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Resveratrol inhibits proliferation and induces apoptosis of nasopharyngeal carcinoma cell line C666-1 through AMPK activation

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Received October 2, 2014, accepted November 7, 2014

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Pharmazie 70: 399–403 (2015) *doi: 10.1691/ph.2015.4815*

Background: Resveratrol, a natural phenolic compound found in red grapes, has been reported to inhibit proliferation and induce apoptosis *via* regulation of AMPK signaling pathways in several cancer cell types. However, little is known about the effect of resveratrol on the human Nasopharyngeal carcinoma (NPC) cell line C666-1. Moreover, the molecular mechanisms of resveratrol-mediated apoptosis in C666-1 cells remain to be clarified. *Methods:* Cell proliferation was measured by CCK8 assay, cell apoptosis rate was evaluated by flow cytometric analysis, and the protein expression alterations of AMPK signaling pathways were detected by Western blotting. *Results:* Treatment of resveratrol inhibited cell viability and promote apoptosis of C666-1 cells. In addition, we showed that resveratrol could also activate caspase-3 and alter the Bax/Bcl-2 apoptotic signaling. Furthermore, all these changes may be due to the activation of AMPK activity by resveratrol treatment, and we also found that the p70S6K and s6 activities, downstream factors of AMPK, were also blocked by resveratrol. *Conclusion:* Our results revealed that resveratrol can be regarded as a new effective and chemopreventive compound for human NPC treatment.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck cancer prevalent in south-east Asia, southern China, the Middle East and Northern Africa (Hui and Chiang 2014). Evidence from epidemiology, clinical and experimental studies suggested that multiple factors, including genetic and non-genetic factors such as Epstein-Barr virus (EBV) infection, genetic susceptibility and exposure to environmental carcinogens, cooperatively contribute to the development of NPC (Kam et al. 2014; Ying et al. 2014). In addition to its rapid growth behavior, NPC has a great tendency to invade adjacent regions and metastasize to regional lymph nodes and distant organs at an early stage (Wang et al. 2014a). Although chemotherapy in combination with radiation has significantly improved overall survival in patients with advanced stage of NPC, adverse effects such as fatal toxicity and the poor outcome of recurrent disease were observed (Luo et al. 2014). Therefore, there is an emergent need for the development of novel anti-nasopharyngeal cancer drugs with more efficiency and less toxicity.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural substance found in red wine, peanuts and certain berries (Chow et al. 2014). A growing body of evidence from numerous *in vitro* and *in vivo* studies has shown that resveratrol is able to prevent the progression of a variety of diseases ranging from cancer and cardiovascular dysfunction to premature aging (Izmirli et al. 2014; Zhang et al. 2013). Resveratrol has also been reported to induce apoptosis of HT-29 colon cancer cells *via* activation of AMPK signaling pathways (Hwang et al. 2007). Moreover, recent studies also showed that resveratrol could inhibit the proliferation and induce apoptosis in CNE-1 and CNE-2Z cells, two established human NPC cell lines, suggesting that resveratrol may play an important role in the regulation of NPC progression (Zhang et al. 2013).

The C666-1 cell line was originally derived from an undifferentiated tumour transplanted into athymic nude mice. It carries the Epstein-Barr virus (EBV) in long-term cultures and expresses EBV-encoded RNAs (Zhao et al. 2011). Because the C666-1 cell line is the only *in vitro* native EBV-infected NPC cell model, it has become an important investigative tool for NPC research (Tso et al. 2013). However, to the best of our knowledge, there is no report about the effect of resveratrol on the human NPC cell line C666-1. Furthermore, the underlying molecular mechanisms pertaining to resveratrol mediated signaling pathways in C666-1 cell line remains to be clarified. In the current study, we aimed to investigate the anti-cancer ability of resveratrol in human C666-1 cell line, and to study the underlying mechanisms by focusing on the AMP-activated protein kinase (AMPK) signaling cascade.

2. Investigations and results

2.1. Resveratrol inhibits the cell viability of C666-1 cells

We first examined the effect of resveratrol on the cell viability of C666-1 cell. C666-1 cells were incubated with various concentrations of resveratrol for 24 h, and the cell viability was assessed by CCK8 assay. As shown in Fig. 1A and B, resveratrol

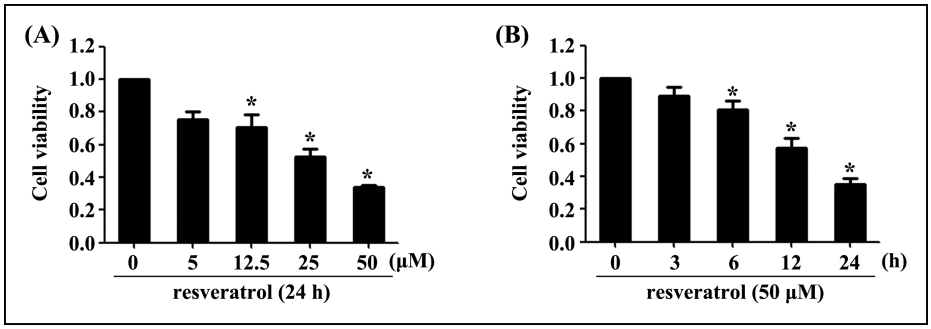

Fig. 1: Resveratrol inhibits cell viability in C666-1 cells. (A and B) C666-1 cells were treated with various concentrations of resveratrol for 24 h or with 50 μM resveratrol for indicated time points. The cell viability was detected by CCK8 assay. *$P < 0.05$ vs. the group without treatment, n = 3.

inhibited C666-1 cells viability in a dose- and time- dependent manner.

2.2. *Resveratrol induces apoptosis in C666-1 cells*

The results above showed that resveratrol inhibited C666-1 cells proliferation; next we tested whether cell apoptosis was involved in such an effect. As shown in Fig. 2A and B, resveratrol (25 and 50 μM) induced apoptosis in C666-1 cells. Meanwhile, resveratrol also caused caspase-3 cleavage and Bcl-2 degradation (Fig. 3A-D).

2.3. *Resveratrol activates AMPK and inhibits mTORC1 in C666-1 cells*

To elucidate the potential molecular mechanisms of resveratrol on C666-1 cells *in vitro*, we assessed the phosphorylation level of AMPK. As shown in Fig. 4A and B, stimulation with 50 μM resveratrol for 24 h resulted in up-regulation of AMPK phosphorylation. Moreover, resveratrol dose-dependently decreased the phosphorylation of S6K1 (T389) and consequently reduced the phosphorylation of ribosomal S6 protein (S235/236), which is a component of the 40S ribosomal subunit (Figure. 4A, C and D).

3. Discussion

In recent years, natural antioxidants such as curcumin, epigallocatechin gallate and resveratrol that are present in food and beverages consumed by humans have attracted extensive attention because of their cancer prevention function (Chow et al. 2010; Lei et al. 2012; Wang et al. 2014b). Among these, resveratrol has gained interest as a non-toxic chemopreventive agent capable of inducing tumor cell death in a variety of cancer types (Sareen et al. 2007). In this present study, we demonstrated that resveratrol suppresses proliferation and promotes apoptosis of human NPC cell line C666-1. This occurs in a concentration-dependent manner. Moreover, we further found that the effect of resveratrol is associated with the activation of the AMPK-dependent signaling pathway. Our findings support the concept that resveratrol could be an effective preventive and therapeutic candidate against NPC.

Previous studies reported that resveratrol exerts a variety of biological functions and its antioxidant and chemopreventive effects are currently evaluated in a wide range of human dis-

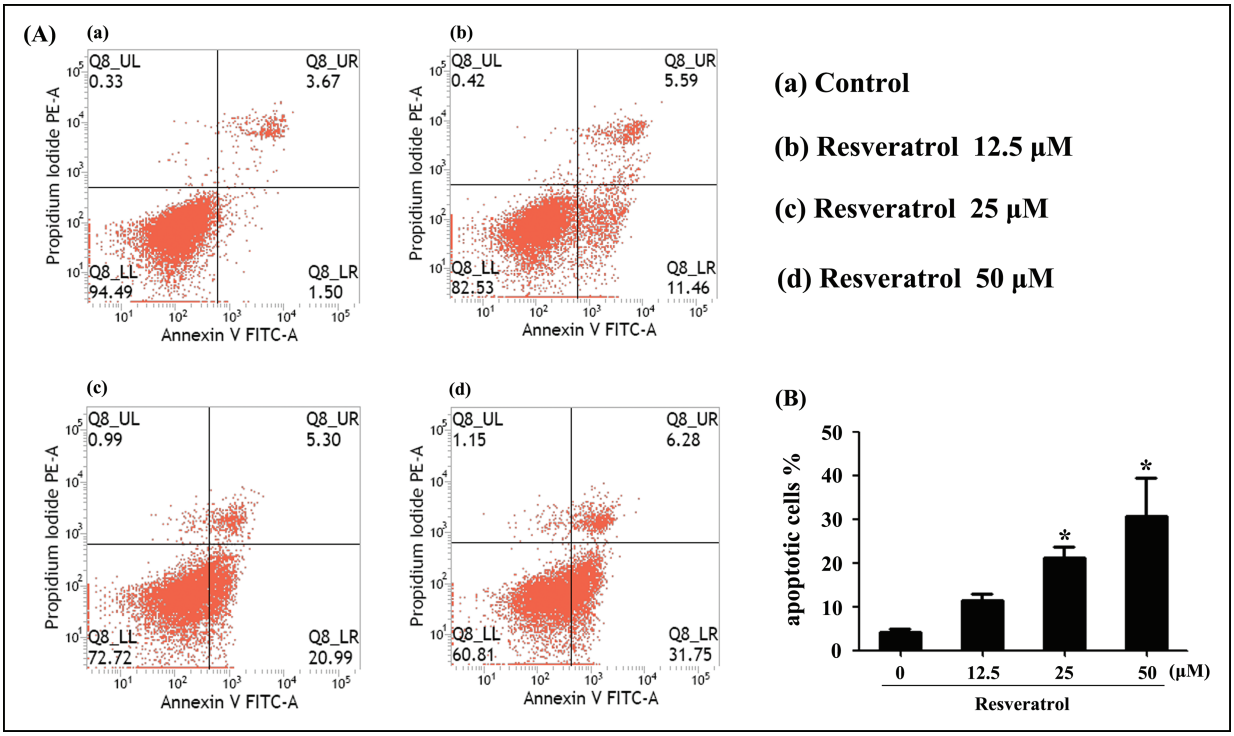

Fig. 2: Resveratrol induces apoptosis in C666-1 cells. (A and B) C666-1 cells were treated with increasing concentrations of EGCG for 24 h. The cell apoptosis was detected by Flow Cytometric Analysis. *$P < 0.05$ vs. the group without treatment, n = 3.

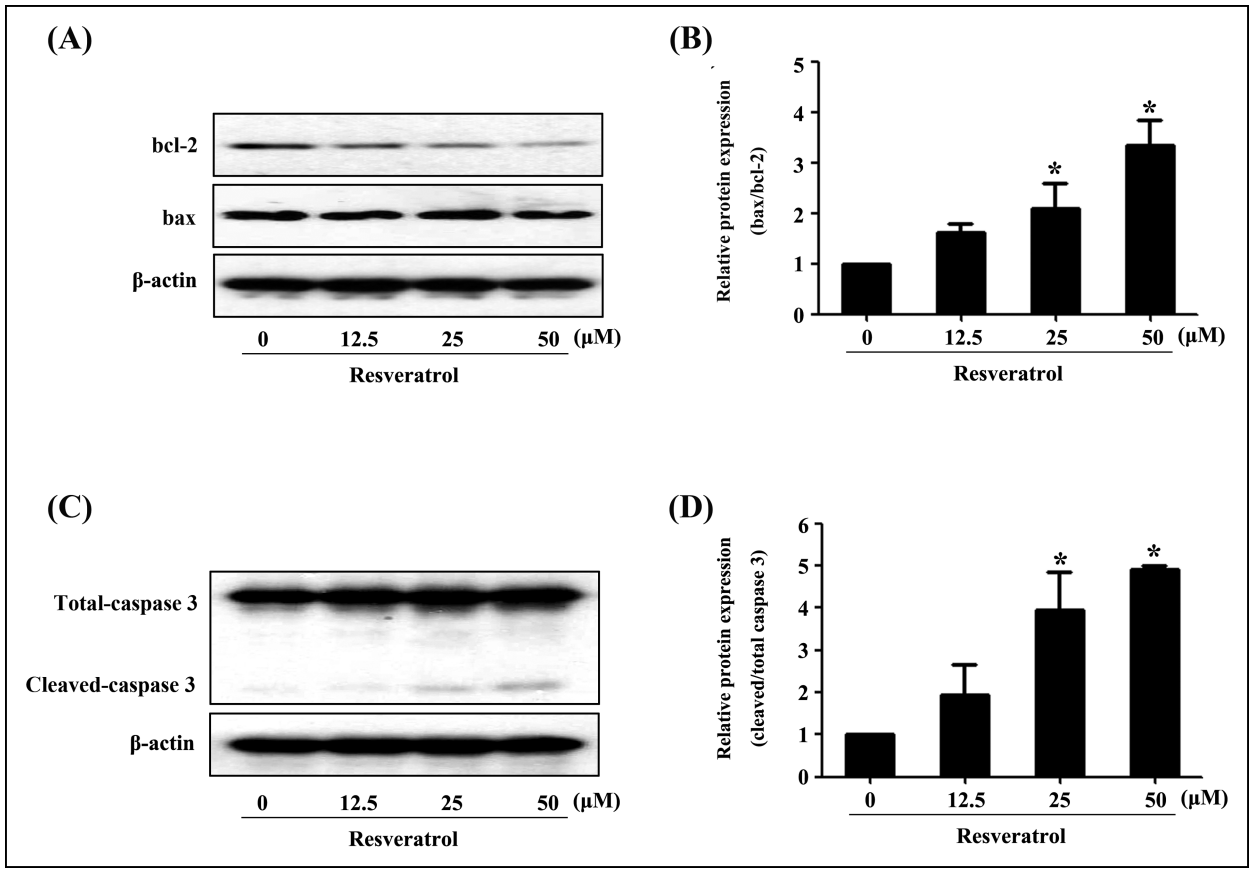

Fig. 3: Effect of resveratrol on the protein expression of bax/bcl-2 and caspase 3. C666-1 cells were treated with increasing concentrations of EGCG for 24 h. The protein expression of bax/bcl-2 (A and B) and caspase 3 (C and D) were detected by western blotting assay. *$P < 0.05$ vs. the group without treatment, n = 3.

eases including cancer (Qian et al. 2009; Rimando et al. 2002). In 2010, Huang et al. first demonstrated that resveratrol induced apoptosis in human NPC cells through regulation of multiple apoptotic pathways, including death receptor, mitochondria, and endoplasmic reticulum (ER) stress (Huang et al. 2011). Recently, Zhang et al. (2013) showed that resveratrol exerted potent anti-proliferative and pro-apoptotic effects on human NPC cells possibly through interfering with the pAkt1/p70S6K signaling pathways. Among human NPC cell lines, the C666-1 cell line is the only *in vitro* native EBV-infected NPC cell model, up to now, little is known about the effect of resveratrol on C666-1 cells. In this work, we found that resveratrol could suppress cell proliferation and induce apoptosis in C666-1 cells. It was reported that resveratrol induces apoptosis in human esophageal carcinoma cells, which may be mediated by down-regulating the expression of Bcl-2 and up-regulating the expression of Bax. Similar to these results, in this study, we found that resveratrol treatment of C666-1 cells is accompanied by down-regulation of Bcl-2. Although Bax protein level did not change, we showed that resveratrol results in a significant increase of the Bax/Bcl-2 ratio which is regarded as a driving force for apoptosis in C666-1 cells. Moreover, resveratrol treatment also promoted the expression of caspase-3, which may contribute to the induction of apoptosis in C666-1 cells. The results of the present study showed that resveratrol treatment could significantly suppress cell growth and promote apoptosis of C666-1 cell line. However, detailed mechanisms mediating resveratrol effects remain unclear.

AMPK, a serine/threonine kinase, can be activated by cellular stress and ATP depletion (Chi et al. 2014). Although AMPK is generally recognized as a critical regulator of energy balance, a great deal of research has suggested that cellular stress-activated AMPK also promotes cell apoptosis, such an effect by AMPK is through regulating AMPK's downstream signals, including mTOR and p53 (Jing et al. 2011; Lee et al. 2012; Miao et al. 2013; Nieminen et al. 2013). Previous studies have shown that overactivation of AMPK by pharmacological drugs such as AICAR and metformin suppresses cell proliferation and induces apoptosis in cancer cells *in vitro* and *in vivo* (Santidrian et al. 2010; Theodoropoulou et al. 2013). In addition, AICAR treatment of the authentic EBV-positive NPC cell line C666-1 not only inhibited cell growth, but also enhanced cell susceptibility to the chemotherapeutic agent 5-FU (Lo et al. 2013), which suggested that AMPK reactivation may offer a new therapeutic approach to improve the efficacy of current conventional chemotherapeutic protocols for NPC treatment. In this present study, we showed that resveratrol is able to activate AMPK in a concentration-dependent manner. Moreover, we also demonstrated that resveratrol could inhibit mTOR signaling, as manifested by dephosphorylation of S6K1 (T389) and S6 (S235/236) in C666-1 cells.

Conversely, our present work demonstrated that resveratrol inhibits proliferation of the human NPC cell line C666-1 in a time and dose-dependent manner. Resveratrol also could induce C666-1 cell apoptosis and this effect is linked to the activation of AMPK-dependent pathway. This study is relevant to the understanding of the inhibitory effect of resveratrol on NPC and related molecular mechanisms. Our findings suggested that resveratrol might be potentially used for prevention or treatment of human NPC.

4. Experimental

4.1. Reagents

Resveratrol (purity > 95%) was purchased from Sigma Aldrich. Dulbecco's Modified Eagle Medium (DMEM) and Newborn Calf Serum (NCS) were

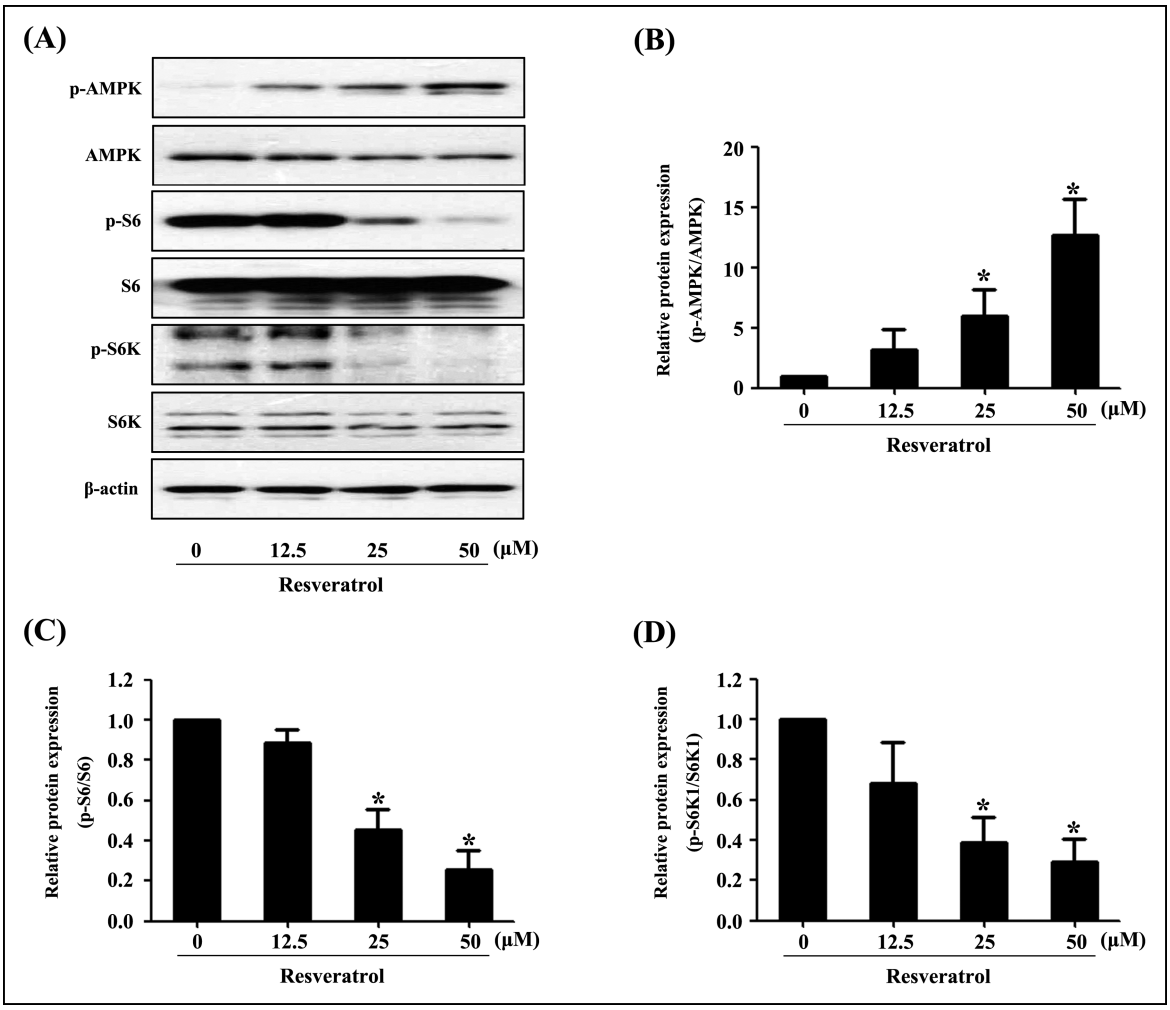

Fig. 4: Resveratrol activates AMPK and inhibits mTORC1 in C666-1 cells. C666-1 cells were treated with increasing concentrations of EGCG for 24 h. The protein expression of p-AMPK, AMPK, p-S6K, S6K, p-S6 and S6 were detected by western blotting assay. $^{*}P < 0.05$ vs. the group without treatment, n = 3.

purchased from Gibco (Logan, UT, USA) and Sijiqing Biological Engineering Materials Co Ltd. (Hangzhou, China), respectively. Antibodies against Bax, Bcl-2, Caspase 3, p-AMPK(T172), P-S6K(T389), S6K1 and p-S6(S235/236) were purchased from Cell Signaling (Cell Signaling Technology); anti-AMPK, S6 and β-actin antibody was purchased from Santa Cruz Biotech (Santa Cruz Biotechnology). Resveratrol was dissolved in absolute ethanol (final concentration 100 mM) and stored at −20 °C until dilution before use.

4.2. Cultures of C666-1 cell line

The undifferentiated human NPC cell line, C666-1 was a gift from Professor Yao Kaitai (Cancer Research Institute of Southern Medical University, Guangzhou, China). The cells were maintained in DMEM supplemented with 10% Newborn Calf Serum (NCS) and incubated in a 37 °C humidified atmosphere containing 95% air and 5% CO_2. Culture was trypsinized upon confluence and propagated to passage 2 before being subcultured into 12, 6, or 96 well plates for further experiments.

4.3. Cell viability analyses

Cell viability was measured by Cell counting kit 8 (Dojindo Molecular Technologies, Inc.). C666-1 cells were seeded at 5×10^3 per well into 96 well microplate and incubated for 24 h. After addition of different concentrations of resveratrol respectively, cells were incubated for another 24 h until testing. CCK8 solution (10 μl) was added to each well and the plate was incubated for an additional 1 h. Finally, the absorbance of each well was measured by BioTek Epoch multi-volume spectrophotometer system (BioTek Instruments Inc., USA) at 450 nm wavelength.

4.4. Flow cytometric analysis

To measure apoptotic cell death, an Annexin V-FITC Apoptosis Detection Kit (BioVision) together with flow cytometry was used according to the recommended protocol to quantify the externalization of inner membrane phosphatidylserine. Briefly, 5×10^5 cells grown to about 60% confluence were treated with 12.5 or 50 μM of resveratrol for 24 h. For Annexin V/PI binding assay, both floating and adherent cells were collected and washed with serum-containing media. Cells collected were washed with serum containing media before being resuspended in 500 ml of 1_binding buffer, followed by addition of 5 ml of Annexin V-FITC and 5 ml of PI to cell suspension. Cells used as controls (0 μM resveratrol) were incubated with the vehicle (DMSO) alone. The mixture was then incubated for 5 min at room temperature in the dark and immediately analyzed with the flow cytometer (BD FACSVerse^{TM}) and BD FASCuite software analysis.

4.5. Western blotting analysis

Western blotting analyses were performed as previously described. Briefly, protein was separated by SDS-PAGE gel electrophoresis, and then transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies, followed by incubation with appropriate horseradish peroxidase (HRP)-labeled second antibodies. Immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescent Substrate (Pierce), and molecular band intensity was determined by densitometry.

4.6. Statistical analysis

Data are presented as mean ± SE. Statistical analyses between two groups were performed by unpaired Student's *t*-test. Differences among groups were tested by one-way analysis of variance (ANOVA). In all cases, differences were considered statistically significant with $P < 0.05$.

Source of Funding: National Natural Science Foundation of China (NO. 81300085; NO. 81302357), the Natural Science of Guangdong Province (NO. S2013040013895; NO. S2013040016493), the scientific research Foundation of Guangzhou Medical University (NO. 2012C03). This work was supported by Guangdong Provincial Key Laboratory of Malignant

Tumor Epigenetics and Gene Regulation, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University.

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