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Effects of fine air particulates on gene expression in non-small-cell lung cancer

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ABSTRACT

Purpose: Airborne particulate matter smaller than 2.5 μm ($PM_{2.5}$) has been shown to induce adverse health effects through various mechanisms. However, its effects on gene expression in non-small-cell lung cancer (NSCLC) remain undefined. The aim of this study was to analyze the expression profile of $PM_{2.5}$-induced adverse health effects on human.
Materials and methods: We performed RNA sequencing to elucidate key molecular effects of $PM_{2.5}$ collected from Shenyang China, to identify potential diagnostic markers or therapeutic targets, and further validated these differences in gene expression by using quantitative PCR in A549 and H1299 human non-small-cell lung cancer cell lines. To investigate the functional changes on $PM_{2.5}$ exposed cells, we carried out the viability assay for the cell counting, and the Boyden chamber assay for invasion.
Results: We found 143 genes that were expressed at least twice as much, or no more than half as much, in NSCLC cells exposed to $PM_{2.5}$ than in unexposed cells. Results showed deregulated genes confronted $PM_{2.5}$ exposure were significantly expressed, but commonly expressed in NSCLC cells. In addition, according to the viability assay and the Boyden chamber assay, $PM_{2.5}$ exposed cells which have more competent on proliferation and invasion can keep the line with the results in RNA-Seq.
Conclusion: Our data may provide a more specific understanding of the signaling patterns associated with pathogenesis, and lead to novel markers and therapeutic targets for NSCLC.
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1. Introduction

Airborne particulate matter (PM) smaller than 2.5 μm ($PM_{2.5}$) has been shown to be hazardous to human health and the natural environment. It is generated by various sources and therefore is a heterogeneous mixture of various substances. Airborne PM is divided into total suspended particulates (TSP), including coarse PM (particles with a median aerodynamic diameter <10 μm [PM_{10}]), fine PM (particles <2.5 μm, i.e., $PM_{2.5}$), ultrafine particles (<0.1 μm [$PM_{0.1}$]), and nano-PM [1,2]. For human health, composition of these mixtures is the most important risk factor [3]. In terms of both number and surface area, fine and ultrafine PM are effectively the most toxic portions of airborne particulates [4]. Therefore, researchers of particulate air pollution focus primarily on $PM_{2.5}$ rather than PM_{10}.

Numerous investigations of the effects of $PM_{2.5}$ on human health have mainly addressed its cytotoxic effects [3,5–7]. Epidemiological surveys have also shown the negative effects of air pollution on the human body, especially in the respiratory system [8–13]. Although lung cancer is the leading cause of cancer-related deaths worldwide-especially non-small-cell lung cancer (NSCLC), which accounts for more than 80% of all lung cancers – the mechanisms that underlie the effects of $PM_{2.5}$ on lung cancer remain unclear [14–16]. Furthermore, the highly variable composition of airborne particulate mixtures complicates this sort of research, although evaluation of changes in gene expression in the respiratory tissues upon exposure to such mixtures is clearly needed.

Our preliminary studies indicated that A549 cells cultured with supernatants from cells exposed to $PM_{2.5}$ had stronger proliferation ability and were more vigorous in a wound-healing assay, which implies that $PM_{2.5}$ exposure induces a cross-talking pathway that promotes survival, proliferation, invasion, and migration of tumor cells.

In this study, we exposed NSCLC cells to $PM_{2.5}$ and analyzed data from RNA sequencing (RNA-seq) to identify differential gene

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expression between exposed and unexposed cells. We used microarray analysis of A549 cells exposed to $PM_{2.5}$ to conduct gene expression profiling, and gene ontology (GO) and analyses using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, and identified those of differentially expressed genes in A549 and H1299 cell lines by using quantitative PCR. Further to investigate the functional changes on $PM_{2.5}$ exposed cells, we carried out the viability assay for the cell counting, and the Boyden chamber assay for invasion.

2. Materials and methods

2.1. Sampling and characteristics of $PM_{2.5}$

$PM_{2.5}$ samples on nitrocellulose filters were prepared as described in our previous studies, and the analysis of $PM_{2.5}$ composition was performed [3,17]. Briefly, $PM_{2.5}$ samples were collected on nitrocellulose filters (Waterman, USA) using a high-volume sampler particle collector equipped with a Hi-Vol $PM_{2.5}$ inlet (Tisch Environmental, USA) at a constant flow rate of 1.13 m^3/min in the center of the downtown area of Shenyang. Particulate matters of the aerodynamics diameter less than 2.5-μm can deposit on filter membrane sampler. $PM_{2.5}$ were extracted from sampled filter strips by immersing them in deionized water and sonication for 30 min. The stock $PM_{2.5}$ solution (5 mg/ml in PBS) was stored at −80 °C until cell exposure. The frozen $PM_{2.5}$ aliquots were thawed and briefly sonicated before being added into the cell culture media for the in vitro experiments. Chemical composition of the $PM_{2.5}$ we collected was analyzed. Briefly, heavy metals and polycyclic aromatic hydrocarbon substances are the compositions of $PM_{2.5}$. The 40 kinds of elements were investigated and the concentration of each was determined.

2.2. Cell culture, in vitro exposure to $PM_{2.5}$

Human NSCLC cell lines A549 and H1299 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin G and 100 μg/ml streptomycin (Hyclone, USA) in a humidified chamber at 37 °C and 5% CO_2. For cell growth, the medium was renewed every 3 days and cells were subcultured twice per week. For RNA Sequencing, before total RNA was extracted, the cells were seeded into 6-well plates, grown to 85% confluence and tackled with $PM_{2.5}$ at the concentration of 50 μg/cm^2 for 72 h, and the same amount of PBS (Phosphate Buffered Saline) was added in the control group. After 3-day incubation, the cells were harvested for the other assays.

2.3. RNA sequencing and enrichment analysis

Transcriptome high-throughput sequencing were performed at the Shanghai Biochip Company, according to the protocols in the HiSeq 2500 Sequencing System. And analyze the FPKM (Fragments Per Kilobase of exon model per Million mapped reads) values that was quantitative normalization, originated from the differential genes on two samples using cufflinks. Differentially expressed genes were identified through fold change ≥2 and false discovery rate ≤0.05. The gene ontology (GO) project aims to describe gene and gene product attributes (http://www.geneontology.org), which covers three domains: biological process, cellular component and molecular function. Pathway analysis is a functional analysis that maps genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (http://www.genome.jp/kegg/). The enrichment level was calculated by transforming the enrichment p values after false discovery rate (FDR) correction to negative log10 values, and the lower the p-value is the more significant the correlation (a p-value cut-off is 0.05).

2.4. Network construction

In our study we used the protein–protein interactions from the STRING database, The integrative network of $PM_{2.5}$-mediated non-small cell lung cancer protein interactions was drawn, which integrates and weighs information from numerous sources, including activation, inhibition, binding, catalysis, reaction, and expression [18]. The scores higher than 0.7 will be considered as high confidence, thus, we used the interactions with combined scores higher than 0.7 for further analysis (Table 1).

2.5. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed in triplicate to detected the fold changes of candidate genes, using an ABI Prism 7500 (Applied Biosystems, USA) and SYBR Select Master Mix (Applied Biosystems, USA) according to the

Table 1
Differentially expressed genes in A549 and H1299 cells after the confrontation with $PM_{2.5}$ exposure.

Genes	Microarray Fold change	Q-PCR (A549 cells) Fold change	Q-PCR (H1299 cells) Fold change
Down-regulated genes			
LAMA5	3.8	1.8	1.5
TGFB2	3.7	2.3	2.1
TNFRSF1A	2.2	1.5	1.8
TP53	3.5	2.8	2.1
Up-regulated genes			
ADM	2.4	1.8	1.7
CYP1B1	3.1	2.8	2.6
EREG	2.5	2.4	2.0
IL1A	9.1	4.6	3.9
IL1B	13.8	7.5	7.0
ITGB3	3.0	1.7	1.6
MMP1	162.7	27.8	23.1
MT1X	3.2	2.3	1.9
PGM3	3.8	2.8	2.7
PPP1R14A	4.8	4.2	3.5
RND3	2.8	1.8	1.9
SPHK1	2.6	1.6	1.7

Table 2
List of primers for RT-PCR.

Gene	Forward	Reverse
LAMA5	GGTTCTCCATTCATTTCAGTGC	GAAGGTTGAGGGTTCAGTGG
TGFB2	CCCATACCTAAGCCCTCTGG	GTTGCCTTCCCTTTCTTTCC
TNFRSF1A	GGTCTCAACGCCATCCTG	GCTCCATTTATCAGAACATCTCC
TP53	ATGGCACTGAGGAAGATGCT	CAGATAATGCGGGAAAGAGG
ADM	CCCGAGACTGACACACTGAA	ACCCTGACTTTGGCGAGTAA
CYP1B1	CTCCTCTCGCTCTCCTCCTC	TTCTGGCTGGCTCTTCTCTC
EREG	ATGCCCGATGAGATCAACA	CGACAGGTTTCCCACATGAC
IL1A	GTGGGCTGTGCCAAGTGT	GGTCACGGTCAGGGTTGTA
IL1B	GAACTCCTGCTTCTCCTTGC	ACTTGGCACAGCCCACAG
ITGB3	GCCAGCACCATCTCTTTACC	GCACTCTCTCCCTTTGAGGA
MMP1	GCCTCTGATTGGTGAATGGT	TCTTGTCCCTCTGGTCCTGT
MT1X	TGGCAGAAAGGGAACAGAAA	CTGGCTGATGGACAGGAGAT
PGM3	AAGACCCAAAGGCAGCAAA	CCCAGGCAAGGCAACATT
PPP1R14A	TTGACACAGGAAATGGGAAA	ATCTCAGCCTCCACAGGAA
RND3	ACCGCAACTTCAGCAACTTC	AGAGAGGATAAAGGCGTCCA
SPHK1	CCGACTATGGACTCACAGCA	AGGCGCTTGTAGCAGTTGAT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

ADM, adrenomedullin; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; EREG, epiregulin; IL1A, interleukin 1, alpha; IL1B, interleukin 1, beta; ITGB3, integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); MMP1, matrix metallopeptidase 1 (interstitial collagenase); MT1X, metallothionein 1X; PGM3, phosphoglucomutase 3; PPP1R14A, protein phosphatase 1, regulatory (inhibitor) subunit 14A; RND3, Rho family GTPase 3; SPHK1, sphingosine kinase 1; LAMA5, laminin, alpha 5; TGFB2, transforming growth factor, beta 2; TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A; TP53, tumor protein p53; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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