Combined BRM270 and endostatin inhibit relapse of NSCLC while suppressing lung cancer stem cell proliferation induced by endostatin

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Endostatin (ES, ENDO) has been reported to suppress the growth of tumors while inducing the proliferation of lung cancer stem cells (LCSCs), causing a poor prognosis for lung cancer. In this study, we aimed to clarify whether BRM270 can inhibit the proliferation of cancer stem cells (CSCs). Endostatin + BRM270 showed anti-tumor effects by reducing tumor volume and increasing survival. Administration of BRM270 reduced the number of aldehyde dehydrogenase-positive (ALDH+) cells and the level of ALDH1A1 expression in tumors by increasing the level of miR-128 while decreasing the levels of BMI-1, ABCC-5, E2F3, and c-MET. The luciferase activity of miR-128 promoter was increased by an increasing concentration of BRM270. In addition, BMI-1, ABCC-5, E2F3, and c-MET were identified as candidate targets of miR-128, and the overexpression of miR-128 significantly reduced mRNA/protein levels of BMI-1, ABCC-5, E2F3, and c-MET in A549 and H460 cells. Administration of BRM270 inhibited the expression of BMI-1, ABCC-5, E2F3, and c-MET in a dose-dependent manner. In this study, we showed for the first time that the combined administration of endostatin and BRM270 achieved anti-tumor effects while suppressing the proliferation of stem cells.

INTRODUCTION
Lung cancer is a malignant cancer which causes 1.3 million deaths per year among adults worldwide.1 Non-small cell lung cancer (NSCLC) accounts for greater than 87% of lung cancer cases. 46% of cases at presentation are at stage IIIB and IV NSCLC, and both stages result in a poor prognosis.2 Standard therapy includes first-line chemotherapy based on platinum.3 It has been reported that endostatin (ES, ENDO) could inhibit the proliferation, migration, and invasion of endothelial cells and tube formation. Though there was not any association between endostatin-based monotherapy and clinical efficacy according to phase II clinical trials, the combination of platinum-based chemotherapy and endostatin was reported to significantly increase overall survival (9.77–13.75 months) and progression-free survival (PFS) (3.59–6.28 months) in a phase III trial involving advanced NSCLC patients.4 The State Food and Drug Administration of China has approved endostatin for treating advanced NSCLC, and, in Europe and the United States, there is a plan for a randomized clinical trial.5 Endostatin treatment was found to inhibit new vasculature formation, lower VEGF levels, and enhance the expression of HIF-1α in tumors, thus worsening hypoxia in the microenvironment of tumors.6 Moreover, endostatin was also reported to exhibit proliferation-promoting effects in vivo, which increased the aldehyde dehydrogenase-positive (ALDH+) cells and cancer stem-like cells (CSCls) population by accelerating the generation of tumor hypoxia and by modulating the secretion of cytokines and growth factors such as IL-6, EGF, and TGF-β into the tumor microenvironment. Therefore, these results collectively implied that endostatin is effective in the treatment of the relapse of cell lung cancer by inducing the production of LCSCs.

BRM270 is a formulated extract of seven plants (Arnebia euchroma, Aloe vera, Citrus unshiu Markovich, Portulaca vulgaris var. lilacina, P. oleracea, Saururus chinensis, and Scutellaria baicalensis), used in traditional medicine in Asia, which inhibits many types of cancer cells from proliferating.7 BRM270 has been reported to suppress tumorigenesis through the negative regulation of the expression of nuclear factor (NF)-κB signaling in cancer stem cells (CSCs) that were multidrug resistant.8 It is reported that BRM270 treatment inhibited the growth, migration, and invasion of normal lung adenocarcinoma cells and chemoresistant ones in humans. Notably, it was found that BRM270 modulated the self-renewal and the tumor-initiating capacity of CSCs via the upregulation of the microRNA (miRNA)-128 in A549 cell lines, which indicated the potentially effective therapy method of combining miRNA-128 and BRM270 in the treatment of chemoresistant NSCLC.8 miRNAs are short RNA molecules, each with 20 to 22 nucleotides, that repress the expression of the target protein through being paired

Received 26 May 2020; accepted 26 May 2021; https://doi.org/10.1016/j.omto.2021.05.011.
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with sites that are partially complementary in the 3' untranslated region (UTR) of mRNA. Many biological mechanisms of cancer cells are reported to be regulated by miRNAs. For example, miR-128 was reported to specifically block glioma self-renewal consistent with BMI-1 reduction, which led to a decrease in H3K27 methylation and modulation of cellular pathways involved in cell cycle exit and cell survival. Accordingly, downregulated miR-128 contributes to chemotherapeutic resistance in breast cancers by increasing the BMI-1 level. And miR128-1 was reported to function as a tumor suppressor in glioblastoma (GBM) by negatively regulating tumor cell proliferation, invasion, and self-renewal through direct targeting of BMI-1 and E2F3. Also, the miR-128/c-Met pathway was reported to enhance the gefitinib sensitivity of the LCSCs by suppressing the PI3K/AKT pathway.

Therefore, despite the fact that endostatin treatment has been reported to suppress the growth of tumors while inducing the proliferation of LCSCs, the administration of BRM270 may upregulate the expression of miR-128 and inhibit the proliferation of CSCs. In this study, we hypothesized that co-administration of BRM270 with endostatin may suppress the LCSC-inducing effect of endostatin to inhibit the potential relapse of lung cancer. To test the hypothesis, we used endostatin to treat cultured lung cancer cells with or without BRM270 and studied the inhibitory effect of BRM270 on the LCSC-inducing phenomena of endostatin as well as its effect on the expression of miR-128, c-MET, ABCC-5, BMI-1, and E2F3.

RESULTS

In vivo anti-tumor efficacy of endostatin and BRM270
To explore the in vivo anti-tumor efficacy of endostatin and BRM270, tumor volume and survival among the control, endostatin, and endostatin + BRM270 groups were determined. As shown in Figure 1, a significant inhibition of tumor growth was observed in both the endostatin treatment and endostatin + BRM270 groups, while the addition of BRM270 significantly increased the anti-tumor efficacy of endostatin (Figure 1A). In addition, the endostatin group had a higher survival compared with the control group, while the presence of BRM270 significantly increased the pro-survival attribute of endostatin (Figure 1B).

BRM270 treatment decreased the number of ALDH+ cells and the level of ALDH1A1 expression in tumors
The activity of ALDH was implicated in LCSCs, a type of cells significantly affecting the prognosis of lung cancer. In this study, an ALDEFLUOR assay was performed to detect the activity of ALDH in tumors. The results showed that the endostatin treatment significantly increased the number of ALDH+ cells, while the presence of BRM270 in the endostatin + BRM270 group reduced the number of ALDH+ cells to a certain extent (Figure 1C). Meanwhile, immunohistochemistry (IHC) assays were used to compare the protein levels of ALDH1A1 among control, endostatin, and endostatin + BRM270 groups. As shown in Figure 1D, the level of ALDH1A1 protein was the highest in the endostatin group and the lowest in the control group.

miR-128, BMI-1, ABCC-5, E2F3, and c-MET were differentially expressed in different groups
Real-time PCR and western blot analysis were carried out to compare the expression of miR-128, BMI-1, ABCC-5, E2F3, and c-MET among control, endostatin, and endostatin + BRM270 groups. As shown in Figure 1E, miR-128 expression was evidently decreased in the endostatin group compared with that in the control group, while the presence of BRM270 increased the level of miR-128 in the endostatin + BRM270 group to a certain extent. By contrast, mRNA (Figures 1F–11) and protein (Figure 1J) levels of BMI-1 (Figures 1F and 11), ABCC-5 (Figures 1G and 11), E2F3 (Figures 1H and 11), and c-MET (Figures 1I and 11) were the highest in the endostatin group and the lowest in the control group.

miR-128 directly targeted BMI-1, ABCC-5, E2F3, and c-MET
Luciferase assays were used to clarify the role of BRM270 and miR-128 in the prognosis of lung cancer. As shown in Figure 2A, the BRM270 treatment increased the luciferase activity of miR-128 promoter in a dose-dependent manner in A549 and H460 cells. Meanwhile, the luciferase activity of luciferase reporter containing the wide-type 3' UTR of BMI-1 (Figure 2B), ABCC-5 (Figure 2C), E2F3 (Figure 2D), and c-MET (Figure 2E) was significantly reduced in A549 and H460 cells co-transfected with miR-128 mimics, whereas the luciferase activity of luciferase reporter containing the mutant 3' UTR constructs was not affected by miR-214 overexpression.

Effect of miR-128 and BRM270 on the expression of BMI-1, ABCC-5, E2F3, and c-MET
Real-time PCR and western blot analysis were used to detect the levels of BMI-1, ABCC-5, E2F3, and c-MET in A549 and H460 cells transfected with miR-128 mimics along with various doses of BRM270 (0.5 μM and 2 μM). As shown in Figure 3, the mRNA (Figures 3A–3D) and protein (Figure 3E) levels of BMI-1 (Figures 3A and 3E), ABCC-5 (Figures 3B and 3E), E2F3 (Figures 3C and 3E), and c-MET (Figures 3D and 3E) in A549 and H460 cells were significantly reduced by transfection with miR-128 mimics. Also, the mRNA (Figures 3F–3I) and protein (Figure 3J) levels of BMI-1 (Figures 3F and 3J), ABCC-5 (Figures 3G and 3J), E2F3 (Figures 3H and 3J), and c-MET (Figures 3I and 3J) in A549 and H460 cells showed a stepwise decline over an increasing concentration of BRM270.

DISCUSSION

In this study, we investigated the effect of endostatin and BRM270 on tumor growth, and our results indicated that both endostatin and BRM270 exerted anti-tumor effects. Meanwhile, we performed a flow cytometry analysis to analyze the effect of BRM270 on the proliferation of CSCs and the expression of ALDH1A1. The results showed that treatment with BRM270 reduced the number of ALDH+ cells and the level of ALDH1A1 in tumors. In 1997, the model of CSCs was proposed when stem cells were discovered in leukemia, and Giangreco et al. reported evidence of the existence of CSCs. CSCs are also known as stem-like cancer cells and tumor-initiating cells, which belong to a subpopulation of tumor cells that have shown immortal production and self-renewal and formed
tumors in xenograft mouse models. It has been shown that CSCs were involved in the growth, metastasis, and relapse of tumor. Endostatin has been shown to increase the formation of CSCs, which is one of the reasons for the resistance to endostatin revealed by the failure in the repression of the tumor volume after 12 days of continuous endostatin treatment. More specifically, endostatin worsens hypoxia in tissues of tumor and improves the secretion of TGF-β1 through activating Notch1, β-catenin, Smad2, and Smad3. TGF-β1 and hypoxia increase the induction of CSC.

BRM270 is a formulated extract from seven medicinal plants that has been reported to contain compounds targeting the signaling pathway of NF-κB and inducing cell cycle arrest and apoptosis, according to the evidence that the expression of the CSC marker CD133 was lowered. Furthermore, it was found that BRM270 modulated epithelial-mesenchymal transition (EMT), the self-renewal of CSCs, and the expression of genes related to stemness in all examined cell lines. The overexpression of miR-128 induced by BRM270 has been reported to decrease the expression levels of

Figure 1. Anti-tumor efficacy of endostatin + BRM270 was much stronger than that of endostatin in vivo
BRM 270 reduced ALDH+ cells in tumors and also regulated expression of miR-128, BMI-1, ABCC-5, E2F3, and c-MET among different cell groups (*p value < 0.05 compared with control group; **p value < 0.05 compared with endostatin group). (A) Tumor volume was the lowest in the endostatin + BRM270 group and the highest in the control group. (B) Survival was the highest in the endostatin + BRM270 group and the lowest in the control group. (C) Endostatin treatment increased the number of ALDH+ cells in tumors, but BRM270 reduced the number of ALDH+ cells in tumors. (D) Treatment with BRM270 downregulated the protein expression of ALDH1A1 in tumors. (E) Endostatin and endostatin + BRM270 treatments repressed miR-128 expression, while the inhibitory effect of endostatin was much stronger than that of endostatin + BRM270. (F) Endostatin and endostatin + BRM270 treatments enhanced the mRNA expression of BMI-1, while the promotion effect of endostatin was much stronger than that of endostatin + BRM270. (G) ABCC-5 mRNA expression was the highest in the endostatin group and the lowest in the control group. (H) E2F3 mRNA expression was the highest in the endostatin group and the lowest in the control group. (I) c-MET mRNA expression was the highest in the endostatin group and the lowest in the control group. (J) Protein levels of BMI-1, ABCC-5, E2F3, and c-MET were the highest in the endostatin group and the lowest in the control group.
A

B

C

D

E

(legend on next page)
Musashi-1 and Bmi-1 in A549 cell lines. It has been shown that miR-128 abolished chemoresistance through suppressing PI3K/AKT signaling. \(^{15}\) miR-128 at high levels inhibited the angiogenesis and progression of tumor-blocked AKT, ERK, and p38 signaling pathways. \(^{20}\) The present study resulted in the conclusion that BRM270-induced miR-128 suppresses the activating effect of VEGFR on ERK/p38/AKT signaling, according to the evidence that the phosphorylation of AKT, p38, and ERK was decreased. In this study, we carried out real-time PCR and western blot analysis to determine the effect of endostatin and BRM270 on the expression of miR-128, BMI-1, ABCC-5, E2F3, and c-MET. Our results showed that BRM270 increased the level of miR-128 while decreasing the levels of BMI-1, ABCC-5, E2F3, and c-MET in the presence of endostatin. Furthermore, we investigated the effect of BRM270 on the expression of miR-128 by improving the translational efficiency of miR-128 promoter, and our results showed that BRM270 treatment dose-dependently increased the luciferase activity of miR-128 promoter. In addition, we identified BMI-1, ABCC-5, E2F3, and c-MET as target genes of miR-128.

In lung cancer, the expression of miR-128 is frequently decreased. \(^{20}\) However, the expression product has also been reported to be a suppressor of tumor in several malignancies through suppressing the proliferation, migration, and invasion of cells. \(^{21}\) It has been reported that miR-128 had effects on glioma cells; for example, it decreased the expression of Bmi-1, resulting in the decrease in the self-renewal of glioma stem cells. Cell proliferation is inhibited by miR-128 through targeting Bmi-1 in order to inhibit the motility of neuroblastoma cells. \(^{12}\) miR-128 has been reported to be directly paired with the 3’ UTR in Bmi-1, which is consistent with a recent study. \(^{12}\) Furthermore, a Bmi-1 \(p\) subpopulation was isolated from malignant pleural mesothelioma and displayed properties similar to those of prominent stem cells and resistance to pemetrexed and cisplatin. \(^{22}\) miR-128 was also reported to play a significant role in regulating the differentiation of prostate cancer cells through suppressing BMI-1, which affects epigenetic gene silencing and the renewal of stem cells. \(^{23}\) miR-128 inhibits CSCs through targeting BMI-1 and other critical molecules. BMI-1 also plays an indispensable role in regulating the self-renewal of NSCLC. In addition, BMI-1 is involved in tumor growth and is required for CSC renewal and differentiation. \(^{24}\) Overexpression of miR-128 significantly blocked CSC self-renewal by directly targeting BMI-1. \(^{12}\) Decrease in miR-128 resulting in the overexpression of Bmi-1 and ABCC-5 is a feature of breast tumor-initiating cells (BT-ICs) similar to that of stem cells. In BT-ICs, the ectopic expression of miR-128 decreased the expression of both ABCC-5 and Bmi-1, while making BT-ICs sensitive to doxorubicin. The present study also indicated that the decreased miR-128 level in invasive breast tumor tissues was associated with poor curative effects of chemotherapy and the poor survival of patients. Therefore, the decrease in miR-128 in BT-ICs is responsible for their resistance to chemotherapy through alleviating its inhibition of the translation of ABCC-5 and Bmi-1. E2F3 is a transcription factor that regulates cell cycle progression and has also been reported to be directly targeted by miR128-1. Accordingly, the overexpression of miR128-1 caused the downregulation of both E2F3 and BMI-1 in glioblastoma stem cells (GSCs) and glioma cells. These results showed that miR128-1 regulates the differentiation and self-renewal of cells by mechanisms, one of which is via the pairing with E2F3 and BMI-1. In LCSCs, the gene \(c\text{-}\text{met}\) has been reported to be overexpressed. The present study also confirmed that the sensitivity of the LCSCs to gefitinib was determined by the miR-128/\(c\text{-}\text{met}\) axis in vivo and in vitro, and \(c\text{-}\text{met}\) is a gene functionally targeted by miR-128 in PC9-CSCs. In summary, these results show that miR-128 can enhance the sensitivity to gefitinib in PC9-CSCs by the downregulation of \(c\text{-}\text{met}\). In this study, we utilized real-time PCR and western blot analysis to examine the effect of miR-128 and BRM270 on the expression of BMI-1, ABCC-5, E2F3, and c-MET. We found that miR-128 and BRM270 both inhibited the expression of BMI-1, ABCC-5, E2F3, and c-MET. There is increasing evidence to confirm that the overexpression of miR-128 can reverse the LCSC resistance to gefitinib by suppressing the \(c\text{-}\text{met}/\text{PI3K}/\text{AKT}\) pathway. Moreover, it was proven that the \(c\text{-}\text{met}\) gene was directly suppressed by miR-128.

However, the results obtained from this study are limited by the lack of validation upon human subjects. The results of the present study shed light on the prevention of relapse of lung cancer, which is associated with activation of LCSCs caused by the treatment with endostatin. Endostatin is a promising chemotherapeutic agent, although its side effects concerning LCSCs have been a concern. The co-administration of endostatin and BRM270 will further improve the inhibitory effect on the proliferation of lung cancer cells, and the side effects of endostatin on LCSCs could be effectively suppressed by BRM270. Furthermore, clinical trials with combination of the two agents are warranted.

In summary, the findings of this study demonstrated that endostatin treatment can suppress the growth of tumors while inducing the proliferation of LCSCs, which contribute to the relapse of lung cancer. In this study, we also combined the administration of \(v\) and BRM270 to achieve an anti-tumor activity while suppressing the promotional effect of endostatin on stem cell proliferation.

**Figure 2. miR-128 directly regulated the expression of BMI-1, ABCC-5, E2F3, and c-MET**

(A) BRM270 apparently and dose-dependently upregulated the luciferase activity of miR-128 promoter (*p value < 0.05 compared with untreated group). (B) miR-128 mimics inhibited the luciferase activity of wild-type BMI-1 3’ UTR but not that of mutant BMI-1 3’ UTR (*p value < 0.05 compared with NC group). (C) Luciferase activity of wild-type ABCC-5 3’ UTR but not mutant ABCC-5 3’ UTR was repressed by miR-128 mimics (*p value < 0.05 compared with NC group). (D) Luciferase activity of wild-type E2F3 3’ UTR but not mutant E2F3 3’ UTR was repressed by miR-128 mimics (*p value < 0.05 compared with NC group). (E) Luciferase activity of wild-type c-MET 3’ UTR but not mutant c-MET 3’ UTR was repressed by miR-128 mimics (*p value < 0.05 compared with NC group).
MATERIALS AND METHODS

Animals and treatment

Operating procedures used in all the mouse experiments were standard ones approved by the Animal Ethics Committee of the university. Wild-type C57BL/6 mice of 5–7 weeks were obtained from the institutional animal center. The injection with $3 \times 10^{5}$ Lewis lung carcinoma (LLC) cells was conducted subcutaneously in the right hindquarters of each mouse. When average volumes of tumors were

Figure 3. Transfection with miR-128 mimics or BRM270 reduced expression of BMI-1, ABCC-5, E2F3, and c-MET in A549 and H460 cells (*p value < 0.05 compared with NC group)

(A) Transfection of miR-128 mimics reduced the mRNA level of BMI-1 in A549 and H460 cells. (B) Transfection of miR-128 mimics reduced the mRNA level of ABCC-5 in A549 and H460 cells. (C) Transfection of miR-128 mimics reduced the mRNA level of E2F3 in A549 and H460 cells. (D) Transfection of miR-128 mimics reduced the mRNA level of c-MET in A549 and H460 cells. (E) Administration of BRM270 inhibited the mRNA expression of BMI-1 in a dose-dependent manner in A549 and H460 cells. (F) Administration of BRM270 inhibited the mRNA expression of ABCC-5 in a dose-dependent manner in A549 and H460 cells. (G) Administration of BRM270 inhibited the mRNA expression of E2F3 in a dose-dependent manner in A549 and H460 cells. (H) Administration of BRM270 inhibited the mRNA expression of c-MET in a dose-dependent manner in A549 and H460 cells. (I) Administration of BRM270 inhibited the protein expression of BMI-1, ABCC-5, E2F3, and c-MET in a dose-dependent manner.
greater than 5 mm³, the mice were randomly divided into three groups (n = 15 in each group): group 1, normal saline (NS) was used in the control group; group 2, the endostatin group received recombinant human endostatin (Simcere Medgen Bio-Pharmaceutical, Yantai City, Shandong Province, China) at a dose of 20 mg/kg; group 3, the endostatin + BRM270 group received recombinant human endostatin at a dose of 20 mg/kg + BRM270 (5 mg/kg/day). Normal saline and the treatments were administered once daily by intraperitoneal (i.p.) injection accordingly, and tumor development was monitored for each of the mice every 3 days. According to the formula V = a × b²/247, the volume was calculated; in this formula, a and b represent the long and short diameters of the tumor, respectively.

RNA isolation and real-time PCR
A Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from cell and tissue samples following a standard protocol. Subsequently, chloroform was added into the lysate, and the mixture was centrifuged at 4°C and 13,000 × g for 15 min. In the next step, the pellet was treated by isopropanol and washed by ethanol before being resuspended in RNase-free water. The RNA concentration was determined by UV spectrophotometry. A reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was utilized to synthesize the cDNA of miR-128, BMI-1 mRNA, ABCC-5 mRNA, E2F3 mRNA, and c-MET mRNA from 2 μg of total RNA in each sample, and a housekeeping gene, 18S RNA, served as an internal control. The expression of miR-128, BMI-1 mRNA, ABCC-5 mRNA, E2F3 mRNA, and c-MET mRNA was measured using SYBR green dye and a light cycler (Roche Diagnostics, Indianapolis, IN, USA) according to the instruction of the manufacturer. The relative expression of miR-128 (forward: 5′-AACCTGGGTAGAGGG-3′; reverse: 5′-GAACATGTCCTGGTATCTC-3′), BMI-1 mRNA (forward: 5′-GGTACTTCTGGTGAGCAACC-3′; reverse: 5′-CTGGTCTTGTGAAC TTGGACATC-3′), ABCC-5 mRNA (forward: 5′-GGCTGTATTA CGGAAAGAGGCAC-3′; reverse: 5′-TCTTCTGTGAACTCCTTT CCC-3′), E2F3 mRNA (forward: 5′-AGGGTCACTGATACCTCT TCAG-3′; reverse: 5′-TGTGAGACGACCAAGAGAGC-3′), and c-MET mRNA (forward: 5′-TGACAGTGTGGCTTGGCCATGA-3′; reverse: 5′-CAGCCATAGGGACCTATTCCC-3′) was determined by calculating the cycle threshold (Ct) values, and U6 (forward: 5′-TACGATACAAGGCTGTTAGAGAG-3′; reverse: 5′-TACGATACAAGGCTGTTAGAGAG-3′) was used as an internal control in such calculations. Three independent reactions were run for each target miRNA.

Luciferase assay
To study the effect of BRM270 on the transcription efficiency of the miR-128 promoter, the full sequence of miR-128 promoter was amplified using PCR, and the PCR product was cloned into a pcDNA3.1 luciferase reporter vector (Promega, Madison, WI, USA) at a position upstream of the firefly luciferase reporter gene. Subsequently, A549 and H460 cells were divided into three groups: an untreated control group, a 0.5 μM BRM270 group, and a 2 μM BRM270 group. In the next step, the cells in the control group were treated with phosphate-buffered saline (PBS), while the cells in the other two groups were treated with corresponding concentrations of BRM270. At 48 h post transfection, the cells were harvested, and the luciferase activity of ABCC-5, E2F3, and c-MET in transfected A549 and H460 cells was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) following the manufacturer’s guide-line. Each assay was repeated three times.

Western blot analysis
Collected cell and tissue samples were washed twice using an ice-cold PBS solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) before being lysed by an RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% SDS, and protease inhibitors (Roche, Diagnostics, Indianapolis, IN, USA). Subsequently, the lysate was centrifuged for 15 min at 15,000 × g and 4°C, followed by boiling in 2-mercaptoethanol for 10 min. The samples were then separated using 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA), which was subsequently incubated at 37°C for 60 min in a Tris-bufffered saline solution containing 10% nonfat dry milk and 0.1% Tween 20 to eliminate nonspecific binding. In the next step, the membrane was incubated with the primary antibodies against BMI-1 (dilution 1:10,000; cat. no. ab126783; Abcam, Cambridge, MA, USA), ABCC-5 (dilution 1:50; cat. no. ab24107; Abcam, Cambridge, MA, USA), and β-actin (internal control; dilution 1:8,000; cat. no. ab8226; Abcam, Cambridge, MA, USA) for 12 h at 4°C, followed by 2 h of addition incubation at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:5,000; cat. no. 8.25 The cells in the NC group were treated with PBS, while the cells in the other two groups were treated with corresponding concentrations of BRM270. For miRNA transfection experiments, the cells were seeded into 24-well plates at a concentration of 1 × 10⁵ cell/well. When the cells reached 80% confluence, they were divided into two groups: a NC group and a miR-128 mimic group. Subsequently, the cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. The cells in the NC group were transfected with a negative control miRNA, while the cells in the miR-128 mimic group were transfected with 30 nM miR-128 mimics. At 48 h after cell transfection or treatment, the cells in various groups were harvested and used for subsequent analyses. Three independent experiments were carried out.
Detection of ALDH+ cells by flow cytometry
Following the instruction provided by the manufacturer, flow cytometry (BD Biosciences, San Jose, CA, USA) was utilized to detect ALDH expression of A549 and H460 cells using a standard staining kit (Thermo Fisher Scientific, Waltham, MA, USA) following the protocol of the kit.

Immunohistochemistry (IHC)
The collected tissue samples were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and cut into 4 μm sections. Immunohistochemical staining of the tissue sections was performed using a rabbit anti-mouse monoclonal antibody against ALDH1A1 (dilution 1:500; Abcam, Cambridge, MA, USA). Subsequently, the tissue sections were incubated with anti-rabbit IgG antibodies (dilution 1:1,000; Abcam, Cambridge, MA, USA) for 60 min at room temperature, followed by peroxidase DAB (3, 3-diaminobenzidine) staining in accordance with the manufacturer’s instructions. Finally, hematoxylin was used to counterstain DAB, and the sections were observed under a microscope.

Statistical analysis
All statistical analyses were carried out in GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA), and all results are shown as means ± SD. The difference between two groups was compared using Student’s t tests, while the difference among multiple groups was compared using one-way ANOVA. The postoperative prognostic analysis of the tissue sections was performed using a rabbit anti-mouse monoclonal antibody against ALDH1A1 (dilution 1:500; Abcam, Cambridge, MA, USA) for 60 min at room temperature, followed by peroxidase DAB (3, 3-diaminobenzidine) staining in accordance with the manufacturer’s instructions. Finally, hematoxylin was used to counterstain DAB, and the sections were observed under a microscope.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS
Y.H.G.: study plan, data collection, data interpretation, and manuscript preparation; Y.O.Y.: data collection and data analysis, and manuscript preparation; F.Q.W.: study director, study plan, and data interpretation. All authors have read and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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