Environmentally Persistent Free Radical Promotes Lung Cancer Progression Via Regulating The Expression Profile of miRNAs

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Abstract

Environmentally persistent free radicals (EPFRs) are generated in the combustion processes of solid waste and can cause adverse influences on human health, especially lung diseases. Lung cancer is one of the most serious malignancies in recent years, which the global deaths rate is about 1.6 million every year. However, the mechanism of EPFRs on lung cancer is still not clear. In this study, we verified that ZnO/MBM EPFRs promote cell proliferation and migration, impedes cell apoptosis in lung cancer. Furthermore, we found that ZnO/MBM could influence the expression of miRNAs (miR-18a and miR-34a). In vivo, ZnO/MBM and ZnO EPFRs can reduce the weight and survival rate of BALB/c male mice more than that of BALB/c female mice. In the ZnO/MBM exposed group, male mice lung became even smaller, while the female mice the lung increased significantly. Taken together, our results provide evidence for assessing the potential health risks of persistent free radicals on fine particles. In conclusion, this study linked toxicity of EPFRs with miRNAs revealed the potential health hazard to human lung cancer.

Introduction

At present, the amount of domestic waste is increasing and waste incineration treatment technology has attracted more and more attention because of its large amount of treatment, harmless treatment, good volume reduction, complete heat recovery and so on. However, the incineration of municipal solid waste (MSW) will produce a large amount of particulates, which will have a certain impact on the health of residents. A large number of epidemiological studies have confirmed that particulate matter produced by incineration, especially fine particles, is related to the morbidity and mortality of human heart and lung diseases. The toxicity of municipal solid waste incineration particulates is not only related to particle size, but also related to the toxic and harmful substances, such as transition metals and organic compounds. These toxic and hazardous substances form persistent free radicals on particulates during incineration. This compound has low reaction activity, which could exist in the environment. When they enter the human body, the organism can produce reactive oxygen species, destroy its normal redox balance, causing a series of inflammatory reactions and even cancer. EPFRs can produced ROS (reactive oxygen species) may affect ecological health or mediate the degradation of pollutant. Among inorganic metal oxides, ZnO materials are believed to be full of promise for biochemical sensors, antibacterial agents, food packaging and photocatalysts. Anti-tumor efficacy of ZnO has also been studied with or without light.

Lung cancer is one of the top cancer deaths in the worldwide, among which non-small cell lung cancer (NSCLC) accounts for nearly 80%. The 5-year survival rate of NSCLC is very low (less than 15%), due to when it is diagnosed at an advanced stage and the disease recurrence or metastasis. The prevalence and mortality of this disease highlight the consequence importance of investigating the mechanisms involved in the tumorigenesis of NSCLC, especially the mechanism related to particulate damage.
microRNA (miRNA) is a class of small non-coding RNA molecules (19–22 nucleotides, nts), which plays an essential role in regulating protein expression through inhibiting translation or inducing mRNA degradation by binding to the 3'-untranslational region (3'-UTR) of target mRNAs. miRNAs are proved to regulate a variety of human diseases including cancer, diabetes, heart diseases, asthma, etc. It has been demonstrated that miRNAs have important functions in tumorigenesis by regulating the cell apoptosis, cell differentiation and cell proliferation. EPFRs can adjust cancer initiation and progression via regulating miRNAs expression. MiRNAs can regulate the expression of tumor associated genes and involved in the tumor progression.

In this research, we detected the diameter of the persistent free radical (ZnO/MCB) were below 2.5 µm, which was fine particle size. Our previous study has analyzed the EPR signal diagram of synthetic samples, the g value of ZnO/MCB was 2.0036, which was consistent with the persistent radical type of oxygen atom with the center of carbon as the center. This study found EPFRs can influence inflammatory condition and apoptosis in cell level and in lung lesions of the in vivo mice model, which though there are still unknowns to explore in regulating tumor. Therefore, we propose that exposure to ZnO/MCB may regulate the expression of related miRNAs. We searched for miRNAs which might influence lung cancer and select the most promising ones for further investigation.

In this study, we explore whether toxicity effects of ZnO/MCB EPFRs can regulate by miRNAs in NSCLC. Our data provide a fundamental understanding of MSW fine particle involvement in NSCLC and demonstrate the toxicity of EPFRs of fine particulates from solid waste incineration plants that potentially threat to the public health.

**Materials And Methods**

**Cell culture**

BEAS-2B, A549, and H1299 cells were gained from the Cell Bank, China Academy of Sciences (Shanghai, China). H1299 cell was cultured in RPMI-1640 medium. BEAS-2B and A549 cells were cultured in DMEM medium. All media were supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT, USA), an antibiotic cocktail of 100 µg/mL streptomycin and 100 U/mL penicillin (Gibco). Cell culture was performed at 37°C in a 5% CO₂ humidified environment.

**RNA extraction and Quantitative RT-PCR**

Total RNA was extracted with TRIzol reagent (Bio Basic Inc., Toronto, Canada). Reverse transcription was carried out with the QuantiMir cDNA Kit (Takara) and PrimeScriptTM RT reagent Kit (Takara). According to the manufacturer's protocols, quantitative PCR was performed with SYBR Premix Ex Taq™ (Takara). U6 snRNA was used as the endogenous controls for mRNA and miRNA. Results were expressed using the relative quantification ($2^{-\Delta\Delta Ct}$) method. Primer sequences are shown in supplementary table 1.

**Cell proliferation assay**
Cell proliferation was performed with the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). Briefly, cells were plated in a 96-well plate at a density of $2 \times 10^3$ cells per well and incubated at 37˚C in a 5% CO$_2$ humidified environment. CCK-8 was added and cells were returned to incubate for 2.5 h. Light absorbance at 450 nm was measured daily with a microplate reader. Experiments with triplicates were performed independently at least thrice.

**Cell apoptosis**

Cell apoptosis assay was performed as previously described $^{20}$. An Annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instruction to determine the cell apoptosis level. Cells were resuspended in 1 × binding buffer solution with Annexin V-fluorescein isothiocyanate and propidium iodide, after then incubated at room temperature for 15 min in the dark. Apoptotic cells were analyzed using a MoFlo XDP flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Experiments with triplicates were performed independently at least thrice.

**Cell migration assay**

Cell migration assay analysis was carried out as previously described $^{29}$. BEAS-2B, A549 and H1299 were seeded at $1 \times 10^5$ cells per well in 24-well plate and allowed to reach confluence. A single-scratch wound was introduced through the middle of each well with a sterile pipette tip. Cell migration across the margins was evaluated and photographed after 24 h. Experiments with duplicates were performed independently at least thrice.

**Mice model establish and experiment**

In this part of the experiment five-week-old female BALB/c mice were purchased from the SLRC Laboratory Animal Centre (Shanghai, China). The mice housed under specific pathogen free conditions at the animal facility of the Institutional Animal Care and Use Committee of Shanghai University (Shanghai, China) were used for the animal experiments. The animals were random allocation to 4 groups: Air, 0.9% NaCl, ZnO, and ZnO/MB. The mice were subjected to nasal drip exposures of 20 µL 0.9% NaCl, ZnO or ZnO/MB at 5 µg/mL or air for 4 times every other day. Eight days later, the mice were euthanized and lung tissues were isolated, in the meantime their weights were evaluated. Lung tissues were subjected to serial sectioning and haematoxylin and eosin (HE) staining. Pathological changes were observed under a light microscope (Nikon, Tokyo, Japan). Each group consisted of 6–8 mice and all experiments were performed in triplicate. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Shanghai University (Shanghai, China). All efforts were made to minimize suffering. The study is reported in accordance with ARRIVE guidelines $^{30}$.

**Statistical analyses**

All experimental data are expressed as the group means ± SEM and analyzed using GraphPad Prism 8 software, using t-tests for 2-group comparisons and one-way ANOVA for three or more group comparisons. Differences were considered statistically significant when $p < 0.05$. 

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Ethics approval

In this study, all the findings data acquisition and processing comply with the ethical standards described in the latest declaration of Helsinki. Animals were handled according to national and institutional guidelines for animal research. The Ethics Committee of Shanghai University approved the experimental study (Authorization No. 2020-001).

Results

ZnO/MCB promotes cell proliferation and impedes cell apoptosis in NSCLC

To verify the role of ZnO/MCB in NSCLC, we first measured its proliferative effects in several NSCLC cell lines such as A549 and H1299, control with normal lung epithelial cell line BEAS-2B. Three cell lines were incubated with different concentrations (10, 20, 30, 40 µg/ cm²) of fine particles for 24 h and the cell viability was then determined by CCK-8. The results showed that ZnO/MCB could significantly inhibit the proliferation of BEAS-2B in a dose-dependent manner, ZnO as control group (Fig. 1a). The cell viability of the ZnO/MCB treatment at the concentration of 20 µg/cm² decreased to 59%. In contrast, cell viability of A549 and H1299 at the concentration of 20 µg/cm² increased to 109.67% and 115.67%, respectively (Fig. 1b, 1c). The data collectively suggest that the ZnO/MCB particles have cytotoxicity on bronchial cells while stimulate the proliferation of NSCLC cells.

Next, to investigate the underlying mechanism of action that ZnO/MCB exerts on the lung cancer cell proliferation, we performed flow cytometry to analyse the effect of ZnO/MCB on cell apoptosis in BEAS-2B, A549 and H1299 cell lines. The percentage of apoptotic cells for the BEAS-2B cell line was increased remarkably (Fig. 1d). Conversely, the percentage of apoptotic cells for the A549 and H1299 cell line significantly decreased. The apoptosis rate of the A549 and H1299 cell lines was 3.27% and 1.80%, respectively upon treatment with 20 µg/cm² of ZnO/MCB for 24 h, decreased by 36% and 39% compared with the control group (Fig. 1d). This demonstrated that ZnO/MCB was more significant in the ability of inducing apoptosis.

ZnO/MCB promotes the migration of NSCLC

Furthermore, ZnO/MCB affected migration ability which was a vital aspect of cancer progression. We performed wound healing assays. After treatment with 20 µg/cm² of ZnO/MCB for 24 h, the A549 and H1299 cells migrated toward the wound at a much faster rate than the NC and ZnO group, as well as BEAS-2B which the migration ability don’t obviously (Fig. 2a-c). Results showed that ZnO/MCB could promote the migration of A549 and H1299 cells (Fig. 2d).

These results suggested that ZnO/MCB could exhibit an important effect in NSCLC tumor cells via promotion of cell proliferation and migration, inhibition of apoptosis.
ZnO/MCB up-regulates the expression of miR-18a-5p and down-regulates the expression of miR-34a

We tested miR-199a-3p, miR-199a-5p, miR-34a, miR-107, Let-7c, Let-7b, Let-7d, because of their explicit antitumor effect in lung cancer and their positions as the miRNAs our group has confirmed[20,31–33]. After treatment with 20 µg/cm² of ZnO/MCB for 24 h in BEAS-2B, we also tested the expression of miR-150, miR-411, miR-18a-5p, miR-183, miR-182, miR-96, which we have demonstrated that miRNAs were oncogenic in lung cancer[29,34–36].

Results from qRT-PCR assays showed that the expression levels of the miRNAs being tested (miR-199a-3p, miR-199a-5p, miR-107, Let-7c, Let-7b, Let-7d) were no significant changes compared with control group. Only the expression level of miR-34a was significantly down-regulated by ZnO/MCB (Fig. 3a). In contrast, the expression levels of the miRNAs being tested (miR-150, miR-18a-5p, miR-183, miR-182) were significantly up-regulated by ZnO/MCB (Fig. 3b).

We next focused on miR-34a and miR-18a-5p, as they have been reported that miR-34a may be an anti-oncogene in lung cancer and miR-18a may be an oncogene in lung cancer[35]. However, the relationship between miR-34a/miR-18a and EPFRs in NSCLC has yet to be brought to light. We set out to uncover the contact between miR-34a or miR-18a and EPFRs in NSCLC.

**MiR-18a-5p promotes cell proliferation and impedes cell apoptosis of NSCLC after exposure to ZnO/MCB**

To investigate the effects of ZnO/MCB whether was mediated by miR-18a-5p or miR-34a, we used A549 cell line, which stably over-expressing miR-18a-5p (pLenti-miR-18a) or miR-34a (pLenti-miR-34a) and negative control (pLenti). We examined the effect of miR-18a-5p and miR-34a on the proliferation after dosing 20 µg/cm² of ZnO/MCB with CCK-8 assay in A549. Results showed that ZnO/MCB significantly increased the proliferation of the A549 (pLenti-miR-18a) (Fig. 3c). There was no influence on A549 (pLenti-miR-34a) proliferation exposed to ZnO/MCB (Fig. 3d). Previous studies indicated that miR-18a may be a tumor promoter gene and our results confirmed that it could promote cell proliferation of tumor cells in NSCLC. But miR-34a had no obvious effect on cell proliferation after exposure to ZnO/MCB in A549 cell line.

Furthermore, the flow cytometry assessed that the apoptosis rate in A549 cell line, which stably overexpressing miR-18a-5p (pLenti-miR-18a) or miR-34a (pLenti-miR-34a). The apoptosis rate was decreased visibly in miR-18a-5p or miR-34a after exposed to ZnO or ZnO/MCB (Fig. 3e). Then, we also performed wound healing assays in miR-18a-5p A549 and miR-34a A549. Results showed that ZnO/MCB could promote the migration of miR-18a-5p A549, but miR-34a could suppress migration which may because of it as a tumor suppressor gene (Fig. 3f, g).
These results demonstrated that ZnO/MCB could promote the proliferation and migration, inhibit the apoptosis of NSCLC cells via increased the level of miR-18a-5p.

**Exposing to ZnO/MCB causes lung lesions in vivo**

To confirm would cause lung lesions, we established a mice model to explore the biological effects of fine particles. ZnO/MCB and ZnO can reduce the mice body-weight (Fig. 4a, b) and lung-weight (Fig. 4c, d) of BALB/c male mice more than BALB/c female mice, and EPFRs (ZnO/MCB) has greater influence. After 28 days of chronic exposure, the survival rate of the ZnO/MCB group was as low as 25% (Fig. 4e, f). The order of influence of the four samples on the lung morphology of BEAL/c mice was: EPFRs > control group. In the ZnO/MCB exposed group, the lung morphology of the female mice became smaller, but the lung morphology of the male mice increased significantly (Fig. 4g, h). H&E staining shows that there was an obvious decrease in the thickness of the alveolar walls in BALB/c mice exposed to ZnO/MCB, causing lung lesions (Fig. 4i). Taken together, the effects of four samples on the pathological changes of lung tissue in BEAL/c mice were: ZnO/MCB > ZnO > Nacl > Blank. These results suggest that fine particles, especially ZnO/MCB, induce inflammation and lung lesions *in vivo*.

**Discussion**

There are many hypotheses about the mechanism of the emission of fine particulate EPFRs, including oxidative damage, inflammatory damage and mitochondrial damage\(^{37}\). The smoke from burning tobacco also has health effects. Oxidative damage refers to a series of reactions in which EPFRs formed on the surface of fine particles enter the cell, stimulating the body to produce a large amount of ROS, destroying the body's redox equilibrium state\(^9,38\), thereby causing lipid peroxidation of the cell membrane, accelerating protein hydrolysis or damage oxidation, resulting in cell damage and even apoptosis\(^10\). Inflammatory injury means that the body stimulates the transcription level of related inflammatory factors in the body under external stimulation, thereby inducing inflammatory cells to produce many inflammatory factors or adhesion factors. Such as interleukins, interferons, growth factors, tumor necrosis factors, etc., the body producing inflammatory response and ultimately cause inflammatory damage\(^{39,40}\).

Inflammation is a common pathological process in the clinic. It can be produced in tissues and organs of various parts of the body, such as pneumonia, hepatitis, and nephritis\(^{41-43}\). The production of inflammation is essentially a reflection of the body's resistance to inflammatory factors. This divergence struggles throughout the inflammatory process. After acting on the body, the inflammatory factor causes damage to the tissue cells, causing local tissue cells to show degeneration and necrosis. On the other hand, it induces an increase in the body's resistance to disease, which is beneficial to clearing the inflammatory factors and repairing the damaged tissue\(^{44}\). The fine particles enter the bronchus, causing the body to produce excessive inflammatory factors, breaking this balance and causing a series of adverse reactions. The fine particles exposed below PM\(_{10}\) are related to the poor health of human body, which will lead to respiratory diseases such as chronic obstructive pulmonary disease and asthma\(^{45}\).
Study have verified that short term exposure to concentrations below the EPA standard ($\text{PM}_{2.5} < 12 \ \text{ug/m}^3$) can lead to decreased lung function. Long-term exposure to particulate matter below $\text{PM}_{10}$ is associated with the occurrence and mortality of lung diseases, and the incidence increases with the longer exposure time.

At present, there are also some studies on the regulatory effect between the relevant environmental fine particles and miRNA in human disease. Studies have found that the longer exposure to $\text{PM}_{2.5}$, the more it can inhibit the expression of some miRNAs (miR-21-5p, miR-187-3p, miR-1-3p, miR-146a-5p and miR-199a-5p) and promote the expression of inflammatory factors such as IL1 and TNF. Zhang et al. demonstrated that when HBE cells are exposed to PM2.5, miR-382-5p triggers lung inflammation through the targeting relationship with CXCL12. Li et al. showed that overexpression of miR-224 inhibited the airway epithelial cell inflammation and airway remodeling induced by $\text{PM}_{2.5}$ in asthmatic mice by reducing the expression of TLR2. However, more in-depth studies are needed on the potential biological mechanisms between exposure to air pollutants and miRNA, exploring the role of miRNA in $\text{PM}_{2.5}$ between normal and cancer cells.

In this study, we used Zinc Oxide (ZnO) as raw material, respectively pumped in chlorobenzene (MCB) gas, synthesis of the persistent free radicals (ZnO/MCB) in vitro and in vivo. In vitro experiment, normal lung cells (BEAS-2B) and lung cancer cells (H1299 and A549) as the research object, researched the cell toxicity difference and its precursor mechanism between ZnO and ZnO-EPFRs. In vivo experiments, BALB/c mice as the research object, explore the chronic toxicity difference between ZnO and ZnO-EPFRs. In conclusion, our findings have showed that ZnO/MCB EPFRs promote cell proliferation and migration, impedes cell apoptosis in NSCLC. ZnO/MCB EPFRs make the expression of miR-18a up-regulate and down-regulates the expression of miR-34a. Besides, exposure to ZnO/MCB EPFRs in NSCLC, miR-18a promotes cell proliferation. Furthermore, our results showed that the EPFRs reduce the body weight and survival rate of male mice was greater than female mice in vivo experiments, which also caused lung lesions. Overall, our findings provide evidence for assessing the potential health risks of persistent free radicals on fine particles within the regulation network (Fig. 5). Nevertheless, fine particulate stimulation of the body’s production and secretion of chemokines and cytokines are important steps in inflammatory damage, and their combination can affect the strength of the inflammatory response.

Declarations

Author contributions

Zhongliang Ma, Jie Lu and Guangren Qian designed the study and approved the final version of the submitted article. Xiaomin Liu and Binshu Chai developed the methodology and researched the data. Xiaomin Liu, Binshu Chai, Xianyi Wang, Zong Wu, Xing Zhang, Heng Zou, Yangyang Liu and Saijing Zheng performed the experiments. Xiaomin Liu, Zong Wu, Xing Zhang and Heng Zou analysed and
interpreted the data. Zhongliang Ma, Xiaomin Liu and Binshu Chai wrote and edited the manuscript. All authors read and approved the contents of the manuscript and its publication.

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Data availability

Authors can confirm that all relevant data are included in the article or its supplementary information files.

Conflict of interest

The authors have no conflict of interest to declare that are relevant to the content of this article.

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**Figures**
ZnO/MCB could promote cell proliferation and impede cell apoptosis in NSCLC. (a-c) The proliferation of BEAS-2B, A549 and H1299 cell lines, after treatment with different concentrations (10, 20, 30, 40 μg/cm²) of fine particles (ZnO, ZnO/MCB) for 24 h, which measured by CCK-8 assay. (d) The rate of apoptosis was analyzed by flow cytometry following treatment with fine particles in BEAS-2B, A549 and H1299 cell lines. *P<0.05, **P<0.01, and ***P<0.001.
ZnO/MCB could promote migration in NSCLC cells. (a-c) BEAS-2B, A549 and H1299 cells incubated with 20 μg/cm² of ZnO/MCB for 24 h were subjected to wound healing assay and images were taken at 0 h and 24 h. (d) The rate of migration was analyzed following treatment with fine particles in BEAS-2B, A549 and H1299 cell lines. *P<0.05, **P<0.01.
Figure 3

ZnO/MCB up-regulates the expression of miR-18a and down-regulates the expression of miR-34a. (a, b) The mRNA levels of miRNAs were detected by qRT-PCR in BEAS-2B cells treatment with fine particles (ZnO, ZnO/MCB). (c, d) The proliferation of A549 (pLenti-miR-34a/pLenti and pLenti-miR-18a/pLenti) cells following treatment with fine particles (ZnO, ZnO/MCB), as measured by CCK-8 assay. (e) The apoptosis rates of A549 (pLenti-miR-34a/pLenti and pLenti-miR-18a/pLenti) cells following treatment with fine particles (ZnO, ZnO/MCB) were analyzed by flow cytometry. (f, g) The wound healing assay and
images were taken at 0 h and 24 h of A549 (pLenti-miR-34a/pLenti and pLenti-miR-18a/pLenti) cells following treatment with fine particles (ZnO, ZnO/MCB). *P<0.05, **P<0.01, and ***P<0.001.

Figure 4

Mouse model exposure to EPFRs (ZnO/MCB). (a, b) The body weights of BALB/c mice which 6-week-old female/male after nasal drip exposures of 20 μL 0.9%NaCl, ZnO or ZnO/MCB at 5 μg /mL or air for 4 times every other day. (c, d) The lung weights of BALB/c mice 6-week-old female/male. (e, f) The survival rate of BALB/c mice. (g, h) Images of lung are displayed. (i) The lungs of mice with metastasis disease are displayed.
Figure 5

The regulatory network of EPFRs (ZnO/MCB) in NSCLC. We present that the potential health risks of persistent free radicals on fine particles.

Supplementary Files

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• SupplementaryTable1.docx