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The inhibition of resveratrol to human skin squamous cell carcinoma A431 xenografts in nude mice

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ABSTRACT

Squamous cell carcinoma (SCC) is one of the commonest dermatological malignancies. Resveratrol (Res) is one type of polyphenolic compound which was first identified from the roots of *Veratrum grandinorum* in 1940. The previous studies found that Res can promote apoptosis of a variety of tumor cell, especially SCC cells. However it is rare to study the inhibition mechanism of Res in the animal model. In this study, through the establishment of human cutaneous SCC A431 xenografts in nude mice, we observed Res inhibition effect and investigated the inhibition mechanism by checking the expression of apoptosis-related factors, p53, ERK and survivin. The results showed that the xenograft volume and weight of Res groups were less than those of the control groups ($P<0.05$), but the net body mass of nude mice of Res groups was not significantly different from the control groups ($P>0.05$). The apoptotic index of Res groups were significantly higher than the control groups ($P<0.05$). The protein and mRNA expression of p53 and ERK were statistically positively correlated ($P<0.05$) and significantly increased in Res high- and medium-dose groups compared with the control groups ($P<0.05$). Moreover, the protein and mRNA expression of SVV were negatively correlated with p53 ($P<0.05$) and lower than the control groups ($P<0.05$). The results demonstrate Res inhibitory effect and indicate that the inhibition mechanism of Res is to upgrade the protein and mRNA expression of p53 and to downgrade the protein and mRNA expression of SVV, thus inducing the apoptosis of tumor cells.

1. Introduction

Squamous cell carcinoma (SCC) is a common dermatological malignancy which keeps increasing year by year. In the global scale, skin cancer accounts for 30% of newly diagnosed cancers every year [1], however the therapeutic method for SCC is currently quite limited. Surgical resection associated with chemotherapy is the most preferred treatment, but it would usually damage the patient's appearance. Moreover the postoperative chemotherapy drug would inevitably cause a series of side effects, not to mention the extremely high cost of treatment, so it is naturally of significant clinical and social importance to search for a new and effective treatment for squamous cell carcinoma by natural medicine.

Resveratrol, 3,4′,5-trihydroxy-trans-stilbene, is a non-flavonoid polyphenol compound containing stilbene structure similar to that of estrogen diethylstilbestrol. It can be widely found in some natural plants or fruits, e.g., grape, pine tree, polygonum, cassia, and peanut, which is a phytoalexin produced by many plants when they are subject to biotic or abiotic stresses, such as fungous infections, ultraviolet radiation, etc. In addition to the improvement of plant disease resistance, Res shows many other biological activity and pharmacological

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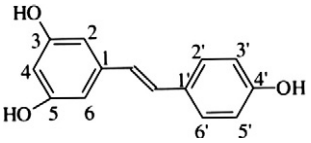
effects such as anti-tumor, anti-cardiovascular disease, anti-inflammatory, antioxidant, liver protection, nervous system protection, etc. It has become a highly important natural active ingredient with great medicinal value and market prospects. As for its anti-tumor effect, Res has become the research focus in cancer chemoprevention and chemotherapy thanks to Jang et al. who published a series of studies about the inhibition effect of Res on the origin, enhancement and development of cancer in *Science* in 1997 [2–8].



In recent years, the relative studies have shown that Res can inhibit a variety of tumor cell growth in vitro [9], and inhibit the human cutaneous squamous cell carcinoma A431 cell proliferation and induce apoptosis [10]. Previously, the inhibitory effect of Res has been reported in skin cancer in nude mice induced either by UVB irradiation or by 7,12-DMBA [1,11], but by now there is no in-vivo study about the inhibition mechanism of Res on the relevant animal model of cutaneous squamous cell carcinoma. This study was to investigate the inhibition mechanism of Res to human skin squamous cell carcinoma A431 xenograft tumor through the establishment of human skin squamous cell carcinoma A431 xenograft model in nude mice, observing the inhibition of Res on the xenograft tumor growth, and observing the impact of Res on the apoptosis-related factors survivin, p53 expressions by Western-blot and real-time PCR assay. We believe that this study would provide the valuable information of further identifying the drug target site.

2. Material and methods

2.1. Reagents

The resveratrol (Res) was a commercial product from Xi'an Oceanside (98%, HPLC); the fetal calf serum was a commercial product from Hangzhou Evergreen Biological Engineering & Materials Co., Ltd.; the DMEM high glucose culture medium was a commercial product from Thermo Fisher Biochemical Products (Beijing) Co., Ltd.; the rabbit anti-human/mouse p53 (BA0521), survivin (BA1420), P-ERK antibody and TUNEL apoptosis detection kit (MK1020) were from Wuhan Boster Biological Engineering Co., Ltd.; ECL chemiluminescence kit and BCA kit were from Amersham Life Sciences; SYBR Premix Ex Taq kit was from Invitrogen; TRIzol kit was from TaKaRa bio Dalian Baosheng; M-MLV reverse transcriptase kit was from Promega; the up- and down-stream primers of the relative genes and β-actin internal reference calibration gene were delegated to be designed and synthesized by TaKaRa Bio company, with the following detailed sequences: p53 (115 bp) up-stream primer 5′-TCTGACTGTACCACCATCCACTA-3′ and down-stream primer 5′-CAAACACGCACCTCAAAGC-3′, survivin (456 bp) up-stream primer 5′-CTTTCTCAAGGACCACCG-3′ and down-stream primer 5′-GCACTTTCTCCGCAGTTT-3′, ERK1 (567 bp) up-stream primer 5′-TCAACACCACCTGCGACCTT-3′ and down-stream primer 5′-GCTCCTTCAGCCGCTCCTTA-3′, β-actin (111 bp) up-stream primer 5′- CGGGAAATCGTGCGTGACAT-3′ and down-stream primer 5′-GAAGGAAGGCTGGAAGAGTG-3′.

2.2. The experimental animals and tumor strain

60 Balb/c (Nu/Nu) nude mice of SPF grade, evenly distributed in male and female, 6–8 weeks old, 18–22 g, purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (the qualified animal license number: SCXK (Beijing) 2006–0009). The human skin squamous cell carcinoma A431 cells were purchased from Nantong Bai Ao Maike (Biomics) Biotechnology Co., Ltd., Batch No.: CRL-1555, from ATCC.

2.3. Cell culture

Human skin squamous cell carcinoma cell line A431 was adherent cultured in DMEM (high glucose type) complete medium (containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin), in the incubator under the condition of 37 °C, saturated humidity and 5% CO_2. The modeling phase was prepared by using the logarithmic growth of the cell.

2.4. The model preparation of human skin squamous cell carcinoma xenograft in nude mice

The nude mice were raised in clean biological laminar flow rack which had been with UV disinfection on a regular basis and maintained at a constant temperature (25 ± 2 °C) and humidity (45% to 50%). The cages and water were sterilized by high-pressure steam, and experimental operations were conducted in a sterile hood. We took the logarithmic phase of A431 cells, after digestion centrifuge-washed with serum-free culture medium twice, then adjusted cell suspension concentration to $4–6 \times 10^7$ unit/ml by serum-free culture medium. Under the sterile conditions, 0.2 ml of suspension was inoculated subcutaneously into the left armpit of each Nu/Nu nude mouse. 7–8 days after planting, the mice with about 1000 mm^3 tumor volume were selected as the experimental model.

2.5. In-vivo inhibition experiment

2.5.1. Experimental groups and drug intervention

The selected nude mice were stochastically divided into six groups: ① saline negative control group: 30 μg/g.d; ② Res low-dosage group: 10 μg/g.d; ③ Res medium-dosage group: 20 μg/g.d; ④ Res high-dosage group: 40 μg/g.d; ⑤ CTX positive control group: 20 μg/g.d; ⑥ blank control group. Each group had been exploiting the intervention of celiac injection for 14 days.

2.5.2. The tumor volume change curve and the inhibition rate calculation

After intervention, the tumor long diameter (a) and maximum perpendicular diameter (b) were measured every 4 days until the day prior to sacrificing the mice. The tumor volume was calculated according to $V = 1/2 \cdot a \cdot b^2$, and the averaged value of each group was recorded to obtain the implanted tumor growth

curve. 7 days after drug intervention the mice were sacrificed by cervical removal, and then the tumor tissues were separated and weighed under sterile conditions. The inhibition rate was calculated in accordance with inhibition rate% = (tumor weight of saline negative control group − tumor weight of experimental group)/tumor weight of saline negative control group × 100%. The Res tumor-inhibition effect was studied statistically.

2.5.3. The changes of mouse body weight in each group

The body weights of nude mice were recorded at the end of the intervention, and the tumor weights were recorded afterwards. The discrepancy was recorded as the nude mice net body mass. The difference of the nude mice net body mass among groups was obtained to understand the adverse effects of Res.

2.6. The tumor histopathological observation and determination of apoptotic index

After being weighed, each tumor tissue specimen was divided into three parts. The first part was fixed by formalin and embedded by paraffin, then sliced and regular HE-stained. The tumor cell morphological changes of each group were observed under light microscope to determine the nature of the tumor and the apoptosis was detected by Terminal-Deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL) technique. The TUNEL results: The apoptotic cells were shown in brown. 5 fields were selected under the ×400 high-powered microscope for each slice, and the positive expression was accounted for as the apoptotic index (Apoptotic Index, AI). The other two parts of tumor tissue specimen were immediately placed in liquid nitrogen tank at −80 °C for the Western-blot and real-time PCR detection.

2.7. Detection of the relevant regulators

2.7.1. Preparation and detection of protein by Western-blot

Each tumor tissue sample frozen in liquid nitrogen was re-warmed at 37 °C, then homogenated, 12,000 r/min centrifugation for 20 min at 4 °C. After the determination of total protein content according to BCA kit instructions, the samples were repacked and stored at −80 °C for later use. 50 μg of protein of each group were prepared and boiled for 10 min to be denatured, then separated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose film by electron transfer method. The nitrocellulose membrane was sealed in PBST containing 10% of skim milk at 4 °C overnight, then washed with the PBST thoroughly (10 min × 3 times), added with rabbit anti-mouse survivin, p53, P-ERK (1:300 dilution) and rabbit anti-mouse β-actin antibody (1:800 dilution), incubated at 37 °C for 2 h, thoroughly washed with PBST (10 min × 3 times), by adding horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution), incubated at 37 °C for 2 h, thoroughly washed by PBST (10 min × 3 times), colored by using chemiluminescence method (ECL kit, Amersham Life Sciences USA product), then conduct semi-quantitative Western-blot results analysis by using Image J analysis software.

2.7.2. The detection of mRNA expressions of survivin, p53 and ERK1 by using real-time PCR

1) The extraction and reverse transcription reaction of total RNA: the total RNA was extracted according to Trizol kit (TaKaRa Company) instruction with a slight modification. A260/A280 was measured by the nucleic acid analyzer, and the quantity of RNA was measured by 1% agarose gel electrophoresis. Reverse transcription reaction was conducted according to the kit instruction. Adding 3 μg of RNA to 30 μL of RT reaction system, 60 min at 42 °C, 30 min at 45 °C, 10 min at 96 °C, inactivating reverse transcriptase, resulted in cDNA which was stored at −20 °C for later use.
2) The template gradient dilution of cDNA: cDNA was diluted into 5 gradients (10^5, 10^4, 10^3, 10^2, 10) to obtain a series of cDNA template.
3) SYBR Green1 real-time quantification of PCR: PCR was amplified in the Roche LightCycler 480 amplifier. The β-actin was used as internal reference for the gene expression in each group. PCR system was 15 μL, SYBR Premix Ex Taq (2×) 7.5 μL, upstream and downstream primers (10 pmol/μL) were 0.5 μL respectively, cDNA template was 0.5 μL, sterile deionized water was 6 μL. The amplification program: 2 min at 50 °C, 2 min at 95 °C, 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, repeating for 40 cycles. The measurement of each sample and reference was repeated 3 times. The reaction was performed in 96-well PCR. The fluorescence emitted by fluorescent dye was real-time monitored.
4) The analysis method: the relative expression rate of each gene in each experimental group compared with blank control group was obtained by $2^{(-\triangle\triangle CT)}$ method based on CT values. CT, Threshold Cycle, was generated automatically by the instrument at end of the experiment. $\triangle\triangle CT$ = Experimental Group ($CT_{\text{target gene}} - CT_{\text{reference gene}}$) − Control Group ($CT_{\text{target gene}} - CT_{\text{reference gene}}$).

2.8. Statistical analysis

SPSS13.0 was exploited for data processing. All the data are in the form of mean ± standard deviation. The variance analysis and Pearson correlation analysis were used, and the repeated measure designed variance analysis was used when conducting statistical comparison of different times. A statistically significant difference was indicated with $P<0.05$.

3. Results

3.1. The impact of Res on the subcutaneous tumor growth in nude mice

From the day of implantation to the end of intervention, all of the mice acted normally without any significant weight loss. However in the period between the end of intervention and the day of operation, the mice ate and moved less than usual. The tumor volume, tumor weight, net body weight and the calculated inhibition rate at the end of treatment of each group are listed in Table 1.

As shown in the tumor growth curve (Fig. 1), CTX and Res of varied dosage showed an inhibition effect on the xenograft

Table 1
The xenograft volume and weight, mice net body weight, inhibition rate and apoptotic index of all the groups.

	Volume (mm^3)	Weight (g)	Net body weight (g)	Inhibition rate (%)	AI (x ± s, %)
CTX	1153.56 ± 255.41 *,#	1.84 ± 0.30 *,#	19.27 ± 1.76*	41.77#	36.79 ± 8.86*
Res high-dosage	1001.69 ± 115.08 *,#	1.72 ± 0.39 *,#	22.37 ± 1.99△	45.57#	33.15 ± 6.00*
Res medium-dosage	1206.80 ± 175.88 *,#	1.96 ± 0.40 *,#	24.00 ± 2.02△	37.97#	18.09 ± 3.92 *,△
Res low-dosage	1342.28 ± 211.12*	2.67 ± 0.73*	24.68 ± 1.14△	15.51	10.53 ± 4.20 *,△
Saline Negative	1642.34 ± 225.85	3.16 ± 0.52	22.76 ± 2.20△	–	3.87 ± 1.63△
Blank	1564.32 ± 156.49	3.33 ± 0.59	23.37 ± 2.32△	–	2.73 ± 1.61△

* $P<0.05$, compared with saline and blank control groups.
\# $P<0.05$, compared with Res low-dosage group.
△ $P<0.05$, compared with CTX group.

growth at different levels compared with the saline negative and blank control groups. Before the intervention, the tumor volume difference among groups was insignificant. In the initial stage of drug intervention, the inhibition effect of CTX and Res was not very obvious. However in the latter stage of drug intervention, the xenograft growth rate of CTX positive group and Res groups, especially the Res high-dosage group, were much lower than those of the saline negative and blank control groups.

At the end of treatment, the tumor volume and weight of CTX and Res groups were statistically significantly different from those of the saline and blank control groups ($F_{tumor\ volume}=15.997$, $F_{tumor\ weight}=19.150$, $P<0.05$). The inhibition rates of CTX and Res groups are 41.77%, 45.57%, 37.97% and 15.51% respectively. The tumor volume and weight decreased with increased Res concentration and those of Res medium- and high-dosage groups were comparable with the CTX group ($P>0.05$). This result shows that Res of varied concentrations could significantly inhibit skin squamous cell carcinoma xenograft growth; moreover the inhibition effect of Res of medium- and high-dosage is comparable with the classic anticancer drug, CTX.

After treatment, the net body weight of CTX group was lighter than that of the saline and blank control groups ($F=9.489$, $P<0.05$), but the net body weight of Res groups showed no significant difference from that of the saline and blank control groups ($P>0.05$). This result indicates that plant-origin Res may be safer than the classic anti-cancer drug, CTX.

3.2. The histological impact of Res to the xenograft

As shown in Fig. 2a & b, under light microscope the HE stained sections showed the tumor necrosis and clear boundaries between necrotic and tumor tissue in CTX and Res groups. By contrast, only very little necrotic tissue could be observed in the saline and blank control groups. The proportion of necrotic tissue increased with increased Res concentration. Furthermore, in the CTX and Res high-dosage groups, the necrotic tissues are even more than tumor tissues. In the saline and blank control groups, the tumor cells were found with intensive number, high vitality, polygonal shape and big deeply-stained nucleus; while in the CTX and Res groups, it was clear to see that the tumor cells were less in number, sparsely distributed, roundly shaped, and some of the tumor cells were even found with liquefaction necrosis and shrinking nucleases.

3.3. The apoptotic effect of Res on tumor tissue

As shown in Table 1, it is clear to see that the AI in CTX and Res groups is significantly ($F=93.257$, $P=0.000$) higher than that in saline and blank groups. Moreover, it is interesting to

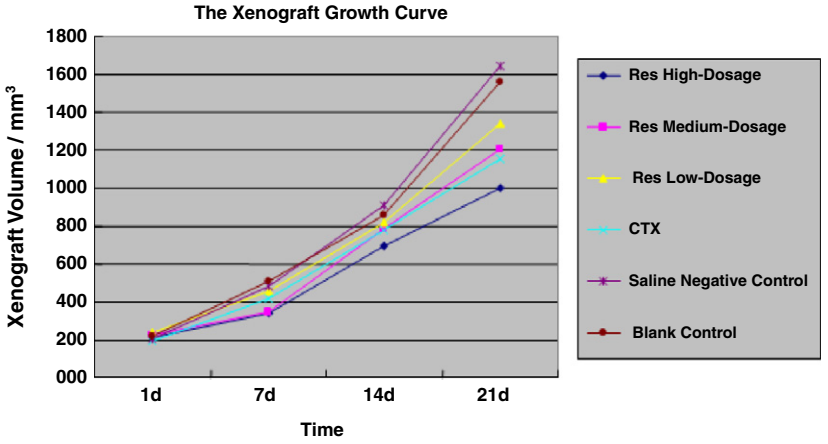


Fig. 1. The xenograft growth curve of all the groups.

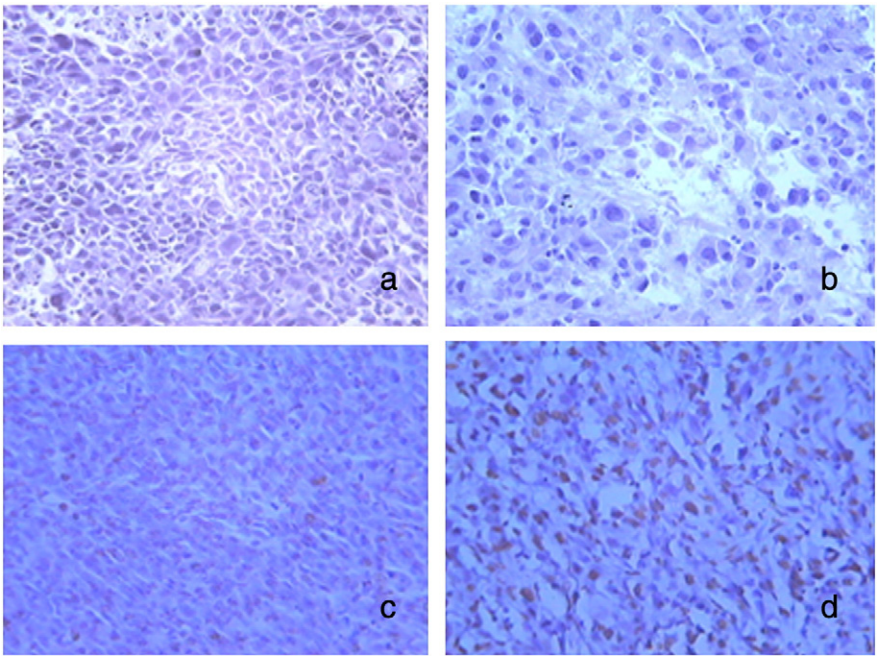


Fig. 2. The pathological changes of a) the blank control group and b) the Res high-dosage group (HE ×400); The apoptosis situation of c) the blank control group and d) the Res high-dosage group (TUNEL ×400).

note an increased AI with increased Res concentration in the Res groups, and even in Res high-dosage group a significant apoptostic effect, shown in Fig. 2c & d. There was no significant difference between the Res group and CTX group ($P>0.05$).

3.4. The impact of Res on the mRNA and protein expressions of the relative apoptotic factors

3.4.1. The detection of mRNA/protein expressions of SVV by real-time PCR and Western-blot assay

As shown in Tables 2, 3 and Fig. 3, it was found that the SVV mRNA/protein expressions of saline and blank control groups were quite high, whereas in CTX and Res groups, by intraperitoneal injection, the expressions decreased significantly ($F_{mRNA}=9.314$, $F_{protein}=7.110$, $P<0.05$). And the expressions decreased further with the increased Res concentration. At the level of mRNA, the Res high-dosage group showed a comparable expression as the CTX group ($P>0.05$), while both of them are significantly lower than in the other groups ($P<0.05$). At the level of protein, the expression in Res groups was even lower than in CTX ($P<0.05$). This result indicates that certain concentration of Res would clearly inhibit the gene expression and protein production of SVV of skin squamous cell carcinoma and such inhibition is even more obvious than with the classic anti-cancer drug, CTX.

3.4.2. The detection of mRNA/protein expressions of p53 by real-time PCR and Western-blot assay

As shown in Tables 2, 3 and Fig. 3, it was found that the p53 mRNA/protein expressions of saline and blank control groups were very low, whereas in CTX and Res groups, by intraperitoneal injection, the expressions increased significantly ($F_{mRNA}=7.024$, $F_{protein}=6.782$, $P<0.05$). And the expressions increased further with increased Res concentration. At the level of mRNA, the Res high- and medium-dosage groups showed a comparable expression as CTX group ($P>0.05$), while all of them are significantly lower than in other groups ($P<0.05$). At the level of protein, the expression

Table 2
The impact of Res on mRNA expression of SVV, p53 and ERK ($2^{-\triangle\triangle CT}$, $x\pm s$).

	SVV	p53	ERK
CTX	$0.56\pm0.13^{*,\#}$	$14.10\pm5.28^{*,\#}$	$5.06\pm0.55^{*,\#,\triangle}$
Res high-dosage	$0.48\pm0.24^{*,\#}$	$12.17\pm3.26^{*,\#}$	$4.66\pm0.97^{*,\#,\triangle}$
Res medium-dosage	0.85 ± 0.07	$8.72\pm2.85^{*,\#}$	$3.08\pm1.01^{*,\#}$
Res low-dosage	0.97 ± 0.04	3.83 ± 2.75	1.31 ± 1.34
Saline negative	0.98 ± 0.15	3.20 ± 2.21	0.93 ± 0.43
Blank	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

* $P<0.05$, compared with saline and blank control groups.
$^{\#}$ $P<0.05$, compared with Res low-dosage group.
$^{\triangle}$ $P<0.05$, compared with Res medium-dosage group.

Table 3
The impact of Res on protein expression of SVV, p53 and P-ERK ($x\pm s$).

	SVV/β-actin	p53/β-actin	P-ERK/β-actin
CTX	$0.48\pm0.20^{*}$	$0.28\pm0.03^{*}$	$0.33\pm0.12^{*,\#}$
Res high-dosage	$0.19\pm0.21^{*,\triangle}$	$0.39\pm0.09^{*}$	$0.36\pm0.09^{*,\#}$
Res medium-dosage	$0.22\pm0.22^{*,\triangle}$	$0.37\pm0.18^{*}$	$0.34\pm0.11^{*,\#}$
Res low-dosage	$0.28\pm0.24^{*,\triangle}$	$0.29\pm0.15^{*}$	$0.27\pm0.08^{*}$
Saline negative	0.98 ± 0.41	0.09 ± 0.09	0.16 ± 0.06
Blank	0.85 ± 0.34	0.07 ± 0.0874	0.12 ± 0.05

* $P<0.05$, compared with saline and blank control groups.
$^{\#}$ $P<0.05$, compared with Res low-dosage group.
$^{\triangle}$ $P<0.05$, compared with CTX group.

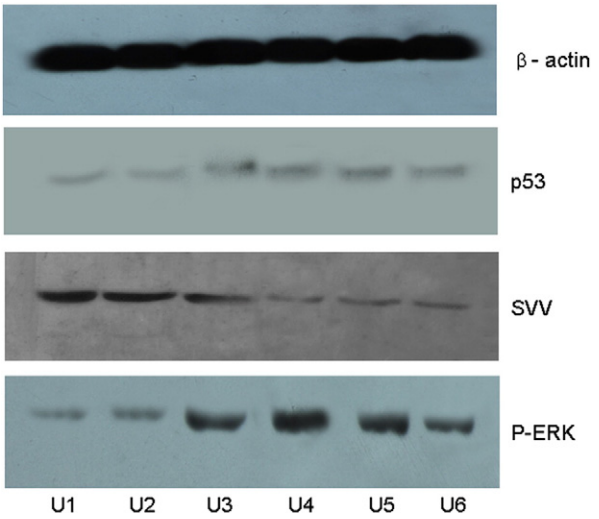


Fig. 3. The expression of apoptotic factors in the blank control group (U1), saline control group (U2), CTX group (U3), Res high-dosage group (U4), Res medium-dosage group (U5) and Res low-dosage group (U6).

in Res groups was comparable with CTX ($P > 0.05$). This result indicates that Res high- and medium-dosage would significantly increase the gene expression and protein production of p53 of skin squamous cell carcinoma. Moreover the Res (of all the tested concentrations) could promote the p53 protein formation as efficiently as CTX.

3.4.3. The detection of mRNA and P-ERK protein expressions of ERK1 by real-time PCR and Western-blot assay

Also as shown in Tables 2, 3 and Fig. 3, it was found that the ERK mRNA/protein expressions of saline and blank control groups were very low, whereas in CTX and Res groups, by intraperitoneal injection, the expressions increased significantly ($F_{mRNA} = 14.840$, $F_{protein} = 6.646$, $P < 0.05$). And the ERK expressions increased further with the increased Res concentration. At the level of mRNA and protein, the Res high-, medium-dosage and CTX groups showed a significant increased expression compared with Res low-dosage group ($P < 0.05$). This result indicates that Res high- and medium-dosage would significantly increase the gene expression and protein production of ERK of skin squamous cell carcinoma. Moreover the Res (of high- or medium dosage) could promote the P-ERK protein formation as efficiently as CTX.

3.4.4. The correlation analysis between SVV and p53 in terms of the mRNA/protein expression in all the groups

It was noticeable that the mRNA/protein expressions of SVV decreased with the increased mRNA/protein expressions of p53. This finding was confirmed by Pearson correlation analysis, the SVV expressions are negatively correlated with the p53 expressions ($r_{mRNA} = -0.599$, $P_{mRNA} = 0.009$; $r_{protein} = -0.416$, $P_{protein} = 0.022$, $P < 0.05$).

3.4.5. The correlation analysis between ERK and p53 in terms of the mRNA/protein expression in all the groups

It was noticeable that the mRNA/protein expressions of p53 increased with the increased mRNA/protein expressions of ERK. This finding was confirmed by Pearson correlation analysis, the ERK expressions are positively correlated with the p53 expressions ($r_{mRNA} = 0.778$, $P_{mRNA} = 0.000$; $r_{protein} = 0.675$, $P_{protein} = 0.000$, $P < 0.05$).

4. Discussion

In this study, the tumor weight, volume and growth curve of each group were recorded. The result showed a clear tumor growth inhibition in the Res groups compared to the negative control group and blank group. Such inhibition effect would be enhanced with the increasing concentration of Res. In the Res high-, medium- and low-dose groups, the tumor volume and weight were significantly lower than those in the negative control group and the blank control group ($P < 0.05$), and the inhibition rates were 45.57%, 37.97% and 15.51% respectively. Furthermore, the inhibitory effect of Res high- and medium-dosage was statistically comparable with the classic anti-cancer drug CTX with the inhibition rate of 41.77% ($P > 0.05$). It indicates that high- and medium-dosage of Res could deliver a comparable effect as CTX for inhibiting tumor growth in human skin squamous cell carcinoma.

As for the side effect of Res, the nude body mass did not significantly decrease in all of the Res treatment groups compared with the saline-negative control or blank control groups ($P > 0.05$), while CTX significantly reduced the mice body weight ($P < 0.05$). It indicates that herbal-originated Res is safer than the classic anti-cancer drug CTX.

The occurrence and development of cancer are associated with many factors among which the abnormal reduced apoptosis and enhanced proliferation ability are two of the key factors. Many studies have shown that Res could inhibit tumor growth by inhibiting tumor cell proliferation and by inhibiting the expression of anti-apoptotic factors while promoting the expression of proapoptotic factors [12]. And during the regulation of the apoptosis process, p53, survivin, Bcl-2 and Bax play important roles by interacting with each other. Detected by TUNEL, the apoptotic effect of Res to A431 xenograft in this study showed that tumor apoptotic index increased with increasing doses of Res. This further validates the pro-apoptotic biological activity of Res.

As a tumor suppressor gene, p53 usually can repair genetic errors, and then induce the cell differentiation and apoptotic functions. It has been reported that p53 could reduce B cell lymphoma 2 (Bcl-2) and increase Bax, hence decrease the ratio of Bcl-2/Bax, and then activate caspase 3 to induce apoptosis [13]. Many research results show that p53 dependent pathway may be one of the important mechanisms for Res inducing apoptosis [14,15]. As Res has estrogen-like effect [16], it would first combine with the estrogen receptors, and then activate the Mitogen-Activated Protein Kinase/Extracellular Regulated Kinase (MAPK/ERK) pathway, of which the principal signaling molecules would react with a variety of apoptosis-related molecules: caspase3, caspase7, p53, etc. And the activation of caspase3, caspase7 and p53 induces tumor cell apoptosis. This hypothesis can be supported by many studies: She et al. found that in the tumor cells treated by Res, p53 formed a complex with activated ERK and p38 kinase [17], and such action would cause the phosphorylation of serine15 of p53 which could activate p53 and increase the expression of p53 protein, and then the apoptosis of tumor cells is induced accordingly. Other in-vitro research results about ovarian

cancer and thyroid cancer have also confirmed that in tumor cells treated by Res, MAPK/ERK1/2 pathway would be activated and transcribed, and then p53-dependent apoptosis would commence [18–20].

In this study, the results showed that the protein and mRNA expression of wild-type p53 and ERK detected by Western-blot and real-time PCR assay significantly increased in Res high- and medium-dosage groups compared with those of saline-negative and blank control groups. And the expression was proportional to the Res dose. The protein/mRNA expression of p53 and ERK were statistically relevant. This result further confirms that Res increases p53 expression to induce skin cancer cell apoptosis through the activation of MAPK/ERK pathway.

Survivin (SVV) is a new member of the inhibitor of apoptosis protein (IAP) family, and it is the strongest apoptotic inhibitor factor found by far. Due to its huge expression difference between malignant and normal tissues, SVV becomes the signature protein of tumor and anti-tumor therapy. SVV can antagonize the exogenous apoptosis which is induced by FAS-activated caspase8, and the endogenous (mitochondrial pathway) apoptosis which is induced by Bax, caspase3, caspase7, and caspase9. Therefore, one of the biological functions of SVV is the carcinogenic potential [21]. The study of cervical cancer by Lu H showed that SVV was negatively related with caspase3 expression [22]. And some studies of non-small cell lung cancer [23] and pancreatic cancer [12] indicated that Res could block the phosphorylation of SVV, hence reduce SVV protein expression, and thereby induce tumor cell apoptosis.

In this study, the results indicated that SVV protein and mRNA expression of the skin squamous cell carcinoma tumor, detected by Western-blot and real-time PCR, could be significantly reduced in the Res high-dosage group compared with the saline and blank control groups. And with increasing doses of Res, the SVV expression decreased further. This finding is consistent with the conclusion in other studies; Res can introduce apoptosis by inhibiting the SVV expression and activating caspase3, caspase7, and caspase9.

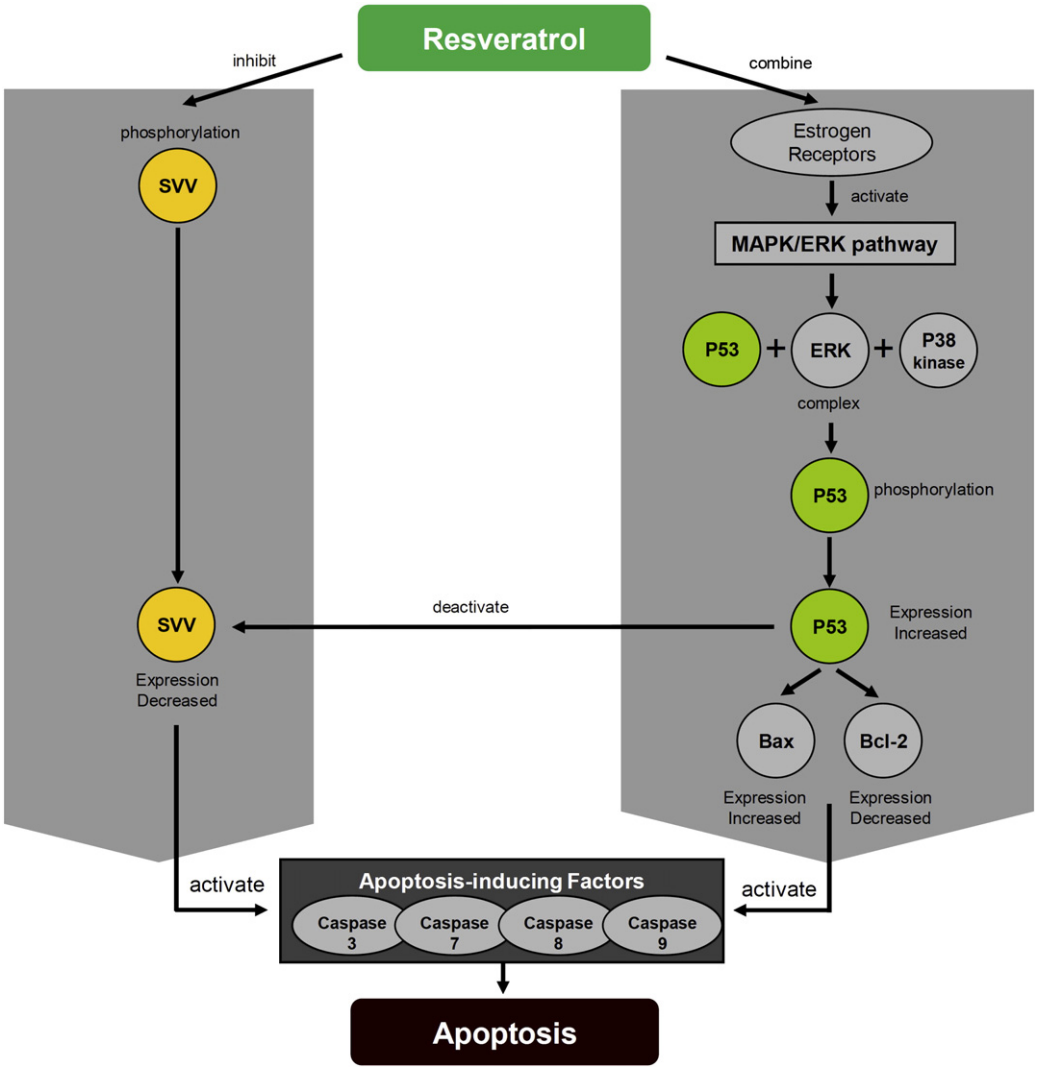


Fig. 4. The scheme of Res inhibitory mechanism.

As for the relationship between SVV and p53, the current findings show that one of the apoptotic mechanisms induced by p53 was against the anti-apoptotic activity of SVV [24,25]. The over-expression of SVV in tumors was closely related with the deficiency of wild-type p53. In human myeloma cells [26], p53 gene could be activated to inhibit the expression of SVV by muting Melanoma Antigen GEnes (MAGEs). In addition, in basal cell carcinoma or lung cancer issues, over-expression of wild-type p53 protein could function in a similar pattern [27,28]. Moreover the descent of SVV could enhance the radio sensitivity of sarcoma cells and the linear expression of wild-type p53 [29].

In this study, the statistical analysis showed that in the skin squamous cell carcinoma treated by Res, SVV and p53 related protein and mRNA would express in a negative correlation manner. This finding is in accord with previous studies indicating that one of the anti-cancer mechanisms of p53 is against SVV anti-apoptotic activity.

Based on previous studies and our findings, the inhibitory mechanism of Res (shown in Fig. 4) can be summarized as: 1) Res could reduce the SVV expression by blocking the phosphorylation of SVV, thus inducing apoptosis of tumor cells; 2) Res could increase the expression of p53 by activating MAPK/ERK pathway, also thus inducing apoptosis; 3) in addition, p53 could further induce apoptosis by countermining SVV anti-apoptotic activity. Therefore, we believe that this clear interaction mechanism could offer us some new opportunities for finding new breakthrough targets for the treatment of SCC.

5. Conclusion

Res showed a significant inhibition of the growth of human squamous cell carcinoma A431 xenografts in nude mice, and this inhibitory effect was safer than the classic anti-cancer drug CTX. Based on previous studies and our findings, the inhibition mechanism could be further clarified and validated.

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The authors declare that there is no conflict of interest in this work.

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