

RESEARCH ARTICLE

Circulating microRNAs as potential biomarkers for smoking-related interstitial fibrosis

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Abstract

Numerous efforts have been made to identify reliable and predictive biomarkers to detect the early signs of smoking-induced lung disease. Using 6-month cigarette smoking in mice, we have established smoking-related interstitial fibrosis (SRIF). Microarray analyses and cytokine/chemokine biomarker measurements were made to select circulating microRNAs (miRNAs) biomarkers. We have demonstrated that specific miRNAs species (miR-125b-5p, miR-128, miR-30e, and miR-20b) were significantly changed, both in the lung tissue and in plasma, and exhibited mainstream (MS) exposure duration-dependent pathological changes in the lung. These findings suggested a potential use of specific circulating miRNAs as sensitive and informative biomarkers for smoking-induced lung disease.

Keywords: Cytokines, inflammation, microRNA, plasmas, smoking-related interstitial fibrosis

Introduction

The incidence of lung interstitial fibrosis is on the increase, and the diagnoses portend poor prognosis, with a mean survival rate of 2–5 years. In 2003, the mortality rate for pulmonary fibrosis was 64.3 deaths per million in men and 58.4 deaths per million in women (Olson et al., 2007). Although the American Thoracic Society and European Respiratory Society published a joint statement on the diagnosis and treatment of lung interstitial fibrosis in 2000 (American Thoracic Society, 2000), and the majority of practicing chest physicians are aware of these recommendations, there continues to exist uncertainty and variability as to the proper diagnosis and management of lung interstitial fibrosis (Peikert et al., 2008).

Smoking-induced chronic lung diseases, especially smoking-related interstitial fibrosis (SRIF) constitute a significant threat to patient health and have

enormous economic impacts on health care expenditures. Furthermore, Katzenstein et al. reported the presence of surprisingly frequent and often severe interstitial fibrosis in cigarette smokers with no clinical evidence of interstitial lung disease (Katzenstein et al., 2010).

Many studies have been conducted to identify more reliable and sensitive early blood markers for smoking-induced lung diseases by using various high throughput technologies. It is known that inflammatory markers have a temporal relationship to smoking (Bakhru & Erlinger, 2005; Leitch et al., 2005; van der Vaart et al., 2005) and that the acute effects of cigarette smoke have an impact on a number of cellular and biochemical measures in the lung (van der Vaart et al., 2004; Koczulla et al., 2010). Marques-Vidal et al. found circulating IL-6, TNF- α , C-reactive protein (CRP) were associated with cigarette smoking in healthy Caucasian populations

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(Marques-Vidal et al., 2011). Bruno et al. (2011) showed that leptin was inversely correlated with TNF- α , whereas sICAM-1 was positively correlated with TNF- α in plasma of chronic obstructive pulmonary disease (COPD) patients. Studies performed in age- and gender-matched patients with stable COPD have demonstrated that the plasma soluble form of sICAM-1 could be considered as a marker of inflammation (Aldonyte et al., 2004) and that its levels were positively correlated with TNF- α .

MicroRNAs (miRNAs) are small regulatory, noncoding RNAs (Bartel, 2004). It has been reported that miRNAs primarily affect the stability of mRNA and/or the initiation and progression of protein translation, but broader regulatory roles have been suggested (Ruvkun, 2008; Tang et al., 2008). Even though the biological functions of miRNAs are yet to be fully understood, it has been shown that the circulating levels of specific miRNAs correlated well with the pathological development of several different cancers (Huang et al., 2010), liver injury (Wang et al., 2009) and myocardial injury (Ji et al., 2009). Recently, a growing number of researches have suggested the potential for miRNAs-based blood biomarkers in disease diagnoses (Ji et al., 2009; Wang et al., 2009; Huang et al., 2010). We hypothesized that the levels of specific circulating miRNAs might also be used to monitor the pathological development of SRIF. Using a 6-month mainstream (MS) exposed mouse experiment, we report here a set of circulating miRNAs whose levels were directly associated with SRIF induced by MS exposure.

Materials and methods

Animals

C57 mice ($n = 50$; 6 weeks old) were purchased from and housed in West China Hospital Animal Center of Sichuan University. Mice were with fed standard rodent diet twice a day and housed in ventilated cages (five per cage) under controlled conditions ($23 \pm 2^\circ\text{C}$; 12/12-h light/dark periods). Experiments on animals were conducted in compliance with protocols approved by the Animal Care and Use Committee of the Sichuan University.

Exposure to MS

C57 mice were divided into five groups: control group ($n = 10$) was kept in a filtered air environment, and 1-, 2-, 3-, and 6M groups were exposed to 1-, 2-, 3-, and 6-month MS ($n = 10$ mice/group), respectively. MS was generated by burning Sunny Pride cigarette (China Tobacco of Chuanyu Industrial Company, Chengdu, China), having a content of 12 mg of tar, 1.2 mg of nicotine, and 13 mg of CO. Whole-body exposure of mice to mixed MS was achieved by using a smoke-producing machine of peristaltic pump (model WT600-1F, Longer Pump, Baoding, China) and a air producing machine of air sampler (model ZGQ-2, Air Sampler, Yancheng, China). A fan in the toxicant exposure cabinet was used to mix the MS and primary air of cabinet thoroughly. Another peristaltic pump was used to aspirate the mixed gas with

the equal flow rate to keep the homeostasis of atmospheric pressure. The CO concentration of cabinet was monitored by pneumatic detector (model AT1-GAMC, Chino Far East Technology, Canada). Those machines were adjusted to keep no more than 50 ppm CO in the cabinet (Support Information 1). Exposure was daily, 6 cigarettes/d divided into six rounds with a 1-h interval.

All mice were weighted daily. At the end of the experiment, the animals were weighted and anesthetized with 350 mg/kg chloral hydrate intraperitoneal injection (IP). Peripheral blood was gained by cardiac puncture. EDTA anticoagulated peripheral blood was used to extract and purify total RNA. Anticoagulation free peripheral blood was used to separate serum. The lungs were removed and embedded in paraffin blocks (LEIKA CM3050, Germany) after being kept in Bouin's fixative for 24 h. Four- μm thick cross-sections of lung were cut (LEIKA RM 2135, Germany) from the blocks. Three- μm thick cross-sections of lung were cut from the blocks and stained with H&E to observe pathological changes and Masson staining to reflect collagen fiber reconstruction. Sections were examined using a Leica DFC 280 light microscope and Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK). For each group, four random areas from each mice were selected. In total, 40 areas for each group under $40\times$ magnification ($40\times$ objective) were examined by two histologists. The histologists who were blind to experimental groups examined all the preparations, and on completion, both histologists made the same comments and graded the preparations with the same numbers. None of the preparations differed with regard to comments of the histologists.

RNA extraction, purification, and microarray analyses

Total RNA, including miRNA from plasma or tissue, was separately isolated and purified with *mirVana*TM miRNA Isolation Kit (Cat#1560, Ambion, Austin, TX) or RecoverAllTM Total Nucleic Acid Isolation Kit (Cat#AM1975, Ambion, Austin, TX) following the manufacturer's instructions and checked for a (RNA integrity number) RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA). The procedures of RNA extraction and purification were described in support information 2.

We used a microarray platform from Agilent to profile the miRNA spectra. The array contains 627 mouse and 39 viral miRNA sequences. miRNA molecular in total RNA was labeled by miRNA Complete Labeling and Hyb Kit (Cat# 5190-0456, Agilent Technologies, Santa Clara, CA) followed the manufacture's instructions, labeling section. Each slide was hybridized with 100 ng Cy3-labeled RNA using miRNA Complete Labeling and Hyb Kit in hybridization Oven (Cat#G2545A, Agilent technologies, Santa Clara, CA). After hybridization and washing, slides were scanned by Agilent Microarray Scanner (Cat#G2565BA, Agilent Technologies, Santa Clara, CA) and Feature Extraction software 10.7 (Agilent

Technologies, Santa Clara, CA) with default settings. Raw data were normalized by quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies, Santa Clara, CA).

Cytokine/chemokine biomarkers measurements

The cytokine/chemokine measurements were as King et al. (2010) reported. In short, the serum samples were harvested by centrifugation (3000 g for 15 min) in order to measure the protein levels of the cytokines/chemokines using the MILLIPLEX™ MAP MOUSE CYTOKINE/CHEMOKINE KIT (IL-6 and TNF- α , Cat# MPXMCYTO-70K, Millipore Corp., Billerica, MA) and the MILLIPLEX™ MAP RAT/MOUSE CRP SINGLE PLEX KIT (Cat# MCVD77K1CRP, Millipore Corp., Billerica, MA). Samples were measured in duplicate under the manufacturer's instructions. Assay plates were run on a Luminex 200 instrument (Luminex, Austin, TX). Five-parameter logistic standard curves were fit using Miraibio MasterPlex QT software (Hitachi Software, South San Francisco, CA).

Statistical analysis

The statistical analyses was performed, including a multiple ANOVA using SPSS (Version 13.0), and differences between groups were determined by the Student's *t*-test. Differences with $p < 0.05$ were taken as being statistically significant. Bioinformatics data were analyzed by SAS system (Shanghai Biotechnology Corporation).

Results

Dynamic changes of circulating cytokine/chemokine biomarkers

Three months exposure to MS caused dramatic inflammatory cells infiltration, which decreased during the 3rd- to 6th-month period (Supporting Information 3, S3). Smoking-related inflammation biomarkers in serum were measured, including IL-6, TNF- α , and CRP, during a 6-month MS exposure. We found some regularly dynamic changes that serum CRP and IL-6 were increasing until 3 months and decreased thereafter. However, the concentration of circulating TNF- α reached a peak level in the 3rd month. It was believed that MS exposure firstly induced CRP and IL-6 secreting, which may lead to the production of TNF- α (Figure 1). Those changes related well with the degree of lung tissue injuries induced by MS exposure, as supported by the histopathological examination (S3).

Dynamic ranges of miRNA expression levels in tissue and plasma

A significant number of miRNAs were either not expressed or expressed at very low levels as described (Liang et al., 2007; Tang et al., 2007). Excluding any miRNA with hybridization intensity <2-folds, the MS exposure resulted to different expression of 131 miRNAs in the 1st month, 117 miRNAs in the 2nd month, 113 miRNAs in the 3rd month, and 120 miRNAs in the 6th month, when comparing with control group (Figure 2B). There were 74 miRNAs species that were observed in both control and MS exposed groups (Figure 2A). By

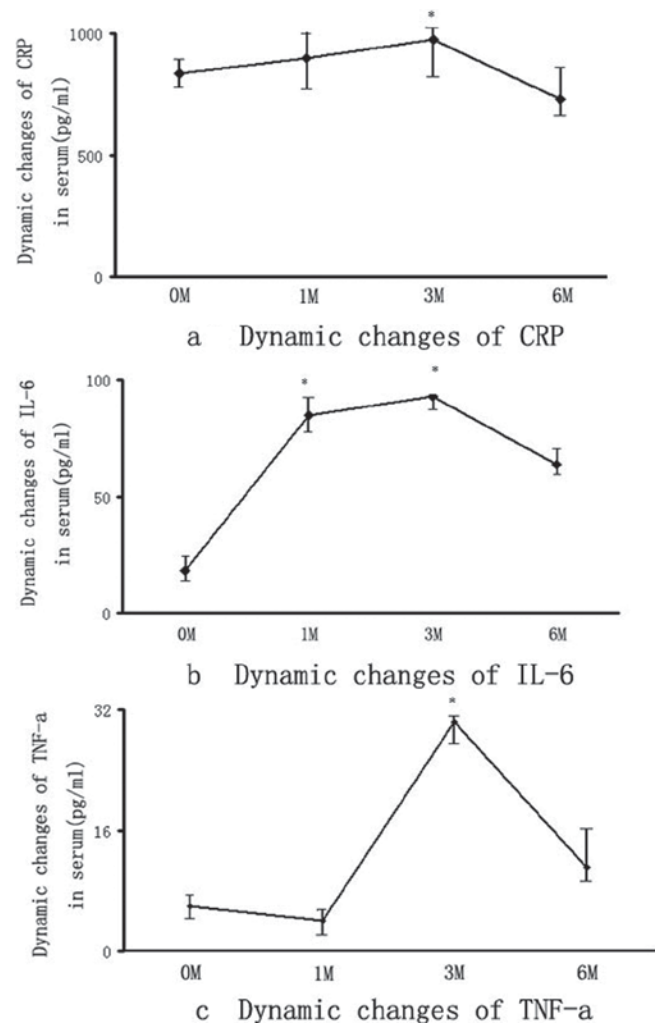


Figure 1. Dynamic changes of selected circulating cytokine/chemokine biomarkers. Levels of serum cytokines were measured by MILLIPLEX™ assay, during chronic 6-month mainstream cigarette smoke exposure. Serum (A) and IL-6 (B) levels increased between 1 and 3 months (1M, 3M) of exposure ($p < 0.05$) and decreased thereafter. The concentration of circulating TNF- α (C) reached its peak by the 3rd month ($p < 0.05$). * $p < 0.05$ comparing with levels at 0 month (0M). C-reactive protein

using the same selection criterion as above, there were 77 miRNAs in the 1st month, 101 miRNAs in the 2nd month, 34 miRNAs in the 3rd month, and 40 miRNAs in the 6th month, when comparing with control group (Figure 2C).

Chronic MS exposure induced changes in the expression of miRNAs in lung and plasma

The changes of different miRNAs in lung tissue were earlier than those in plasma. The peak level of different miRNAs numbers was in 1st month and decreased thereafter in lung tissue (Figure 2B). The peak of level of circulating different miRNAs numbers was in 2nd month, which was later than those in lung tissue (Figure 2C). Furthermore, when analyzing upregulation or downregulation different miRNAs numbers in each group, the results supported those dynamic changes.

The changes of miRNA levels in the lung may be due to cellular responses toward chronic MS exposure.

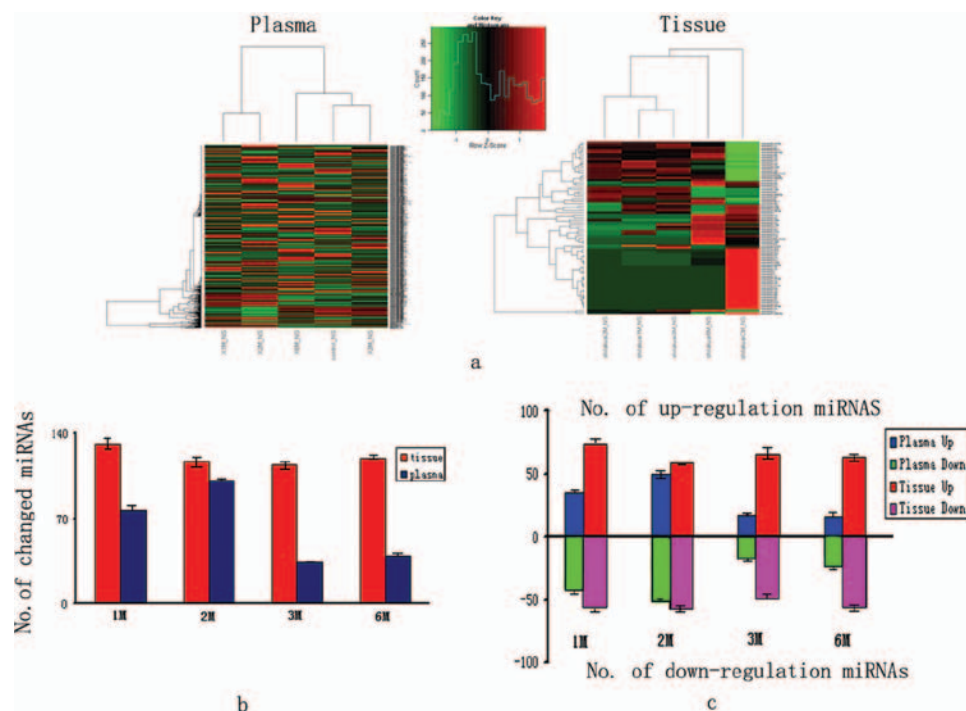


Figure 2. Dynamic ranges of miRNA expression levels in serum and lung tissue. (A) The hierarchical cluster analysis of miRNAs in serum and lung tissue. We chose any miRNA with a hybridization intensity >2-fold during the 6-month mainstream (MS) cigarette smoke exposure. (B) Increased expression of lung tissue miRNAs was observed over 6 months of MS cigarette smoke exposure as follows: 131 miRNAs in the 1st month (1M), 117 miRNAs in the 2nd month (2M), 113 miRNAs in the 3rd month (3M), and 120 miRNAs in the 6th month (6M), compared with the control group. Increased expression of serum miRNAs was observed over 6 months of MS cigarette smoke exposure as follows: 77 miRNAs in the 1st month (1M), 101 miRNAs in the 2nd month (2M), 34 miRNAs in the 3rd month (3M), and 40 miRNAs in the 6th month (6M), compared with the control group. The total level of different miRNAs in the serum (B) or the extent of upregulation or downregulation (C) were later than those changes in the lung tissue after chronic MS cigarette smoke exposure.

There are many different cell types and tissues in the body might contribute to the composition and the level of circulating miRNAs. Combining the detectable miRNA from lung and plasma samples revealed a total of 10 different miRNA species, miR-125b-5p, miR-128, miR-192, miR-30e, miR-423-5p, miR-484, miR-21, miR-20b, miR-350, miR-149, which were observed both in lung and in plasma among chronic MS exposed groups. Using Genespring Expression software, we found those miRNAs were response to drug/toxin/stimulus, oxidoreductase activity, inflammatory/immunity response, cell adhesion, extracellular matrix organization, fibroblast proliferation, apoptosis, lung development, integral to membrane, regulation of proliferation/differentiation, regulation of transcription (Table 1). This specific cluster of targeted gene functions suggested a general breakdown of tissue integrity and an increase in inflammation/immune responses, along with tissue repairing processes, following chronic MS exposure (Wang et al., 2009).

Discussion

The spectra and levels of some miRNAs have emerged as biomarkers for various pathological conditions (Waldman & Terzic, 2008). An increasing number of research suggested that circulating miRNAs may be promising plasma biomarkers for the diagnosis of different cancers (Huang

et al., 2010), liver injury (Wang et al., 2009), myocardial injury (Ji et al., 2009). We used chronic MS-induced lung injury in a mouse as a model system and detected changes in the spectra and levels of miRNAs both in plasma and lung tissue. There were 10 different miRNAs, selected from both plasma and lung tissue, discovered by the use of microarray gene chip technology.

Katzenstein et al. (2010) used 23 lobectomy specimens excised for neoplasms, including three from nonsmokers and 20 from smokers. Interstitial fibrosis involving greater than 25% of the specimens was identified in 12 of 20 smokers (60%), but in none of the three never-smokers. Furthermore, nine cases did not fit with a named interstitial lung disease and were considered to represent examples of SRIF. The pathological changes included alveolar septal thickening by collagen deposition with minimal inflammation, or random interstitial fibrosis in deep parenchyma with or without emphysema. Previous work by our group (Huang et al., 2012) showed a significant inflammation response during the 3-month MS exposure. However, the inflammation response dramatically decreased during the 3rd- to 6th-month period with increasing collagen deposition. These histopathological changes were characteristic for those changes observed in SRIF.

Proinflammatory cytokines, such as IL-6, TNF- α , and CRP, have important effects in inflammation. Several

Table 1. Targeted gene functions of selected miRNAs.

GO term	miR-125b-5p	miR-128	miR-192	miR-30e	miR-423-5p	miR-484	miR-21	miR-20b	miR-350	miR-149
Response to drug/toxin/ stimulus	Y	Y	<i>N</i>	Y	<i>N</i>	<i>N</i>	<i>N</i>	N	<i>Y</i>	<i>N</i>
Oxidoreductase activity	Y	Y	<i>N</i>	Y	<i>N</i>	<i>N</i>	<i>N</i>	Y	<i>Y</i>	<i>N</i>
Inflammatory/immunity response	Y	Y	<i>N</i>	Y	<i>N</i>	<i>N</i>	<i>Y</i>	Y	<i>N</i>	<i>Y</i>
Cell adhesion	Y	Y	<i>Y</i>	Y	<i>Y</i>	<i>Y</i>	<i>N</i>	Y	<i>N</i>	<i>Y</i>
Extracellular matrix organization	Y	N	<i>N</i>	N	<i>N</i>	<i>N</i>	<i>Y</i>	Y	<i>Y</i>	<i>N</i>
Fibroblast proliferation	N	N	<i>N</i>	Y	<i>Y</i>	<i>Y</i>	<i>N</i>	N	<i>Y</i>	<i>N</i>
Apoptosis	Y	Y	<i>N</i>	Y	<i>Y</i>	<i>N</i>	<i>N</i>	Y	<i>N</i>	<i>Y</i>
Lung development	N	Y	<i>N</i>	Y	<i>N</i>	<i>Y</i>	<i>N</i>	Y	<i>N</i>	<i>N</i>
Integral to membrane	Y	Y	<i>Y</i>	Y	<i>Y</i>	<i>Y</i>	<i>Y</i>	Y	<i>Y</i>	<i>Y</i>
Regulation of proliferation/ differentiation	Y	Y	<i>Y</i>	Y	<i>Y</i>	<i>Y</i>	<i>N</i>	N	<i>Y</i>	<i>Y</i>
Regulation of transcription	Y	Y	<i>Y</i>	Y	<i>Y</i>	<i>N</i>	<i>N</i>	Y	<i>Y</i>	<i>Y</i>

Summary of the relationship between miRNA and targeted gene functions. There are 10 miRNAs that were upregulated or downregulated by chronic mainstream cigarette smoke exposure and which overlapped between serum and lung tissue samples. Using Genespring Expression software to summarize the relationship between miRNAs and targeted gene functions, miRNAs were shown to be involved in a number of pathways including: drug/toxin/stimulus responses, oxidoreductase activity, inflammatory/immunity responses, cell adhesion, extracellular matrix organization, fibroblast proliferation, apoptosis, lung development, membrane integrity, regulation of proliferation/differentiation, and regulation of transcription.

Boldface text indicates most promising circulating biomarkers of SRIF and text in italics indicates abnormal expression miRNAs both in serum and tissue.

Y: Yes.

N: No.

studies have shown that cytokine levels can be mediated by several lifestyle factors, including cigarette smoking (Bermudez et al., 2002). Marques-Vidal et al. suggested that IL-6, TNF- α , and CRP were associated with cigarette smoking (Marques-Vidal et al., 2011). The dynamic changes of IL-6 and CRP were in conjunction with the occurrence of an inflammatory response. Furthermore, TNF- α expression levels did not change until the 3rd month, then, decreased to the normal levels. Therefore, it may be concluded that chronic MS exposure was the main reason for an inflammation response in the lung, and circulating IL-6, CRP, and TNF- α may act as promising biomarkers of MS exposure and subsequently induced inflammation. Collagen deposition was ascribed to reconditioning of foci of destroyed lung tissue (Peikert et al., 2008).

Challenges for developing protein-based biomarkers from body fluids, such as plasma, serum, and urine, include the complexity of protein composition, the assorted posttranslational modifications, the low abundance of protein of interest, the difficulty of developing suitable high-affinity capture agents, the complexities of proteolysis and protein denaturation, and potentially complex assay methods (Cowan & Vera, 2008). The biological function of circulating miRNAs is largely unknown; but, unlike proteins, there are far fewer known miRNA species, thus obtaining a complete profile is relatively easy. Currently, there are approximately 1000 known miRNAs for humans compared with perhaps a million or more serum proteins, including various processing and posttranslationally modified proteins (Khan

et al., 2010). In addition, miRNAs do not have known postprocessing modifications, with their small size (~20 nt) and excellent stability, their chemical composition is much less complex than most other biological molecules (Huang et al., 2010). Detecting specific miRNA species, although somewhat challenging, is inherently a much easier task than detecting proteins.

Accordingly, numerous researches have identified aberrant miRNAs expression profiles in many types of human diseases (Ji et al., 2009; Wang et al., 2009; Huang et al., 2010). Characteristics of miRNA expression are different between normal tissues and tumor tissues and vary among tumor types (Davalos & Esteller, 2010). In addition, circulating miRNAs in plasma may have a special tissue origin. Furthermore, the high stability of miRNAs constitutes an enormous advantage from a clinical diagnostic point of view. It is efficient isolation from clinical specimens including sputum (Xie et al., 2010), plasma (Mitchell et al., 2008), serum (Chen et al., 2008), and even formalin-fixed paraffin-embedded tissue samples stored for 10 years (Xi et al., 2007). We chose plasma and formalin-fixed paraffin-embedded tissue samples to exact total RNA, including miRNAs. Interestingly, the peak levels of different miRNAs in plasma, total different expression, upregulation or downregulation, were later than those changes in lung tissue after chronic MS exposure. This may imply that the cellular damage in the lung tissue resulted in transport or release of cellular miRNAs into the plasma, which may be a similar process by which cellular enzymes are released after cellular damage (Wang et al., 2009). We cannot, however, rule out the possibility

of a specific transport mechanism, as part of an inherent cellular process that releases specific miRNAs as a response to the chronic MS exposure.

Our results, although preliminary, strongly suggest that a more comprehensive study of circulating miRNAs and their association with various physiopathological conditions may lead to another dimension in the discovery of biomarkers in the blood from many physiological and pathological conditions. Furthermore, a more comprehensive program in mice and humans is needed to determine the specificity and sensitivity of selected miRNA species.

Declaration of interest

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