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Investigating The effect of Particulate Matter (PM2.5) On The expression Profile and Methylation of EGR Gene Family and *P53* Gene in The lung Tissue of Rat Exposed to Air Pollution

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PARTICLES smaller than 2.5 µm in size and diameter of particulate matter (PM2.5) are known to be associated with several cancers, lung and cardiovascular disease, cognitive, hormonal and reproductive disorders. The present study investigated the effect of PM2.5 exposure on the expression level and methylation of the transcription factors EGR1 and EGR2 and the well-known proto-oncogene P53 in lung tissue of rat model of air pollution exposure.48 adult Wistar albino rats (190±35 g) were classified into three treatment and control groups. The current study results were showed a significantly low expression of *EGR-1*, *EGR-2*, and *P53* in both exposure groups (exposure 1 and 2) compared with the control group. Also, significant down expression of *EGR-1* and *EGR-2* in group exposure one was revealed versus group exposure 2. MS-HRM assays detected hypermethylation of exposure one and exposure two groups compared to control in six months (for *EGR-1*, *EGR-2*, and *P53*). Our findings revealed that PM2.5 exposure could provoke downregulation and hyper-methylation on transcription factors and proto-oncogenes that, in turn, may alter the expression level and function of several essential genes downstream of molecular pathways. PM2.5 exposure could potentially accelerate several respiratory diseases and cancers and should be considered as a health concern.

Keywords: PM2.5 exposure, EGR-1, EGR-2, P53.

Introduction

One of the most important and serious problems in environmental health is air pollution that led to a variety of health issues, such as pulmonary diseases [1], abnormalities of the heart and cardiovascular system [2], and death [3]. Modernization and industrialization of world society accelerated urban development as well as air pollution escalation. Exposure to air pollutants such as gaseous pollutants and particulate matter (PM), particularly in the lungs, can be the cause of several lung diseases [4]. The lung is a sensitive organ and composed of a complex assemblage of more than 40 different cell types [5]. Research by researchers showed that PM is associated with

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hospitalization due to respiratory and cardiac diseases [5]. Air pollutants are a combination of gaseous pollutants and particulate matter (PM), among which small particles with a diameter of 2.5 micrometers, known as PM2.5, are the main cause of diseases and could play the main role impact on human health [6]. PM2.5 with a small diameter can easily pass through the respiratory system, on the other hand, due to its toxicity and its accumulation at the end of the respiratory tract and bronchi and its spread in the body causes serious physical injury that the lungs are main members are exposed. While the PM2.5 impact are chronic, exposure to heavy air pollution, for example exposure to PM10 and coarse particles cause respiratory diseases to increase and worsen [7]. Enrichment Analysis of gene expression profiling assessments revealed that metabolic processes, stress response, and inflammation are significantly up-regulating in response to lung and pulmonary diseases [8, 9]. Animal models exposed to PM2.5 and PM10 components showed a high inflammatory response [10].The Release of cytokines and matrix metalloproteinase such as MMP-12 from activated macrophages and lung epithelial cells is one of the main parts of the inflammatory response, leading to neutrophil recruitment [11-14]. PM2.5 exposure could induce respiratory inflammation, acute asthma, and lung carcinoma; taking together these pieces of evidence, the acute and chronic PM2.5 effects on the respiratory tract should be studied [15-17].

The early growth response (EGR) family is a group of transcription regulatory factors, which is involved in orchestrating the changes in gene expression that underlie several molecular pathways in different tissues including differentiation and mitogenesis. Early growth response gene 1 (EGR-1) one of the EGR family of zinc finger proteins, Cys2His2, is a nuclear protein and acts as a transcriptional regulator and potential tumor inhibitor gene (18, 19). *E*GR-1, which is produced by tobacco and has a strong function in pro-inflammatory mechanisms, can develop chronic obstructive pulmonary disease (COPD) in the smoker's lungs. *EGR-1* is known as a hypoxic transcription factor and is overexpressed in the lungs of COPD patients with history of smoking [13, 20, 21]. EGR proteins have shared highly conserved zinc-finger DNA-binding domains which in turn make them able to bind shared target genes [22]. Early growth response-2 (EGR-2, alias Krox20) protein structurally and functionally is similar to *EGR-1* But *EGR-2* plays an important role in myelination of peripheral nerve, lipid production, tissue regeneration, fibrosis, and tolerance of immune system, and the regulatory role of *EGR-1* [23].

P53 has been identified as a tumor-inhibiting gene that regulates important genes downstream of the target and its mutation or epigenetic alteration is related to the oncogenic driver in lung cancer [24, 25]. Previous reports have indicated that gene-specific DNA hypermethylation and genome-wide DNA hypomethylation play role in carcinogenesis of the lung and PM2.5 exposure develops epigenetic silencing of *P53* and may help to PM2.5 induced lung carcinogenesis [25].

In this study, researchers aimed to evaluate the lung alterations in gene expression and promoter methylation level of *EGR-1*, *EGR-2*, and *P53* genes in rat models exposed by PM2.5 in six months.

Material and Methods

Chemicals

Chloroform was purchased from Merck (Germany). SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) #K0221, Fermentas, Latvia)**,** Fast 96-Well Block Module (Applied Biosystems, sFoster City, CA, USA)**,** QIAamp DNA tissue kit (Qiagen, Netherlands) and EpiTec Bisulfite Kit (Qiagen, Netherlands) were used. GeneJET™ RNA Purification Kit#K0732, (Fermentas, Latvia) and RevertAid Premium First Strand cDNA Synthesis Kit #K1652, (Fermentas, Latvia) have traded. Syto9 dye (Invitrogen, Carlsbad, CA), HotStarTaq polymerase (Qiagen, Netherlands) were used.

PM2.5 Sampling

Sampling for PM2.5 was performed continuously with the Echo PM compact air sampler in the pilot environment of the adjacent animal room (EPA 2017a) [26, 27]. According to previous researches, PM2.5 collection and maintenance procedures were implemented [28]. Analysis of metals and polycyclic aromatic hydrocarbons (PAHs) were repeated triplicate, and the mean concentration was obtained. Based on our previous studies, the analysis was evaluated using standard reference materials (SRM 1648) [29].

Assessing of PM2.5 and gaseous pollutants

The ambient air of the pilot animal room was continuously analyzed for PM2.5 concentration along with SO2, O3 and NO2 during each period. PM2.5 concentration based on beta-attenuation

monitoring method and gaseous pollutant concentration were obtained with ultraviolet (UV) fluorescence (Horiba AP-370 series) at the time of exposure (5 hours: 9:00 to 14:00) method based on previous studies.

Period exposure time

The animal testing room was located in the north of Tehran, the capital of Iran. A follow-up including six months was designed for induction of exposure. In six months period, exposure was carried out for five hours per day (9.00 am to 2.00 pm), four days a week in December.

Experimental animals and ethical aspects

Forty-eight male Wistar rats in four-weeks of age (n¼18) weighing from 155 to 225 g were provided from Pasteur Institute of Iran (Pasteur Institute of Iran No. 69, Pasteur Ave, Tehran, Iran). The rat habituation was performed under standard conditions of water supply, food, and 12h/12h light/dark cycles for 1 week. In addition, the mice were kept in standard environmental conditions for one week prior to the study. These conditions included temperature (20-25°C), indoor air quality, and relative humidity (40-60%). This research has the approval of the Central Ethics Committee of Islamic Azad University and the Ethics Committee of Shahid Beheshti University of Medical Sciences. All the process has been performed based on the "Guide for the Care and Use of Laboratory Animals" protocols of Shahid Beheshti University of Medical Sciences (ethical code: IR.IAU.SRB.REC.1398.056).

Animal study design

The number of rats was kept to a minimum as much as possible, and in the end, eight male rats were included in each group. Three groups have been defined in the pilot study room for animals, including the following:

"Exposure 1" group that was exposed by PM2.5 plus gaseous pollutants.

"Exposure 2" group that exposed only by gaseous pollutants.

"Control" group that used air with the clean standard condition.

Real-Time PCR

RNA extraction

All animals were sacrificed through beheading, and the left lungs were quickly severed and immediately used to prepare RNA in cold conditions. The left lung was removed and placed in liquid nitrogen for freezing. Lung tissue was homogenized and complete RNA was prepared with RNA Purification Kit based on the manufacturer's instructions and then treated with DNase I without RNase to remove genomic DNA contamination. Eventually, RNA was suspended in water without RNase and stored at -80 °C. Integrity and quality/amount of RNA was detected using intact ribosomal RNA (28S and 18S bands) using 1% agarose gel electrophoresis and NanoDrop ND1000 spectrophotometer (NanoDrop Technologies) based on a previous study [23].

cDNA synthesis

One µg of total RNA was inverted in a 20 µl reaction mixture containing 0.5 µg of oligo (dT) as a primer and 200U of the reverse transcript of Maloney leukemia virus, according to the Transcription First cDNA synthesis kit.

Primer design

The particular primers for *EGR-1*, *EGR-2*, *P53*, and *GAPDH* (as an internal control) were designed through the National Center for Biotechnology Information (NCBI) website (Table 1).

Quantitative Real-Time PCR

Serial (1:4) dilutions of cDNA from total RNA extracted from blood samples of 10 non-psychiatric subjects were used to construct standard curves for each gene. In each experiment, the R2 of the standard curve was above 0.99 with no detectable signal in the unpatterned control measurements. Quantitative Real-Time PCR was conducted using SYBR Green. Specimens with

TABLE 1. primer sequences used to analysis of gene expression by Real-time PCR

Gene	Forward primer	Reverse primer
EGR-1	5'AGCCCTTTCACTTAGCGTCC3'	5'ACTTGGACGTGGCTGTTTCA3'
EGR-2	5'ATCCACTCGAGCTGCAAACA3'	5'TTGCCCAGGATCACAGGTTC3'
P53	5'CATAATGCAGTGTGCGCAGG3'	5'GCAATGGGGTCCATCCATCA3'
GAPDH	5'TCATCGTCACTGCACCTTCC3'	5'TTGCTGACAACGGTCATGGA3'

Ct values within the linear range of the standard curve were measured on a plate for a target gene. Round or failure samples were repeated for each gene in qPCR experiments. The ratio is calculated according to the pfaff formula. GAPDH is used for normalization as an endogenous internal control gene. The QPCR method is based on the order of previous studies [30].

DNA extraction and Sodium bisulfite modification

Genomic DNA was extracted using a QIAamp DNA tissue kit (Qiagen) according to the manufacture's protocol. The quality and integrity of the extracted DNA were evaluated by agarose gel electrophoresis and UV spectroscopy (ND1000 NanoDrop Technologies). One microgram of genomic DNA was exposed to bisulfite conversion using the Bisulfite EpiTec kit (Qiagen) based on the manufacturer's protocol by removing the second 70% ethanol wash. The converted pure bisulfite samples were washed in a volume of 40 µL. DNA bisulfates were stored at -20 ° C for methylation analysis.

Primer design for High resolution melting analysis (HRM)

PCR bias compensation was used to design the primer sets for all MS-HRM assays. Both methylated and unmethylated templates were amplified with the primers. Primers for methylation assessments were designed by Methyl Primer Express® Software v1.0.

Methylation-sensitive high resolution melting (MS-HRM) assay

PCR amplification and high resolution melting analysis had been conducted sequentially on Rotor-GeneTM 6000 (Corbett Research, Mortlake, Australia). PCR reactions were performed in a 20 microliter total volume containing: Buffer, 4mM Mg2, 200mM from each dNTPs, and 250 nM of each primer, 5 mM Syto9 dye (Invitrogen, Carlsbad, CA), 1U HotStarTaq polymerase (Qiagen), and 1 ml of bisulphate modified template (theoretical concentration 20 ng/ml). PCR was performed at 95.8°C for 15 minutes, followed by 45 cycles of 95.8°C for 5s, primer annealing temperature (Ta) for 5s, and 72.8°C for 10s. High-resolution melting analyses were performed using the manufacturer's suggested temperature ramp and fluorescence acquisition settings, which are temperature ramps from 70 to 95.8°C with a 0.18°C/sec increase. All reactions were conducted three times. Melting plots were normalized using the software provided with the Rotor-GeneTM 6000 by calculating the line of best fit between two normalization regions before and after the major fluorescence decrease

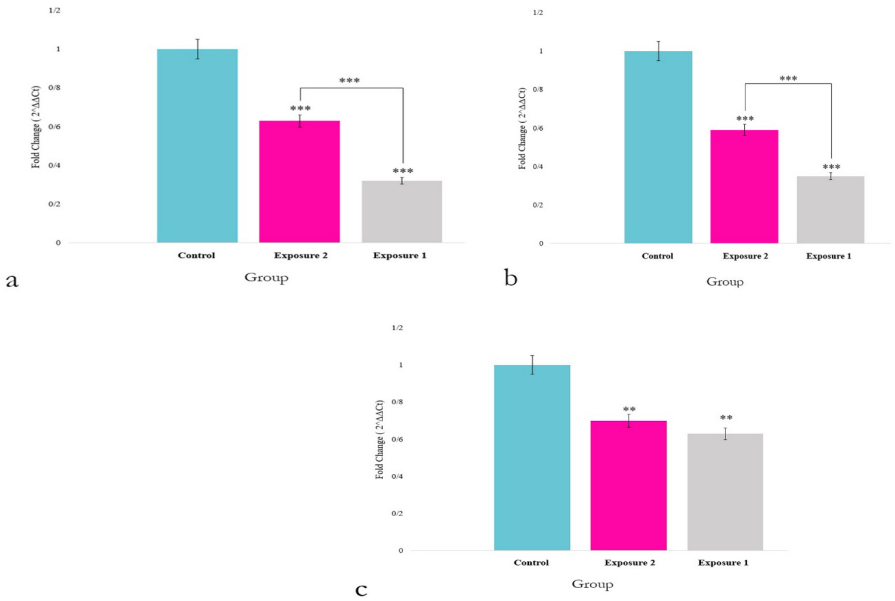

Fig. 1. The gene expression changes after six months period; *EGR-1* (a), *EGR-2* (b) and *P53* (c) genes expression level in exposure 1 that exposed by PM2.5 plus gaseous pollutants (showed by orange color), and exposure 2 that exposed only by gaseous pollutants (showed by turquoise color) and control group that used air with clean standard condition (showed by blue color); Data are expressed as means ± SEM. The mean values indicated by different superscripts are significantly different from each other (P≤0.05).

representing melting of the PCR product. This algorithm makes it possible to compare samples with different fluorescent intensities.

Statistical evaluations

One-sample Kolmogorov-Smirnov test was used to test for normality of continuous variables. For statistical differences in multiple group comparisons, a one-way ANOVA analysis was used. To determine the relationship between variables, Pearson correlation analysis was performed. Descriptive data are presented as mean ± SD (range). $P < 0.05$ was set as the level of statistical significance. Statistical analysis was conducted by using the SPSS version24.

Results

Real-time PCR

Gene expression comparisons between groups ratio ($2^{\wedge\Delta\Delta Ct}$) after exposure periods showed that the treated exposure 1 and exposure 2 played an essential role in the changes the expression of *EGR-1*, *EGR-2*, and *P53* genes in six months of treatment. As can be seen in (Figure 2a, 2b, and 2c), showed the expression levels of *EGR-1*, *EGR-2*, and *P53* gene in lung tissues treated by exposure 1 and exposure2 for six months. According to the figure, EGR-1 was expressed at lower levels in both treated groups compared to control (P<0.001) (Figure 2a). Moreover, *EGR-2* gene expression in groups treated by exposure1 and 2, show a statistically significant decrease in comparison with control (P<0.001) (Figure 2b). There are some results for *P53* gene expression level in groups treated by exposure1 and 2, the *P53* gene was decreased in both groups in comparison with control (P<0.001) (Figure 2c). Comparing the expression level of *EGR-1* and *EGR-2* genes between the groups treated by exposure 1 and exposure 2 statistically significant decrease was observed (P<0.001) but no statistically significant changes were observed in *P53* gene expression.

In a general conclusion, quantitative PCR findings showed a significant down expression of *EGR-1*, *EGR-2*, and *P53* in exposure groups (exposure 1 and 2) compared with the control group. Comparison of two exposure groups, exposure 1 and exposure 2 revealed significant down expression of *EGR-1* and *EGR-2* in group exposure 1 versus group exposure 2.

MS-HRM results

The consistency of normalized melting profiles derived from samples with different percentages of methylated and unmethylated template was examined in the methylation profiling of specimens by MS-HRM. Consistency between replicates and different runs was observed in the normalized melting profiles of the PCR products. Based on the similarity of normalized HRM profiles, MS-HRM can be designed to estimate the methylation percentage of unknown samples. The length and number of differences between methylated and unmethylated PCR products must be taken into account. Short products have high sensitivity and low resolution between various levels of methylation, longer products will give readily detectable HRM profiles for PCR products from samples with different ratios of methylated and un-methylated template which estimate the methylated proportion of an unknown sample more accurate. The annealing temperature at which PCR amplification is at equilibrium between methylated and non-methylated products must be determined experimentally before analysis. In the study of methylation of studied genes after six months, significant changes were observed in the groups. The group treated by exposure 1 and exposure 2 showed hypermethylation compare to control for *EGR-1*, *EGR-2* (P<0.001), and *P53* (P<0.01). According to the reported results of methylation of *EGR-1*, *EGR-2*, and *P53* genes after six months of exposure 1 and 2 did not demonstare statistically significant differences in comparison with each other (Table 2).

Correlation between epigenetic data and mRNA level evaluations:

Statistical analysis revealed a significant association between hypermethylation and low expression of genes in *EGR-1* and *EGR-2* in six months period. No significant correlation was

TABLE 2. Comparison of genes' promoter methylation level between groups after six months of the exposure period

Gene	Exposure 1 vs. Control	Exposure 2 vs. Control	Exposure 1 vs. Exposure 2
EGR-1	*p-v*alue (0.001)	*p-v*alue (0.001)	*p-v*alue (0.54)
EGR-2	*p-v*alue (0.001)	*p-v*alue (0.001)	*p-v*alue (0.26)
P53	*p-v*alue (0.001)	*p-v*alue (0.01)	*p-v*alue (0.47)

found between methylation percent of the *P53* gene and mRNA level of the *P53* gene. Details of methylation assessments results' have been presented in and details of correlations between results of gene expression evaluations and methylation assessments have been presented in (Table 3).

Discussion

Air pollution is becoming an increasing public health concern around the world. Particulate matter (PM), a combination of solid and liquid particles in the air, affects the health of people, according to the World Health Organization (WHO). The link between pollutants and respiratory diseases has been confirmed by epidemiologic and biological studies. It has been observed that increase in the concentration of particulate matter (PM2.5) increases various lung diseases such as cancer [31].

EGR-1 protein binding site is a GC-rich sequence, which is highly shared with Sp1 (specificity protein 1) DNA binding sequence. Either activation or repression of common target genes results from competition between Sp1 and EGR-1 [19, 32]. On the other hand, *EGR-1* and *EGR-2* are provoked by several stimuli such as cytokines, growth factors, and stress signals like ultraviolet radiation, exposure to chemical factors, industrial metals, injury, and stretch. Effects of these stimuli on *EGR* family members, in turn, cause a dramatically widespread alteration in apoptosis, cell proliferation, differentiation, and survival regulations [19, 33].

Previous researches revealed the association of *EGR-1* in lung diseases caused by smoking. Besides, it has been shown that the EGR-1 protein is induced in the epithelial cells of the small airways after exposure to an extract of cigarette smoke [21]. Our findings showed similar but more severe effects of PM2.5 on *EGR-1* expression level. Pm2.5 was able to confirm the findings of previous studies by significantly reducing the expression of *EGR-1* and *EGR-2* in the exposure 1 receiving group compared to the exposure 2 and control groups. Also, the same alteration of *EGR-2* mRNA level with *EGR-1* could confirm the constant and mechanistically effects of PM2.5 of these two structurally and functionally related members of the *EGR* transcription factor family. PM2.5 can influence several gene expression patterns including cytochromes, oxide reductase, glutathione transferase, and MAP-kinases signaling in different organs [34]. Moreover, PM2.5 treatment can alter the IL-17 signaling pathway in the pulmonary cells which leads to abnormal immune and inflammation response in the lung tissue [34].

On the other hand methylation assays were showed that hypermethylation of *EGR-1* and *EGR-2* is attributed to downregulation of expression level. *EGR-1* down expression is associated with dysfunction in growth, development, differentiation, apoptosis, inflammation, and fibrosis [35-37]. According to studies, hypermethylation and decreased expression of the *P53* gene have been observed in patients with lung cancer [38, 39]. In human bronchial epithelial cells, decreased DNA methylation was observed after exposure to PM2.5 [40, 41]. The present study may provide evidence for the hypothesis that repeated exposure of PM2.5 may lead to epigenetic silencing of *P53* via ROS-Akt-DNMT3B pathway-mediated promoter hypermethylation [25]. Our findings in mRNA level evaluations of P53 also confirmed epigenetic analysis. Environmental chemicals are introduced into the air from different sources and loss of function observed in P53 as a major proto-oncogene may explain air pollution-

TABLE 3. Correlation assessments between epigenetic data and mRNA level evaluations after six months exposure period

Gene	Group A	Group B	Group C
EGR-1	*p-v*alue 0.004 r: - 0.79	*p-v*alue (0.006) r: -0.61	*p-v*alue (0.2) r: -0.26
EGR-2	*p-v*alue 0.002 r: -0.78	*p-v*alue (0.01) r:- 0.55	*p-v*alue (0.12) r:0.1
P53	*p-v*alue 0.04 r: - 0.47	*p-v*alue (0.13) r: - 0.3	*p-v*alue (0.45) r:0.11

induced and lung cancer caused by PM2.5 [42-48]. The present in vivo study of mouse models showed severe changes in the expression of *EGR-1*, *EGR-2*, and *P53* genes in both exposure 1 and exposure 2 recipient groups over six months of treatment. The studied genes were expressed in healthy groups, while in the groups treated with exposure 1 and exposure 2, their expression was reduced. Therefore, the six months treatment with exposure 1 and exposure 2 had a direct impact on the expression of the mentioned genes in inducing negative effects. Also, in comparison with the two groups treated with exposure 1 and 2 in two treatment periods, the group receiving exposure 1 showed a higher decrease in the expression of genes *EGR-1* and *EGR-2* than the other groups. PM2.5 exposure caused chronic epigenetic changes associated with P53 inhibition. Our study provides molecular evidence for epidemiological researches and the pathway for lung carcinogenesis induced via pm2.5. The results of our study can confirm the findings of previous studies.

Conclusion

In conclusion, the present study indicated that PM2.5 is the most dangerous substance of air pollution that affects a variety of signaling pathways that in turn. While increase in polluting industry may increase the rate of air pollutions at least strict public policies and governmental legislations for reduction of PM2.5 may decrease the rate and burden of PM2.5-induced respiratory diseases.

Authors' Contributions

M.F.M., P.M. developed the idea and designed the experiments. P.M., S.M.Z. conducted the experiments. M.F.M., A.E. analyzed the data. M.F.M. wrote the manuscript. P.M., A.E., S.M.Z. helped through characterization of the samples. All authors confirmed the final manuscript before the submission.

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Availability of data and materials

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

Ethics approval and consent to participate

There are no "human subjects" in this study.

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