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Comprehensive analysis of the alteration of plasma miRNA expression level in mice exposed to diesel exhaust

Ken Tachibana[1,2], Iori Kodaira[2], Noriko Kuroiwa[2], Ryo Uzuki[2], Yusuke Shinkai[2] and Ken Takeda[1,2]

[1]Division of Toxicology and Health Science, Faculty of Pharmaceutical Sciences, Sanyo-Onoda City University, 1-1-1 Daigaku-dori, Sanyo-onoda, Yamaguchi 756-0884, Japan
[2]The Center for Environmental Health Science for the Next Generation, Research Institute for Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

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ABSTRACT — MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides in length that play important roles in controlling a huge range of eukaryotic cell functions. Many studies have shown that abnormal expression levels of miRNAs are associated with many diseases and detrimental health effects caused by exposure to environmental pollutants and particulate matter. As a number of reports suggest that profiles of miRNAs in body fluids reflect physiological and pathological status, extracellular miRNAs, especially in plasma and serum, are being focused on as candidate disease biomarkers. Although these phenomena suggest that expression levels of plasma miRNAs can also be used as biomarkers for the detection of adverse health effects caused by exposure to environmental pollutants, there are still few studies on this subject. In the present study, we used diesel exhaust (DE) and filtered-DE (F-DE), which is DE with the particulate matter removed, as a model for environmental pollutant exposure and comprehensively analyzed alteration of the expression levels of plasma miRNAs in mice using a LNA miRNA microarray. MiRNA microarray analyses showed altered expression level of 5 plasma miRNAs (miR-1983, miR-720, miR-1957, miR-335-3p, and miR-1897-5p) in the DE-exposed group and F-DE-exposed group. The results show both the possibility that exposure to various environmental chemicals including DE alters plasma miRNAs and the potential for plasma miRNAs to be used as biomarkers of exposure to these chemicals.

Key words: Plasma microRNA, Diesel exhaust, Environmental chemical

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides in length that modulate intracellular expression of various target genes primarily by regulating their translation (Bartel, 2018; Jonas and Izaurralde, 2015; Kim *et al*., 2009). MiRNAs play important roles in controlling a huge range of eukaryotic cell functions such as cell growth, cell proliferation, cellular senescence, embryonic development, tissue differentiation, and apoptosis (Benhamed *et al*., 2012; Esquela-Kerscher and Slack, 2006). Many studies have shown that abnormal expression levels of miRNAs are closely related to diseases such as cancer, diabetes, stroke, and Alzheimer's disease (Hironaka-Mitsuhashi *et al*., 2017; Kumar *et al*., 2017; Liang *et al*., 2020; Vijayan *et al*., 2018). Furthermore, several reports indicated that exposure to environmental pollutants and particulate matter such as arsenic,

Correspondence: Ken Tachibana (E-mail: k_tachibana@rs.socu.ac.jp)

lead, cadmium, cigarette smoke, $PM_{2.5}$, and nickel nanoparticles affect the expression levels of miRNAs (An *et al*., 2014; Chao *et al*., 2017; Hassan *et al*., 2012; Mo *et al*., 2020; Ren *et al*., 2015). These reports suggest that dysregulation of miRNA expression is associated with detrimental effects caused by exposure to environmental chemicals.

Recent findings have shown that miRNAs, which are encapsulated into exosomes or lipoprotein or which form a complex with argonaute2 protein, are secreted in body fluids such as plasma, urine, and saliva (Arroyo *et al*., 2011; Valadi *et al*., 2007; Vickers *et al*., 2011). Because extracellular miRNAs are easily detectable and remarkably stable in body fluids (Arroyo *et al*., 2011; Li *et al*., 2012; Mitchell *et al*., 2008; Valadi *et al*., 2007; Vijayan and Reddy, 2020), various studies have been conducted on their potential as disease biomarkers. Furthermore, several reports have suggested that extracellular miRNAs have advantages as biomarkers because their profiles accurately reflect physiological and pathological statuses, including expression levels of miRNA of cells or tissues (Cortez *et al*., 2011; Pritchard *et al*., 2012; Yokoi *et al*., 2019). Although these phenomena suggest that expression levels of plasma miRNAs can also be used as biomarkers for the detection of adverse health effects, especially the functional alteration of cells and tissues, caused by exposure to environmental chemicals, there are still few studies on this subject. Use of miRNAs would also be considered useful as a method of minimally invasive inspection of the adverse health effects of exposure to not only environmental chemical pollutants but also chemical substances and particulate matter, such as engineered nanomaterials, which are used in the work environment.

In the present study, we used exposure to diesel exhaust (DE) and filtered-DE (F-DE), which is DE with particulate matter > 10 nm in size removed, as a model for exposure to environmental pollutant and comprehensively analyzed the alteration of the expression level of plasma miRNAs in mice using a locked nucleic acid (LNA) miRNA microarray.

MATERIALS AND METHODS

Animals and exposure

C57BL/6J male mice were purchased from SLC (Shizuoka, Japan) and acclimated until 8 weeks old. Mice were housed under controlled conditions (temperature: 22 ± 1ºC, humidity: 50 ± 5%) with a 12-hr light/12-hr dark cycle and *ad libitum* access to food and water. Mice were exposed to DE or F-DE in an inhalation chamber for 8 hr per day (10:00-18:00) for 2 weeks. Mice of the control group were housed in a clean air chamber. All experiments were performed in accordance with institutional and Japanese national guidelines for the care and use of laboratory animals.

Diesel exhaust

DE was produced by same procedure as described in the previous paper (Shaheen *et al*., 2016). A four-cylinder 2179-cc diesel engine (Isuzu Motors, Tokyo, Japan) was operated at a speed of 1500 rpm and 80% load with diesel fuel. The exhaust was introduced into a stainless-steel dilution tunnel (216.3 mm diameter × 5250 mm) in which the exhaust was mixed with clean air. To produce F-DE, DE was passed through a HEPA filter (kindly provided by Cambridge Filter Japan, Ltd. [Tokyo, Japan]) that can remove particles having a size of more than 10 nm. The mass and number concentrations of DE particles (DEP) were measured by a Piezobalance Dust Monitor (model 3521, Kanomax Japan, Osaka, Japan) and a condensation particle counter (model 3007, TSI Inc., Shoreview, MN, USA), respectively. Concentrations of gas components, (sulfur dioxide [SO_2] and carbon monoxide [CO]) in the chambers were measured by an Enhanced Trace Level SO_2 Analyzer (model 43i-TLE; Thermo Fisher Scientific, Franklin, MA, USA) and a CO Analyzer (model 48i; Thermo Fisher Scientific), respectively.

RNA extraction

One day after exposure of the mice to DE, blood samples were collected from the inferior vena cava and transferred to Capiject Capillary Micro Collection Tubes (Terumo, Tokyo, Japan), and then the plasma samples were separated by centrifugation.

For microarray analysis, 150 µL of plasma samples from each mouse in each treatment group (Control air: n = 9, DE: n = 9, F-DE: n = 9) were pooled and then total RNA was extracted using a mirVana PARIS kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. For quantitative reverse transcription polymerase chain reaction (qRT-PCR), total RNA was extracted from the plasma sample of each mouse in each treatment group (Control air: n = 6, DE: n = 5, F-DE: n = 5) using ISOGEN II (Nippon Gene, Tokyo, Japan) with slight modification. One mL of ISOGEN II and 400 µL of nuclease-free water were added to 50 µL of plasma from each mouse and mixed well by vortexing. Subsequently, 10 µL of 100 pg/µL *Caenorhabditis elegans* miR-39 RNA (*cel*-miR-39), which was synthesized by Hokkaido System Science (Hokkaido, Japan), was spiked to each sample for use in normalization of extraction efficiency. Then, total RNA

Table 1. Characteristics of diesel exhaust exposure.

	CO (ppm)	SO_2 (ppb)	DEP ($\mu g/m^3$)	DEP (number/cm^3)
Control air	0.37 ± 0.09	0.46 ± 0.26	8.2 ± 4.3	18.6 ± 8.6
DE	3.32 ± 0.69	6.76 ± 1.32	92.8 ± 12.3	50618.1 ± 29296.4
F-DE	2.26 ± 0.82	4.18 ± 1.47	24.7 ± 7.1	16.7 ± 6.1

Values are the average concentration of each component in control air, diesel exhaust (DE) and filtered-diesel exhaust (F-DE) expressed as mean ± SD.

was extracted in accordance with the manufacturer's instructions. Extracted total RNA samples were stored at -80°C until use.

MiRNA microarray analysis

MiRNA expression profiles of the plasma samples were obtained using the miRCURY LNA microarray (5th generation; Exiqon, Vedbaek, Denmark). The RNA samples were submitted to a miRCURY LNA microarray analysis service (Cosmo Bio, Tokyo, Japan). Each sample was labeled with Hy3 and double hybridized against a pool (blends of equal amounts of each sample) of the three samples (Control, DE, F-DE) labeled with Hy5, which was used as a reference. The obtained Hy3 signal was normalized with Hy5 signal in each array. The raw data was filtered to exclude probes with weak signals (average Hy3 signal was less than 10 in all samples) as well as probes with large percent coefficient of variation (%CV) values for replicate spots (%CV was higher than 50).

qRT-PCR

Total RNA was reverse transcribed using a Universal cDNA Synthesis kit (Exiqon) according to the manufacturer's instructions. Synthesized cDNA was diluted 20 times with ultrapure water and then applied to qRT-PCR, which was performed using miRCURY LNA SYBR Green Master Mix (Exiqon) with an MX-3000P system (Agilent Technologies, Santa Clara, CA, USA). PCR primers for miR-720 and *cel*-miR-39 were purchased from Exiqon. The obtained miR-720 signal was normalized with the *cel*-miR-39 signal.

Statistical analysis

Significant differences in qRT-PCR data from the Control air, DE-exposed group, and F-DE-exposed group were assessed using one-way ANOVA with the Tukey-Kramer *post hoc* test, and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

In the present study, we used DE, which is known to cause various detrimental health effects (Steiner *et al*., 2016; Tachibana *et al*., 2015; Taxell and Santonen, 2017), and F-DE as a model for exposure to environmental pollutants. The average number and mass concentration of the DEP of the DE-exposed group were approximately 5.1×10^4 particles/cm^3 and 0.1 mg/m^3, respectively. The average concentration of exhaust gases of the DE-exposed group was maintained at 3.32 ± 0.69 ppm for CO and 6.76 ± 1.32 ppb for SO_2 (Table 1). We produced a mass concentration of DEP of about 100 $\mu g/m^3$, which is environmentally relevant (Tachibana *et al*., 2015). The average concentration of exhaust gases in the F-DE-exposed group showed a value close to that of the DE-exposed group (2.26 ± 0.82 ppm for CO and 4.18 ± 1.47 ppb for SO_2, respectively) (Table 1). We confirmed that DEP were eliminated in the F-DE group to an extent comparable to that of the control level (Table 1).

First, to screen the plasma miRNAs whose expression levels are altered by DE exposure, we comprehensively profiled miRNA expression using a miRNA microarray (Supplemental Table 1). The plasma miRNA obtained from 9 mice of each group were pooled and applied for analysis. Differential expression of miRNAs was characterized by a fold change threshold of > 2.0 or < 0.5 compared to the Control group. Furthermore, miRNAs that showed a Hy3 signal of 100 or more in any of the three groups (Control, DE, or F-DE) were extracted. Finally, 5 miRNAs (miR-1983, miR-720, miR-1957, miR-335-3p, and miR-1897-5p) showed different expression levels in the DE-exposed group or F-DE-exposed group (Table 2). Especially, the expression levels of miR-720 and miR-1983 seemed to apparently depend on the number of particles in DE because the expression levels of these miRNAs in the F-DE group were equivalent to those in the Control group. We focused on miR-720 because the Hy3 signal from the microarray analysis was much higher than those of the other differentially expressed miRNAs.

To confirm the altered expression levels of miR-720,

Table 2. Plasma miRNAs that the expression level was altered by exposure to DE or F-DE.

microRNA	Signal intensity						Normalized signal (Hy3/Hy5)			Fold change	
	Control		DE		F-DE		Control	DE	F-DE	DE/ Control	F-DE/ Control
	Hy3	Hy5	Hy3	Hy5	Hy3	Hy5					
mmu-miR-1983	78.07	110.31	175.52	98.67	36.11	67.75	0.70	1.77	0.53	2.51	0.75
mmu-miR-720	324.35	459.74	590.23	393.49	126.57	172.78	0.70	1.49	0.73	2.13	1.04
mmu-miR-1957	53.32	87.00	111.85	90.51	11.78	39.80	0.61	1.23	0.29	2.02	0.48
mmu-miR-335-3p	111.45	133.03	177.46	121.03	29.20	73.67	0.83	1.46	0.39	1.75	0.47
mmu-miR-1897-5p	152.43	134.73	93.31	107.62	30.88	67.99	1.13	0.86	0.45	0.77	0.40

the plasma miRNAs obtained from each mouse, which were independent sample from the microarray analysis, were applied to qRT-PCR analysis. The results showed that the expression level of plasma miR-720 in the DE-exposed group was increased more than that in the Control group consistent with the results of the microarray analysis (Fig. 1). The F-DE-exposed group also showed increased expression of plasma miR-720 compared with that in the Control group, but the results were not consistent with those of the microarray analysis (Fig. 1). As multiple samples were pooled and used in the microarray analysis, it is presumed that inaccurate results were obtained in the F-DE-exposed group due to the influence of several different samples. From these results, we considered miR-720 to be a miRNA that shows an increased plasma expression level with exposure to DE regardless of the presence of particles.

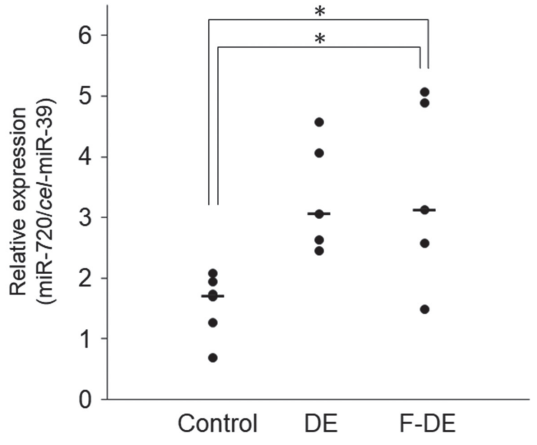

Fig. 1. Effects of diesel exhaust (DE) or filtered-DE (F-DE) exposure on the expression level of plasma miR-720. The expression level of plasma miR-720 was analyzed by qRT-PCR (Control air: n = 6, DE: n = 5, F-DE: n = 5). All values of miR-720 were normalized to the value of *cel*-miR-39. Points depict the value of each sample and bars indicate the median value. Significant differences were assessed using one-way ANOVA with the Tukey-Kramer *post hoc* test (*P < 0.05).

Recently, it was reported that several miRNAs are differentially expressed in the plasma of patients with cancer, liver diseases, and type 2 diabetes (Chen *et al*., 2008; Mitchell *et al*., 2008; Szabo and Momen-Heravi, 2017). Furthermore, several reports indicated that some plasma miRNA expression levels are altered in liver or kidney injury (Vliegenthart *et al*., 2015; Wolenski *et al*., 2017). These reports show that not only diseases but also tissue injury can alter plasma miRNA expression levels. The increase in the plasma miR-720 expression level could be derived from the tissue in which the expression level of this miRNA was affected by exposure to DE. To show the relationships between detrimental health effects and changes in plasma miR-720 expression levels following exposure to DE, alterations of the expression level of miR-720 and functions in each tissue and cell type will need to be clarified in a future study. Schopman *et al*. (2010) reported that miR-720 is a small RNA derived from transfer RNA (tRNA). RNA fragments derived from tRNAs were associated with the regulation of gene silencing (Haussecker *et al*., 2010) and were suggested to be involved in biological roles such as cell proliferation (Lee *et al*., 2009). Furthermore, tRNA-derived small RNAs are also associated with cancer, neurodegenerative diseases, and acquired metabolic diseases (Li *et al*., 2018). Although several reports suggest that miR-720 is associated with T-cell function, the progression of non-alcoholic fatty liver disease-non-alcoholic steatohepatitis-hepatocellular carcinoma, and tumor cell invasion and migration (Bhat *et al*., 2017; Tessitore *et al*., 2016; Wang *et al*., 2015), it is not fully understood what function of miR-720 is affected by exposure to DE. It will be necessary to clarify the alterations of miR-720 expression and accompanying functional changes in each tissue that are caused by exposure to DE.

In the present study, we showed that exposure to DE altered the expression level of several plasma miRNAs in mice. These results suggest the possibility that expo-

sure to various environmental chemicals including DE alters plasma miRNAs and also show the potential for plasma miRNAs to be used as biomarkers of exposure to these chemicals and potentially of exposure to chemical substances used in the workplace. Further studies are required to use plasma miRNAs as functional biomarkers indicative of detrimental health effects caused by chemical exposure. It will be especially important to clarify the relationship between exposure to various chemicals and alterations in plasma miRNA and to investigate what kind of functions the altered miRNAs exhibit.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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