ORIGINAL RESEARCH

Resveratrol Suppresses Tumor Progression via Inhibiting STAT3/HIF-1α/VEGF Pathway in an Orthotopic Rat Model of Non-Small-Cell Lung Cancer (NSCLC)

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Background: The STAT3/HIF-1α/VEGF pathway is associated with the development and progress of various tumors including NSCLC. The aim of the present study was to investigate whether resveratrol (RES) could suppress NSCLC progression via inhibiting the expressions of STAT3, HIF-1α, and VEGF in a nude rat model.

Methods: Twenty-four nude rats were randomly divided into control, NSCLC, and NSCLC+RES groups. An orthotopic rat model of NSCLC was established. The animals in the NSCLC+RES group received the same operation as the NSCLC group and were intragastrically administered RES at 250 mg/kg/day for 12 weeks. Lung tissue samples were harvested for gross tumor burden measurement, histological examinations, RT-PCR, and Western blot assays.

Results: In the NSCLC+RES group, significant decreases in lung weight index, lung tumor burden, STAT3/HIF-1α/VEGF mRNA, and protein levels were observed when compared with the NSCLC group (all $P<0.05$). The structural integrity of the lung was less affected and the apoptotic index was significantly higher in the NSCLC+RES group, when compared to the NSCLC group ($P<0.05$).

Conclusion: RES suppresses NSCLC partly through inhibiting the expressions of STAT3, HIF-1α, and VEGF. The STAT3/HIF-1α/VEGF pathway might be a candidate drug target for developing new chemotherapy agents derived from RES for the treatment of NSCLC.

Keywords: resveratrol, non-small-cell lung cancer, STAT3, HIF-1α, VEGF, rat

Introduction

Lung cancer is a serious threat to human health. Currently, both the incidence and mortality of lung cancer rank first in all malignant tumors. Lung cancer can be classified into small-cell lung cancer and non-small-cell lung cancer (NSCLC) based on histopathologic characteristics.[1] More than 80% of lung cancer is NSCLC, which includes mainly adenocarcinoma and squamous cell carcinoma.[2] Though great progress has been made in the past few decades in its diagnosis and treatment, the prognosis of NSCLC is still unsatisfactory, with a 5-year overall survival rate of only 15%.[3] Therefore, it is an urgent need to further explore the mechanism of the occurrence and development of NSCLC and seek novel targets for NSCLC therapy.

Signal transduction and transcription activator 3 (STAT3) is a well-characterized oncogene, which regulates various fundamental cellular processes, including

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proliferation, angiogenesis, and apoptosis.[4] Hypoxia inducible factor-1α (HIF-1α), an important endogenous hypoxia marker,[5] and its direct target gene vascular endothelial growth factor (VEGF), are two prominent transcription targets for STAT3 and important proangiogenic factors.[6] It has been reported that the STAT3/HIF-1α/VEGF pathway was closely associated with tumorigenesis and progress of various tumors, including hepatocellular carcinoma, gastroenteropancreatic neuroendocrine tumor, hemangioma, malignant peripheral nerve sheath tumor, and NSCLC.[7,12]

Resveratrol (trans-3,4′,5-trihydroxystilbene, RES), a natural polyphenolic compound, is found in a large number of edible plants, especially grapes and peanuts.[13] Numerous studies have demonstrated its diverse pharmacological activities, such as immune regulation, neuroprotection, antioxidant and anti-inflammatory properties.[14] Its antitumor activity has also been widely studied and the results demonstrated that RES could exert potent antitumor effects via inhibiting cytochrome enzyme and COX-2, regulating cell cycle and NF-κB, inducing apoptosis and autophagy, and suppressing angiogenesis and metastasis.[15,16] A recent study demonstrated that RES could inhibit the hepatic expressions of HIF-1α and VEGF in a rat model of liver ischemia-reperfusion injury.[17] Another recent research showed that RES downregulated STAT3 expression in primary astrocyte cultures of rats.[18] However, if RES could exert its antitumor activity in NSCLC through regulating the STAT3/HIF-1α/VEGF pathway is still unknown.

Therefore, our present study is aimed at investigating whether RES could suppress NSCLC progression via inhibiting the STAT3/HIF-1α/VEGF pathway in a nude rat model.

Materials and Methods
Cell Line and Reagents
The human NSCLC cell line A549 was purchased from Shanghai Cell Biology Medical Research Institute (Shanghai, China). Cells were cultured in RPMI 1640 containing 10% FBS at 37°C in a 5% CO_2 incubator. RES was obtained from Xi'an Jinheng Chemical Co., Ltd (Xi'an, China) and dissolved in sterile saline to a final concentration of 5 mg/mL. Anti-STAT3 (ab76315), anti-HIF-1α (ab2185), anti-VEGFA (ab46154), anti-β-actin (ab8227), and HRP Goat Anti-Rabbit (IgG) secondary antibody (ab6721) were all obtained from Abcam (Cambridge, MA, USA).

Animals
Male Rowett nude rats (rnu/rnu) (age, 8 weeks old; weight, 240–260 g) were purchased from Beijing Vital River

Laboratory Animal Technology Co., Ltd. (Beijing, China). Experimental rats were kept under specific-pathogen-free (SPF) conditions at 22±2°C, 45–60% humidity, with 12-hour light/dark cycles and free access to autoclaved deionized water and irradiated pelleted food, except for an overnight fast before surgery. All experiments were conducted in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Ethics Committee of Xi'an Jiaotong University Health Science Centre.

Model of NSCLC
An orthotopic rat NSCLC model described previously[19] was adopted. Briefly, rats were anesthetized using 64% N_2O-32% O_2-4% isoflurane and a total of $20×10^6$ A549 cells were inoculated into the rat lung by intranasal drip. For the control group, animals underwent the same surgical procedure and received the same amount of solvent only (0.5 mL RPMI-1640 medium containing 10% FBS). Animals were monitored constantly until fully recovery from anesthesia.

Experimental Design
In total, 24 experimental nude rats were randomized into three groups (n=8 in each group): 1) control; 2) NSCLC; and 3) NSCLC+RES group. Animals in the NSCLC+RES group received the same operative procedure as NSCLC rats, and were intragastrically administered RES at 250 mg/kg/day for 12 weeks. Twelve weeks after inoculation of A549 cells, rats were euthanized by administering an overdose of intravenous pentobarbital sodium (150 mg/kg), and the lungs were harvested for subsequent experiments.

Lung Weight Index and Gross Tumor Burden Measurement
After the rats were euthanized, the body weights and the wet weights of the lungs were measured and recorded to calculate the lung weight index using the formula: lung weight (mg) divided by body weight (g). The gross tumor burden was determined using an arbitrary scoring system from 1 to 4, based on the surface tumors and palpable parenchymal tumors (grade 1: minimal; grade 2: mild; grade 3: moderate; and grade 4: marked tumor burden, respectively).[20]

H-E Staining and TUNEL Assay
Immediately after weighing, the lung tissue was fixed in 10% formalin (fixation time 24 hours, room temperature) and embedded in paraffin. Then the tissue blocks were cut

into 5 μm-thick sections and routinely stained with hematoxylin (15 minutes)-eosin (5 minutes) at room temperature. These sections were examined under light microscopy with 400x magnification (Olympus, Olympus LX70, Japan).

For TUNEL assay, the sections were dewaxed and rehydrated, and the apoptosis rate in lung tissue was evaluated using a commercial TUNEL assay kit (Shanghai Ruisai Biotechnology Co., Ltd., China) in accordance to manufacturer's instructions. Ten fields were selected randomly from each tumor or lung tissue slide and the rate of positive stained cells was calculated under light microscopy with 400x magnification (Olympus).

Quantitative Real-Time PCR

The tumor or lung tissue was taken to extract the total RNA by using TriPure Reagent Isolation Reagent (Roche Diagnostic, Penzberg, Germany). After determining the RNA concentration using a spectrophotometer, 500 nanograms of total RNA were reversely transcribed using the PrimeScript RT Reagent Kit (Takara, Japan). Then real-time qPCR was conducted with the SYBR@remix Dimer Eraser™ kit (Takara) in accordance to the manufacturer's protocol. The expression levels of mRNA were standardized by that of β-actin. STAT3 primer: sense 5-′AGAGGCGGCAGCAGATAGC-3′ and anti-sense 5-′TTGTTGGCGGGTCTGAAGTT-3′; VEGF primer: sense 5-′CCATGCAGATCATGCGGATCA-3′ and anti-sense 5-′CCTTGGCTTGTCACATCTGCAA-3′; HIF-1α primer: sense 5-′ACCTATGACCTGCTTGGTGCTGAT-3′ and anti-sense 5-′CAGTTTCTGTGTCGTTGCTGCCAA-3′; and β-actin primer: sense 5-′ATCGTGGGCCGCCCTAGGCA-3′ and antisense 5-′ TGGCCTTAGGGTTCAGAGGGG-3′.

Western Blot Analysis

Total protein was extracted from the tumor or lung tissue using T-PER™ tissue protein extraction buffer (Pierce; Thermo Fisher Scientific, Inc.) containing protease inhibitors. Equal amounts of proteins (50 μg/well) from each sample were separated by 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE), and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). After being blocked with 5% bovine serum albumin, the membranes were incubated with primary antibodies at 37°C for 2 hours, followed by the addition of HRP-conjugated secondary antibodies at 37°C for 1 hour. The primary and secondary antibodies used were listed in Cell line and reagents. Then the signal was detected by enhanced chemiluminescence method. Protein expression was quantified by densitometry with β-actin as the loading control.

Statistical Analysis

Data were presented as mean±SD. Comparisons among groups were analyzed using one-way ANOVA followed by Tukey's post hoc test using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). A P-value<0.05 was considered statistically significant.

Results

Lung Weight Index and Lung Tumor Burden Measurement

Compared to the NSCLC group, the lung weight index in rats treated with RES was significantly decreased (11.0±1.2 vs. 15.1±1.4, P<0.05, Table 1). Besides, our results indicated that RES significantly reduced tumor burden in lungs when compared to the NSCLC group (1.88±0.85 vs. 2.88±0.90, P<0.05) (Table 1).

H-E Staining and TUNEL Assay in Lung Tissue

The histological changes of lung tissues were examined by H-E staining (Figure 1). In the NSCLC group, tumor lesions could be found in the lung, and the tumor cells were large, irregular in shape, squeezed, and deformed, and the ratio of nucleus to cytoplasm increased, showing the characteristics of NSCLC. Compared to the NSCLC group, the structural integrity of the lung was less affected in the NSCLC+RES group. TUNEL staining was used to determine cell apoptosis in rat lung tissue (Figure 2). Compared to NSCLC animals, the apoptotic index was significantly elevated in the NSCLC+RES group (P<0.05).

The Expression of STAT3, HIF-1α, and VEGF in Lung Tissue

The expressions of STAT3, HIF-1α, and VEGF in lung tissues of animals were examined by RT-qPCR and Western blot assay. Compared to control animals, the mRNA/protein levels of STAT3, HIF-1α, and VEGF were all markedly increased in NSCLC group rats (all P<0.05). However, compared to the NSCLC group, treatment with RES led to a significant decrease in these parameters in the NSCLC+RES group (all P<0.05) (Figure 3).

Discussion

STAT3 is the most commonly activated member of the STAT family in human malignant tumors.[21] It can be

**Table 1** Effect of RES on the Lung Weight Index and Gross Tumor Burden in the Lung

Groups	N	Wet Lung Weight (g)	Body Weight (g)	Lung Weight Index	Tumor Burden (0–4, Arbitrary Size)
Control	8	2.14±0.62	294.82±24.32	7.26±0.64	0
NSCLC	8	4.25±0.83	278.75±21.27	15.25±1.43	2.88±0.90
NSCLC+ RES	8	3.18±0.54	280.61±19.88	11.33±1.25*	1.88±0.85*

**Note:** *$P<0.05$, compared to NSCLC group.

activated by multiple proinflammatory factors and growth factors and be implicated in the proliferation, differentiation, invasion, metastasis, angiogenesis, and apoptosis of various tumor cells.[22] STAT3 can induce the expression of factors that contribute to cellular proliferation and survival, such as Bcl-2; invasiveness and metastasis, such as E-cadherin and MMP-9; immunosuppression, such as

TGFβ and IL-10, and angiogenesis, such as HIF-1α and VEGF.[23,25] HIF-1α and VEGF are two prominent transcriptional targets for STAT3.[7,26] Besides, STAT3 was proposed to be required for the function of the HIF-1α complex.[27]

The rapid growth of solid tumors may cause hypoxia of tumor microenvironment and HIF-1α is one of the main

Control   NSCLC   NSCLC+RES
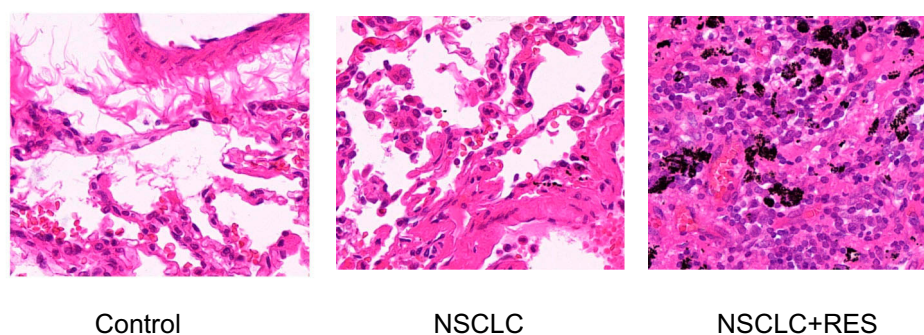
Figure 1 H-E staining of lung tissues in different groups.
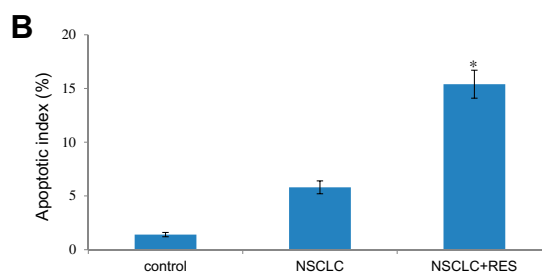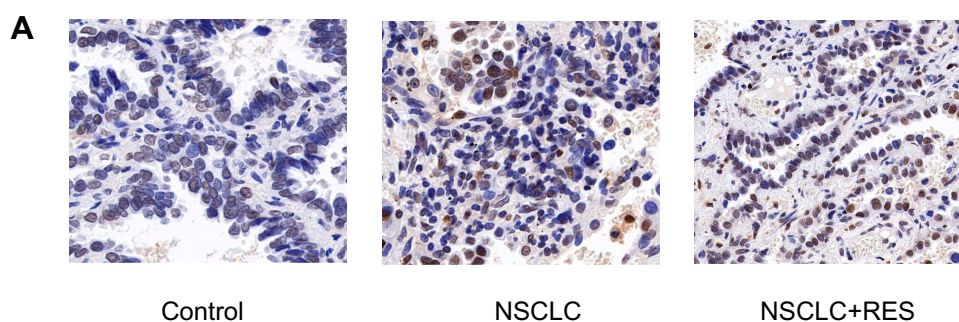
Figure 2 Effect of RES on cell apoptosis in lung tissue of different groups. (**A**) Cell apoptosis detected by TUNEL staining (×400). (**B**) Cell apoptosis index. Values are represented as the mean±SD (n = 8) (*$P<0.05$ vs. NSCLC group).
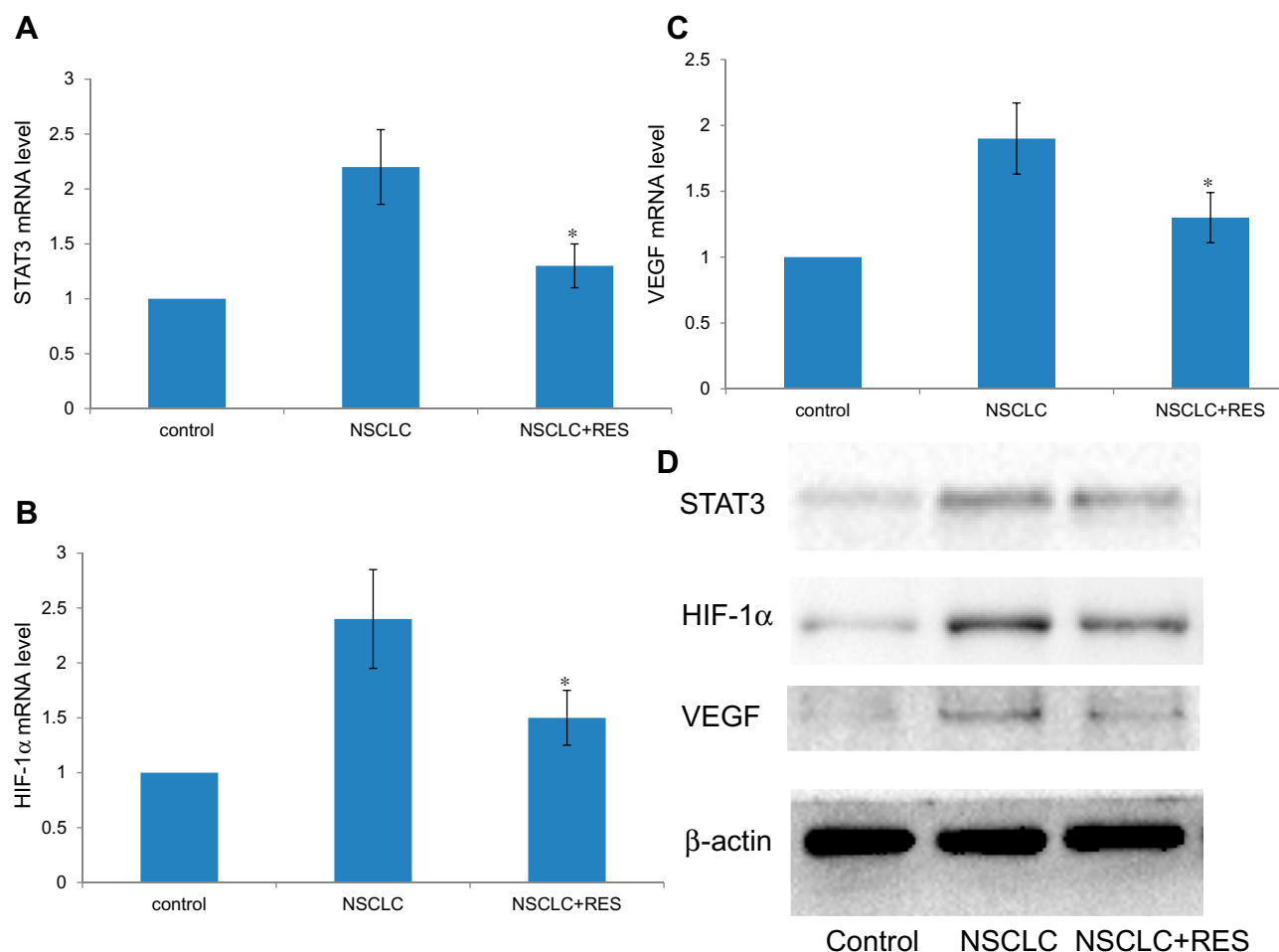
Figure 3 Effect of RES on the expression of STAT3, HIF-1α, and VEGF in lung tissue. (**A–C**) STAT3, HIF-1α, and VEGF mRNA levels were determined by RT-qPCR. Relative fold induction for STAT3, HIF-1α, and VEGF mRNA (means±SD) in NSCLC and NSCLC+RES group rat lungs is presented relative to the expression in control group rat lungs (n=8) (*$P<0.05$ vs. NSCLC group). (**D**) Western blot analysis for STAT3, HIF-1α, and VEGF protein expressions in the indicated groups. β-actin was used as a loading control.

factors for promoting tumor cells' adaptation to the low oxygen tension.[28] In a hypoxic environment, accumulated HIF-1α entered the nucleus to form a HIF-1 complex by binding to HIF-1β. Then the complex bound with the hypoxia responsive elements of HIF target genes and activates the expression of these genes, such as VEGF.[29] The expression of VEGF was closely related to both developmental and pathological angiogenesis,[30] which can promote the proliferation of endothelial cells, increase vascular endothelial permeability, and stimulate the formation of new blood vessels.[31] Numerous studies had demonstrated the role of STAT3, HIF-1α, as well as VEGF in the occurrence and development of various cancers.[32,34] More importantly, some recent studies have revealed that the STAT3/HIF-1α/VEGF pathway was activated in NSCLC.[11,12,35]

The relationship between STAT3 expression and apoptosis had been reported in many studies. Generally, while

the expression, production, phosphorylation, or activation of STAT3 was found in various cancer cells, the apoptosis process in these cells was inhibited. Evidences showed that STAT3 regulates multiple genes that could suppress the cellular apoptosis pathway and contribute to oncogenesis.[36]

In recent years, studies on resveratrol as a potential antitumor agent have been paid more and more attention, and a variety of cellular pathways have been reported to be involved in the anti-tumor effects of RES.[37,39] Sun et al[40] reported that RES significantly inhibited the progression of xenografted lung cancer in mice, and this effect was associated with decreased expression of p-STAT3 in a tumor. Another study revealed that RES could efficiently inhibit breast cancer lung metastasis in mice and the inactivation of STAT3 by RES is one of the underlying mechanisms.[41] Zhang et al[42] showed that RES obviously suppressed both basal and hypoxia-induced expressions of HIF-1α, as well

as the expression of VEGF, in human oral squamous cell carcinoma line SCC-9 and human hepatocellular carcinoma cell line HepG2. Cao et al[43] [43]However, so far there is no experimental study that has determined whether RES could exert its antitumor activity in NSCLC through regulating the STAT3/HIF-1α/VEGF pathway.

As expected, in the present study we found that, compared to the NSCLC group, treatment with RES (intragastrically administered at 250 mg/kg/day for 12 weeks) could lead to significant decreases in lung weight index and lung tumor burden, as well as a less affected lung structural integrity in the NSCLC+RES group. These findings affirmed the results of previous studies showing the antitumor effects of RES in NSCLC. The morphology of the lung in the NSCLC+RES group was different as compared with control and NSCLC groups. We speculated that there are two reasons: the first is due to the sampling position; the second is related to the antiapoptotic activity of RES on human pulmonary alveolar epithelial cells.[44] Further studies on the precise role of RES in this phenomenon are needed in the future.

We further found that compared to the NSCLC group, the apoptotic index was significantly higher, while the STAT3/HIF-1α/VEGF mRNA and protein levels were all significantly decreased in the NSCLC+RES group. These findings were consistent with that of previously published research demonstrating the anti-tumor effects of RES in NSCLC through promoting cell apoptosis.[45,47] More importantly, we suggested for the first time that RES might exert its antitumor activity in NSCLC, at least partly, through inhibiting the STAT3/HIF-1α/VEGF pathway.

In conclusion, our current study has provided the first evidence that RES exerts its anti-tumor effects, at least in part, through inhibiting the expressions of STAT3, HIF-1a, and VEGF in a rat model of NSCLC. The STAT3/HIF-1α/VEGF pathway might be a candidate drug target for developing new chemotherapy agents derived from RES for the treatment of NSCLC. However, the deeper mechanism underlying the involvement of RES in suppressing NSCLC are still unknown and further studies, including gene knockout and rescue experiments, as well as PDX experiment, need to be performed in the future.

Disclosure

The authors report no conflicts of interest in this work.

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