Effect of inhibiting ACAT-1 expression on the growth and metastasis of Lewis lung carcinoma

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Abstract. Accumulating evidence suggests that acetyl-CoA acetyltransferase 1 (ACAT-1) may mediate tumor development and metastasis. However, the specific function served by ACAT-1 in lung cancer is not well understood. Therefore, the present study initially verified that ACAT-1 was overexpressed in Lewis lung carcinoma (LLC) tissues compared with non-LLC mice and that this overexpression promoted the proliferation, invasion and metastasis of these LLC samples. Western blotting, immunofluorescence microscopy and flow cytometry allowed the present study to determine that the ACAT-1 inhibitor avasimibe significantly reduced the expression of ACAT-1 in LLC compared with LLC cells that are not treated with avasimibe (P<0.05). A combination of Cell Counting Kit-8 and wound healing assays demonstrated that downregulating ACAT-1 expression sufficiently inhibited the proliferation of LLC cells. Avasimibe promoted LLC cell apoptosis as assessed by a Annexin V/propidium iodide double staining assay. Furthermore, avasimibe inhibited tumor growth in vivo and improved immune responses, with tissue biopsies from LLC model mice exhibiting higher levels of ACAT-1 compared with in healthy controls. Altogether, the results of the present study reveal that avasimibe may inhibit the progression of LLC by downregulating the expression of ACAT-1, which may thus be a potential novel therapeutic target for lung cancer treatment.

Introduction

Lung cancer remains the most common form of cancer, with the highest mortality rate globally, making it a major public health threat (1,2). Surgery, chemotherapy, radiotherapy and molecular targeted therapies are currently the primary treatment options for this disease (3). Whilst early lung cancer may be treated surgically, the treatment options for advanced metastatic lung cancer are limited. Although radiotherapy and chemotherapy postpone the progression of these advanced lung cancer types, the survival rate of patients is low, and patients are often unable to tolerate the side effects of these therapies. Molecular targeted therapies are only effective in patients with lung adenocarcinoma who have specific mutations in genes including epidermal growth factor receptor (EGFR) or ALK receptor tyrosine kinase (ALK) (4, 5). Patients who have wild-type EGFR or ALK and non-adenovirus-associated non-small cell lung cancer lack effective targeted therapies. Thus, there remains a clear need for the identification of novel therapeutics suitable for treating patients with advanced lung cancer.

The enzyme acetyl-CoA acetyltransferase 1 (ACAT-1) is a central mediator of the cholesterol esterification pathway (6). Previous studies have identified that ACAT-1 is abnormally expressed in certain cancer types, including prostate cancer, pancreatic cancer, leukemia, glioma, breast cancer and colon cancer (7-12). However, the functions of ACAT-1 in lung cancer are not well understood. In the present study, the effects of the inhibition of ACAT-1 on Lewis lung cancer (LLC) growth and metastasis were investigated using in vitro cellular experiments and in vivo animal models. The effect of downregulation of ACAT-1 expression on proliferation, migration and apoptosis of LLC cells was observed at the cell level in vitro. The effects of ACAT-1 inhibitor avasimibe on tumor growth and metastasis in LLC mice were observed in an animal model in vivo, and the expression of ACAT-1 in Lewis lung carcinoma tissues was detected to add new content for lung cancer research and provide new strategies for clinical lung cancer treatment.
Materials and methods

Ethics statement. The Research Ethics Committee of Bengbu Medical College (Bengbu, China) ethically approved this study.

Cell culture. The LLC cell line is a malignant murine lung cancer cell line which was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). LLC cells were grown in 25 cm² cell culture vessels containing Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 10% foetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) and penicillin/streptomycin in standard growth conditions (37°C). After culturing for approximately 3 days, cell subculture was performed when the cell density reached ~80% of the bottom of the culture bottle.

Cell viability assessment. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; BioSharp; Beijing Lanjieke Technology Co., Ltd., Hefei, China) assay, as previously described (13). Briefly, LLC cells in the logarithmic phase of growth were harvested, resuspended at 5x10⁴/ml, and 100 µl cells were added to a 96 well plate (5,000 cells/well) with five replicates per condition. Once adherence to the plates was achieved, a concentration gradient of avasimibe (Med Chem Express LLC, Monmouth Junction, NJ, USA) of 0.0, 2.5, 5.0, 10.0 and 20.0 µM was added to the corresponding wells. Following 24, 48, 72 or 96 h of incubation at 37°C, CCK-8 solution was added to each well followed by a 1-4 h incubation at 37°C. The optical density at 450 nm was then determined via a microplate reader (BioTek Instruments, Inc.) for 30 min at 37°C. The wound healing rate was determined based on the ratio of the change in the scratch width to the initial scratch width.

Wound healing assay. This assay was conducted as previously described (14). Briefly, LLC cells were plated in 6-well plates (5x10⁴ cells/well). Subsequent to achieving 90% confluency, a vertical scratch (width, ~900 µm) was created in the monolayer using a sterile pipette tip. Plates were then washed using phosphate buffered saline (PBS) and serum-free DMEM containing either 0, 5 or 10 µM avasimibe was added to the appropriate wells. After 48 h, cell migration was observed and photographed at 0 and 48 h after scratching. The width of the healing wound was calculated using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA). The wound healing rate was determined based on the ratio of the change in the scratch width to the initial scratch width.

Flow cytometry. LLC cells were plated in a 6 well plate (5x10⁴ cells/ml) and harvested after 72 h treatment with 0, 10 or 20 µM avasimibe. Cells were then fixed at room temperature in 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min, prior to permeabilization using 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 20 min. The cells were then blocked in 5% goat serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) and 0.3% Triton X-100 for 30 min at 37°C. Cells were incubated with anti-ACAT-1 antibody (pAb; 1:200; catalog no. #44276; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at 37°C followed by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (IgG; 1:200; catalog no. #4412; Cell Signaling Technology, Inc.) for 30 min at 37°C. The cells were washed 2-3 times with 3 ml TBPS buffer. A Cytomix FC 500 flow cytometer (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) was used to detect labeled cells. Mean fluorescence intensity was analyzed using FlowJo software (version 7.6; FlowJo LLC, Ashland, OR, USA).

Immunofluorescence. LLC cells were seeded onto coverslips (1x10⁵ cells/ml). Following treatment with 0 and 10 µM avasimibe for 72 h, the coverslips were fixed using 4% paraformaldehyde and permeabilized as aforementioned, and were then blocked in 5% goat serum and 0.3% Triton X-100 for 30 min at 37°C. The coverslips were then incubated with anti-ACAT-1 antibody (pAb; 1:200; catalog no. #44276; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at 37°C and then incubated with FITC-labeled goat anti-rabbit IgG (1:50; catalog no. BL003A; Biosharp; Beijing Lanjieke Technology Co., Ltd.) for 30 min at 37°C. DAPI (Beyotime Institute of Biotechnology, Shanghai, China) was used to harvest the cells, and protein was quantified via a BCA assay (Beyotime Institute of Biotechnology). Samples were then separated via SDS-PAGE (10% gel) with 30 µg per lane and then transferred to a polyvinylidene fluoride membrane (Beyotime Institute of Biotechnology). A total of 5% skim milk was used for membrane blocking for 2 h at room temperature, and membranes were incubated at 4°C with the primary antibody (rabbit anti-mouse ACAT-1 polyclonal antibody; 1:1,000; catalog no. #44276; Cell Signaling Technology, Inc.) overnight. A horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (goat anti-rabbit IgG; 1:8,000; catalog no. BL003A; Biosharp; Beijing Lanjieke Technology Co., Ltd.) was then used for detection for 2 h at 37°C. ACAT-1 was then visualized using an ECL (EMD Millipore, Billerica, MA, USA) chromogenic reaction in a dark room. Bands were quantified using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Apoptosis detection. Apoptosis was detected using an Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Subsequent to LLC cell treatment with avasimibe (0 and 10 µM) for 72 h,
the cells were digested with EDTA-free trypsin and resuspended in 500 µl binding buffer. Samples were then stained with 1:100 annexin V and propidium iodide for 10 min at 4°C in the dark, and cells were detected using flow cytometry as described above. The rate of apoptosis was analyzed using FlowJo software (version 7.6; FlowJo LCC).

In vivo mouse model experiments. A total of 24 male C57BL/6 mice (age range, 8-10 weeks; weight, 18-22 g) were obtained from the Experimental Animal Center of Bengbu Medical College (Bengbu, China). The mice were housed in a pathogen-free central animal facility (temperature, 20-26°C; relative humidity, 40-70%; light-dark alternate time, 12/12 h; food and water were disinfected and sterilized, the mice had continuous access to the food and water) at the Bengbu Medical College. The animal experiments were performed based on the recommendations provided in the National Institutes of Health Laboratory Animal Care and Use Guidelines (15). LLC cells were washed twice with PBS and filtered through a 40 µm filter membrane prior to being resuspended at a density of 2x10^7 cells/ml and subcutaneously injected into the left forelimbs of these mice. Following 10 days, a total of 34 mice meeting the experimental requirements, that the tumor sizes were similar, were identified.

In the avasimibe treatment trial, 24 mice were randomized into 4 groups: A control group, a cyclophosphamide (CTX; 20 mg/kg, once every other day) group, an avasimibe (15 mg/kg, once every 2 days) group and a CTX + avasimibe group. From days 10-35 following tumor inoculation, CTX or avasimibe were injected intraperitoneally and mouse body weight was monitored once weekly. On day 35, all mice were euthanized and tumors, livers, lungs and spleens were harvested. Spleens were then weighed, tumor volume was determined, and liver and lung samples were inspected for evidence of metastases. For the remaining 10 tumor model mice, the lungs were collected on day 35. In parallel, lung samples from 10 healthy tumor-free mice were also collected. Lung tissue protein was then extracted and ACAT-1 expression was detected by western blotting.

Statistical analysis. All in vitro experiments were conducted at least three times. SPSS v16.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Data are provided as the mean ± standard deviation, and were assessed using one-way analyses of variance and Student's t-tests. The least significant difference-t test was used as a post-hoc test for comparison between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Decreased expression levels of ACAT-1 inhibit LLC cell proliferation. LLC cells were treated with different avasimibe concentrations to inhibit ACAT-1, revealing a significant dose- and time-dependent suppression of proliferation as revealed using a CCK-8 assay (P<0.05; Fig. 1A and B). The cell viability rates in the blank group and the avasimibe (2.5, 5, 10 and 20 µM) groups were 100.00±0.00, 63.57±4.88, 45.47±5.35, 37.66±3.72 and 30.59±1.24%, respectively (Fig. 1A). In addition, the viability of the control group and the groups at 1, 2, 3 and 4 days were 100.00±0.00, 72.21±4.50, 58.60±5.25, 46.11±3.9 and 39.02±3.04%, respectively (Fig. 1B). These results therefore demonstrated that avasimibe inhibits LLC cell proliferation compared with the controls.

Avasimibe downregulates ACAT-1 expression in LLC cells. LLC cells were treated with different avasimibe doses for 72 h, following which the ability of avasimibe to inhibit ACAT-1 expression was validated using flow cytometry, western blotting and immunofluorescence microscopy. As presented in Fig. 3, it was revealed that avasimibe treatment resulted in the significant downregulation of ACAT-1 protein expression levels in LLC cells compared with the control groups (P<0.05).

Avasimibe promotes the apoptosis of LLC cells. Based on a flow cytometry-based analysis, avasimibe (10 µM)
treatment of LLC cells for 72 h significantly increased the apoptotic rate of these cells compared with the control group (P<0.05; Fig. 4).

**Avasimibe inhibits LLC growth and metastasis in mice.** In the present study, a subcutaneous mouse LLC model was established in order to validate the anti-tumor effects of ACAT-1 inhibition *in vivo*. The results revealed that avasimibe may significantly reduce tumor size (P<0.01; Fig. 5A and B), and resulted in a significant increase in spleen weights and spleen indexes (P<0.01; Fig. 5F and G) compared with the control groups. Compared with the CTX and avasimibe mono-treatment groups, the combination of CTX+avrasimibe was able to significantly inhibit tumor growth more effectively than either single treatment (P<0.01; Fig. 5B). Avasimibe treatment did not result in notable changes in mouse body weight over time (Fig. 5C). Metastatic lesions in distant organs (liver and lung) were also assessed at the study endpoint, with at least one metastatic lesion being observed in the liver and lung of each control mouse, compared with the lack of evidence of metastatic lesions in other groups (Fig. 5D and E). These results further confirmed that avasimibe inhibited tumor growth and enhanced immune responses in mice with LLC tumor types. Furthermore, avasimibe enhanced the anti-tumor effects of CTX.

In the present study, an association between ACAT-1 and lung cancer growth and metastasis was identified. ACAT-1 has primarily been studied in the context of cardiovascular diseases, with numerous ACAT inhibitors having been developed to treat diseases including cardiovascular diseases and Alzheimer's disease (16,17). Recently, researchers have determined that ACAT-1 expression and activity are upregulated in numerous tumor cells, and ACAT-1 inhibitors exhibit anti-tumor activity in certain experimental models *in vivo* and *in vitro* (9,18-22), including in renal cell carcinoma, colon cancer, breast cancer, glioma, pancreatic ductal adenocarcinoma, chronic myelogenous leukemia and lung cancer. A previous paper studied the function of ACAT-1 in lung cancer cells, uncovering results which differed from those of the present study (19). This may be due to a number of reasons; firstly, this may be that this previous study did not perform migration experiments to investigate the effect of avasimibe on lung cancer cell migration. Secondly, this previous study used A549 cells, which are distinct from LLC cells. Thirdly, this previous study did not conduct animal model experiments assessing lung cancer. Finally, this previous study investigated the anti-tumor effects of avasimibe by detecting cholesterol in cells, whereas the present study focused specifically on the expression of ACAT-1. Here, it was demonstrated that the inhibition of ACAT-1 may be effective in treating lung cancer.

The present study provides novel insights into the importance of ACAT-1 in LLC cells, suggesting that this protein serves an important function in the growth and metastasis of LLC cells and the development of LLC in mice. Targeting ACAT-1 is thus a potential novel therapeutic strategy for treating lung cancer. By treating LLC cells with avasimibe, an ACAT-1 inhibitor, it was revealed that avasimibe reduced the expression of ACAT-1 and significantly inhibited LLC cell proliferation and migration compared with the control cells (P<0.05), further promoting the apoptosis of these LLC cells. The present study additionally established a mouse model of LLC to verify the anti-tumor effects of ACAT-1 inhibitors *in vivo*. Mice were treated with saline, CTX (20 mg/kg), avasimibe (15 mg/kg) or CTX+avrasimibe. It was revealed that avasimibe did not cause weight loss in mice. No evidence of avasimibe toxicity in mice was observed, and avasimibe alone or in combination with the existing chemotherapy CTX was sufficient to inhibit tumor growth and metastasis, with avasimibe further increasing tumor sensitivity to CTX. Studies have reported that avasimibe may enhance the efficacy of gemcitabine as a means of combatting pancreatic ductal adenocarcinoma proliferation (22), and may additionally enhance the efficacy of imatinib as a means of disrupting the growth of chronic myelogenous leukemia (9). The present study further observed that the inhibition of ACAT-1 enhanced immune responses in mice, as evidenced
by measuring the spleen weight and spleen indexes of treated mice. Additionally, it was revealed that the expression of ACAT-1 in the lung tissue of the LLC mice was higher compared with in healthy mice. These results will provide the basis for further biological research to fully understand the function of ACAT-1 in the occurrence and development of cancer.

The mechanisms linking ACAT-1 to lung cancer growth and metastasis require further study. One potential link is the fact that ACAT-1 may reduce the levels of the pyruvate dehydrogenase complex (PDC) (23). As early as the 1950s, German scientists proposed the Warburg effect (24); unlike the oxidative metabolism of normal cells, tumor cells undergo extensive aerobic glycolysis to provide the energy necessary for growth in an oxygen‑rich environment. PDC is extremely important mediator of the interface between glycolysis and oxidative phosphorylation (25). Studies have demonstrated that decreased PDC levels are able to enhance glycolysis in tumor cells, thus providing a metabolic advantage for tumor growth (26-28). ACAT-1 is an upstream regulator of pyruvate dehydrogenase and pyruvate dehydrogenase phosphatase (PDP), which acetylates pyruvate dehydrogenase E1-a subunit and PDP-1 to downregulate PDC levels (23). In the present study, the expression of ACAT-1 was successfully inhibited using avasimibe, which in theory may increase PDC levels and thereby inhibit tumor growth.

In addition to promoting glycolysis, ACAT-1 serves a key role in cholesterol metabolism, catalyzing free cholesterol conversion into cholesterol esters (29). ACAT-1 may increase cholesterol ester levels and elevate blood sugar in the body, resulting in insulin secretion which may in turn increase the activity of insulin-like growth factor (IGF). Increased IGF function may promote cell mitosis, thereby accelerating cell proliferation and promoting tumor growth (30-32). Studies have demonstrated that IGF is closely associated with lung cancer progression (33,34). Thus, the inhibition of ACAT-1

Figure 3. Avasimibe regulates the expression levels of ACAT‑1 in LLC cells. (A) ACAT‑1 protein levels were quantified by flow cytometry. The effect of avasimibe (10 and 20 µM) on the expression levels of ACAT‑1 in LLC cells subsequent to treatment for 72 h was determined, with 0 µM used as a control. (B) ACAT‑1 protein levels in LLC cells were quantified by western blotting (normalized to β-actin) in order to assess the effects of treatment with 10 µM avasimibe for 72 h, with 0 µM used as a control. (C) ACAT‑1 protein levels were quantified by immunofluorescence microscopy following 72 h treatment with 10 µM avasimibe, with 0 µM used as a control. Scale bars=20 µm. *P<0.05 and **P<0.01 with comparisons shown by lines. ACAT-1, acetyl-CoA acetyltransferase 1; LLC, Lewis lung cancer; FITC, fluorescein isothiocyanate; NS, not significant.
Figure 4. Effect of 72 h treatment with 10 µM avasimibe on the apoptotic rate of Lewis lung cancer cells, with 0 µM used as a control. *P<0.05 with comparisons shown by lines. FITC, fluorescein isothiocyanate.

Figure 5. Therapeutic effect of avasimibe in a mouse model of Lewis lung carcinoma. (A) Images of the tumor; (B) ex vivo measurements of the tumor size; (C) monitoring of the mouse body weight over time (0-5 weeks); (D) Images of metastatic lesions of the lung; (E) ex vivo measurements of spleen weights; (F) Images of metastatic lesions of the liver; (G) ex vivo measurements of spleen indexes. **P<0.01 with comparisons shown by lines. CTX, cyclophosphamide.
expression reduces cholesterol ester levels, and may thereby result in decreased IGF activity, resulting in the inactivation of phosphoinositide 3 kinase/protein kinase B or RAS type GTPase family/mitogen-activated protein kinase signaling pathways, thereby inhibiting tumor growth and metastasis (35).

Recent studies (36‑38) have also identified an association between ACAT-1 and the immune system, as inhibiting ACAT-1 expression may enhance the anti-tumor activity of cluster of differentiation (CD)8+ T cells. During T cell activation, lipid metabolism is required for membrane biosynthesis and cell growth. Cholesterol regulates membrane protein function, participates in membrane trafficking and regulates transmembrane signaling (39,40). Previous studies have also reported that ACAT-1 inhibitors potentiate the anti-tumor effects of human chimeric antigen receptor-modified T cells, and that CD8+ T cell responses to melanoma may be enhanced by inhibiting cholesterol esterification in mice via ACAT-1 inhibition (41,42). The present study similarly observed that avasimibe may improve immune responses in tumor-bearing mice, which may additionally be associated with ACAT-1 affecting immune system function. The avasimibe and CTX+avasimibe group exhibited increased spleen weights and spleen index values compared with the control group, but these values were higher in the avasimibe treatment group compared with the CTX+avasimibe group, which may be due to the effects of CTX on the immune system in these mice. CTX is commonly used as an immunosuppressant to inhibit tumor growth, which may inhibit immune system function (43-45). Furthermore, the inhibition of ACAT-1 by avasimibe may improve the immune function in mice (38,41,42). Therefore, the regulation of cholesterol metabolism may have a profound impact on anti-tumor responses through these other signaling pathways. Further research will therefore be required to fully elucidate the underlying molecular mechanisms.

The safety and toxicity of avasimibe and CTX have been evaluated in clinical trials. In prior clinical trials for the treatment of atherosclerosis, avasimibe proved to have good safety in humans (46,47). CTX is a commonly used chemotherapeutic drug, and its clinical safety has also been confirmed (48-50). At present, to the best of our knowledge there has been no clinical trial assessing the combination of avasimibe and CTX. It is therefore necessary to conduct clinical trials to confirm the effectiveness and safety of this combination.

In summary, a preliminary conclusion may be drawn from the present study: that inhibiting the expression of ACAT-1 may weaken the proliferation and metastasis of LLC cells. Avasimibe may not only inhibit the tumor growth and development of distant metastases in mice, but may additionally enhance the anti-tumor efficacy of chemotherapeutic drug treatment and enhance immune responses in vivo. Therefore, targeted blocking of the ACAT-1 metabolism pathway has potential for use as a novel treatment strategy for lung cancer that may be combined with chemotherapy to provide novel treatment strategies for this disease. This treatment has the potential to delay the progression and metastasis of cancer, thereby prolonging patient survival time and improving quality of life. However, the further study of ACAT-1 is still required in order to fully understand the function of ACAT-1 in the development and progression of lung cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MB designed the study, contributed to the conception of the study and the analysis and interpretation of the experimental data, revised the manuscript, and gave final approval of the version to be published. XuxQ, WL, YL and SZ performed the experiments. HaoZ, ZG, HW and JH performed the data analysis. HaiZ, MS, YW, JY, XujQ and ZZ participated in developing the animal model. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.
All experiments were performed in accordance with a protocol ethically approved by the Institutional Animal Care and Use Committee of Bengbu Medical College (Bengbu, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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