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Plasma microRNA Expression and Micronuclei Frequency in Workers Exposed to Polycyclic Aromatic Hydrocarbons

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Abstract

Background: Ubiquitous polycyclic aromatic hydrocarbons (PAHs) have been shown to alter gene expression patterns and elevate micronuclei (MN) frequency, but the underlying mechanisms are largely unknown. MicroRNAs (miRNAs) are key gene regulators that may be influenced by PAH exposures and mediate their effects on MN frequency.

Objectives: We sought to identify PAH-associated miRNAs and evaluate their associations with MN frequency.

Methods: We performed a two-stage study in healthy male coke oven workers to identify miRNAs associated with PAH exposures quantified using urinary monohydroxy-PAHs and plasma benzo[a]pyrene-r-7,t-8,c-10-tetrahydrotetrol-albumin (BPDE-Alb) adducts. In the discovery stage, we used Solexa sequencing to test differences in miRNA expression profiles between pooled plasma samples from 20 exposed workers and 20 controls. We then validated associations with eight selected miRNAs in 365 workers. We further evaluated associations between the PAH-associated miRNAs and MN frequency.

Results: In the discovery stage, miRNA expression profiles differed between the exposed and control group, with 68 miRNAs significantly downregulated [fold change (FC) \leq -5] and three miRNAs mildly upregulated ($+2 \leq$ FC $<$ +5) in exposed group. In the validation analysis, urinary 4-hydroxyphenanthrene and/or plasma BPDE-Alb adducts were associated with lower miR-24-3p, miR-27a-3p, miR-142-5p, and miR-28-5p expression ($P < 0.030$). Urinary 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyphenanthrene, and the sum of

monohydroxy-PAHs were associated with higher miR-150-5p expression ($P < 0.030$). These miRNAs were associated with higher MN frequency ($P < 0.005$), with stronger associations in drinkers ($P_{\text{interaction}} < 0.015$).

Conclusions: Associations of PAH exposures with miRNA expression, and of miRNA expression with MN frequency, suggest potential mechanisms of adverse effects of PAHs that are worthy of further investigation.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that are mainly generated through incomplete combustion of carbon-containing materials. One of the major sources of environmental PAHs is industrial activities which constantly emit high concentrations of PAHs, such as tar distillation and coke production. Substantial epidemiological evidence suggests that long-term exposure to PAH-rich emissions is associated with a higher lung cancer risk in exposed workers (IARC 2005). Before exerting their carcinogenic effects, PAHs are metabolically activated to form stable PAHs-DNA adducts and cause DNA oxidation, eventually leading to DNA damage (Xue and Warshawsky 2005). DNA damage can result in chromosome aberration and genetic instability, and might eventually trigger cancers. Micronuclei (MN) frequency is extensively used as a biomarker of chromosomal damage, genome instability, and cancer risk (El-Zein et al. 2006; Fenech 2007). Previous studies have reported that occupational exposure to PAHs is associated with higher MN frequency (Liu et al. 2006; Pavanello et al. 2008). However, the underlying mechanisms still need to be clarified.

In experimental systems, PAHs have been shown to alter the expression patterns of genes that are relevant to the carcinogenic effects of PAHs (Hewitt et al. 2007; Staal et al. 2006). Effects on microRNA (miRNA) expression have been proposed as a mechanism through which environmental exposures might affect gene expression (Jirtle and Skinner 2007; Schembri et al. 2009). miRNAs are a class of small noncoding RNAs that function as gene regulators by base pairing with the 3'-untranslated regions of target mRNAs and leading to translational repression

or degradation of target mRNAs (Ambros 2004). There is growing evidence that exposure to environmental pollutants may significantly disrupt miRNA expression patterns (Bollati et al. 2010; De Flora et al. 2012; Izzotti et al. 2009; Jardim et al. 2009; Schembri et al. 2009). However, few epidemiological studies have examined potential effects of PAH exposures on miRNA expression profiles. miRNAs play crucial roles in a broad range of physiologic and pathologic processes, including the processes that may affect genetic damage levels, such as DNA damage response (Lal et al. 2009; Pothof et al. 2009) and oxidative stress (Sangokoya et al. 2010). Previously, miRNAs were shown to regulate cellular responses to ultraviolet-induced genetic damage *in vitro* (Pothof et al. 2009). However, no studies have demonstrated effects of miRNAs on PAH-related chromosome damage specifically.

Circulating miRNAs in plasma have the potential to serve as stable non-invasive biomarkers of physiological and pathological conditions (Mitchell et al. 2008). Thus in the present study, we sought to identify plasma miRNAs that are associated with PAH exposures by genome-wide miRNA sequencing and subsequent validation in healthy male coke oven workers with well characterized PAH exposures. We further evaluated associations between the PAH-associated miRNAs and MN frequency.

Materials and methods

Airborne PAHs monitoring

We collected airborne samples from different workplaces in the coke oven factory of a steel company located in southern China and determined the concentrations of 16 PAHs by high-performance liquid chromatography (Li et al. 2012).

Study subjects

In our previous study (Li et al. 2012), we enrolled 1333 coke oven workers (1126 males and 207 females) who were employed at the coke oven factory for at least one year and worked at the top, side, and bottom of the coke ovens, adjunct workplaces (such as blower operation room and recycling workshops), or in offices. The present study population is a subset of the previous study consisting of male workers between 20-60 years of age. We excluded workers with a self-reported history of chronic diseases, including cancers, cardiopulmonary diseases, chronic inflammation, and hypertension, and excluded workers who reported taking any medicines in the preceding three months. We also excluded workers who did not provide urine and/or blood samples. Finally, we enrolled a total of 391 workers in this study. After participants provided informed consent, we administered a questionnaire to collect information on demographic characteristics, smoking and drinking habits, medical history, and occupational experiences. Individuals who had smoked an average of less than 1 cigarette per day for less than 1 year in their lifetime were defined as nonsmokers; otherwise, they were considered as smokers. Individuals who had drunk alcoholic beverages less than once a week for less than 1 year in their

lifetime were defined as nondrinkers; otherwise, they were considered as drinkers. Each worker donated ~ 20 ml morning urine, ~ 4 ml fasting heparin-anticoagulated and ~1ml fasting EDTA-anticoagulated venous blood. This study was approved by the Medical Ethics Committee of the School of Public Health, Tongji Medical College.

Study design

Based on the previously measured ambient concentrations of total PAHs [mean \pm standard deviation (SD), $\mu\text{g}/\text{m}^3$] (Li et al. 2012), we classified 256 workers working in offices (1.13 ± 0.37) or at adjunct workplaces (3.72 ± 2.09) as the control group, and 135 workers working at the top of the coke oven (90.30 ± 69.51) or at the side and bottom of the coke oven (11.08 ± 7.29) as the exposed group. As urinary monohydroxy-PAHs (OH-PAHs) and plasma benzo[a]pyrene-r-7,t-8,c-10-tetrahydrotetrol-albumin (BPDE-Alb) adducts were all significantly correlated with the sum of OH-PAHs ($\Sigma\text{OH-PAHs}$) ($P < 0.001$), and the sum of their correlation coefficients with $\Sigma\text{OH-PAHs}$ was the highest (see **Supplemental Material, Table S1**), we used $\Sigma\text{OH-PAHs}$ as the representative PAH internal exposure biomarker for sample selection for miRNA sequencing in the discovery stage. We selected 20 workers with higher $\Sigma\text{OH-PAHs}$ from the exposed group, and 20 matched workers with lower $\Sigma\text{OH-PAHs}$ from the control group. We intentionally frequency-matched the distribution of important general characteristics, including age (± 5 years), smoking status, pack-years of smoking (± 5 pack-years), drinking status, working years (± 2 years), and body mass index (BMI) (± 2), between these two groups to minimize their confounding effects on miRNA expression profiles. We prepared a 5-ml pooled

plasma sample for each group that included 250µl of plasma from each subject. Two plasma pools were then subjected to miRNA sequencing, and miRNA expression profiles were compared between these two groups. In order to focus on the most likely related miRNAs in the validation stage, we selected miRNAs based on the following criteria: demonstrated at least a five-fold lower or higher expression in the exposed group compared with the control group, expressed at least 50 copies in at least one group, and were found based on an extensive literature review to be associated with PAH response, DNA damage, or other DNA damage-related mechanisms, such as oxidative stress. In the validation stage, we measured the expression levels of selected miRNAs in the remaining 351 subjects by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). In addition, 14 subjects in the discovery stage (5 in the control group and 9 in the exposed group) had enough plasma (200µl) for validation, and were also included in the validation stage. For the miRNAs that were significantly associated with at least one PAH exposure biomarker, we further evaluated their associations with MN frequency.

Urinary creatinine and OH-PAHs measurements

We measured the concentration of urinary creatinine according to Jaffe's colorimetric method on an automated clinical chemistry analyzer. We measured 12 urinary OH-PAHs (10 non-carcinogenic metabolites: 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 9-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, 9-hydroxyphenanthrene, and 1-hydroxypyrene; 2 carcinogenic metabolites: 6-hydroxychrysene and 3-hydroxybenzo[a]pyrene) by gas chromatography-mass

spectrometry (Li et al. 2012). Limits of quantification (LOQ) were in the 0.1-1.4 $\mu\text{g/L}$ range, and we replaced default values for each OH-PAH with 50% of the corresponding LOQ (see **Supplemental Material, Table S2**). The molar concentrations of OH-PAHs were calibrated by urinary creatinine and expressed as $\mu\text{mol/mmol}$ creatinine.

Determination of plasma BPDE-Alb adducts

We measured albumin adducts of the carcinogenic metabolite BPDE (i.e. BPDE-Alb adducts) in heparin-anticoagulated plasma with sandwich enzyme-linked immunosorbent assay (Chung et al. 2010). We assayed each sample in duplicate. The average concentration of BPDE-Alb adducts for each sample was calibrated by plasma albumin and expressed as ng/mg albumin. The LOQ was 1ng/mg albumin, and we replaced the default values with 50% of the LOQ (see **Supplemental Material, Table S2**).

Cytokinesis-block micronucleus (CBMN) assay

We carried out a CBMN assay with fresh heparin-anticoagulated whole blood according to the standardized protocol developed by Fenech (2007). We prepared two duplicative slides for each subject. We examined 1000 binucleated cells in each slide microscopically, and identified the numbers of binucleated cells containing MN according to scoring criteria. The MN frequency (%) of each subject was recorded as the mean number of CBMN cells per 1000 binucleated cells.

RNA isolation

We isolated total RNAs from the two EDTA-anticoagulated plasma pools in the discovery stage for Solexa sequencing and from 200 μ l EDTA-anticoagulated plasma of each subject in the validation stage for qRT-PCR, with mirVana PARIS miRNA Isolation Kit (Ambion, Austin, TX). For RNA isolation in the validation stage, we added *Canorhabditis elegans* miRNA cel-miR-39 (synthesized by Qiagen) into the denatured plasma samples to normalize the sample-to-sample variation in the isolation step.

Solexa sequencing

Purified small RNA molecules under 30 bases were ligated with Solexa adaptors and reverse transcribed into cDNA. We used the purified cDNA for cluster generation and sequencing analysis by Illumina's Solexa Sequencer (San Diego, CA). Then we converted the generated image files into digital-quality data. Subsequently, we obtained clean reads by removing the adaptors, low-quality reads and contaminated reads, and compared them with miRBase 18.0 to identify miRNAs. We calculated the normalized copy number for each miRNA by the equation $(C \times 10^6) / N$, in which C means the number of reads mapped to individual miRNA, and N means the total number of aligned reads.

qRT-PCR assay

RNAs were first reverse transcribed by TaqMan miRNA Reverse Transcription Kit (ABI, Foster City, CA), and then subjected to real-time polymerase chain reaction in duplicate with TaqMan miRNA Assay Kit (ABI, Foster City, CA) and ABI Prism 7900HT Sequence Detection System

(ABI, Foster City, CA). The miRNA expression levels were normalized against cel-miR-39 and calculated by the equation $2^{-\Delta Ct}$, in which $\Delta Ct = \text{cycle threshold } (Ct)_{\text{miRNA}} - Ct_{\text{cel-miR-39}}$.

Target gene prediction and enriched biological function analysis

We incorporated miRanda (<http://www.microrna.org/microrna/home.do>) and TargetScan (<http://www.targetscan.org/>) to predict the potential target genes of miRNAs, and conducted Gene Ontology (GO) function enrichment analysis of these target genes with DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov>).

Statistical analyses

As 6-hydroxychrysene and 3-hydroxybenzo[a]pyrene were always below their LOQ, we excluded them from further analyses. The concentrations of 10 other urinary OH-PAHs and plasma BPDE-Alb adducts were normalized by natural logarithm (ln) transformed, and the miRNA expression levels measured by qRT-PCR were normalized by log2 transformed. Thus, we used the transformed urinary OH-PAHs, plasma BPDE-Alb adducts, and miRNA expression levels in statistical analyses. We evaluated the differences of general characteristics between different groups (control vs. exposed groups in discovery stage; discovery vs. validation) by Student *t*-test (for continuous variables) and Chi-square test (for categorical variables). We analyzed the differences of urinary OH-PAHs, plasma BPDE-Alb adducts, and MN frequency between different groups by multivariate covariance analysis and Poisson regression analysis, respectively, with adjustment for several primary confounding factors, including age (continuous), smoking status (yes/no), pack-years of smoking (continuous), drinking status

(yes/no), all working years (continuous), BMI (continuous), and/or workplace (control/exposed group), when appropriate.

In discovery stage, we performed Hierarchical clustering for the miRNAs that showed differential expression in two groups using PermutMatrix clustering tool (Caraux and Pinloche 2005). In validation stage, we used multivariate linear regression models to evaluate associations between miRNA expression levels (as the dependent variable) and creatinine-corrected urinary OH-PAHs or plasma BPDE-Alb adducts, with adjustment for the above confounding variables. To compare the magnitude of these associations, we reported standardized coefficients (β_{std}) that represent the estimated difference in log₂-transformed miRNA expression (in SD units) associated with a 1-SD increase in the ln-transformed PAH exposure levels.

In our unpublished work (Zhang X et al. unpublished data) and in the present study (see **Supplemental Material, Table S3**), OH-PAHs and BPDE-Alb adducts were significantly associated with MN frequency. Thus, in order to minimize the confounding effects of PAH exposure, we additionally adjusted for ln-transformed Σ OH-PAHs (continuous) and BPDE-Alb adducts (continuous) in Poisson regression models to evaluate the associations of log₂-transformed miRNA expression levels (in SD units) with unstandardized MN frequency (as the dependent variable). Frequency ratio (FR) was calculated based on the equation $e^{\beta_{\text{std}}}$ and represented the proportional changes of MN frequency due to 1-SD increase in log₂-transformed miRNA expression. We evaluated the differences of the associations between miRNA and MN frequency in workers with different drinking status, smoking status or age groups by modeling

interaction terms of (miRNA*stratum variables) in Poisson regression models. We carried out all data analyses using SPSS (version 12.0). Two-side $P < 0.05$ was considered statistically significant.

Results

Subject characteristics

As shown in **Table 1**, in the discovery stage, the distribution of age, smoking status, pack-years of smoking, drinking status, all working years, and BMI were matched between the control and exposed group (all $P > 0.05$), whereas the PAH exposure levels and MN frequencies were all significantly different between these two groups (all $P \leq 0.005$). The distribution of general characteristics, most of the PAH internal exposure biomarkers (except for 1-hydroxynaphthalene, 3-hydroxyphenanthrene, and BPDE-Alb adducts), and MN frequency for the 40 subjects in the discovery stage were not significantly different from the other 351 workers in the validation stage (all $P > 0.05$).

miRNA expression profiles and miRNAs selection for validation

In the discovery stage, we detected a total of 217 and 153 miRNAs in the plasma pools of the control group and the exposed group, respectively (**Figure 1A**). The miRNA expression levels in these two groups were strongly correlated ($R^2 = 0.844$) (**Figure 1B**). Notably, compared with the expression levels in the control group, 68 miRNAs demonstrated at least a five-fold lower expression in the exposed group [all fold changes (FC) ≤ -5] (**Figure 2**), while no miRNA

showed at least a five-fold higher expression in the exposed group. Based on the miRNA selection criteria (see **Materials and methods**), we selected seven highly differentially expressed miRNAs from these 68 downregulated miRNAs. Moreover, there were three miRNAs that showed at least a two-fold higher expression in the exposed group compared with the expression in control group ($+2 \leq \text{all FC} < +5$) (**Figure 2**). We also selected one miRNA from these three mildly upregulated miRNAs, which was expressed at least 50 copies in at least one group. The expression levels and the related functions of eight selected miRNAs were shown in **Table 2**.

Identification of PAH-associated miRNAs

In the validation stage, we used multivariable linear regression analysis to estimate confounder-adjusted associations of urinary OH-PAH concentrations and plasma BPDE-Alb adducts with the expression of eight selected miRNAs (**Table 3**). Urinary 4-hydroxyphenanthrene concentration was associated with significantly lower miR-24-3p, miR-27a-3p, and miR-142-5p expression (all $P < 0.030$), with the strongest association estimated with miR-27a-3p ($\beta_{\text{std}} = -0.141$; 95% CI: -0.246, -0.037; $P = 0.008$). Plasma BPDE-Alb adducts were associated with lower miR-24-3p and miR-28-5p expression (all $P \leq 0.004$), with the strongest association estimated with miR-28-5p ($\beta_{\text{std}} = -0.180$; 95% CI: -0.290, -0.071; $P = 0.001$). Notably, miR-24-3p was significantly associated with both 4-hydroxyphenanthrene ($\beta_{\text{std}} = -0.117$; 95% CI: -0.222, -0.013; $P = 0.028$) and BPDE-Alb adducts ($\beta_{\text{std}} = -0.166$; 95% CI: -0.277, -0.055; $P = 0.004$). miR-150-5p was the only selected miRNA showing a mild higher

expression in the exposed group ($+2 < FC < +5$) in the discovery stage. In the validation stage, urinary 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyphenanthrene, and Σ OH-PAHs all were associated with higher miR-150-5p expression (all $P < 0.030$), with the strongest association estimated with 1-hydroxynaphthalene ($\beta_{\text{std}} = 0.190$; 95% CI: 0.076, 0.303; $P = 0.001$). These five PAH-associated miRNAs were not significantly associated with drinking status, smoking status, and age (all $P > 0.05$) (see **Supplemental Material, Table S4**).

PAH-associated miRNAs and MN frequency

We then investigated whether there were associations between the expression of the five PAH-associated miRNAs and MN frequency. As shown in **Table 4**, the five PAH-associated miRNAs all were associated with higher MN frequency (all $P < 0.005$), with the strongest association estimated for miR-24-3p (FR = 1.152; 95%CI: 1.086, 1.222; $P = 2.59 \times 10^{-6}$). We further performed stratification analyses by drinking status, smoking status, and age groups, respectively. We found that the associations of all five miRNAs with MN frequency remained significant in drinker (all $P < 0.005$), and most associations were significantly stronger in drinkers compared with nondrinker (all $P_{\text{interaction}} < 0.015$), except for the association between miR-142-5p and MN frequency ($P_{\text{interaction}} = 0.288$) (**Table 4**). Although the associations between miRNA expression and MN frequency also remained significant in smokers and workers between 41-60 years of age (all $P < 0.025$), most associations were not significantly different between the corresponding subgroups (all $P_{\text{interaction}} > 0.05$), except for the association

of miR-27a-3p with MN frequency between smoking strata ($P_{\text{interaction}} = 0.049$) (see **Supplemental Material, Table S5**).

Enriched biological functions of the target genes

To explore the potential functions of these five miRNAs, we predicted their target genes. There were 733 putative targets for miR-24-3p, 452 for miR-27a-3p, 66 for miR-142-5p, 432 for miR-28-5p, and 721 for miR-150-5p. GO function enrichment analysis showed that these target genes were mainly involved in 13 enriched biological functions ($P < 0.002$), including cellular physiological process, external stimulus response, enzyme activity regulation, and metabolism (**Table 5**).

Discussion

To the best of our knowledge, this is the first study to explore associations between PAH exposure and miRNA expression, and between the same miRNAs with MN frequency, in an occupational population. Our genome-wide miRNA sequencing revealed that miRNA expression profiles were different between two occupational groups with high and low PAH exposure levels, with most miRNAs significantly downregulated in the higher exposed group compared with the low-exposed controls. In a detailed validation study, we identified five miRNAs that were associated with at least one of the PAH exposures, including four that were negatively associated with urinary non-carcinogenic 4-hydroxyphenanthrene or plasma carcinogenic BPDE-Alb adducts or both (miR-24-3p, miR-27a-3p, miR-142-5p, and miR-28-5p) and one (miR-150-5p)

that was positively associated with exposure to three different urinary non-carcinogenic OH-PAHs and with the sum of all measures urinary OH-PAHs. The same miRNAs also were associated with chromosome damage, as reflected by higher MN frequency, with stronger associations among drinkers than nondrinkers.

Many studies have reported evidence suggesting that environmental stimuli can induce changes in miRNA expression (Bollati et al. 2010; Jardim et al. 2009; Schembri et al. 2009). Our work provided novel information about associations of PAH exposures with plasma miRNA expression in exposed workers. Our genome-wide miRNA sequencing and subsequent validation revealed that most miRNAs were negatively associated with PAH exposure levels, which is similar to the results reported in prior studies regarding the effects of PAH-rich cigarette smoke on miRNA expression patterns (De Flora et al. 2012). Izzotti et al. (Izzotti et al. 2009) demonstrated that cigarette smoke would mainly result in a remarkable downregulation of miRNA expressions in rat lung. Schembri et al. (Schembri et al. 2009) have observed 28 miRNAs differentially expressed in the bronchial airway epithelium in smokers with the majority being downregulated. Moreover, miRNA expressions are generally downregulated in multiple different types of cancers, including lung cancer (Lu et al. 2005).

In our study, miR-24-3p, miR-27a-3p, miR-142-5p, and miR-28-5p were all negatively associated with exposure to urinary 4-hydroxyphenanthrene concentrations or plasma BPDE-Alb adducts (or both), and positively associated with MN frequency. These miRNAs have been reported to regulate genes that could protect against adverse effects of PAH exposures.

miR-24-3p has been reported to negatively regulate *H2AX* which is crucial in double-stranded break repair, and thus reduced expression might increase cellular sensitivity to DNA damaging agents and genomic instability (Lal et al. 2009; Wang and Taniguchi 2013). In addition, miR-24-3p has also been reported to negatively regulate *ARNT*, the protein product of which forms a heterodimer with the aryl hydrocarbon receptor that mediates PAH responses, and downregulate the metabolism gene *CYP1A1* (Oda et al. 2012). miR-27a-3p has been proposed to operate with miR-24-3p in a cooperative cluster. It can be downregulated by reactive oxygen species (Pathi et al. 2011). miR-27a-3p is a key regulator of *TFIIH* which displays activities involved in DNA repair processes (Portal 2011). miR-27a-3p downregulation may elevate TFIIH and DNA repair capacity, and thus decrease chromosome damage. miR-142-5p is repressed in lung cancer (Liu et al. 2009) and downregulated following benzo[a]pyrene exposure (Halappanavar et al. 2011). miR-142-5p downregulation in healthy CD4⁺ T cells can lead to upregulation of *SAP* expression, and increase T cell function and IgG production (Ding et al. 2012); thus it may protect individuals against the deleterious effects of PAHs. miR-28-5p is linked to frequent chromosomal alterations (Wilting et al. 2013). miR-28-5p negatively regulates *Nrf2*, the protein product of which is an important transcription factor that regulates the expression of detoxifying enzymes (Yang et al. 2011). miR-28-5p downregulation may elevate the expression of *Nrf2* and detoxifying enzymes, and protects cells from carcinogen-induced DNA damage.

In addition, miR-150-5p expression, which was increased in association with several biomarkers of internal non-carcinogenic PAH exposure in our study, also was associated with higher MN frequency. However, a previous study found that miR-150-5p was downregulated following benzo[a]pyrene exposure in rat lung (Halappanavar et al. 2011). miR-150-5p is a key regulator of *c-Myb* which is important for immune cell differentiation and activation, and miR-150 deficiency can lead to enhanced immune response (Xiao et al. 2007). Thus, miR-150-5p upregulation may decrease immune response to PAH exposure and make individuals more vulnerable to the deleterious effects of PAHs.

The stratification analyses in our study showed that the associations between miRNAs and MN frequency were more prominent in drinkers. These results provided some clues that can be used for more detailed risk assessment.

Our study has several strengths. First, to identify PAH-associated miRNAs, we first screened and compared hundreds of miRNAs in pooled plasma samples from high and low exposure groups, and then validated several miRNAs to reduce the false-positive probability. Second, we systematically evaluated the associations of miRNAs with non-carcinogenic and carcinogenic PAH internal exposure biomarkers. Our study recruited workers who had been regularly exposed to PAH-rich emissions for at least one year and their major PAH exposure sources and concentrations did not fluctuate much. Thus, it was reasonable to use OH-PAHs and BPDE-Alb adducts as biomarkers of PAH chronic exposure in this occupational population (Sobus et al. 2009). Moreover, BPDE-Alb adducts cannot be repaired and have a mean residence time of 28

days that is sufficiently long to dampen the day-to-day variability in exposure levels (Chung et al. 2010). Besides, our study subjects had been working in the same factory, minimizing the confounding effects of other PAH exposure sources from the day-to-day environment, socioeconomic factors and other characteristics.

However, as our study is a cross-sectional study in which we measured biomarkers of PAH exposures, miRNA expression levels, and MN frequency at the same time point, it is not possible to determine whether differences in miRNA expression preceded or followed PAH exposures or chromosome damage. Moreover, we validated only a subset of the miRNAs found in the discovery stage, and other miRNAs should be further validated.

Conclusions

We identified five potentially PAH-associated miRNAs in plasma, and found that the same miRNAs were associated with a marker of chromosome damage in coke oven workers, suggesting that miRNAs might be a novel mechanism mediating the effects of PAH exposure on chromosome damage. Further studies are warranted to verify our findings and determine their underlying mechanisms.

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Table 1. General characteristics, PAH exposures, and MN frequency in the discovery and validation populations.

Variables^a	Discovery stage: controls (n=20)	Discovery stage: exposed (n=20)	<i>P</i>	Validation population (n=365)	<i>P^c</i>
General characteristics					
Age (years)	39.85±5.97	41.74±7.82	0.394 ^d	42.15±8.12	0.314 ^d
Smoking status, yes/no (%yes)	15/5 (75.0)	14/6 (70.0)	0.723 ^d	252/113 (69.0)	0.619 ^d
Pack-years of smoking	10.69±11.18	15.07±15.13	0.305 ^d	12.88±15.14	0.965 ^d
Drinking status, yes/no (%yes)	6/14 (30.0)	9/11 (45.0)	0.327 ^d	160/205 (43.8)	0.441 ^d
Working years (years)	18.55±6.20	22.04±8.59	0.149 ^d	20.94±9.59	0.720 ^d
BMI	24.18±3.30	23.85±3.43	0.754 ^d	24.10±3.19	0.832 ^d
PAH internal exposure biomarkers^b					
1-hydroxynaphthalene ($\times 10^{-2}$)	0.54 (0.44, 0.65)	4.28 (3.53, 7.32)	<0.001 ^e	1.11 (0.67, 1.82)	0.045 ^e
2-hydroxynaphthalene ($\times 10^{-2}$)	0.64 (0.33, 0.86)	3.68 (2.54, 6.15)	<0.001 ^e	1.13 (0.62, 1.68)	0.148 ^e
2-hydroxyfluorene ($\times 10^{-3}$)	2.19 (1.79, 3.65)	13.22 (8.22, 27.19)	<0.001 ^e	4.73 (2.96, 7.41)	0.450 ^e
9-hydroxyfluorene ($\times 10^{-3}$)	0.69 (0.06, 1.78)	7.15 (3.39, 19.84)	<0.001 ^e	2.72 (1.02, 5.87)	0.730 ^e
1-hydroxyphenanthrene ($\times 10^{-3}$)	0.79 (0.50, 1.51)	16.81 (9.93, 25.59)	<0.001 ^e	3.96 (1.93, 7.47)	0.557 ^e
2-hydroxyphenanthrene ($\times 10^{-3}$)	0.71 (0.52, 1.03)	6.30 (3.78, 12.11)	<0.001 ^e	1.46 (0.85, 2.77)	0.127 ^e
3-hydroxyphenanthrene ($\times 10^{-3}$)	1.19 (0.68, 1.62)	7.46 (3.45, 16.03)	<0.001 ^e	1.81 (1.01, 3.21)	0.031 ^e
4-hydroxyphenanthrene ($\times 10^{-3}$)	1.69 (0.32, 2.46)	4.00 (2.84, 12.98)	0.005 ^e	1.73 (0.68, 3.31)	0.408 ^e
9-hydroxyphenanthrene ($\times 10^{-3}$)	1.21 (0.73, 1.78)	12.18 (10.22, 18.93)	<0.001 ^e	3.51 (2.00, 6.66)	0.963 ^e
1-hydroxypyrene ($\times 10^{-2}$)	0.68 (0.52, 0.90)	7.50 (4.95, 11.15)	<0.001 ^e	1.31 (0.82, 2.39)	0.052 ^e
Σ OH-PAHs ($\times 10^{-2}$)	3.16 (2.59, 3.41)	21.09 (17.37, 37.44)	<0.001 ^e	6.12 (4.27, 9.43)	0.134 ^e
BPDE-Alb	3.94 (3.43, 4.51)	6.33(4.41, 10.01)	0.002 ^e	4.20 (3.45, 5.17)	0.005 ^e
Chromosome damage					
MN frequency (‰)	2 (1,3)	4 (3,6)	<0.001 ^e	3 (2, 5)	0.092 ^e

^aValues are mean \pm SD, median (P25, P75) or n (%); ^bThe unit for OH-PAHs is $\mu\text{mol}/\text{mmol}$ creatine, and the unit for BPDE-Alb adducts is ng/mg albumin; ^cComparisons between 40 workers in the discovery stage and the other 351 workers in the validation stage; ^dStudent t-test for continuous variables and Chi-square test for categorical variables; ^eMultivariate covariance analysis for PAH internal exposure biomarkers and Poisson regression analysis for MN frequency, with adjustment for age, smoking status, pack-years of smoking, drinking status, working years, BMI, and/or workplace when appropriate.

Table 2. Expression levels and related functions of the eight selected miRNAs.

miRNA	Discovery: Control ^a	Discovery: Exposed ^a	FC ^b	Validation stage ^c	Related Functions	Sources
miR-24-3p	1109	10	-110.9	0.221 (0.024, 1.193)	DNA damage response; ARNT regulation	(Lal et al. 2009; Oda et al. 2012; Wang and Taniguchi 2013)
miR-27a-3p	1106	10	-110.6	0.247 (0.054, 0.924)	ROS-mediated repression; TFIIH regulation	(Pathi et al. 2011; Portal 2011)
miR-142-5p	5901	470	-12.56	0.012 (0.003, 0.048)	Benzo[a]pyrene response	(Halappanavar et al. 2011)
miR-16-5p	24442	2080	-11.75	0.622 (0.240, 1.659)	Regulation of DNA damage signaling in response to PAHs	(Niziolek-Kierecka et al. 2012)
miR-451a	5493	475	-11.56	0.165 (0.077, 0.477)	Regulation of susceptibility to oxidative damage	(Yu et al. 2010)
miR-28-5p	131	18	-7.28	0.006 (0.001, 0.025)	Associated with frequent chromosomal alteration; antioxidant response	(Wilting et al. 2013; Yang et al. 2011)
let-7b-5p	532	83	-6.41	0.102 (0.028, 0.307)	Downregulated by cellular stress; protecting cells from oxidant injury	(Hou et al. 2012; Saleh et al. 2011)
miR-150-5p	88	252	+2.86	0.015 (0.008, 0.037)	Benzo[a]pyrene response	(Halappanavar et al. 2011)

Abbreviations: FC: fold change.

^aMean copy numbers based on Solexa sequencing; ^bNegative values indicate that miRNA expression was lower in the exposed group, positive values indicate that miRNA expression was higher in the exposed group; ^cRT-PCR expression relative to cel-miR-39. Values are median (P₂₅, P₇₅).

Table 3. Associations of PAH exposures with miRNA expression levels (as the dependent variable) [standardized β (95% CI)].

PAH biomarker ^a	miR-24-3p ^b	<i>P</i> ^c	miR-27a-3p ^b	<i>P</i> ^c	miR-142-5p ^b	<i>P</i> ^c	miR-16-5p ^b	<i>P</i> ^c	miR-451a ^b	<i>P</i> ^c	miR-28-5p ^b	<i>P</i> ^c	let-7b-5p ^b	<i>P</i> ^c	miR-150-5p ^b	<i>P</i> ^c
1-hydroxynaphthalene	-0.007 (-0.125, 0.110)	0.902	-0.024 (-0.138, 0.091)	0.686	0.039 (-0.075, 0.153)	0.503	0.059 (-0.056, 0.174)	0.317	0.074 (-0.040, 0.187)	0.202	0.033 (-0.082, 0.148)	0.576	0.089 (-0.026, 0.204)	0.130	0.190 (0.076, 0.303)	0.001
2-hydroxynaphthalene	-0.007 (-0.133, 0.118)	0.908	-0.041 (-0.164, 0.083)	0.517	0.018 (-0.105, 0.141)	0.776	0.078 (-0.047, 0.203)	0.219	0.089 (-0.034, 0.213)	0.156	0.013 (-0.111, 0.137)	0.839	0.080 (-0.046, 0.206)	0.214	0.153 (0.029, 0.277)	0.016
2-hydroxyfluorene	-0.058 (-0.163, 0.046)	0.274	-0.037 (-0.142, 0.067)	0.483	-0.074 (-0.178, 0.030)	0.161	-0.031 (-0.136, 0.075)	0.565	-0.033 (-0.137, 0.070)	0.527	0.043 (-0.062, 0.148)	0.424	0.043 (-0.063, 0.149)	0.423	0.013 (-0.093, 0.118)	0.813
9-hydroxyfluorene	-0.010 (-0.116, 0.096)	0.857	-0.040 (-0.145, 0.066)	0.461	-0.038 (-0.143, 0.067)	0.482	0.031 (-0.076, 0.138)	0.566	-0.008 (-0.113, 0.097)	0.874	0.037 (-0.069, 0.143)	0.491	0.041 (-0.067, 0.148)	0.459	0.050 (-0.057, 0.156)	0.361
1-hydroxyphenanthrene	0.037 (-0.075, 0.149)	0.517	-0.046 (-0.157, 0.065)	0.414	0.011 (-0.099, 0.121)	0.846	0.024 (-0.088, 0.136)	0.674	0.022 (-0.088, 0.132)	0.695	0.016 (-0.095, 0.128)	0.771	0.027 (-0.085, 0.139)	0.639	0.074 (-0.038, 0.185)	0.195
2-hydroxyphenanthrene	0.000 (-0.106, 0.107)	0.996	-0.004 (-0.111, 0.103)	0.945	-0.014 (-0.120, 0.092)	0.799	0.031 (-0.077, 0.139)	0.573	0.007 (-0.099, 0.114)	0.892	0.075 (-0.032, 0.182)	0.171	0.038 (-0.070, 0.146)	0.488	0.121 (0.014, 0.228)	0.026
3-hydroxyphenanthrene	-0.034 (-0.139, 0.072)	0.529	0.027 (-0.079, 0.134)	0.615	-0.007 (-0.112, 0.099)	0.901	-0.014 (-0.121, 0.093)	0.797	0.016 (-0.089, 0.121)	0.763	0.038 (-0.069, 0.144)	0.488	0.069 (-0.038, 0.176)	0.206	0.046 (-0.061, 0.152)	0.402
4-hydroxyphenanthrene	-0.117 (-0.222, -0.013)	0.028	-0.141 (-0.246, -0.037)	0.008	-0.121 (-0.225, -0.018)	0.022	-0.065 (-0.171, 0.040)	0.224	-0.035 (-0.139, 0.070)	0.516	-0.063 (-0.168, 0.043)	0.244	-0.029 (-0.134, 0.076)	0.588	-0.053 (-0.158, 0.053)	0.329
9-hydroxyphenanthrene	-0.063 (-0.168, 0.043)	0.244	-0.030 (-0.136, 0.076)	0.574	-0.019 (-0.125, 0.086)	0.719	0.006 (-0.101, 0.113)	0.917	0.004 (-0.101, 0.108)	0.946	0.004 (-0.103, 0.110)	0.944	0.000 (-0.107, 0.107)	0.998	0.086 (-0.021, 0.193)	0.114

PAH biomarker ^a	miR-24-3p ^b	<i>P</i> ^c	miR-27a-3p ^b	<i>P</i> ^c	miR-142-5p ^b	<i>P</i> ^c	miR-16-5p ^b	<i>P</i> ^c	miR-451a ^b	<i>P</i> ^c	miR-28-5p ^b	<i>P</i> ^c	let-7b-5p ^b	<i>P</i> ^c	miR-150-5p ^b	<i>P</i> ^c
1-hydroxypyrene	-0.055 (-0.166, 0.056)	0.329	-0.087 (-0.196, 0.023)	0.121	-0.061 (-0.171, 0.048)	0.269	-0.009 (-0.120, 0.102)	0.878	0.017 (-0.092, 0.126)	0.759	-0.017 (-0.128, 0.093)	0.756	-0.027 (-0.138, 0.085)	0.638	0.091 (-0.020, 0.202)	0.107
ΣOH-PAHs	-0.030 (-0.143, 0.082)	0.593	-0.061 (-0.173, 0.050)	0.279	-0.017 (-0.128, 0.094)	0.758	0.035 (-0.078, 0.147)	0.544	0.015 (-0.096, 0.126)	0.793	0.014 (-0.098, 0.126)	0.810	0.048 (-0.066, 0.162)	0.407	0.150 (0.038, 0.261)	0.009
BPDE-Alb	-0.166 (-0.277, -0.055)	0.004	-0.084 (-0.194, 0.026)	0.134	-0.091 (-0.202, 0.019)	0.104	-0.050 (-0.163, 0.064)	0.389	-0.029 (-0.139, 0.081)	0.607	-0.180 (-0.290, -0.071)	0.001	-0.030 (-0.140, 0.080)	0.590	-0.067 (-0.182, 0.047)	0.249

^aLn-transformed; ^bLog2-transformed; ^cMultivariable linear regression analysis with adjustment for age, smoking status, pack-years of smoking, drinking status, working years, workplace, and BMI.

Table 4. Associations between miRNA expression and MN frequency (as the dependent variable) [FR (95% CI)].

miRNAs ^a	All samples (n=365)	<i>P</i> ^b	Nondrinkers (n=205)	<i>P</i> ^b	Drinkers (n=160)	<i>P</i> ^b	<i>P</i> _{interaction} ^c
miR-24-3p	1.152 (1.086, 1.222)	2.59×10 ⁻⁶	1.035 (0.954, 1.122)	0.407	1.293 (1.182, 1.415)	2.34×10 ⁻⁸	3.36×10 ⁻⁴
miR-27a-3p	1.093 (1.032, 1.157)	0.002	1.021 (0.944, 1.103)	0.608	1.180 (1.080, 1.290)	2.47×10 ⁻⁴	0.007
miR-142-5p	1.102 (1.041, 1.166)	8.59×10 ⁻⁴	1.076 (0.996, 1.163)	0.064	1.120 (1.027, 1.221)	0.010	0.288
miR-28-5p	1.147 (1.083, 1.216)	3.69×10 ⁻⁶	1.050 (0.965, 1.144)	0.259	1.236 (1.137, 1.344)	6.68×10 ⁻⁷	0.013
miR-150-5p	1.092 (1.033, 1.155)	0.002	0.997 (0.917, 1.082)	0.936	1.172 (1.084, 1.266)	6.16×10 ⁻⁵	0.009

Abbreviations: FR: frequency ratio.

^aLog2-transformed; ^bPoisson regression analysis with adjustment for age, smoking status, pack-years of smoking, working years, workplace, BMI, ΣOH-PAHs, BPDE-Alb adducts, and/or drinking status when appropriate; ^c*P*_{interaction} was calculated by entering the interaction term (miRNA*drinking status) into Poisson regression models, with adjustment for age, smoking status, pack-years of smoking, working years, workplace, BMI, ΣOH-PAHs, and BPDE-Alb adducts.

Table 5. Enriched biological functions of the target genes of five identified miRNAs^a.

ID	Description	P
GO:0030036	Actin cytoskeleton organization	1.49×10^{-4}
GO:0030029	Actin filament-based process	2.78×10^{-4}
GO:0006468	Protein amino acid phosphorylation	3.44×10^{-4}
GO:0065009	Regulation of molecular function	5.46×10^{-4}
GO:0009605	Response to external stimulus	5.78×10^{-4}
GO:0007242	Intracellular signaling cascade	6.37×10^{-4}
GO:0065008	Regulation of biological quality	7.81×10^{-4}
GO:0051347	Positive regulation of transferase activity	1.00×10^{-3}
GO:0010646	Regulation of cell communication	1.13×10^{-3}
GO:0007243	Protein kinase cascade	1.72×10^{-3}
GO:0050790	Regulation of catalytic activity	1.73×10^{-3}
GO:0051017	Actin filament bundle formation	1.88×10^{-3}
GO:0044267	Cellular protein metabolic process	1.95×10^{-3}

^amiRNAs for enriched biological function analysis: miR-24-3p, miR-27a-3p, miR-142-5p, miR-28-5p, and miR-150-5p.

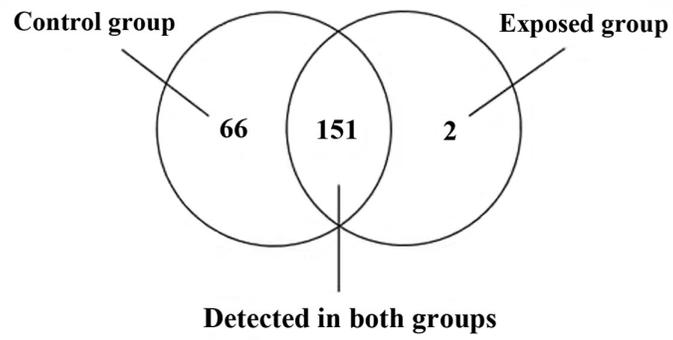
Figure legends

Figure 1. miRNA expression profiles in the discovery stage. (A). Distribution of miRNAs in control group and exposed group; (B). Scatter plots showing the relationship of the log₂-transformed expression levels of miRNAs between two groups.

Figure 2. Heat map of the log₂-transformed expression levels of 68 miRNAs (up) significantly downregulated ($FC \leq -5$) in the exposed group compared with the controls, and three miRNAs (below) mildly upregulated ($+2 \leq FC < +5$) in the exposed group compared with the controls.

Figure 1

A



B

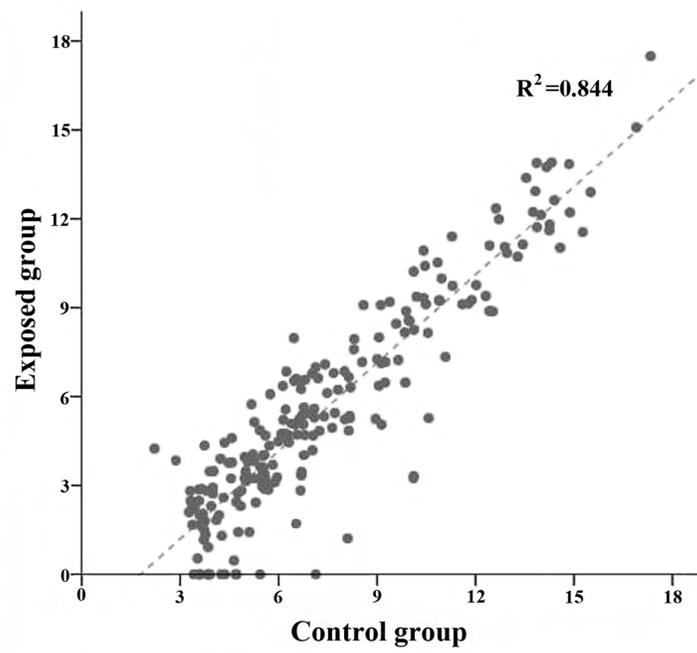


Figure 2

