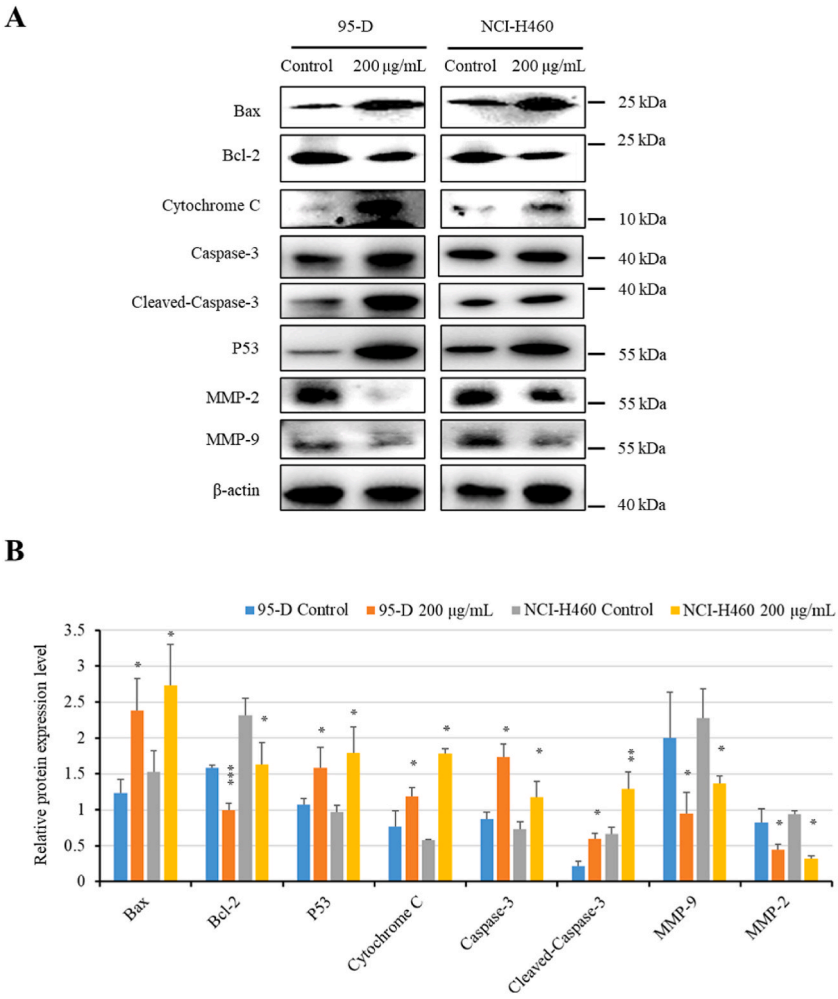

Fig. 9. Key protein expression in NSCLC cells after treated by GS-Rd (48h). (A) Western blotting of key proteins (The original image is provided in the Supplementary file); (B) Statistical analysis of relative expression levels of key proteins. Mean ± SEM, one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

Table 3
Effects of GS-Rd on the expression of proteins in NSCLC cells after 48h treatment (%).

Apoptotic proteins	95-D		NCI–H460	
	Control	200 μg/mL	Control	200 μg/mL
Bax	1.23 ± 0.19	2.38 ± 0.45*	1.53 ± 0.29	2.73 ± 0.57*
Bcl-2	1.58 ± 0.04	1.00 ± 0.09***	2.32 ± 0.23	1.63 ± 0.31*
P53	1.07 ± 0.09	1.58 ± 0.29*	0.97 ± 0.09	1.79 ± 0.36*
Cytochrome C	0.77 ± 0.22	1.19 ± 0.12*	0.58 ± 0.01	1.78 ± 0.07*
Caspase-3	0.87 ± 0.10	1.74 ± 0.18*	0.73 ± 0.10	1.18 ± 0.21*
Cleaved-Caspase-3	0.22 ± 0.06	0.60 ± 0.07*	0.66 ± 0.10	1.29 ± 0.24**
MMP-9	2.00 ± 0.64	0.95 ± 0.29*	2.28 ± 0.41	1.37 ± 0.10*
MMP-2	0.82 ± 0.19	0.44 ± 0.08*	0.94 ± 0.05	0.32 ± 0.04*

Mean ± SEM, *one-way ANOVA*. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

weights of 17 kDa (p17) and 12 kDa (p12), respectively, and translocated to the nucleus [48]. After activation by cleavage, Caspase-3 can activate caspase-6, -7, and -9, forming an apoptotic cascade reaction. This research has found that caspase-3 is activated, further promoting the occurrence of apoptosis in NSCLC cells. MMP-2 and MMP-9 are associated with various tumor progressions and play a pivotal role in the invasion of tumor cells [49].

Migration and invasion are the main characteristics of tumor cells. Cell migration refers to the process by which cells move from one region to another, while cell invasion refers to the entry of cells into areas where they should not otherwise exist. The invasion of tumor

cells refers to the entry of tumor cells from the primary tumor into surrounding tissues and blood vessels, and their spread to other organs in the body. Matrix metalloproteinases (MMPs) belong to the zinc-dependent endopeptidase family and are one of the most important protease families. They can degrade the extracellular matrix (ECM), with MMP-2 and MMP-9 being the most widely studied metalloproteinases. These enzymes can degrade type IV collagen in the basement membrane, providing conditions for invasion and metastasis of cancer cells. MMP-2 and MMP-9 are also involved in processes such as angiogenesis and tumor cell apoptosis. High expression of MMP-2 and MMP-9 is associated with disease progression and reduced survival in various cancer patients. This study confirms that GS-Rd can significantly inhibit the expression of MMP-2 and MMP-9, indicating that GS-Rd has a clear inhibitory effect on the invasion of NSCLC cells.

Cell proliferation, apoptosis, migration, and invasion are typical characteristics of tumor cells, and there are varying degrees of correlation between these characteristics. For example, apoptosis of tumor cells can lead to the inhibition of tumor cell proliferation and migration, but apoptosis is not the only factor affecting tumor cell proliferation. The migration ability of tumor cells is the fundamental guarantee of invasion ability. Drugs that induce apoptosis of tumor cells can also indirectly affect their migration and invasion ability. Therefore, the migration and invasion of tumor cells are often studied together. In this study, the phenotype study of GS-Rd on NSCLC cells found that cell proliferation was inhibited due to cell cycle arrest. Molecular detection revealed significant changes in the expression of Bax, Bcl-2, and Cytochrome C in tumor cells, indicating programmed apoptosis of the cells. At the same time, the migration and invasion ability of cells are affected, and the reason is that GS-Rd participates in regulating the expression of the matrix metalloproteinase family MMP-2 and MMP-9, limiting the ability of cells to degrade the matrix. A schematic diagram of the potential mechanism is shown in Fig. 10. Further studies are needed on the mechanism of GS-Rd inhibiting NSCLC.

Panax Ginseng, an important traditional Chinese medicine with food and medicine homology, has been widely used in many countries. Based on our research findings, ginseng and its monomeric saponins have great potential for future applications in basic research and clinical settings. Currently, widely used types of ginsenosides include Rg1, CK, and others. Research has shown that GS-Rd has a significant intervention effect on various phenotypes of NSCLC cells, and its impact on the expression of key proteins in cells has been discovered. Next, we can conduct in vivo intestinal metabolism and mechanism research. We hope to explore the pharmacological activities and mechanisms of GS-Rd in vivo and in vitro, as well as to discover new applications of GS-Rd.

5. Conclusion

GS-Rd, a tetracyclic triterpenoid active substance widely present in *Panax ginseng*, was the focus of a series of antitumor experiments carried out in this study. The research revealed that GS-Rd effectively promotes cell apoptosis and inhibits cell proliferation, migration, and invasion. Furthermore, it was observed that GS-Rd activates the expression of caspase family members and inhibits the expression of matrix metalloproteinases family members. According to our findings, GS-Rd may directly inhibit the growth of NSCLC cells through the mitochondrial apoptosis pathway. However, further study is needed to understand the inhibitory mechanism and molecular targets of GS-Rd against NSCLC in vivo and in vitro.

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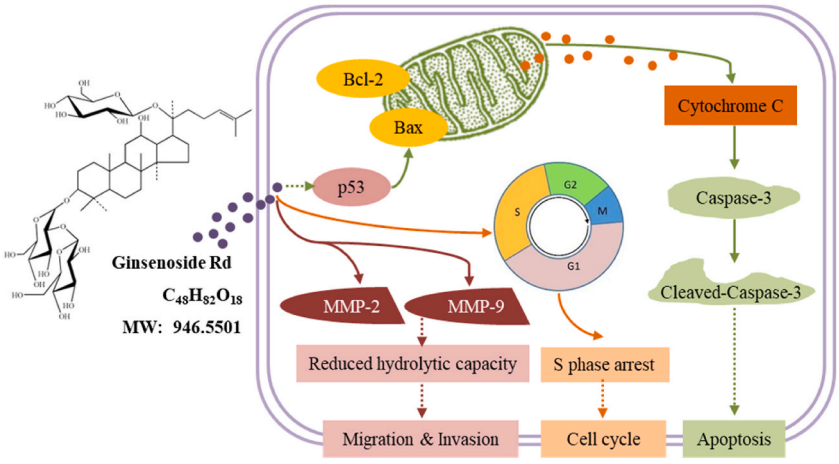

Fig. 10. A series of antitumor mechanism pathways for GS-Rd in NSCLC cells.

Ethics statement

The cell lines 95-D (CL-0011) and NCI–H460 (CL-0299) were obtained from Procell Life Science & Technology (Wuhan, China).

Data availability statement

All the original blot and gel images can be found in Figshare database (https://doi.org/10.6084/m9.figshare.24196485).

CRediT authorship contribution statement

Xilin Wan: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Xin Jin:** Writing – review & editing, Writing – original draft. **Xinmin Wu:** Methodology. **Dan Dong:** Resources. **Hongmei Yang:** Visualization. **Renbo Tan:** Software. **Ying Sun:** Formal analysis. **Xinze Liu:** Data curation. **Kaijing Sun:** Software. **Wei Wu:** Project administration, Investigation. **Changbao Chen:** Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32483.

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