D-Limonene Inhibits the Occurrence and Progression of LUAD by Suppressing Lipid Metabolism Dysregulation Induced by PM_{2.5} Exposure

Tengteng Zhu (✉ zhutengteng@gmail.com)

Shanghai Jiao Tong University <https://orcid.org/0000-0001-7589-7228>

Yangyang Li

Shanghai Jiao Tong University School of Medicine

Tienan Feng

Shanghai Jiao Tong University School of Medicine

Yuqing Yang

Shanghai Huangpu District center for disease control and prevention

Kai Zhang

Shanghai Jiao Tong University School of Medicine

Ying Qian

Shanghai Jiao Tong University School of Medicine

Jing Gao

Shanghai Jiao Tong University School of Medicine

Xiaowei Quan

Shanghai Jiao Tong University School of Medicine

Herbert Yu

University of Hawai'i Cancer Center

Biyun Qian

Shanghai Jiao Tong University School of Medicine

Research

Keywords: PM_{2.5}, Lung adenocarcinoma cancer, lipid metabolism dysregulation, D-limonene, miR-195

Posted Date: October 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-942067/v1>

Abstract

Background: PM_{2.5} exposure is associated with lung adenocarcinoma (LUAD), but the mechanism is unclear. The lack of understanding impedes our effort on prevention. This study examined a possible mechanism of lung cancer caused by PM_{2.5} exposure, and aimed to find a potential intervention for people living in PM_{2.5} polluted regions.

Methods: PM_{2.5} was collected near South-North elevated way in Shanghai city. Lung adenocarcinoma cells, pulmonary epithelial cell and mice were exposed to PM_{2.5} or D-limonene for 48h and two month separately. Cell morphology, abilities of invasion, migration and proliferation, pulmonary fibrosis level were assessed. Lipid metabolism changes were also studied in 11,564 lung cancer patients.

Results: PM_{2.5} exposure induced accumulation of lipid droplets in LUAD cells which was accompanied by increased malignant cellular behaviors and disrupted lipid metabolism. PM_{2.5} exposure of LUAD led to cleaved N-SREBP1 translocation from the cytoplasm to nucleus, which upregulated the expression of FASN and ACACA. These changes increased lipid accumulation in LUAD. D-limonene, a natural monoterpene, was found to inhibit the changes in lipid metabolism through upregulating the expression of *miR-195*, a reported tumor suppressor that inhibited the expression of SREBP1, FASN and ACACA specifically induced by PM_{2.5} exposure. Same changes were also observed in normal lung epithelial cells and normal lung tissue from mice after PM_{2.5} exposure, and the changes were inhibited by D-limonene treatment. Furthermore, in a cohort of 11,564 lung cancer patients, significant lipid metabolism disorders were observed in high, compared to low, PM_{2.5} polluted areas, and a small human intervention trial showed that serum *miR-195* was upregulated after oral intake of D-limonene.

Conclusion: Our study reveals a potential intervention target for LUAD that is related to PM_{2.5} exposure-induced lipid metabolism disturbance and that D-limonene may inhibit the disruption through the upregulation of *miR-195*, which suggests a novel low-cost preventive intervention for people living in PM_{2.5} polluted regions.

Background

Lung cancer is the second most common malignant tumor after breast cancer and the leading cause of cancer-related death worldwide [1]. Due to the reduction in tobacco smoking, the incidence of lung squamous cell carcinoma (LUSC) had decreased, while the incidence of lung adenocarcinoma (LUAD) has increased in never-smokers [2]. Aside from smoking, epidemiological data show that outdoor ambient air pollution, especially fine particulate matter (PM_{2.5}) pollution, is another risk factor for lung cancer and has been classified as a level I carcinogen by the International Agency for Research on Cancer [3]. A meta-analysis indicated that lung cancer risk increased 9% when the PM_{2.5} concentration increased by 10 µg/m³ (95% CI = 1.04–1.14) [4]. Because of the small particle size (diameter < 2.5 µm) and large surface area in a given concentration, PM_{2.5} can easily invade the human alveolar and blood circulation and carry

a variety of hazardous materials, such as carcinogens, promoting the occurrence of disease. PM_{2.5} increases proinflammatory reaction and lipid metabolism in the lung both of which are closely associated with cancer, and continuous de novo fatty acid synthesis is a common feature of cancer [5–9].

It has been observed that PM_{2.5} exposure induces lipid droplet formation in the liver due to the increases in lipid synthesis and decreases in lipid transport and catabolism. These changes suggest that PM_{2.5} exposure may cause lipid metabolism reprogramming [10, 11]. Lipid metabolism reprogramming includes de novo fatty acid generation, fatty acid oxidation, and changes in phospholipid and neutral lipid metabolism, which are widely involved in the development of cancer. Sterol regulatory element-binding protein-1 (SREBP-1) is a key molecule in regulating the production of fatty acids and triglycerides through stimulating the expression of fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACACA), which increases cancer cell viability and promotes tumor growth [12]. Inhibition of SREBP-1 can suppress tumor growth and promote cell death [13]. Therefore, blocking or correcting lipid metabolism reprogramming caused by PM_{2.5} exposure may be a potential target for intervention to reduce the risk of cancer associated with PM_{2.5} exposure.

D-limonene (monocyclic monoterpene, extracted from Chinese herb *Pericarpium citri reticulatae*) has been found to regulate lipid metabolism [14]. Now, D-limonene can be extracted or chemically synthesised. Previous studies have reported its antitumor property *in vivo* and *in vitro* [15, 16], including cancers of the breast [17], liver [18], kidney [19] and skin [20]. An early clinical trial showed that D-limonene at a dosage of 0.5 g/m²/day could halt the progression of cancer for nine months [21]. We reported previously that D-limonene exerts its antitumor activity by inducing autophagy and apoptosis and by regulating lipid metabolism in cancer cells [22, 23]. Evidence also suggests that miRNAs may play an important role in the development of malignant tumors and respiratory diseases related to PM_{2.5} exposure [24–26]. The role of miRNAs as central regulators of lipoprotein metabolism and cholesterol homeostasis is well established [27]. Furthermore, miRNAs can mediate the biologic effects of xenobiotics [28, 29], including PM_{2.5} [30]. We and others have found that *miR-195* can inhibit non-small-cell lung cancer by regulating cell cycle and lipid metabolism [11, 31, 32].

In the present study, we demonstrated that PM_{2.5} promoted LUAD occurrence and progression through disrupting lipid metabolism resulting in increased lipid droplet formation. We also found that D-limonene could inhibit the occurrence and progression of LUAD caused by PM_{2.5} exposure through upregulating the expression of *miR-195*, which targets the activity of lipogenic genes. Collectively, our study reveals a possible mechanism of lung cancer caused by PM_{2.5} exposure and suggests a novel low-cost preventive intervention for people living in PM_{2.5} polluted regions.

Materials And Methods

PM_{2.5} sample collection and preparation

The sampling site was located at Shanghai Jiao Tong University School of Medicine in Shanghai City, China. PM_{2.5} was sampled continuously using an Echo PM Low Volume Sampler near the air intake for the exposure chambers (MSP HFI-129, China). Every 3 days, a new filter was used for sampling. Additionally, field blanks were used to control for possible contamination during the sampling procedures. After sampling, the filters were cut into tiny pieces and placed in an ultrasonicator for 2 h. After filtration, PM_{2.5} solution was purified in a vacuum freeze drier for 7 days, and PM_{2.5} powder was stored in an ultracold storage freezer. Before frozen, Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Gas Chromatography Mass Spectrometer (GC-MS) and Carbon analyser were used to analysis the compositions of PM_{2.5} powder.

Cell culture and treatments

A549 cells, H1975 cells and Beas-2b cells were obtained from the American Type Culture Collection. Cells were kept in DMEM/high-glucose medium supplemented with 10% FBS and 1% penicillin / streptomycin at 37°C in a 5% CO₂ humidified culture incubator. The cells were exposed to PM_{2.5} for 48 h at a final concentration of 100 µg/ml. Beas-2b cells were treated with the same concentration and maintained for 30 passages. Treated cells were then analysed in subsequent assays.

siRNA transfection

Specific siRNAs (Jima Gene, China) were used for the knockdown of *SREBF1* and *FASN*. A549 and H1975 cells were seeded in 6-well plate and transfected by the 1 µM *SREBF1* or *FASN* siRNA via Lipofectamine 2000 (Thermo Fisher Scientific). After siRNA transfection for 24 h, the cells were stimulated with 100 µg/ml PM_{2.5} for 48 h. Finally, the expression of *SREBF1* and *FASN* was confirmed by RT-qPCR.

Cell migration and cell invasion assays

A549 and H1975 cells were seeded into 6-well plates. After treatment, scratch wounds were made by scraping the cell layer in each culture well using the tip of a 200-µl pipette when the cell density reached > 90%. Then, the cells were washed with PBS and cultured for 24 h or more, individually or together, after which three fields were randomly chosen from each scratch wound and visualized by microscopy to evaluate the cell migration ability. The experiments were performed in triplicate.

Invasion assays were performed with Transwell inserts (8-µm pores, Millipore). The upper chambers of the Transwells were precoated with diluted Matrigel (BD Biosciences, Sparks, MD). A total of 10⁴ cells were seeded onto the upper chamber in serum-free medium, and medium containing 10% serum was added to the lower chamber as a chemoattractant. After 24 h of incubation, the upper surface of the insert was wiped with a cotton swab, and cells that migrated to the lower surface were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet. The cells were counted in three random fields per well, and the quantity is expressed as the mean ± SD of triple assessments.

Colony forming assay

Cells were exposed to the indicated treatments accordingly and seeded into 10 cm dishes (500 cells/dish). After culturing for 14 days, colonies were fixed with 4% PFA and stained with 0.4% crystal violet solution for analysis. Experiments were performed in triplicate.

RNA-Seq and data analysis

Cells were treated in triplicate per group with PM_{2.5}, D-limonene or both. Total RNA was extracted and subsequently sequenced on an Illumina HiSeq 3000 machine. The RNA-Seq reads were aligned to the human genome (GRCh37/hg38) using TopHat version v2.1.0 with the hg38 iGenome annotation guide and default parameters. TopHat was also run permitting novel junctions for the purpose of novel transcript assembly. Cufflinks v2.2.1 was used to assemble transcript models and compare these to the reference hg38 iGenome annotation. Differentially expressed mRNAs were identified in cuffdiff 2.2.1 based on the criterion of being significantly altered in the experimental vs. control condition ($P < 0.1$ and absolute fold change > 1).

Electron microscopy

Cells and lung tissues were fixed with 1% osmic acid for 1 h. The samples were transferred into a penetrant prepared according to a ratio of embedding agent and acetone volume of 1:1. After using a 60-nm ultrathin microtome and staining in 1% uranyl acetate lead citrate for double staining, samples were observed and captured with an electron microscope.

Western blotting

After treatment, the cells and the mouse lung tissues were lysed by RIPA with protease phosphate inhibitor. The proteins were separated by 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked for 2 h at room temperature with 5% BCA and incubated overnight with rabbit or mouse polyclonal antibodies against GAPDH (CW0100M, CWBIO, China), ACACA (ab45174, Abcam, UK), FASN (ab22759, Abcam, UK) and SREBP1 (sc-365513, SCBT-Santa, Japan) at 4°C. After washing with PBST for 0.5 h, the PVDF membrane was incubated for 2 h with antibody-conjugated goat anti-rabbit IgG or anti-mouse IgG. The PVDF membrane was washed with PBST and then visualized via a protein imaging system.

Luciferase reporter assay

Luciferase reporter plasmids containing the 3' untranslated region (3' UTR) of *SREBP1*—either the wild-type (WT) coding sequence (CDS) region or a sequence with a mutation in the predicted binding site of the *FASN* and *ACACA* genes—were constructed by Jima Gene (China). A549 and H1975 cell lines were seeded in 12-well plates, and then the reporters (1 µg) and *miR-195*-mimics or negative control (NC) (25 nM) were co-transfected into the cells with Lipofectamine 2000. After 24 h, the cells were harvested, and luciferase activities were detected by the Dual Luciferase Reporter Assay Kit.

RT-qPCR analysis

Total RNA was extracted from cells after treatment using TRIzol. Subsequently, RNA was reverse-transcribed using PrimeScript™ RT Master Mix. RT-qPCR amplification was performed with an ABI 7500 system with PowerUP™ SYBR™ Green® Master Mix. The expression level of the target gene was normalized to that of GAPDH, and the $2^{-\Delta\Delta CT}$ method was used to calculate the expression results. Each group included 3 duplicate wells.

Masson's trichrome staining

Mouse lung tissues embedded in wax were cut into 6-μm thick slices. The slices were immersed in dimethylbenzene and gradient ethanol for deparaffinization and rehydration. After staining in iron haematoxylin solution, 1% hydrochloric acid alcohol was used for differentiation. The slices were immersed in Ponceau staining solution, phosphomolybdate aqueous solution and aniline blue solution for 10 min, 3 min and 6 min, respectively, for redyeing. The slices were sealed with Permount™ mounting medium after dehydration and vitrification in dimethylbenzene and gradient ethanol. Pictures were captured with a microscope, and data were analysed with Image Pro-Plus.

miR-195 knockout (KO) mice construction

We used CRISPR/Cas9 technology to construct a *miR-195* KO mouse model via non-homologous recombination. The brief process is as follows: Cas9 mRNA and guide RNA (gRNA) (AAGACTCTACTTTGCTCTGT GGG) were obtained by in vitro transcription; Cas9 mRNA and gRNA were microinjected into the fertilized eggs of C57BL/6J mice to obtain F0 generation mice. After confirmation by PCR product sequencing, the obtained F0 generation mice with target gene deletion were mated with C57BL/6J mice to obtain positive F1 generation mice.

Animals and treatments

C57BL/6 male mice weighing 22–26 g were purchased from the Experimental Animal Center of Chinese Military Medical Sciences Academy. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (IACUC-SIFDC17082). After one week of acclimatization, *miR-195* KO and WT mice were randomly distributed into equal groups with six mice in each group, including the control group, PM_{2.5} group and PM_{2.5} plus D-limonene group. The treatment groups were instilled with a 0.1 mL PM_{2.5} suspension, while the control group was instilled with 0.1 mL physiological saline after the mice were anaesthetized with diethyl ether. Exposure was performed once every day, and this practice continued for 2 months. After every treatment, the mice were euthanized, and their lungs were rapidly removed. The mice were sacrificed by cervical dislocation. The removed lungs were placed in liquid nitrogen instantly.

Sample collection and oil red staining

Human LUAD cancer and corresponding adjacent noncancerous tissues were collected with informed consent from patients who underwent radical resections from Shanghai City and Hebei Province. The

tissues were cut into 10- μ m-thick slices after flash freezing in liquid nitrogen. The sections were fixed in ice-cold 4% PFA for 30 min and then placed into oil red solution for 10 min at 37°C after washing in PBS. An 85% propylene glycol solution was used for 2 min for differentiation, and then the sections were rinsed in 2 changes of PBS.

Recruitment and inclusion/exclusion criteria

Ten people were recruited into the D-limonene intervention group after strict inclusion and exclusion criteria were met (Trial number: ChiCTR2000030200), and 1 g D-limonene was administered every day for 4 weeks. Ten millilitres of whole blood was captured every two weeks, including at baseline. The inclusion criteria were as follows: healthy subjects more than 18 years old; body mass index (BMI) more than 18.5 kg/m³; no history of allergy to citrus fruits; and voluntarily participation with sign informed consent. The exclusion criteria were as follows: the presence of acute or chronic gastrointestinal diseases; intake of more than 50 g citrus fruits one day in a recent week; the presence of heart, liver, kidney or haematopoietic system disease or other serious diseases; severe allergic constitution; pregnant or lactating women; the use of anti-inflammatory drugs; surgery was not scheduled in the next three months.

Cohort analysis

We constructed a PM_{2.5} exposure cohort based on the SuValue database. The SuValue database included 221 hospitals from 23 provinces, municipalities or autonomous regions across China. The 221 hospitals included more than one million patients from 176 general hospitals, 28 traditional Chinese medicine hospitals, 14 maternal and childcare hospitals and 3 specialized hospitals. People in this cohort had at least two tests for triglycerides (TGs), which are the main component of lipid droplets. The PM_{2.5} dataset of each province across China was collected from the Atmospheric Composition Analysis Group.

Statistical analysis

The results of laboratory experiments are presented as the mean $\pm$ SD. Student's t test was used to compare means between two groups, and ANOVA was employed for comparison of more than two groups when samples were normally distributed. Otherwise, the rank sum test was used for comparison between the two groups, and the Kruskal-Wallis test was used for comparison between multiple groups. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (version 19.0) and GraphPad Prism version 8.0 (GraphPad Software, San Diego CA, USA). $P < 0.05$ indicates a significant difference (* means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$). All experiments were repeated three times.

Results

PM_{2.5} Compositions analysis

As shown in Table1, the dominant components of metal ions and part non-metallic ions in PM_{2.5} were Al (1255.33 ± 5.51 ng/mg), Ca (9351.68 ± 69.97 ng/mg), Fe (2691.15 ± 20.84 ng/mg), Na (1567.68 ± 13.40

ng/mg), K (1567.43 ± 2.98 ng/mg), Mg (1074.41 ± 6.25 ng/mg) and S (12688.06 ± 66.99 ng/mg). The average concentrations of OC and EC in the PM_{2.5} were 527.11 ± 144.62 µg/mg and 119.71 ± 50.11 µg/mg, and the OC/EC ratio was about 4.61 ± 1.16 . Among the total of 16 PAHs elements measured, Naphthalene, Acenaphthylene and Pyrene were the most abundant elements in the PM_{2.5}.

Table 1
Metals, Carbon and organic compositions detected in PM_{2.5}

Metals	Concentration (ng/mg)	OC\EC	Concentration (µg/mg)(µg/mg)
Al	1255.33 ± 5.51	OC	527.11 ± 144.62
As	11.53 ± 0.46	EC	119.71 ± 50.11
B	28.59 ± 0.39	OC/EC	4.61 ± 1.16
Ba	95.20 ± 1.00	PAHs	Concentration (ng/mg)
Be	0.11 ± 0.00		
Bi	1.69 ± 0.97	Naphthalene	32.38 ± 1.34
Ca	9351.68 ± 69.97	Acenaphthylene	1.16 ± 0.49
Cd	2.96 ± 0.01	Acenaphthene	0.56 ± 0.32
Co	2.02 ± 0.15	Fluorene	0.42 ± 0.30
Cu	94.05 ± 1.18	Phenanthrene	0.14 ± 0.06
Fe	2691.15 ± 20.84	Anthracene	0.01 ± 0.01
Hg	1.16 ± 0.07	Fluoranthene	0.21 ± 0.15
K	1567.43 ± 2.98	Pyrene	0.66 ± 0.14
Li	4.28 ± 0.21	Benzo [A] Anthracene	0.07 ± 0.01
Mg	1074.41 ± 6.25	Chrysene	0.16 ± 0.12
Mn	181.71 ± 1.58	Benzo [B] Fluoranthene	0.39 ± 0.16
Mo	8.43 ± 0.04	Benzo [K] Fluoranthene	0.15 ± 0.11
Na	1567.68 ± 13.40	Benzo [A] Pyrene	0.15 ± 0.06
Ni	27.37 ± 0.16	Indene (1, 2, 3-cd) Pyrene	0.39 ± 0.06
P	291.58 ± 0.15	Diphenyl Anthracene (A, H)	0.06 ± 0.08
Pb	106.62 ± 0.70	Benzo [G, H, I] Pyrene	0.17 ± 0.13
S	12688.06 ± 66.99		
Sb	14.08 ± 0.58		
Se	12.61 ± 1.52		
Si	1019.26 ± 7.59		

OC: organic carbon; EC: elemental carbon; PAHs: Polycyclic Aromatic Hydrocarbons.

Metals	Concentration (ng/mg)	OC\EC	Concentration (µg/mg)(µg/mg)
Sn	13.56 ± 0.49		
Sr	35.02 ± 0.33		
Ti	62.17 ± 0.22		
V	73.25 ± 0.36		
W	18.56 ± 1.70		
Zn	493.88 ± 0.89		
Zr	4.23 ± 0.39		
OC: organic carbon; EC: elemental carbon; PAHs: Polycyclic Aromatic Hydrocarbons.			

PM_{2.5} induces lipid droplet formation in and promotes the aggressive phenotypes of lung cancer cells.

To study the effect of PM_{2.5} on lung cancer, we treated A549 and H1975 LUAD cells with PM_{2.5}. After the treatment, we observed clear increases in lipid droplets in both cell lines evaluated with electron microscope or Oil Red O staining compared to those untreated cells (Fig. 1a). Cell migration assays showed that A549 and H1975 treated with PM_{2.5} migrated faster than those untreated cells, although A549 showed large variation in migration at 24 h (Fig. 1b). Colony formation assays showed increased colony formation in both A549 and H1975 after the cells were treated with PM_{2.5} for 14 days (Fig. 1c). Transwell assays demonstrated that A549 and H1975 cells treated with PM_{2.5} became more aggressive, and the number of cells penetrating through the Matrigel membrane increased by 2 ~ 4 times (Fig. 1d).

PM_{2.5} upregulates the expression of lipogenic genes and induces lipid metabolism disorder.

To understand the mechanisms by which PM_{2.5} induces lipid droplet formation and promotes LUAD progression, we analyzed the transcriptomes of A549 cells with RNA-seq after PM_{2.5} treatment. The transcriptomic analysis revealed 263 differentially expressed genes (DEGs) between cells treated with and without PM_{2.5}. Of these DEGs, 148 were upregulated, and 115 were downregulated (Fig. 2a). Using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) algorithm to interrogate the DEG profiles, we found significant signal enrichment in fatty acid metabolism (Fig. 2b). The top two genes involved were SREBFs (Fig. 2c). We confirmed these findings with RT-qPCR analysis on *SREBF1* and its downstream targets, *FASN* and *ACACA*, all of which were upregulated significantly after PM_{2.5} treatment (Fig. 2d).

PM_{2.5} promotes N-SREBP1 translocation into the nucleus, inducing lipid droplet formation and LUAD progression.

Following the above findings, we speculate that PM_{2.5} may upregulate the expression of SREBP1 and its downstream genes, which leads to changes in lipid metabolism, resulting in increases in lipid droplets and aggressive cell behaviors. However, the level of total SREBP1 protein was not elevated after PM_{2.5} exposure in either lung cancer cell lines based on western blot analysis, while its downstream proteins FASN and ACACA were clearly upregulated (Fig. 3a). Using fluorescence immunohistochemical staining for SREBP1, we found that the protein was enriched in the cell nucleus (Fig. 3b). Western blot analysis showed that N-SREBP1 and SREBP cleavage activating protein (SCAP) were upregulated after PM_{2.5} exposure (Fig. 3a). Next, we used siRNA to knock down *SREBF1* and the main downstream gene *FASN*. Oil Red O staining showed that the number of intracellular lipid droplets in the knockdown cells after PM_{2.5} exposure was reduced (Fig. 3c). Additionally, knocking down the two genes inhibited the PM_{2.5}-induced increases in cell invasion, migration and colony formation (Fig. 3d-3f), suggesting that SREBP1 plays an important role in the accumulation of lipid droplets and the aggression of lung cancer cells induced by PM_{2.5} exposure.

D-limonene inhibits PM_{2.5}-induced lipid metabolism disorder in lung cancer cells by upregulating miR-195.

To investigate the molecular targets and mechanisms of D-limonene in lung cancer, we analysed the transcriptomes of A549 cells treated with or without D-limonene after PM_{2.5} exposure. Compared to those without D-limonene treatment, cells with D-limonene treatment showed downregulation of the genes involved lipid biogenesis, such as *SREBF1*, *FASN* and *ACACA* (Fig. 4a). These observations were confirmed by our RT-qPCR analyses (Fig. 4b). Interestingly, we found that the expression of *miR-195*, a known tumor suppressor, was substantially upregulated after D-limonene treatment (Fig. 4c). Using miRWalk [33] and miRSystem [34], we found that *miR-195* was predicted to interact with *SREBF1* in the CDS region when energy < -20, accessibility < 0.0001, me < -8. The *in silico* prediction also indicated that the miRNA could interact with *ACACA* and *FASN* mRNAs at their 3' UTRs. The prediction that the seed sequence of *miR-195* can directly bind to the CDS region of *SREBF1* and the 3' UTR of *FASN* and *ACACA* was verified by our dual luciferase assays. Our experiments showed that the relative luciferase activity was reduced in the WT groups (*SREBF1*, *FASN*, *ACACA*) compared with the mutant groups (Fig. 4d). After *miR-195* was overexpressed in A549 cells, western blot analysis showed that SREBP1, FASN and ACACA were significantly inhibited (Fig. 4e).

To verify the effects of D-limonene and *miR-195* on inhibiting lipid metabolism disorder caused by PM_{2.5} exposure, A549 cells were treated with or without *miR-195* after PM_{2.5} exposure. We found that *miR-195* treatment significantly inhibited the upregulated expression of the *SREBF1*, *FASN* and *ACACA* genes

induced by PM_{2.5} exposure (Fig. 4f). After treatment with D-limonene or *miR-195*, lipid droplet formation induced by PM_{2.5} exposure was decreased dramatically in the cells (Fig. 4g). Western blot analysis showed that D-limonene or *miR-195* decreased the protein levels of SREBP1, FASN and ACACA, which were upregulated in A549 and H1975 cells after PM_{2.5} exposure (Fig. 4h). Colony formation and Transwell experiments showed that both D-limonene and *miR-195* inhibited the PM_{2.5} exposure-induced proliferation and invasion of lung cancer cells (Fig. 4i-4j).

D-limonene prevents pulmonary fibrosis caused by long-term exposure to PM_{2.5} in normal lung epithelial cells and mice by repairing lipid metabolism disorder.

Following our observations that PM_{2.5} could promote tumor cell aggressive behaviors and that D-limonene could inhibit this process by reducing lipid metabolism disorder caused by PM_{2.5} exposure, we further explored whether PM_{2.5} also had a detrimental effect on normal lung tissue *in vitro* and *in vivo* and whether D-limonene can block or inhibit this effect. Normal lung epithelial cells (Beas-2b) were cultured under PM_{2.5} exposure for 30 passages. The PM_{2.5} treatment led to significant lipid droplets accumulation in the cells, and this phenomenon did not appear after the cells were treated with both PM_{2.5} and D-limonene (Fig. 5a). We also found a large amount of lipid droplets in the lung tissue of C57BL/6J mice after the animals were treated with PM_{2.5} inhalation for 60 days, but no lipid droplets were observed in the animals when D-limonene was orally administered simultaneously with PM_{2.5} inhalation (Fig. 5a). RNA-Seq analysis was performed on the lung tissues of mice treated with PM_{2.5} or PM_{2.5} plus D-limonene. The expression of SREBF1, FASN and ACACA were upregulated by PM_{2.5} exposure, and the upregulations were suppressed by D-limonene treatment. Similar results were also seen in normal lung epithelial cells, and the findings were verified by RT-qPCR (Fig. 5b, 5d). Our animal models also showed that long-term PM_{2.5} exposure significantly increased serum levels of TGs in the mice, and D-limonene treatment attenuated this effect (Fig. 5e).

To assess time-dependent variations, we collected Beas-2b cell samples every 5 passages, and the samples were analysed with western blotting. As shown in Fig. 5c, the expression of SREBP1, FASN and ACACA were significantly higher in PM_{2.5}-exposed than in control groups, and the increases appeared as early as in the fifth generation of cells. These proteins were also upregulated in the mouse lung tissue after a long period of exposure to PM_{2.5}. Similar to the observations in LUAD cells, the expression of SREBP1, FASN and ACACA, which were promoted by PM_{2.5} exposure, were inhibited by D-limonene treatment to the mice (Fig. 5c).

The RNA-Seq results of *miR-195* KO and WT mice treated with PM_{2.5} with or without D-limonene showed that D-limonene inhibited the increases in expression of *SREBF1*, *FASN* and *ACACA* induced by PM_{2.5} exposure in the KO mice (Fig. 5f), but the inhibitory effect was weaker than that in the WT mice (Fig. 5g).

Furthermore, Masson's trichrome staining and western blotting analysis revealed that pulmonary fibrosis occurred in both WT mice and *miR-195* KO mice after long-term exposure to PM_{2.5}. This phenotype could be rescued by D-limonene treatment. The level of pulmonary fibrosis in *miR-195* KO mice treated with D-limonene was significantly higher than that in WT mice (Fig. 5h-5k). The above results suggest that the presence of *miR-195* in WT mice facilitates the D-limonene's suppression and attenuation on PM_{2.5}-induced lipid-related gene expression and of pulmonary fibrosis development, respectively.

PM_{2.5} exposure increased TG levels in human plasma and lipid droplets in the lung tissue of cancer patients, while D-limonene intervention upregulated the expression of miR-195 in human plasma.

We assembled a cohort of 11,712 lung cancer patients with information on serum TG levels and their residences where air monitoring data were available on ambient PM_{2.5}. Based on their residence, 7,663 individuals were classified as living in high exposure areas, and 4,049 were in low exposure regions. More patients living in high exposure regions had higher TG levels than those living low exposure regions, 60% vs. 52% (Fig. 6a). Multivariate logistic regression analysis showed that high PM_{2.5} exposure were associated with elevated serum TG levels after adjusting for sex and age (HR = 1.39, 95% CI: 1.29–1.50) (Fig. 6b). Compared to those in Shanghai City, lung cancer patients from Hebei Province where PM_{2.5} pollution was higher than Shanghai, had more lipid droplets in their lung tissues according to the Oil Red O staining (Fig. 6c). A single-arm D-limonene intervention trial was conducted in ten people. Over a 4-week oral administration of D-limonene, individuals in the trial had substantial increases in serum levels of miR-195 (Fig. 6d).

Discussion

In this study, we first confirmed an already known phenomenon that long-term environmental exposure to ambient PM_{2.5} leads to lipid droplet accumulation in pulmonary cells and then identified a possible mechanism that may explain the phenomenon. We found that PM_{2.5}-induced lipid droplet accumulation was caused by the translocation of SREBP1 from cytoplasm to nucleus where it upregulated the expression of FASN and ACACA and increased the lipid anabolism resulting in dysregulation of lipid metabolism and accumulation of lipid droplets in the cells. We further demonstrated that D-limonene administration, a fruit extract, could inhibit the dysregulation of lipid metabolisms, reduce PM_{2.5}-induced lipid droplet accumulation and that these effects were mediated through its upregulation of *miR-195* which is known to have tumor suppression properties. Use of D-limonene was also found to suppress PM_{2.5}-induced pulmonary fibrosis and aggressive tumor behaviors, including cell proliferation, invasion and colony formation. All these findings are summarized in Fig. 7.

Erin *et al.* estimated that 3.8 million deaths worldwide in 2017 were related to ambient PM_{2.5} exposure, and this number increased to 4.1 million in 2019. Chronic obstructive pulmonary disease (COPD) and lung cancer were two primary causes of these deaths followed by ischemic heart disease [35]. PM_{2.5} is a known risk factor for lung cancer [36, 37], and has been found to be associated with increased lung cancer mortalities in many countries or regions, including Iran [38, 39], Europe [40], Eastern Thailand [41], North America [42] and China [43]. Rats developed pulmonary fibrosis after exposure to PM_{2.5} for one month [44]. Pulmonary fibrosis also appeared in C57BL/6J mice along with upregulated collagen I when treated with PM_{2.5} for 4 weeks [45]. These observations are consistent with the results of our animal experiment. In addition, we also found increased lung cancer cell proliferation, migration and invasion after PM_{2.5} exposure.

In our previous studies, we found that PM_{2.5} influenced lung cancer cells by increasing oxidative stress and autophagy and dysregulating certain long non-coding RNAs (lncRNAs) [46, 47]. Other researchers reported that the Wnt3a/ β -catenin pathway was upregulated by PM_{2.5}, which influenced the progression of lung cancer [48]. In this study, we found that PM_{2.5} could facilitate the development and progression of LUAD through disrupting lipid metabolism and increasing lipid droplet formation. Lipid droplets have been reported to be involved in several types of cancer [49, 50], including pancreatic ductal adenocarcinoma [51], colorectal cancer [52], breast cancer [53], and ovarian cancer [54]. However, there has been little research on the association between lipid droplet and lung cancer in previous study. We found that lipid droplets became increasingly larger in lung cancer cells after exposure to PM_{2.5}. Lipid droplets also appeared in normal lung epithelial cells and mouse lung tissues after PM_{2.5} exposure. Our patient cohort also showed that circulating levels of TG were higher in people living in PM_{2.5} heavily polluted regions than in those living in low polluted areas. According to the data from a cohort study of 39,057 individuals in Henan, China, ambient air pollutants were associated with blood lipid levels, and higher pollution linked to dyslipidaemia [55].

In comparison of cell transcriptomes with and without PM_{2.5} treatment, we found that lipid metabolism-related genes, including *SREBF1*, *FASN* and *ACACA*, were differentially expressed as part of the top signature upregulated by PM_{2.5}. Our follow-up experiments showed that PM_{2.5} exposure led to the nuclear entry of N-SREBP1 which upregulated *FASN* and *ACACA*. Liu *et al.* found that PM_{2.5} exposure could upregulate the expression of SREBP-1 by activating CCR2, which promoted fatty acid uptake, causing lipid accumulation and insulin resistance in the liver [56]. Mouse experiments also showed that PM_{2.5} exposure increased the expression of *ACACA* which in turn stimulated lipid deposit in white adipose tissue [57]. Our *in vitro* experiments demonstrated that lipid droplets were decreased, and cell invasion, migration and proliferation were inhibited when we used siRNA to knock down the expression of *SREBF1* or *FASN*. These observations suggest that dysregulated lipid metabolism after PM_{2.5} exposure may play an important role in the occurrence and progression of lung cancer and that blocking or correcting lipid metabolism dysregulation may be a potential intervention strategy for reducing lung cancer risk associated with PM_{2.5} exposure.

D-limonene was found to induce apoptosis and increase the expression of BAX and cleaved PARP *in vivo* and *in vitro* [58]. Our experiments showed that D-limonene could regulate lipid metabolism and suppress the aggression of lung cancer cells [23]. Morse *et al.* found that D-limonene could inhibit the metabolic activation of nitrosamine (NNK) and thus prevent the development of NNK-induced lung cancer through its interaction with cytochrome P450 (CYP450) detoxification enzymes [59]. Chow *et al.* reported that D-limonene supplement in diet could prevent breast cancer in animal models and small clinical trials [60]. In this study, we further demonstrated that D-limonene inhibited the expression of *SREBF1*, *FASN* and *ACACA* and decreased lipid droplet accumulation in LUAD cells. D-limonene could also suppress the colony formation, proliferation, invasion, and migration of lung cancer cells. These results suggest that D-limonene may target the genes affected by PM_{2.5} and regulate the synthesis and metabolism of lipid droplets. Furthermore, we found that the expression of *miR-195* was increased significantly when the A549 and H1975 cell lines were treated with D-limonene. This miRNA reported by us and others before can suppress the growth of lung cancer and other cancers, including the breast, prostate, stomach, colorectum, liver and bladder [61–65]. We also found that *miR-195* could directly bind to the mRNAs of *SREBF1*, *FASN* and *ACACA* in lung cancer cells resulting in decreases in lipid droplet formation induced by PM_{2.5} exposure.

In addition to inhibiting cancer cell growth, migration and invasion induced by PM_{2.5} exposure, D-limonene may also prevent or slower the development of lung cancer. Our study showed that D-limonene treatment could block or slow the formation of pulmonary fibrosis caused by long-term PM_{2.5} exposure both *in vivo* and *in vitro*. These observations suggest that D-limonene may be used as a potential chemopreventive agent for people who have experienced long-term exposure to environmental pollution.

To understand the significance of *miR-195* in D-limonene intervention, a *miR-195* KO mouse model was established with the CRISP-Cas9 technology. As shown in Fig. 5, when *miR-195* was knocked out, the effect of D-limonene became weaker, and more fibrosis was indicated by high collagen fibre staining and collagen I expression in the animals compared to the control mice without *miR-195* KO. Li *et al.* found that *miR-195* may serve as a biomarker to assist the diagnosis of lung cancer and classification of high-risk populations [11]. In our small trial, we found that the expression of *miR-195* was dramatically increased in human serum after D-limonene treatment for 4 weeks, which suggests that *miR-195* may serve as a potential biomarker for monitoring the effect of D-limonene administration.

In addition to cell and animal experiments, we have also conducted human studies. In a cohort of 11,712 lung cancer patients, we found that high TG levels were associated with high PM_{2.5} exposure. Analysis of tumor specimens from lung cancer patients living in Hebei Province, where there is heavy ambient PM_{2.5} pollution due to the presence of many iron and steel mills, showed more lipid droplets in their tissue samples compared to those living in Shanghai, confirming that PM_{2.5} exposure may disrupt lipid metabolism in human lung tissues.

Conclusion

In summary, our study showed that PM_{2.5} exposure caused accumulation of lipid droplets in lung cancer cells, normal lung epithelial cells and normal pulmonary tissue. This phenomenon resulted from PM_{2.5}-induced dysregulation of several proteins (SREBP1, FASN and ACACA) which control lipid metabolism. Our study also showed that a monoterpene D-limonene could block or reduce the disruption of lipid metabolism caused by PM_{2.5} exposure, indicating its potential value as a chemopreventive agent for reducing the risk of lung cancer associated with PM_{2.5} exposure. Finally, we found that *miR-195* could mediate the effect of D-limonene on lipid metabolism, suggesting that the miRNA may serve as a biomarker to monitor the use of D-limonene. Although our findings are supported by multiple lines of evidence from human to laboratory, more studies are needed to replicate and validate these observations before D-limonene can be tested in clinical trial for preventive intervention of lung cancer.

Abbreviations

LUAD: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma, COPD: Chronic obstructive pulmonary disease, SREBP1: Sterol Regulatory Element Binding Proteins 1, FASN: Fatty acid synthase, ACACA: Acetyl-CoA carboxylase, PVDF: Polyvinylidene difluoride, KO: Knock out, BMI: Body mass index, ICP-MS: Inductively Coupled Plasma Mass Spectrometry, GC-MS: Gas Chromatography Mass Spectrometer, CDS: coding sequence, WT: Wide type, NC: Negative control, 3' UTR: 3' untranslated region, DEGs: Differentially expressed genes, KEGG: Kyoto Encyclopaedia of Genes and Genomes, HB: Hebei province, SH: Shanghai city, OC: organic carbon, EC: elemental carbons, PAHs: Polycyclic Aromatic Hydrocarbons.

Declarations

Acknowledgments

We gratefully acknowledge Jianwei Xuan for supporting the SuValue database and Haixin Chen for supplying lung cancer samples in Hebei province.

Authors' contributions

T.T.Z., Y.Y.L., T.N.F. and B.Y.Q. conceived the project and designed the experiments. Y.Y.L., K.Z. and Y.Q.Y. designed, performed, and analysed cell and animal experiments. T.T.Z., X.W.Q. and Y.Q. designed, performed, and analysed human studies. T.N.F., J.G. and Y.H. performed bioinformatics and biostatistics analyses. T.T.Z., H.Y. and B.Y.Q. wrote the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (Grant NO. 81973135) and SJTU Medicine-Engineering Research Fund (Grant No. YG2019QNA71).

Availability of data and materials

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

Ethics approval and consent to participate

This study was approved by the Institutional Review Board at Tongren Hospital, Shanghai, China (No. 2019-077-02). Ten people were recruited into the D-limonene intervention group after strict inclusion and exclusion criteria were met (Trial number: ChiCTR2000030200). The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (IACUC-SIFDC17082). Collected data included clinical information and single nucleotide polymorphism results. No private information was used in this study, and patients did not receive extra medical intervention. Data were used only for research purposes. The content of the study was written in the informed consent form, which was signed by all patients. All acquired records and tissue used in the study were anonymized and could not be linked to any of the patients. Methods carried out in this study were in accordance with the approved guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021.
2. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature* 2018, 553(7689):446-454.
3. International Agency for Research on Cancer. Outdoor Air Pollution a Leading Environmental Cause of Cancer Deaths. *IARC* 2013, 109.
4. Chen J, Hoek G. Long-term exposure to PM and all-cause and cause-specific mortality: A systematic review and meta-analysis. *Environ Int* 2020, 143:105974.
5. Zhang S-Y, Shao D, Liu H, Feng J, Feng B, Song X, *et al.* Metabolomics analysis reveals that benzo[a]pyrene, a component of PM_{2.5}, promotes pulmonary injury by modifying lipid metabolism in a phospholipase A2-dependent manner in vivo and in vitro. *Redox Biology* 2017, 13:459-469.
6. Ku T, Zhang Y, Ji X, Li G, Sang N. PM_{2.5}-bound metal metabolic distribution and coupled lipid abnormality at different developmental windows. *Environmental Pollution* 2017, 228:354-362.

7. Salvador MM, de Cedrón MG, Rubio JM, Martínez SF, Martínez RS, Casado E, *et al.* Lipid metabolism and lung cancer. *Critical Reviews in Oncology/Hematology* 2017, 112:31-40.
8. Turner MC, Andersen ZJ, Baccarelli A, Diver WR, Gapstur SM, Pope CA, *et al.* Outdoor air pollution and cancer: An overview of the current evidence and public health recommendations. *CA Cancer J Clin* 2020.
9. Svensson RU, Parker SJ, Eichner LJ, Kolar MJ, Wallace M, Brun SN, *et al.* Inhibition of acetyl-CoA carboxylase suppresses fatty acid synthesis and tumor growth of non-small-cell lung cancer in preclinical models. *Nat Med* 2016, 22(10):1108-1119.
10. Yan R, Ku T, Yue H, Li G, Sang N. PM_{2.5} exposure induces age-dependent hepatic lipid metabolism disorder in female mice. *Journal of Environmental Sciences* 2020, 89:227-237.
11. Li L, Feng T, Zhang W, Gao S, Wang R, Lv W, *et al.* MicroRNA Biomarker hsa-miR-195-5p for Detecting the Risk of Lung Cancer. *International Journal of Genomics* 2020, 2020:7415909.
12. Lewis CA, Brault C, Peck B, Bensaad K, Griffiths B, Mitter R, *et al.* SREBP maintains lipid biosynthesis and viability of cancer cells under lipid- and oxygen-deprived conditions and defines a gene signature associated with poor survival in glioblastoma multiforme. *Oncogene* 2015, 34(40):5128-5140.
13. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, *et al.* Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A* 2007, 104(49):19345-19350.
14. Castro MA, Llanos MA, Rodenak-Kladniew BE, Gavernet L, Galle ME, Crespo R. Citrus reticulata peel oil as an antiatherogenic agent: Hypolipogenic effect in hepatic cells, lipid storage decrease in foam cells, and prevention of LDL oxidation. *Nutr Metab Cardiovasc Dis* 2020.
15. Berliocchi L, Chiappini C, Adornetto A, Gentile D, Cerri S, Russo R, Bagetta G, *et al.* Early LC3 lipidation induced by d-limonene does not rely on mTOR inhibition, ERK activation and ROS production and it is associated with reduced clonogenic capacity of SH-SY5Y neuroblastoma cells. *Phytomedicine* 2018, 40:98-105.
16. Nair SA, Sr RK, Nair AS, Baby S. Citrus peels prevent cancer. *Phytomedicine* 2018, 50:231-237.
17. Chen TC, Cho H-Y, Wang W, Barath M, Sharma N, Hofman FM, *et al.* A novel temozolomide–perillyl alcohol conjugate exhibits superior activity against breast cancer cells in vitro and intracranial triple-negative tumor growth in vivo. *Molecular cancer therapeutics* 2014, 13(5):1181-1193.
18. Santiago JVA, Jayachitra J, Shenbagam M, Nalini N. Dietary d-limonene alleviates insulin resistance and oxidative stress–induced liver injury in high-fat diet and L-NAME-treated rats. *European journal of nutrition* 2012, 51(1):57-68.
19. Dietrich DR, Swenberg JA. The presence of α₂u-globulin is necessary for d-limonene promotion of male rat kidney tumors. *Cancer research* 1991, 51(13):3512-3521.
20. Chaudhary S, Siddiqui M, Athar M, Alam MS. D-Limonene modulates inflammation, oxidative stress and Ras-ERK pathway to inhibit murine skin tumorigenesis. *Human & experimental toxicology* 2012, 31(8):798-811.

21. Vigushin DM, Poon GK, Boddy A, English J, Halbert GW, Pagonis C *et al.* Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. *Cancer chemotherapy and pharmacology* 1998, 42(2):111-117.
22. Yu X, Lin H, Wang Y, Lv W, Zhang S, Qian Y, *et al.* D-limonene exhibits antitumor activity by inducing autophagy and apoptosis in lung cancer. *OncoTargets and therapy* 2018, 11:1833.
23. Feng T, Zhang Q, Li Q, Zhu T, Lv W, Yu H, *et al.* LUAD transcriptomic profile analysis of d-limonene and potential lncRNA chemopreventive target. *Food & Function* 2020, 11(8):7255-7265.
24. Badyda AJ, Grellier J, Dąbrowiecki P. Ambient PM_{2.5} Exposure and Mortality Due to Lung Cancer and Cardiopulmonary Diseases in Polish Cities. *Adv Exp Med Biol* 2017, 944:9-17.
25. Wang L, Xu J, Liu H, Li J, Hao H. PM_{2.5} inhibits SOD1 expression by up-regulating microRNA-206 and promotes ROS accumulation and disease progression in asthmatic mice. *Int Immunopharmacol* 2019, 76:105871.
26. Wang Y, Zou L, Wu T, Xiong L, Zhang T, Kong L, *et al.* Identification of mRNA-miRNA crosstalk in human endothelial cells after exposure of PM_{2.5} through integrative transcriptome analysis. *Ecotoxicol Environ Saf* 2019, 169:863-873.
27. Desgagné V, Bouchard L, Guérin R. microRNAs in lipoprotein and lipid metabolism: from biological function to clinical application. *Clinical Chemistry and Laboratory Medicine (CCLM)* 2017, 55(5):667.
28. Balasubramanian S, Gunasekaran K, Sasidharan S, Jeyamanickavel Mathan V, Perumal E. MicroRNAs and Xenobiotic Toxicity: An Overview. *Toxicology Reports* 2020, 7:583-595.
29. Burgos-Aceves MA, Cohen A, Paoletta G, Lepretti M, Smith Y, Faggio C, *et al.* Modulation of mitochondrial functions by xenobiotic-induced microRNA: from environmental sentinel organisms to mammals. *Science of the Total Environment* 2018, 645:79-88.
30. Ning J, Li P, Zhang B, Han B, Su X, Wang Q, *et al.* miRNAs deregulation in serum of mice is associated with lung cancer related pathway deregulation induced by PM_{2.5}. *Environmental Pollution* 2019, 254:112875.
31. Liu B, Qu J, Xu F, Guo Y, Wang Y, Yu H, *et al.* MiR-195 suppresses non-small cell lung cancer by targeting CHEK1. *Oncotarget* 2015, 6(11):9445-9456.
32. Yu X, Zhang Y, Cavazos D, Ma X, Zhao Z, Du L, *et al.* miR-195 targets cyclin D3 and survivin to modulate the tumorigenesis of non-small cell lung cancer. *Cell Death & Disease* 2018, 9(2):193.
33. Sticht C, De La Torre C, Parveen A, Gretz N. miRWalk: An online resource for prediction of microRNA binding sites. *PLoS One* 2018, 13(10):e0206239.
34. Lu TP, Lee CY, Tsai MH, Chiu YC, Hsiao CK, Lai LC, *et al.* miRSystem: an integrated system for characterizing enriched functions and pathways of microRNA targets. *PLoS One* 2012, 7(8):e42390.
35. McDuffie EE, Martin RV, Spadaro JV, Burnett R, Smith SJ, O'Rourke P, *et al.* Source sector and fuel contributions to ambient PM_{2.5} and attributable mortality across multiple spatial scales. *Nat Commun* 2021, 12(1):3594.

36. Badyda AJ, Grellier J, Dąbrowiecki P. Ambient PM_{2.5} exposure and mortality due to lung cancer and cardiopulmonary diseases in Polish cities. In: *Respiratory treatment and prevention*. edn.: Springer, 2016: 9-17.
37. Guo H, Li W, Wu J. Ambient PM_{2.5} and annual lung cancer incidence: a nationwide study in 295 Chinese counties. *International Journal of Environmental Research and Public Health* 2020, 17(5):1481.
38. Hadei M, Hashemi Nazari SS, Yarahmadi M, Kermani M, Farhadi M, Shahsavani A. Estimation of gender-specific lung cancer deaths due to exposure to PM_{2.5} in 10 cities of Iran during 2013-2016: A modeling approach. *International journal of cancer management* 2017, 10(8).
39. Turner MC, Krewski D, Pope CA, Chen Y, Gapstur SM, Thun MJ. Long-term ambient fine particulate matter air pollution and lung cancer in a large cohort of never-smokers. *Am J Respir Crit Care Med* 2011, 184(12):1374-1381.
40. Badyda AJ, Grellier J, Dąbrowiecki P. Ambient PM_{2.5} Exposure and Mortality Due to Lung Cancer and Cardiopulmonary Diseases in Polish Cities. In: *Respiratory Treatment and Prevention*. edn. Edited by Pokorski M. Cham: Springer International Publishing, 2017: 9-17.
41. Zhang H, Tripathi NK. Geospatial hot spot analysis of lung cancer patients correlated to fine particulate matter (PM_{2.5}) and industrial wind in Eastern Thailand. *Journal of Cleaner Production* 2018, 170:407-424.
42. Ghazipura M, Garshick E, Cromar K. Ambient PM_{2.5} exposure and risk of lung cancer incidence in North America and Europe. *Environmental Research Communications* 2019, 1(1):015004.
43. Cao Q, Rui G, Liang Y. Study on PM_{2.5} pollution and the mortality due to lung cancer in China based on geographic weighted regression model. *BMC Public Health* 2018, 18(1):925.
44. Sun B, Shi Y, Li Y, Jiang J, Liang S, Duan J, *et al.* Short-term PM_{2.5} exposure induces sustained pulmonary fibrosis development during post-exposure period in rats. *J Hazard Mater* 2020, 385:121566.
45. Xu Z, Li Z, Liao Z, Gao S, Hua L, Ye X, *et al.* PM_{2.5} induced pulmonary fibrosis in vivo and in vitro. *Ecotoxicol Environ Saf* 2019, 171:112-121.
46. Deng X, Zhang F, Rui W, Long F, Wang L, Feng Z, *et al.* PM_{2.5}-induced oxidative stress triggers autophagy in human lung epithelial A549 cells. *Toxicology in vitro* 2013, 27(6):1762-1770.
47. Deng X, Feng N, Zheng M, Ye X, Lin H, Yu X, *et al.* PM_{2.5} exposure-induced autophagy is mediated by lncRNA loc146880 which also promotes the migration and invasion of lung cancer cells. *Biochimica et Biophysica Acta (BBA)-General Subjects* 2017, 1861(2):112-125.
48. Xu H, Jiao X, Wu Y, Li S, Cao L, Dong L. Exosomes derived from PM_{2.5}-treated lung cancer cells promote the growth of lung cancer via the Wnt3a/β-catenin pathway. *Oncology reports* 2019, 41(2):1180-1188.
49. Tirinato L, Pagliari F, Limongi T, Marini M, Falqui A, Seco J, *et al.* An overview of lipid droplets in cancer and cancer stem cells. *Stem cells international* 2017, 2017.

50. Chowdhury R, Amin MA, Bhattacharyya K. Intermittent Fluorescence Oscillations in Lipid Droplets in a Live Normal and Lung Cancer Cell: Time-Resolved Confocal Microscopy. *The Journal of Physical Chemistry B* 2015, 119(34):10868-10875.
51. Sunami Y, Rebelo A, Kleeff J. Lipid metabolism and lipid droplets in pancreatic cancer and stellate cells. *Cancers* 2018, 10(1):3.
52. Tirinato L, Liberale C, Di Franco S, Candeloro P, Benfante A, La Rocca R, *et al.* Lipid droplets: a new player in colorectal cancer stem cells unveiled by spectroscopic imaging. *Stem cells* 2015, 33(1):35-44.
53. Jarc E, Kump A, Malavašič P, Eichmann TO, Zimmermann R, Petan T. Lipid droplets induced by secreted phospholipase A2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2018, 1863(3):247-265.
54. Li J, Condello S, Thomes-Pepin J, Ma X, Xia Y, Hurley TD, *et al.* Lipid Desaturation Is a Metabolic Marker and Therapeutic Target of Ovarian Cancer Stem Cells. *Cell Stem Cell* 2017, 20(3):303-314.e305.
55. Mao S, Chen G, Liu F, Li N, Wang C, Liu Y, *et al.* Long-term effects of ambient air pollutants to blood lipids and dyslipidemias in a Chinese rural population. *Environ Pollut* 2020, 256:113403.
56. Liu C, Xu X, Bai Y, Wang TY, Rao X, Wang A, *et al.* Air pollution-mediated susceptibility to inflammation and insulin resistance: influence of CCR2 pathways in mice. *Environ Health Perspect* 2014, 122(1):17-26.
57. Mendez R, Zheng Z, Fan Z, Rajagopalan S, Sun Q, Zhang K. Exposure to fine airborne particulate matter induces macrophage infiltration, unfolded protein response, and lipid deposition in white adipose tissue. *Am J Transl Res* 2013, 5(2):224-234.
58. Jia SS, Xi GP, Zhang M, Chen YB, Lei B, Dong XS, *et al.* Induction of apoptosis by D-limonene is mediated by inactivation of Akt in LS174T human colon cancer cells. *Oncol Rep* 2013, 29(1):349-354.
59. Morse MA, Toburen AL. Inhibition of metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by limonene. *Cancer Lett* 1996, 104(2):211-217.
60. Miller JA, Lang JE, Ley M, Nagle R, Hsu CH, Thompson PA, *et al.* Human breast tissue disposition and bioactivity of limonene in women with early-stage breast cancer. *Cancer Prev Res (Phila)* 2013, 6(6):577-584.
61. Qian B, Nag SA, Su Y, Voruganti S, Qin J-J, Zhang R, *et al.* miRNAs in Cancer Prevention and Treatment and as Molecular Targets for Natural Product Anticancer Agents. *Current Cancer Drug Targets* 2013, 13(5):519-541.
62. Luo J, Pan J, Jin Y, Li M, Chen M. MiR-195-5p Inhibits Proliferation and Induces Apoptosis of Non-Small Cell Lung Cancer Cells by Targeting CEP55. *OncoTargets and therapy* 2019, 12:11465-11474.
63. Yongchun Z, Linwei T, Xicai W, Lianhua Y, Guangqiang Z, Ming Y, *et al.* MicroRNA-195 inhibits non-small cell lung cancer cell proliferation, migration and invasion by targeting MYB. *Cancer Letters*

2014, 347(1):65-74.

64. Yu X, Zhang Y, Wu B, Kurie JM, Pertsemlidis A. The miR-195 axis regulates chemoresistance through TUBB and lung cancer progression through BIRC5. *Molecular Therapy-Oncolytics* 2019, 14:288-298.
65. Liu B, Qu J, Xu F, Guo Y, Wang Y, Yu H, *et al.* MiR-195 suppresses non-small cell lung cancer by targeting CHEK1. *Oncotarget* 2015, 6(11):9445.

Figures

Figure 1

Lipid droplets were clearly detected, and LUAD cells became more aggressive after PM_{2.5} exposure. a Compared to the untreated cells (blank), lipid droplets were substantially increased in A549 and H1975 after the cells were treated with PM_{2.5}, as shown by electron microscope and oil red O staining. b-d PM_{2.5} treatment increased the migration, colony formation and invasion of A549 and H1975 cells according to the wound healing assays, plate colony formation and Transwell experiments.

Figure 2

Transcriptomic analysis showed that lipogenic genes were upregulated after PM_{2.5} treatment of A549 and H1975 cells. a Heat map showing the differential expression of lipid metabolism-related genes. b KEGG results showing enrichment of the DEGs in the lipid metabolism pathway. c Results of transcriptional regulatory relationships according to sentence-based text mining. d Verification of the target genes by RT-qPCR. SREBF1, FASN and ACACA were upregulated after PM_{2.5} exposure. AP, A549-blank group, AB, A549-PM_{2.5} group.

Figure 3

PM_{2.5} promoted N-SREBP1 translocation into the nucleus, inducing lipid droplet formation. a Western blot analysis showed that N-SREBP1, FASN, ACACA and SCAP proteins were upregulated in H1975 and A549 cells after PM_{2.5} treatment. b Fluorescent staining showed that N-SREBP1 in the nucleus of A549 and H1975 cells was increased significantly after PM_{2.5} treatment. c Oil red O staining showed that the production of intracellular lipid droplets was reduced after SREBF1 or FASN was knocked down. d-f After

SREBF1 or FASN was knocked down, the migration, colony formation and invasion abilities induced by PM2.5 treatment were inhibited.

Figure 4

D-limonene inhibited lipid metabolism disorder and lung cancer progression caused by PM2.5 exposure. a RNA-Seq data analysis showed that PM2.5-induced expression of SREBF1, FASN and ACACA was inhibited by D-limonene. b RT-qPCR verification of the RNA-Seq results. c The expression of miR-195

increased after treatment with D-limonene. d Dual luciferase experiments showed the binding of miR-195 with SREBF1, FASN and ACACA mRNAs. e The protein expression of SREBP1, FASN and ACACA was inhibited by miR-195. f PM2.5-induced expression of SREBF1, FASN and ACACA was inhibited by D-limonene. g Oil red O staining showed that PM2.5 exposure-induced lipid droplet formation was reduced after D-limonene or miR-195 treatment. h Western blot analysis showed that SREBP1, FASN and ACACA upregulated by PM2.5 exposure were attenuated by D-limonene or miR-195 treatment. i-g Plate colony formation and Transwell experiments showed that PM2.5-induced proliferation and invasion of lung cancer cells were inhibited by D-limonene or miR-195 treatment. PM, A549- PM2.5 group, PD, A549-PM2.5 + D-limonene group.

Figure 5

PM_{2.5} promotes pulmonary fibrosis, while D-limonene and miR-195 inhibit pulmonary fibrosis by attenuating lipid metabolism disorder. **a** Electron microscopic views of lipid droplets in normal lung epithelial cells (Beas-2b) and normal mouse lung tissue after long-term exposure to PM_{2.5}. The number of lipid droplets decreased after treatment with D-limonene. **b** RNA-Seq analysis showed that PM_{2.5}-induced upregulation of SREBF1, ACACA and FASN was suppressed after treatment with D-limonene. **c**

Western blot analysis showed that PM2.5-induced upregulation of SREBF1, ACACA, and FASN was attenuated by treatment with D-limonene in Beas-2b cells and normal mouse lung tissue. d PM2.5-induced upregulation of SREBF1, ACACA and FASN was inhibited by D-limonene inhalation in WT mice according to RT-qPCR. e TG levels in mouse serum increased after exposure to PM2.5, and the increase was suppressed after D-limonene treatment. f-g The upregulation of SREBF1, ACACA and FASN by PM2.5 in miR-195 KO mice was attenuated after D-limonene treatment (f), and the attenuation was more evident in WT mice (g). h-i Masson's trichrome staining showed that D-limonene repaired (or remove) the fibrosis caused by PM2.5 exposure (h), and the level of pulmonary fibrosis in miR-195 KO mice treated with D-limonene was significantly higher than that of WT mice (i). j Western blot analysis showed that PM2.5-induced upregulation of collagen I and vimentin was inhibited by D-limonene in WT mice. k Western blot analysis showed that PM2.5-induced upregulation of collagen I and vimentin was inhibited by D-limonene in miR-195 KO mice. CPM: C57BL/6J mice exposed to PM2.5. CPD: C57BL/6J mice exposed to PM2.5 and treated with D-limonene. MPM: miR-195 KO mice exposed to PM2.5. MPD: miR-195 KO mice exposed to PM2.5 and treated with D-limonene.

Figure 6

Serum levels of TG and tissue levels of lipid droplets in people living in regions with different PM2.5 pollution, and increases in miR-195 levels after oral administration of D-limonene. a 60% of people living in regions with high PM2.5 pollution had higher serum levels of TG serum levels of TG, whereas 52% of people living in low pollution areas had the same levels of TG. b Multivariate logistic regression analysis showed that PM2.5 exposure were associated with high TG levels. c Oil red O staining results showed

more lipid droplets in the lung tissue of cancer patients living in high than in low PM2.5 pollution areas. d
Changes in miR-195 levels in people taking D-limonene orally. SH, Shanghai City, HB, Hebei Province.

Figure 7

Diagram of the study. A possible mechanism underlying the PM2.5-induced lipid droplet accumulation, pulmonary fibrosis and aggressive tumor cell behaviors, as well as a potential intervention strategy to control these adverse effects.