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Anti-tumor effects of resveratrol on malignant melanoma is associated with promoter demethylation of RUNX3 gene

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The natural phytoalexin resveratrol (RES) has exhibited excellent anti-tumor effects on a variety of tumors including malignant melanoma. However, its specific mechanism of anti-melanoma needs to be further explored. It has been reported, that the expression of tumor suppressor gene RUNX3 was lost or substantially decreased in melanoma. Whether RES exerts its anti-tumor effect by regulating the expression of RUNX3 gene in melanoma is worthy of study. In the present study, we found the RUNX3 promoter is hypermethylated and the expression of RUNX3 mRNA and protein are absent in melanoma cells B16F10. After intervention with RES, promoter hypermethylation of RUNX3 in B16F10 cells could be significantly decreased and mRNA and protein expression of it was upregulated in a dose-dependent manner. We further investigated the effects of RES on B16F10 xenograft models. The intervention of RES and treatment of melanoma positive drug dacarbazine (DTIC) both could significantly inhibit tumor growth in xenograft mice, but only RES could upregulate the expression of RUNX3 mRNA and protein in peripheral blood and tumor tissues. Therefore, the upregulation of mRNA and protein expression of RUNX3 resulting from promoter demethylation might be one of the mechanisms of RES inhibiting melanoma. This research has revealed a novel mechanism for RES against melanoma from the epigenetic perspective, which is helpful to improve the understanding of the anti-tumor mechanism of RES and provide new insights for the treatment of melanoma.

1. Introduction

Malignant melanoma, a neoplasm derived from melanocytes, is one of the most aggressive forms of cutaneous cancers with increasing incidence and alarming mortality rates (Eggermont et al. 2014). Malignant melanoma is also a leading cause of cancer deaths between the ages of 20–35 (Houghton and Polsky 2002). Consequently, there is an urgent need to explore efficacious therapeutic treatment for melanoma.

Runt related transcription factor 3 (RUNX3) is generally identified as a tumor suppressor gene (Chuang and Ito 2010), which has been found to be decreased or lost in a variety of tumors, including gastric, bladder, colon, breast, lung, and pancreas carcinomas (Li et al. 2002; Kim et al. 2005; Ku et al. 2004; Subramaniam et al. 2009; Li et al. 2004; Wada et al. 2004). Kitago et al. (2009) found that the expression of RUNX3 was significantly decreased in malignant melanoma cell lines compared to normal melanocytes. Additionally, a clinical analysis of 440 melanoma patients showed that the expression of RUNX3 in tumor tissues was correlated to tumor stages, pathological types and five-year survival rate, and the expression of RUNX3 was decreased during cancer onset and progression (Zhang et al. 2011). Accordingly, RUNX3 has the potency to become a novel target for malignant melanoma treatment.

Resveratrol (trans-3,4',5-trihydroxystilbene, RES) is a non-flavonoid polyphenolic compound, which mainly exists in grapes, peanuts, veratryl and *Polygonum cuspidatum* (Jang et al. 1997). RES has been proved to exhibit effective anti-tumor effects on a variety of tumors including malignant melanoma. Its complicated anti-tumor mechanisms comprises anti-oxidantion, proliferation inhibition, apoptosis induction, autophagy triggering and anti-angiogenesis, etc (Athar et al. 2007; Hsieh et al. 2005; Niles et al. 2003; Wang et al. 2014; Trapp et sl. 2010). However, little is known about the specific anti-tumor mechanism of RES in malignant melanoma. Whether RES exerts its anti-tumor effects by regulating the expression of RUNX3 gene in malignant melanoma seems worth exploring.

A growing number of studies have suggested that epigenetic factors, especially methylation, play a pivotal role in the pathogenesis of melanoma. Liu et al.(2008) identified that the DCR, DCR2, LOX and TPM1 genes were aberrantly hypermethylated to different extents in multiple melanoma cell lines and tumor tissues. Chen et al. (2010) found that downregulation of RUNX3 in gastric cancer cell line MKN28 was closely correlated with hypermethylation, which could be reversed by 5-Aza-CdR and then induced upregulation of p27 and caspase 3. Deng and Zhang (2009) also demonstrated that 5-Aza-CdR could reactivate the expression of RUNX3 in colorectal Lovo cells and induce caspase-independent apoptosis. Moreover, RES was found to be intimately associated with DNA methylation in cancer (Qin et al. 2005). Thirty-nine adult women at increased breast cancer risk were treated with different doses of RES; the methylation of the tumor suppressor gene Ras-related region family 1A gene (RASSF-1a) decreased with increasing doses of RES (Zhu et al. 2012). It has been reported previously that RES can prevent epigenetic silencing of the tumor suppressor protein BRCA1 in breast cancer cells (Hardy and Tollefsbol 2011). Therefore, we investigated whether RUNX3 is inactivated by methylation in melanoma cells, and whether RES can reverse its methylation and restore RUNX3 gene expression warrants further investigation.

In this experiment, the B16F10 melanoma cells and C57BL xenograft models were used to investigate the expression and promoter methylation status of RUNX3 gene in melanoma, and to study whether RES plays a role in RUNX3 expression, and further to explore its mechanism of action.

2. Investigations and results

2.1. RES causes melanoma cell B16F10 morphological changes

In this study, B16F10 cells were treated with different concentrations of RES for 48 h, and the effect of RES on the proliferation inhibition and cell morphology was observed under an inverted microscope. As shown in Fig. 1, compared with the strong growing status of high cell density and full shape in control group (Fig. 1A), after intervention with positive drug 10 μM 5-Aza-CdR (Fig. 1B) or different concentrations of RES (Fig. 1C-E) for 48 h reduced cell density and caused cell shrinkage, indicating an inhibition effect of cell growth. What is more, the inhibition increased gradually with the dose-escalation of RES, which shows a dose-dependent effect. Overall, these results evidently indicated that RES can result in significant proliferation inhibition of B16F10 cells, which is consistent with the well-known anti-tumor effect of RES.

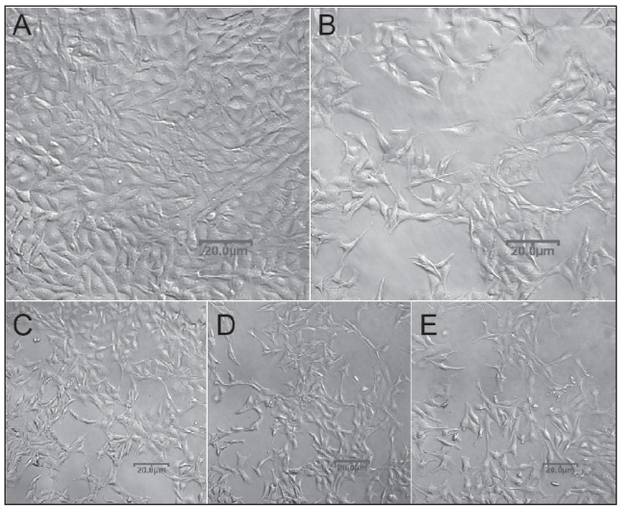

Fig. 1: Cell morphology of B16F10 cells after treatment of resveratrol (RES). Pictures were observed and photographed under an inverted microscope. A: control; B: 10μM 5-Aza-CdR treatment for 48 h; C , D and E are morphology of low-, middle-, and high-dose of RES, respectively.

2.2. RES can reverse promoter hypermethylation of RUNX3

In order to evaluate the correlation between RES and promoter hypermethylation of RUNX3, promoter methylation status of RUNX3 after different treatments was examined using MSP method. The results (Fig. 2) showed that RUNX3 was strongly methylated in B16F10 cells, whereas there were also weakly unmethylated bands which indicate incomplete methylation. After exposure to incremental concentrations of RES for 48 h, promoter methylation of RUNX3 was gradually weakened, unmethylated strips generally stronger. These results effectively manifested that the promoter region of RUNX3 is hypermethylated in B16F10 cells and RES can induce considerable demethylation of RUNX3 promoter in a dose-dependent manner.

2.3. RES can elevate RUNX3 mRNA and protein expression in B16F10 cells

We further detected the expressional levels of RUNX3 with TaqMan probe real-time PCR and western blotting. The expression levels of

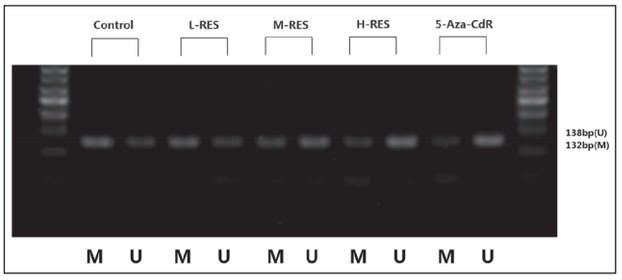

Fig. 2: MSP analysis of RUNX3 promoter in B16F10 cells after treatment of RES or 5-Aza-CdR. Cells were exposed to 5-Aza-CdR (10μM) or RES (20μM, 40μM, 80μM) for 48 h, and were then harvested and analyzed by MSP. L-RES was short of low-dose of RES, and M-RES, H-RES were like this. M, methylated; U, unmethylated.

RUNX3 mRNA (Fig. 3A) and protein (Fig. 3B and Fig. 3C) were extremely low in B16F10 cells. Compared with control group, 10 μM 5-Aza-CdR can significantly elevate RUNX3 mRNA and protein expression, which is 3.06 fold ($P < 0.01$) and 4.8 fold ($P < 0.01$), respectively. Meanwhile, 40 μM and 80 μM of RES can also enhance RUNX3 expression, which comes to 1.81 fold ($P < 0.05$) and 2.09 fold ($P < 0.05$) in mRNA, 2.57 fold ($P < 0.05$) and 3.32 fold ($P < 0.05$) in protein. Nevertheless, 20 μM RES did not show any significant escalation. The reason why low concentration RES has little effect may attributed to that the concentration is too low or the treatment time is not long enough. Maybe a longer time will show more significant results. Altogether, RES can elevate the expression of RUNX3 mRNA and protein in a dose-dependent manner.

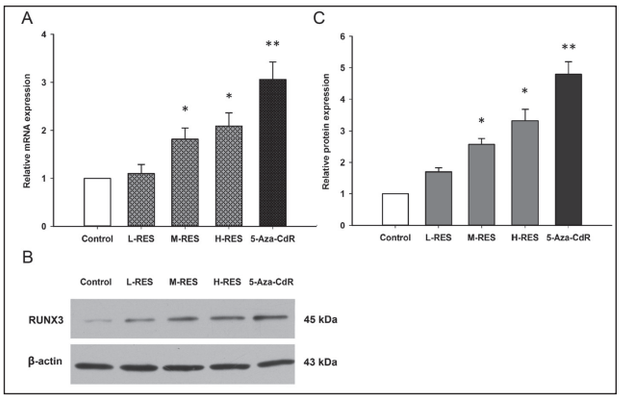

Fig. 3: Effects of RES on RUNX3 expression in B16F10 cells. Cells were exposed to 5-Aza-CdR (10μM) or RES (20μM, 40μM, 80μM) for 48 h, and were then harvested and analyzed by real-time PCR (A) and Western Blotting (B). Values in graph C indicated the relative density of the bands. Results were shown as relative expression ratio of RUNX3 in B16F10 cells. Data were the average results of three independent experiments. * ($P<0.05$) and ** ($P<0.01$) denoted statistically significant difference compared to control.

2.4. Inhibitory effects of RES on tumor growth of xenograft mice

To further identify the anti-tumor effect of RES on melanoma, xenograft models were established. After 21 days of continuous i.g administration, we found that both DTIC (positive drug) and RES could inhibit tumor growth of xenograft significantly. Compared with control group, the increment speed of tumor volume was greatly inhibited in the DTIC group (Fig. 4A and Fig. 4C). RES administration could reduce tumor size and weight significantly, and the high dose group was more obvious than the low dose group. As shown in Fig. 4B and Table 2, DTIC administration could reduce tumor weight obviously, which was 0.27 fold ($P <0.01$) that of control group with an inhibition rate of 72.66 %. While in the low and high doses of RES group, weight of tumor tissues were 0.83 fold ($P <0.05$) and 0.59 fold ($P <0.05$) that of the control group with the inhibition rate of 17.47 % and 40.98 %, respectively. Our results effectively demonstrated that RES can inhibit tumor growth of melanoma.

Table 1: PCR primers used for MSP and RT-PCR

Primer set	forward	reverse	An. Temp	Frg.Size, bp
RUNX3 MSP-M	CGTCGGGTTAGCGAG GTTTC	GCCGCTACCGCGAAA AACGA	62	132
RUNX3 MSP-U	GTGGGTGGTTGTTGG GTTAGT	TCCTCAACCACCACTA CCACA	59	138
RUNX3	TCAGCACCACGAGCC ACTT	AAGCGGCTCTCTGTGA GGC	60	133
TaqMan probe	FAM-CAGACCTGAACCCCTTCTCCGACCC-TAMRAA		–	–

MSP, methylation-specific polymerase chain reaction; MSP-M, methylated-specific primers; MSP-U, unmethylated-specific primers; Frg. Size, fragment size; An. Temp, annealing temperature (°C). Template sequences are referenced to NCBI (RefSeq ID NM_019732.2).

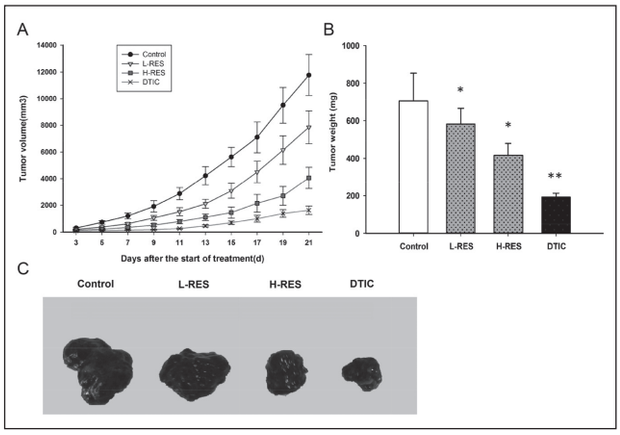

Fig. 4: Effects of RES on tumor growth of C57BL mice xenografts. Low and high-dose of RES could inhibit tumor volume (A) and tumor weight (B) obviously compared with the control group. Represent tumors of four groups were listed in graph C. Significance was analyzed with a one-way analysis of variance (ANOVA). * (P<0.05) and ** (P<0.01) denoted statistically significant difference compared to control.

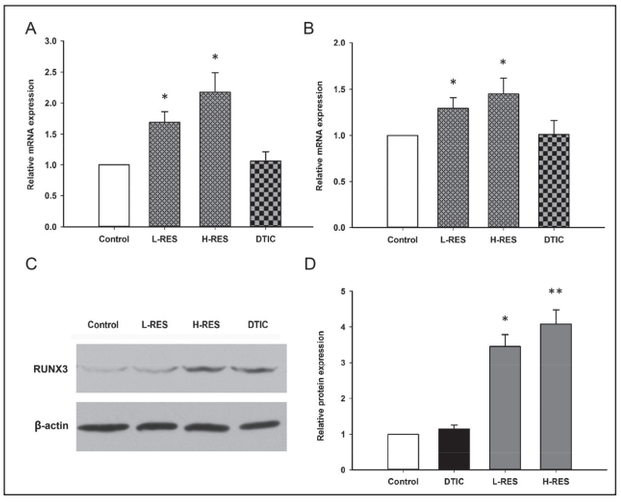

Fig. 5: Effects of RES on RUNX3 expression in C57BL mice xenografts. RUNX3 mRNA levels in peripheral blood (A) and tumor tissues (B) of xenograft mice were performed by real-time PCR. Western Blotting results of RUNX3 protein expression in tumor tissues(C) and Values indicated the relative density of the bands(D). Results were shown as relative expression ratio of RUNX3. * (P<0.05) and ** (P<0.01) denoted statistically significant difference compared to control.

2.5. Effects of RES on RUNX3 expression in vivo

Previous studies suggested that RES has strong anti-tumor effects in melanoma both *in vitro* and *in vivo*, but whether this effect is associated with expression of RUNX3 *in vivo* is unknown. We further detected RUNX3 expression both in peripheral blood and tumor tissues of xenograft mice. Compared with control group, low and high doses of RES could upregulate RUNX3 mRNA expression by 1.69-fold (P <0.05) and 2.18-fold (P <0.05) in peripheral blood (Fig. 5A), 1.29 fold (P<0.05) and 1.45 fold (P<0.05) in tumor tissues(Fig. 5B). However, DTIC had no significant effect on RUNX3 expression either in peripheral blood or in tumor tissues. Since the peripheral blood sample was not enough to identify protein expression, we only detected RUNX3 protein expression in tumor tissues. As shown in Fig 5C and Fig 5D, RUNX3 expression was quite low in control group while DTIC had little effect on RUNX3 protein expression either. Moreover, low and high doses of RES could elevate RUNX3 expression in tumor tissues to 3.46-fold (P <0.05) and 4.08-fold (P<0.01) respectively. In conclusion, RES can significantly upregulate RUNX3 expression in peripheral blood and tumor tissues. Taken together, RES can demethylate RUNX3 promoter, thus led to the promotion of RUNX3 mRNA and protein expression both *in vitro* and *in vivo*.

3. Discussion

According to modern oncology theory, cancer pathogenesis mainly exists in two aspects: genetic mechanisms and epigenetic mechanisms. In the occurrence and metastasis of melanoma, epigenetics play a more important role than gene mutations (Muthusamy et al. 2006). In contrast to changes in genetic factors, epigenetic changes can be reversed or repaired by drugs, it also lays a new theoretical foundation for the treatment of melanoma, provides new ideas and methods (Chu et al. 2013).

Table 2: Average tumor weight and inhibition rate of C57BL mice xenografts

Groups	Tumor weight (mg)	Inhibition rate (%)
Control	705.55±149.08	–
Positive	192.92±20.74	72.66
L-RES	582.31±83.24	17.47
H-RES	416.43±63.91	40.98

Data are presented as mean±SD (n=12). Inhibition rate was calculated according to the following formula: Inhibition rate (%) = (tumor weight of the control group - tumor weight of the experimental group) / tumor weight of the control group × 100% .

Human RUNT related transcription factor 3 (RUNX3), as a newly concerned tumor suppressor gene, was originally found by Levanon et al. in 1994, located on human chromosome l short arm lp36.1 (Bangsow et al. 2001). A large number of studies showed that RUNX3 is closely related to the development of gastric, bladder, colon, breast, lung, pancreatic carcinomas and other tumors (Li et al. 2002; Kim et al. 2005; Ku et al. 2004; Subramaniam et al. 2009; Li et al. 2004; Wada et al. 2004). Large-scale clinical case analysis has shown that RUNX3 expression in melanoma patients decreased, and its expression level was negatively correlated with tumor stage and 5-year survival rate, suggesting that RUNX3 might play a pivotal role in the development of malignant melanoma (Chuang and Ito 2010). Meanwhile, many studies have confirmed aberrant methylation of a variety of genes in melanoma, such as RSSSF1A , PTEN, and HLA-I (Spugnardi et al. 2003; Stahl et al. 2003; Coral et al. 1999). Our findings indicated that RUNX3 promotor regions was aberrantly hypermethylated in melanoma

B16F10 cells, and the levels of mRNA and protein expression *in vitro* and *in vivo* were quite low. This effectively suggested that the methylation of RUNX3 promoter was the major cause of reduced expression of RUNX3 in melanoma cells, which also confirmed the role of RUNX3 as a tumor suppressor gene in melanoma.

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a kind of natural non-flavonoid polyphenolic compound, which is mainly found in plants (Jang et al. 1997). It is also a potential anti-tumor drug with complex mechanism and demethylation is one of its anti-tumor mechanisms. Some studies suggested that RES has a demethylating effect on the methylation status of Ras related domain family 1A (RASSF1A) gene in gastric cancer SGC-7901 cells (Qin et al. 2005). RES could induce mitogen-activated protein kinase (MAP) and melanoma cell A537 apoptosis, upregulate quinone reductase 2 (NQO2) and p53 to inhibit melanoma cell proliferation , downregulate vascular endothelial growth factor growth factor (VEGF) and upregulate thrombospondin-1 (TSP1) antiangiogenesis to inhibit its metastasis (Niles et al. 2003; Hsieh et al. 2005; Trapp et al. 2010). RES could also upregulate p53 to induce doxorubicin (DOX) resistant melanoma cells cell cycle arrest and elevate survivin protein to increase the sensitivity of malignant melanoma to radiotherapy (Gatouillat et al. 2010; Johnson et al. 2008). Our results show that RES could also demethylate the RUNX3 promoter to varying degrees depending on the concentration, and increase RUNX3 mRNA and protein expression. Taken together, we believe that intervention of RES can have a demethylation effect on the RUNX3 promoter.

This study investigated the effect of different concentrations of RES on B16F10 melanoma cells and found that RES could inhibit the proliferation of B16F10 cells in a dose-dependent manner. Meanwhile, RUNX3 gene promotor was almost completely methylated, and rarely expressed RUNX3 mRNA and protein in the melanoma cells; after intervention with RES, the promoter methylation of RUNX3 gene was decreased and the expression of mRNA and protein was higher than that of the control group in a dose-dependent manner. Overall, our results implied that the high methylation status of the RUNX3 gene promoter in melanoma B16F10 cells inhibits the transcription of the RUNX3 gene, which in turn affects the expression of its protein and affects a series of anti-tumor mechanisms associated with RUNX3. RES could demethylate the RUNX3 gene promoter in melanoma B16F10 cells in a dose-dependent manner so that the RUNX3 gene, which was originally in a hypermethylation state, regains its transcription and protein expression of the RUNX3 gene, so as to restore its function as tumor suppressor gene, and play its role in inhibiting cell proliferation.

In summary, our findings indicate that the demethylation of the RUNX3 promoter and the promotion of its mRNA and protein expression might be one of the mechanisms by which RES inhibits melanoma. However, Niles' research showed that RES is unlikely to help inhibiting tumor growth (Niles et al. 2003), which we believe may be due to differences in route of administration and dosage. We did not check the effect of RES on normal cells in this experiment. However, according to other studies, human melanoma cells and normal human fibroblasts have a relative resistance to RES-induced apoptosis and cell cycle arrest at 25-100 μM dose of RES (Ivanov et al. 2008).

This study explores the new mechanism of RES inhibiting melanoma from the perspective of epigenetics, provides a new experimental basis for the study of RES anti-tumor mechanism, and also provides a new direction for the improvement of melanoma therapy. However, we still do not know what the specific mechanism is and the effects of RES on other genes or even oncogenes are, so further studies are needed.

4. Experimental

4.1. Chemicals and reagents

RES (purity>99%) and 5-Aza-CdR were purchased from Sigma Aldrich (St. Louis, Mo, USA). RES and 5-Aza-CdR were dissolved in DMSO for 50 mM and 10 mM respectively, stored at -20 °C. Dacarbazine (DTIC) was from Nanjing Pharmaceutical Co. Ltd. DMEM medium and fetal bovine serum (FBS) were from Gibco (Pittsburgh, PA). The trizol reagent and RNA extraction kit were obtained from Invitrogen. First-strand cDNA synthesis kit was purchased from Fermentas. The RUNX3 antibody and β-actin antibody were from Abcam and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The secondary antibody was from Wuhan Google Biotechnology Co. Ltd. Cell genomic DNA extraction kit was from TIANGEN BIOTECH (Beijing) Co. Ltd. EZ DNA Methylation Gold Kit was purchased from Zymo Research Co. Ltd. Agarose agarose G-10 was from Biowest. All the other reagents used in this research were analytical pure.

4.2. Cell culture and animals

Murine B16F10 melanoma cells were kindly provided by Union Hospital Cancer Center, Huazhong University of Science and Technology. Cells were cultured in DMEM high glucose medium supplemented with 10 % fetal bovine serum and 1 % antibiotics at 37 °C in a humid atmosphere with 5 % CO_2. Experimental animals of C57BL mice (n=48, half male and female) were purchased from The Animal Center of Wuhan University, 6~8 weeks old, weighting 20±4 g. All the mice were housed at 24±2 °C, illuminated daily for 12 h and fed with regular diet and sterile water. Four weeks after the experiment, the mice were sacrificed by cervical dislocation. This study has been performed with approval of the local ethical committee and all the operations were performed according to the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

4.3. Cell morphology

Cells in the logarithmic growth phase were inoculated into 10×10cm dish at a density of 1×10^5 per dish and randomly divided into five groups. Culture medium was replaced by DMEM medium containing RES with concentrations of 20 μM, 40 μM, and 80 μM after 24 h. Medium containing 5 ‰ DMSO was added into the control group, and the positive group was added with medium containing 10 μM 5-Aza-CdR. After 48 h incubation, cell morphology of B16F10 cells was observed and photographed under an inverted microscope.

4.4. Methylation-specific PCR (MSP)

Groups and administration were identical with 2.3. Total cellular DNA was extracted with cellular genomic DNA extraction kit after 48 h incubation. Bisulfite modification of template DNA was operated following the instructions of the EZ DNA Methylation Gold Kit. The referenced primer sequences are listed in Table 1, synthesized by Invitrogen Biotechnology Co. Ltd. Then this template was processed for PCR amplification. The reaction system was 20 μL and reaction cycles for methylated fragment were as follows: 95 °C for 10 min, 94 °C 20 s, 62 °C 20 s, 72 °C 15 s for 35 cycles, 72 °C 10 min for the extension. The cycles for unmethylated were the same except for 59 °C 20 s. Finally, 5 μl PCR product was added to 1.7 % agarose gel for electrophoresis and then observed and photographed under an UVP gel imaging analysis system. The length of methylated fragment is 132bp while 138bp for unmethylated.

4.5. Real-time quantitative PCR (RT-PCR)

Total RNA was extracted using Trizol reagent after administration and incubated for 48 h. Purity of RNA samples was determined on a ND-1000 spectrophotometer and then 2 μg RNA was used for reverse transcription. The sequences of the primers and probes were in the list of Table 1, which were synthesized and labeled by Shanghai Gene Core Bio Technologies Co. Ltd. Reaction of the real-time quantitative PCR was conducted in an ABI7900HT system. Then Ct values of the samples were analyzed automatically with supporting software and the relative quantity of RUNX3 mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method. The reaction was measured with at least three or more parallel samples.

4.6. Western blotting

Cells were washed and lysed in RIPA buffer containing 1 % PMSF. Cellular protein concentration was determined using BCA assay. Identical amounts of protein were then separated on a 10 % SDS-PAGE gel. Subsequently, protein bands were transferred onto a 0.22 μm PVDF membrane, which was blocked with TBST containing 5 % skim milk, incubated with primary antibody at 4 °C overnight, followed by HRP-conjugated secondary antibodies. Protein bands were visualized by an ECL kit. Quantification was performed using the Gel-Pro image analysis software.

4.7. Establishment of xenograft model and treatment

To further investigate the effects of RES on xenograft model, about 2×10^6 B16F10 cells were subcutaneously inoculated into each mouse. Three days later, the mice could grow tumors gradually and were then randomly divided into four groups. The control group received 10 % CMC-Na solution 50 mg/kg/d, while the positive group was administered with DTIC 40 mg/kg/3d, RES was given 50 mg/kg/d and 100 mg/kg/d, respectively. Tumors were measured with a caliper every four days. On the 21th day, approximately 250 μl blood was collected from the orbital vein plexus and placed in EP tubes without RNase but heparin. After blood collection, the mice were euthanized, isolating and weighing the tumors immediately. Total protein and RNA were extracted and processed for QPCR and Western blotting.

4.8. Statistical analyses

All the results were presented as mean±S.D. Statistical analyses were performed using SPSS19.0 software. Comparisons among groups were determined by two-tailed Student's t-test. $P<0.05$ was considered statistically significant. All the experiments were carried out in triplicate.

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Conflict of interest: The authors have no conflicts of interest to disclose

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