Effect of SIRT1 activators and inhibitors on CD44$^+$/CD133$^+$-enriched non-small cell lung cancer cells

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Abstract. Lung cancer is one of the most commonly diagnosed cancers and it is associated with high rates of morbidity and mortality. Metastasis and relapse of the tumor depend on the survival and proliferation of lung cancer stem cells (LCSCs). The ability to identify CSCs may prevent recurrence and lead to more effective treatments. Sirtuins are a group of deacetylases that include seven variants (SIRT1-7), with sirtuin 1 (SIRT1) being the most intensively investigated. Evidence suggests that *SIRT1* is both a tumor-suppressor gene and an oncogene. SIRT1 can deacetylate the tumor-suppressor protein p53 to decrease its activity. SIRT1 activators increase the deacetylation of p53, whereas SIRT1 inhibitors can stimulate p53 by inhibiting deacetylation. In the present study, CD44$^+$ and CD133$^+$-enriched A549 (non-small cell lung cancer) cells collected using the CD44 and CD133 CSC surface markers by fluorescence-activated cell sorting method were treated with SIRT1 inhibitors (tenovin-6 and sirtinol) and SIRT1 activators (resveratrol and SRT1720), and their effects on apoptosis, as well as the mRNA and protein expression of SIRT1 and p53 were investigated. Of these agents, it was found that resveratrol increased p53 expression by 4.1-fold, decreased SIRT1 expression by 0.2-fold, and it was the most potent inducer of apoptosis.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide due to the increase in cigarette smoking (1). Among the different types of lung cancer, non-small cell lung cancer (NSCLC) has the highest mortality, at ~85% (2). Lung cancer tumor cell populations have a heterogeneous structure consisting of cancer cells and a small numbers of cancer stem cells (CSCs) (3). A CSC originates from a mutated normal stem cell or a differentiated progenitor cell that has accumulated various types of damage (3). Several studies have suggested that identification of CSCs will enable a definitive treatment of the tumor (4-6).

The fluorescence-activated cell sorting (FACS) method can be used to identify and differentiate lung CSCs (LCSCs) (7). LCSCs can be isolated using specific surface markers such as CD44 and CD133. Using this method, stem cells can be separated by molecules capable of irradiation at certain wavelengths by binding to antibodies specific to these surface markers (8).

Sirtuins are enzymes that act as nicotinamide adenine dinucleotide (NAD$^+$)-dependent deacetylases (9). The mammalian sirtuin family consists of seven homologs, SIRT1-7, classified by NAD$^+$ binding and catalytic domain (9). Sirtuin 1 (SIRT1), the most investigated of these variants, has been reported to act as both a tumor promoter and a tumor suppressor (10). In the present study, the effects of SIRT1 activators (resveratrol and SRT1720) and inhibitors (tenovin-6 and sirtinol) on SIRT1 and p53 expression in CD44$^+$/CD133$^+$-enriched cells isolated from the A549 cell line (NSCLC) were investigated to explore potential new treatment approaches for lung cancer.

In the present study, cell sorting was performed to enrich the cancer stem cells. Subsequently, proliferation and cytotoxicity experiments were used to determine the optimum SIRT1 activator-inhibitor concentrations to use to treat the cells. Flow cytometric analysis was used to analyze the apoptotic rate of cells treated with the SIRT1 activators and inhibitors, and quantitative (q)PCR and western blotting analysis was used to analyze the expression levels of SIRT1 and p53 mRNA and protein, respectively.

Materials and methods

Cell cultures and reagents. The cell culture media used in this study were obtained from Biological Industries USA, Inc. Allophycocyanin (APC)-labeled CD44 (cat. no. 130-098-110) and phycoerythrin (PE)-labeled CD133/2 (cat. no. 130-098-046) antibodies were obtained from Miltenyi Biotec GmbH. Cell sorting consumables were

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Abbreviations: SIRT1, sirtuin 1; NSCLC, non-small cell lung cancer; CSCs, cancer stem cells; FACS, fluorescence-activated cell sorting; LCSCs, lung cancer stem cells; NAD, nicotinamide adenine dinucleotide; CD, cluster of differentiation; FBS, fetal bovine serum; CDK, cyclin-dependent kinase

Key words: cancer stem cells, lung cancer, sirtuin 1, p53, resveratrol

procured from BD Biosciences. Antibodies for western blotting were purchased from Cell Signaling Technology, Inc. SIRT1 activators and inhibitors were obtained from Santa Cruz Biotechnology, Inc., and stock solutions were prepared for each substance (Table SI). The A549 cell line was grown in F-12 Nutrient Mixture (Ham's) medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin under standard culture conditions.

Cancer stem cell sorting. $CD44^+/CD133^+$ cells were sorted using a BD FACSAria™ device (BD Biosciences). A549 cells were incubated with 1% trypsin-EDTA for 5 min at 37˚C and then collected and centrifuged at room temperature for 10 min at 300 x g to obtain a cell pellet. The cell pellet was washed with PBS containing 2% FBS and 2 mM EDTA, centrifuged at room temperature for 10 min at 300 x g and then incubated in 100 μl PBS buffer with 10 μl each of CD44 and CD133 antibodies (1:11) in the dark for 10 min at 4˚C. After incubation, PBS was added, the suspension was centrifuged at room temperature for 10 min at 300 x g, and the supernatant was discarded. The pellet was resuspended in 3 ml PBS and the APC- and PE-labeled $CD44^+CD1\ 33^+$ cells were sorted using the BD FACSAria™ device. The gating strategy used for the separation of APC- and PE-marked $CD44^+$ and $CD133^+$ cells is demonstrated in Fig. S1.

Cell proliferation and cytotoxicity assays. The xCELLigence® Real-Time Cell Analyzer (Roche Applied Science) was used for cell proliferation and cytotoxicity analyses. After seeding $CD44^+/CD133^+$-enriched A549 cells into an E-Plate at a density of 10,000 cells/well, the impedance values were measured for 72 h to obtain proliferation curves. For cytotoxicity assays, cells were seeded into the E-Plates at the same density and incubated for 24 h at 37˚C. After this incubation, SIRT1 activators (resveratrol and SRT1720) and inhibitors (tenovin-6 and sirtinol) were applied, and the impedance values were measured for 72 h. Each concentration was run in triplicate. Data analysis was performed using the software included in the xCELLigence device, and the IC_{50} of each active substance was obtained.

Apoptosis analyses. Annexin V/PI staining was performed to evaluate the apoptotic effects of the active substances. Cells were seeded onto 12-well plates at a density of $2x10^5$ cells/well and incubated for 24 h at 37˚C to allow adhesion. After this incubation, the active ingredients were applied at the IC_{50} dose. After 72 h, the cells were released by trypsinization, incubated with Annexin V/PI for 1 min at room temperature, and then examined in the BD Accuri™ C6 flow cytometry device and analyzed via BD Accuri™ C6 software (version 227.4; BD Biosciences).

Quantitative (q)PCR analysis. After treating the $CD44^+/CD133^+$-enriched A549 cells with IC_{50} dose for 72 h, the cells were harvested, and RNA was obtained using the MagNA Pure LC RNA Isolation kit (Roche Applied Science). cDNA synthesis was performed using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc.). The following reverse transcription temperature protocol was used: 30 min at 42˚C, 85˚C for 5 min and then maintained at 4˚C. SIRT1 and p53 gene expression levels were analyzed using Hs01009006-SIRT1 and Hs01034249-TP53 TaqMan™ primers and TaqMan® FAM-MGB dye probes (Applied Biosystems; Thermo Fisher Scientific, Inc.). GADPH (Hs03929097-GAPDH) was used as a housekeeping gene. For qPCR, the reaction mixture was prepared according to Table SII. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 10 min; followed by 40 cycles of denaturation at 95˚C for 10 sec and extension at 60˚C for 60 sec; prior to cooling to 40˚C for 30 sec. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (11) and presented as fold change.

Western blot analysis. Western blot analyses of proteins collected from cells treated with IC_{50} doses of the active substances for 72 h and the control cells were performed using SIRT1, p53 and β-actin antibodies. Proteins were obtained using Complete Lysis-M Buffer (Roche Applied Science) and quantified using Bradford solution (Fermentas; Thermo Fisher Scientific, Inc.) and the Pierce™ Bovine Serum Albumin Standard Pre-Diluted set (Fermentas; Thermo Fisher Scientific, Inc.). For each sample, 23.8 μg of protein was run on 8% SDS-PAGE gel and transferred to a PVDF membrane using the iBlot system (Invitrogen; Thermo Fisher Scientific, Inc.; Table SIII). The membranes were blocked with 5 ml blocking buffer (Western Breeze® Chromogenic kit; Invitrogen; Thermo Fisher Scientific, Inc.) for 45 min at room temperature. The membranes were washed with 10 ml antibody washing solution diluted in 150 ml distilled water three times for 5 min. The membranes were subsequently incubated with the anti-SIRT1 (1.5:1,000; cat. no: 9475T; Cell Signaling Technology, Inc.), anti-p53 (1.5:1,000; cat. no: 2527T; Cell Signaling Technology, Inc.) and anti-β-actin (1.5:1,000; cat. no. 8457S; Cell Signaling Technology, Inc.) primary antibodies at room temperature for 90 min. Following the primary antibody incubation, the membranes were washed three times with a washing solution and incubated with the ready to use secondary antibody solution containing alkaline phosphatase-conjugated secondary antibody (WesternBreeze™ Chromogenic kit; cat. no: WB7105; Invitrogen; Thermo Fisher Scientific, Inc.) for 45 min at room temperature. The membranes were washed three times with washing solution. Western Blot Chromogenic Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to visualize the protein bands and the expression levels were analyzed using ImageJ software version 1.8 (National Institutes of Health).

Statistical analysis. All experiments were performed at least in triplicate. The results are expressed as the mean ± SD. Data were analyzed by ANOVA followed by post-hoc Tukey's multiple comparisons test. In the case of $P<0.05$, the difference was considered statistically significant. GraphPad Prism 8.0 software (GraphPad software, Inc.) was used for analyses.

Results

Cell proliferation and cytotoxicity test. For the cell proliferation analysis, the proliferation curve and the slope values

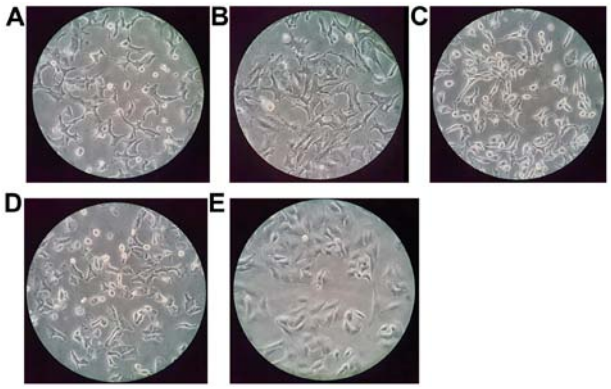

Figure 1. A549 cancer stem cells after 48 h of treatment. Images show treatment with (A) resveratrol, (B) sirtinol, (C) tenovin-6 and (D) SRT1720 compared with (E) untreated control cells. Magnification, x600.

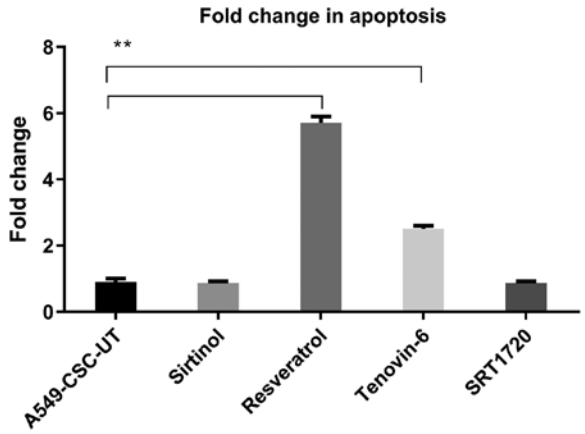

Figure 2. Fold changes in apoptosis rates in cancer stem cells treated for 72 h with IC_{50} doses of sirtuin 1 activators/inhibitors compared with untreated A549 cells. $^{**}P<0.01$ vs. control group.

of $CD44^+/CD133^+$-enriched A549 cells were determined to be $9x10^3$, $18x10^3$ and $36x10^3$ cells/well, respectively, and $12x10^3$ cells/well was calculated as the optimum cell density (Figs. S2 and S3). The IC_{50} values for each agent were then determined in cells treated for a total of 72 h (Table SIV).

When calculating the IC_{50} values at 24, 48 and 72 h for each agent, the IC_{50} values at 72 h were preferred for apoptosis and protein expression experiments, while 48 h values were preferred for mRNA expression experiments. The IC_{50} values of the $CD44^+/CD133^+$-enriched A549 cell line treated with resveratrol, sirtinol, tenovin-6 and SRT1720 for 48 h were 196, 37, 13.9 and 6.57 μM, respectively. The IC_{50} values obtained with the treatment with resveratrol, sirtinol, tenovin-6 and SRT1720 for 72 h were 605, 35.8, 15.3 and 6.64 μM, respectively. The cytotoxicity curves and the IC_{50} plots of the A549-CSC line treated with the active agents are shown in Figs. S4-S11. When the effects of the agents on cell morphology were examined, the most significant change was observed in the cells treated with sirtinol (Fig. 1); the cell membrane surface was rough and furcation had occurred.

Apoptosis assay. As shown in Fig. 2, there was no significant difference in the apoptosis rates in the $CD44^+/CD133^+$-enriched A549 cells treated with sirtinol and SRT1720 compared with the control group, whereas treatment with resveratrol and tenovin-6 significantly increased the apoptosis rate by 5.7- and 2.5-fold, respectively.

As shown in Table I and Fig. 3, the rate of necrosis was 19.75% with SRT1720 treatment and ~11% in cells treated with tenovin-6 and resveratrol. Resveratrol was the strongest inducer of apoptosis (14.64%).

In the flow cytometric analysis, gates were drawn to create four sections. According to these four sections, the lower right quadrant represents early apoptosis (Q3-LR), the upper right quadrant represents late apoptosis (Q3-UR), the lower left quadrant represents the normal state (Q3-LL), and the upper left quadrant represents necrosis (Q3-UL; Fig. 4).

Analysis of mRNA expression. Analysis of p53 mRNA expression at 48 h revealed that sirtinol, resveratrol and tenovin-6 treatments caused significant differences in p53 expression (Fig. 5). SIRT1 expression decreased significantly after sirtinol and SRT1720 compared to the resveratrol treatment group. The most significant difference was a 4-fold decrease in p53 expression in resveratrol-treated cells.

Bradford protein quantification and western blot analysis. Changes in the protein expression of SIRT1 and p53 in CSCs treated for 72 h were evaluated by western blotting (Fig. S12). When the SIRT1 and p53 protein expression levels of CSCs treated for 72 h with SIRT1 inhibitory agents were examined, it was observed that SIRT1 expression decreased by 79% after sirtinol treatment and by 78% after treatment with tenovin-6. After treatment with resveratrol, SIRT1 expression decreased by 58% and p53 expression increased by 37%, whereas treatment with SRT1720 decreased the SIRT1 expression by 31% and increased the p53 expression by 25% (Fig. S13).

Discussion

To the best of our knowledge, this is the first study to investigate the effects of SIRT1 activators and inhibitors on the mRNA and protein expression of sirtuin 1 (SIRT1) and p53 in $CD44^+/CD133^+$-enriched A549 non-small cell lung cancer (NSCLC) cells. Different studies on NSCLC cells have demonstrated that subpopulations with high expression of CD44 and CD133 surface markers exhibit stem cell characteristics (12). In a study on NSCLC, Leung *et al* (13) screened the expression profiles of five stem cell markers (CD34, CD44, CD133, BMI1 proto-oncogene, polycomb ring finger and octamer-binding transcription factor 4) in 10 lung cancer cell lines by flow cytometry and reported that cells rich in CD44 may have a role in spheroid and tumor formation *in vitro*. In another study, Tirino *et al* (14) investigated the role of CD133 in the identification and characterization of NSCLC tumor-initiating cells and found that 72% of NSCLC samples contained $CD133^+$ cells. In another *in vivo* study, $CD133^+$-rich cells isolated from non-obese diabetic mice with severe combined immunodeficiency were found to be more tumorigenic (14). Based on this information, a $CD44^+/CD133^+$ cell subpopulation was obtained from the A549 NSCLC cell line by the FACS method using APC- and PE-labeled antibodies to CD44 and CD133 surface markers.

Table I. A549 cell apoptosis rates with Annexin/PI treatment of cancer stem cells as determined by flow cytometry.

	Healthy		Early apoptosis		Late apoptosis		Necrosis	
Treatment	% of gated area (%)	% of all (%)	% of gated area (%)	% of all (%)	% of gated area (%)	% of all (%)	% of gated area (%)	% of all (%)
A5469-CSCs-UT blank	99.99	61.82	0.00	0.00	0.00	0.00	0.01	0.01
Siritinol	94.20	49.51	1.03	0.54	1.32	0.69	3.45	1.82
Resveratrol	73.87	6.88	1.85	0.17	12.79	1.19	11.49	1.07
Tenovin-6	81.95	0.77	1.62	0.02	4.72	0.04	11.71	0.11
SRT1720	78.25	16.87	0.49	0.11	1.50	0.32	19.75	4.26
A549-CSCs-UT Annexin-PI	93.60	67.73	1.49	1.08	1.09	0.79	3.82	2.76

CSCs, cancer stem cells; UT, untreated; PI, propidium iodide.

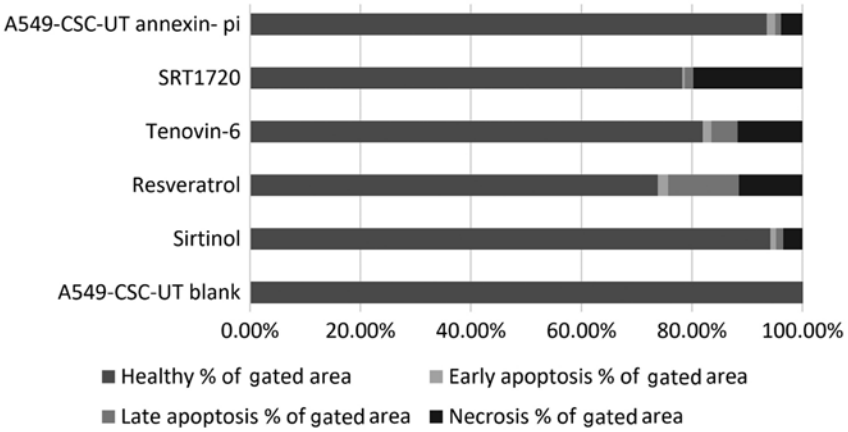

Figure 3. Apoptosis and necrosis rates of the A549 cell line were determined with Annexin and propidium iodide (PI) by flow cytometry. CSC, cancer stem cell; UT, untreated.

Resveratrol, a natural polyphenolic phytoalexin, has been reported to have an antitumor effect by inducing cell apoptosis via the activation of SIRT1 in human chondrosarcoma (15). In addition, according to a study by Wang *et al* (16) resveratrol exhibited an inhibitory effect on proliferation, while inducing apoptosis of NSCLCs. Similarly, the present findings demonstrated that resveratrol had the maximum antitumor effect among all agents on the cell subpopulation, which had cancer stem cell (CSC) features, by increasing the rate of apoptosis by 5.7-fold after 72 h of treatment. However, this effect was observed to occur with a 4.1-fold increased p53 expression and a 0.2-fold decreased SIRT1 expression in the $CD44^+$- and $CD133^+$-enriched A549 cells. Although most of the studies have reported that resveratrol is an activator of SIRT1, the class III NAD-dependent histone/protein deacetylase (17-19), some studies have reported that the interactions between resveratrol, SIRT1 and apoptosis, in fact, are more complex (20-22). Frazzi *et al* (20) found that resveratrol exerted an apoptosis-promoting effect by decreasing the activity of SIRT1 in Hodgkin's lymphoma cells, which is consistent with the present study. Furthermore, although the IC_{50} dose of resveratrol in A549 cells enriched with CSCs was calculated at 173 μM (Table SIV), this value for normal A549 cells was determined to be 98 μM by Wang *et al* (23). Altogether, the possible reasons for all these findings could be that resveratrol may have a different pathway from SIRT1 by affecting apoptosis due to the impact of CSCs.

Yuan *et al* (24) performed cell cycle analysis and found that resveratrol downregulated the expression levels of cyclin D1, cyclin-dependent kinase (CDK) 4 and CDK6, but it upregulated the expression levels of the CDK inhibitors p21 and p27, and induced cell cycle arrest in the G0/G1 phase. Similarly, the increased expression of the tumor-suppressor protein p53 observed in the present study suggests that cancer cells could be more easily forced into apoptosis. In fact, resveratrol produced a 5.7-fold increase in the rate of apoptosis as measured using the Annexin method. On the other hand, a 1.37-fold increase in p53 protein expression was measured by western blotting. Yuan *et al* (24) reported high levels of p53 expression in the A549 cell line. This demonstrates the difficulty of increasing p53 expression in CSCs. However, the significant suppressive effect of resveratrol on the expression of SIRT1, hypothesized to be a tumor promoter, makes it a significant anti-tumorigenic agent.

Regarding the other SIRT1 activator, SRT1720, some researchers have demonstrated that it has SIRT1-independent effects and also indirectly activates SIRT1 (21). Therefore, the cancer stem-like A549 cells treated with SRT1720 experienced

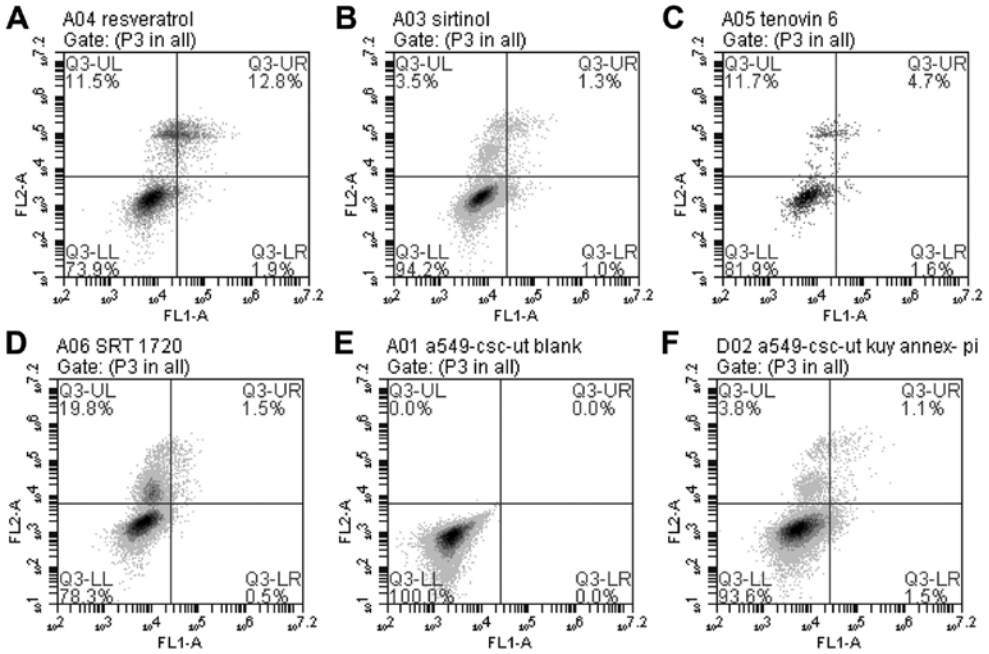

Figure 4. Apoptotic stages in the flow cytometric analysis. Flow cytometry analysis of the A549 cell line treated with (A) resveratrol, (B) sirtinol, (C) tenovin-6 and (D) SRT1720 compared with (E) untreated blank controls and (F) Annexin-PI-treated controls for 72 h.

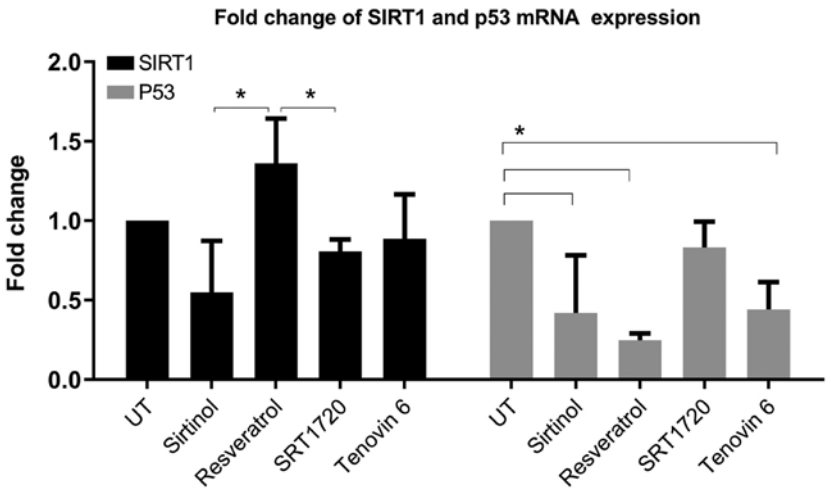

Figure 5. Fold changes in SIRT1 and p53 mRNA expression after 48 h of treatment at the IC_{50} dose compared with untreated A549 cells. *P<0.05, as indicated. SIRT1, sirtuin 1; UT, untreated.

a higher necrosis rate than apoptosis rate, which was 19.75%. Similar to the present result, Lahusen and Deng (25) found that SRT1720 induced necrosis by increasing lysosomal membrane permeabilization in breast cancer cells.

In the present study, the SIRT1 inhibitors sirtinol and tenovin-6 decreased the SIRT1 protein expression by 79 and 78% and decreased the SIRT1 mRNA expression by 1.8- and 1.1-fold, respectively, compared to those in the control group. These results suggest that sirtinol and tenovin-6 block another intermediate step in which SIRT1 protein expression does not suppress the level of mRNA expression effectively. p53 mRNA expression decreased by 2.4- and 2.3-fold upon treatment with sirtinol and tenovin-6, respectively, whereas, sirtinol decreased the rate of apoptosis by 0.1-fold, and tenovin-6 increased the apoptosis rate by 2.5-fold. When protein expressions of SIRT1 and p53 were evaluated using western blotting, it was found that resveratrol, sirtinol and tenovin-6 significantly decreased SIRT1 protein expression, whereas there was no significant change in p53 expression.

Inhibition of SIRT1 with the activation of p53 is associated with the pro-apoptotic effects of both tenovin-6 and sirtinol in several tumors; therefore, in the present study it was expected that these agents would increase the expression of p53-dependent proteins, resulting in apoptosis (26-28). On the other hand, any significant changes in p53 mRNA or protein expression were not observed after treatment with these agents, and there were also no marked changes in apoptosis. However, the present results, in particular for tenovin-6, might be compatible with the literature when looking at a study conducted by MacCallum *et al* (29). These authors observed

that although tenovin-6 did not induce cellular apoptosis or p53 mRNA expression activity, it increased apoptosis in acute lymphoblastic leukemia cells (29).

Due to the budget limitation in the current study, only the A549 cell line in the NSCLC family was used, and acetylated-p53 could not be evaluated in the western blot assay. Although this study did not show acetylated-p53 in Fig. S12, it should be considered that the total p53 also contains acetylated-p53 regulated by SIRT1 (30).

Overall, although the current literature describes the relationship between cancer, SIRT1 and SIRT1 activators/inhibitors, the available data are still controversial and far from being definitive, in particular for CSCs. The results of this study showed that resveratrol is an important agent for inducing apoptosis in a $CD44^+/CD133^+$-enriched A549 cell line.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZE conceived and coordinated the study, and wrote the manuscript; CE maintained the cells and performed the western blotting and qPCR experiments; GO designed and supervised the cell sorting experiments; VBC analyzed the data and revised the figures; and ZD designed and performed the experiments, created the figures and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2018. CA Cancer J Clin 68: 7-30, 2018.
2. Zappa C and Mousa SA: Non-small cell lung cancer: Current treatment and future advances. Transl Lung Cancer Res 5: 288-300, 2016.
3. Rich JN: Cancer stem cells: Understanding tumor hierarchy and heterogeneity. Medicine (Baltimore) 95 (1 Suppl 1): S2-S7, 2016.
4. Leeman KT, Fillmore CM and Kim CF: Lung stem and progenitor cells in tissue homeostasis and disease. Curr Top Dev Biol 107: 207-233, 2014.
5. Salama R, Tang J, Gadgeel SM, Ahmad A and Sarkar FH: Lung cancer stem cells: Current progress and future perspectives. J Stem Cell Res Ther S7: 007, 2012.
6. Luo J, Zhou X and Yakisich JS: Stemness and plasticity of lung cancer cells: Paving the road for better therapy. Onco Targets Ther 7: 1129-1134, 2014.
7. Wang Y, Jiang M, Du C, Yu Y, Liu Y, Li M and Luo F: Utilization of lung cancer cell lines for the study of lung cancer stem cells. Oncol Lett 15: 6791-6798, 2018.
8. Halim NHA, Zakaria N, Satar NA and Yahaya BH: Isolation and characterization of cancer stem cells of the non-small-cell lung cancer (A549) cell line. Methods Mol Biol 1516: 371-388, 2016.
9. Haigis MC and Sinclair DA: Mammalian sirtuins: Biological insights and disease relevance. Annu Rev Pathol 5: 253-295, 2010.
10. Mei Z, Zhang X, Yi J, Huang J, He J and Tao Y: Sirtuins in metabolism, DNA repair and cancer. J Exp Clin Cancer Res 35: 182, 2016.
11. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
12. Zhang HZ, Lin XG, Hua P, Wang M, Ao X, Xiong LH, Wu C and Guo JJ: The study of the tumor stem cell properties of CD133+CD44+ cells in the human lung adenocarcinoma cell line A549. Cell Mol Biol (Noisy-le-grand) 56 (Suppl): OL1350-OL1358, 2010.
13. Leung EL, Fiscus RR, Tung JW, Tin VP, Cheng LC, Sihoe AD, Fink LM, Ma Y and Wong MP: Non-small cell lung cancer cells expressing CD44 are enriched for stem cell-like properties. PLoS One 5: e14062, 2010.
14. Tirino V, Camerlingo R, Franco R, Malanga D, La Rocca A, Viglietto G, Rocco G and Pirozzi G: The role of CD133 in the identification and characterisation of tumour-initiating cells in non-small-cell lung cancer. Eur J Cardiothorac Surg 36: 446-453, 2009.
15. Chao SC, Chen YJ, Huang KH, Kuo KL, Yang TH, Huang KY, Wang CC, Tang CH, Yang RS and Liu SH: Induction of sirtuin-1 signaling by resveratrol induces human chondrosarcoma cell apoptosis and exhibits antitumor activity. Sci Rep 7: 3180, 2017.
16. Wang J, Li J, Cao N, Li Z, Han J and Li L: Resveratrol, an activator of SIRT1, induces protective autophagy in non-small-cell lung cancer via inhibiting Akt/mTOR and activating p38-MAPK. Onco Targets Ther 11: 7777-7786, 2018.
17. Zhang X, Wan F, You W, Tan X, Liu G, Jin Q, Wei C, Liu X, Zhao H, Liu Y and Zhang C: Comparison of apoptosis between bovine subcutaneous and intramuscular adipocytes by resveratrol via SIRT1. Anim Biotechnol 1-9: 2019.
18. Yun JM, Chien A, Jialal I and Devaraj S: Resveratrol up-regulates SIRT1 and inhibits cellular oxidative stress in the diabetic milieu: Mechanistic insights. J Nutr Biochem 23: 699-705, 2012.
19. Chai R, Fu H, Zheng Z, Liu T, Ji S and Li G: Resveratrol inhibits proliferation and migration through SIRT1 mediated post-translational modification of PI3K/AKT signaling in hepatocellular carcinoma cells. Mol Med Rep 16: 8037-8044, 2017.
20. Frazzi R, Valli R, Tamagnini I, Casali B, Latruffe N and Merli F: Resveratrol-mediated apoptosis of hodgkin lymphoma cells involves SIRT1 inhibition and FOXO3a hyperacetylation. Int J Cancer 132: 1013-1021, 2013.
21. Pacholec M, Bleasdale JE, Chrunyk B, Cunningham D, Flynn D, Garofalo RS, Griffith D, Griffor M, Loulakis P, Pabst B, *et al*: SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J Biol Chem 285: 8340-8351, 2010.
22. Bi X, Ye Q, Li D, Peng Q, Wang Z, Wu X, Zhang Y, Zhang Q and Jiang F: Inhibition of nucleolar stress response by Sirt1: A potential mechanism of acetylation-independent regulation of p53 accumulation. Aging Cell 18: e12900, 2019.
23. Wang X, Wang D and Zhao Y: Effect and mechanism of resveratrol on the apoptosis of lung adenocarcinoma cell line A549. Cell Biochem Biophys 73: 527-531, 2015.
24. Yuan L, Zhang Y, Xia J, Liu B, Zhang Q, Liu J, Luo L, Peng Z, Song Z and Zhu R: Resveratrol induces cell cycle arrest via a p53-independent pathway in A549 cells. Mol Med Rep 11: 2459-2464, 2015.

25. Lahusen TJ and Deng CX: SRT1720 induces lysosomal-dependent cell death of breast cancer cells. Mol Cancer Ther 14: 183-192, 2015.
26. Hirai S, Endo S, Saito R, Hirose M, Ueno T, Suzuki H, Yamato K, Abei M and Hyodo I: Antitumor effects of a sirtuin inhibitor, tenovin-6, against gastric cancer cells via death receptor 5 up-regulation. PLoS One 9: e102831, 2014.
27. Ueno T, Endo S, Saito R, Hirose M, Hirai S, Suzuki H, Yamato K and Hyodo I: The sirtuin inhibitor tenovin-6 upregulates death receptor 5 and enhances cytotoxic effects of 5-fluorouracil and oxaliplatin in colon cancer cells. Oncol Res 21: 155-164, 2013.
28. Wang J, Kim TH, Ahn MY, Lee J, Jung JH, Choi WS, Lee BM, Yoon KS, Yoon S and Kim HS: Sirtinol, a class III HDAC inhibitor, induces apoptotic and autophagic cell death in MCF-7 human breast cancer cells. Int J Oncol 41: 1101-1109, 2012.
29. MacCallum SF, Groves MJ, James J, Murray K, Appleyard V, Prescott AR, Drbal AA, Nicolaou A, Cunningham J, Haydock S, *et al*: Dysregulation of autophagy in chronic lymphocytic leukemia with the small-molecule sirtuin inhibitor tenovin-6. Sci Rep 3: 1275, 2013.
30. Solomon JM, Pasupuleti R, Xu L, McDonagh T, Curtis R, DiStefano PS and Huber LJ: Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. Mol Cell Biol 26: 28-38, 2006.