Expression profile of plasma microRNAs in premature infants with respiratory distress syndrome

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Abstract. As well-known regulators of gene expression, microRNAs (miRNAs) are important not only in cell proliferation and differentiation, but also in tumorigenesis and organ development. It has been estimated that miRNAs may be responsible for regulating the expression of almost one third of the human genome. Simultaneously, with advances in neonatal care in the clinic, an increased number of premature infants are being saved and, thus, respiratory distress syndrome (RDS) has become more common. However, previous non-miRNA studies have suggested their connection with RDS. In the present study, a miRNA microarray, including >1,891 capture probes was used to compared the expression profiles of plasma miRNAs between RDS and control groups. miRNAs, which were observed to have consistent fold-changes (fold-change ≥1.3) between the two groups were selected and validated using reverse transcription-quantitative polymerase chain reaction. As a result, 171 differentially expressed miRNAs were identified, including two upregulated and seven down-regulated miRNAs. Of these miRNAs, four were selected as having higher fold-changes between the two groups. This is the first time, to the best ouf our knowledge, that these nine miRNAs have been reported in RDS. It was hypothesized that these novel miRNAs may be important in RDS, and may provide meaningful biomarkers for the diagnosis of RDS.

Introduction

Respiratory distress syndrome (RDS) is a severe type of respiratory disease, characterized by a lack of pulmonary surfactant (PS). As an increasingly common syndrome in preterm infants, without effective treatment, progressive dyspnea, expiratory groaning and respiratory failure can appear just a few hours following birth (1). Treatment of this disease has remained difficult and although the assistance of PS and mechanical ventilation has resulted in a marked reduction in mortality rates, the incidence of RDS does not follow a downward trend due to increased survival rates of low birth weight premature infants (2).

The development of respiratory system begins at a gestational age (GA) of 3-4 weeks, which originated from the endodermal germ layer of the embryo. In humans, this is divided into six stages (3): Embryonic stage (GA of 3-7 weeks), pseudoglandular stage (GA of 7-16 weeks), canalicular stage (GA of 16-24 weeks), saccular stage (GA of 24 weeks to birth), alveolar stage and microvascular maturity stage. Among these, the earlier four stages occur in the uterus and are more likely to be affected by the complex extrauterine environment (3). The entire process of lung development is highly regulated, with the involvement of signal transduction molecules secreted from lung epithelial cells and interstitial cells, including WNT, BMP-4, TGF-β, SHH, FGF (3). In several situations, these signal molecules are further regulated by certain types of RNAs to guarantee developmental coordination in time and space. Micro (mi)RNAs are one of the small RNAs involved in lung development and diseases (4-8).

miRNAs are a type of endogenous, non-coding, single-stranded RNA molecule. It modulates the activity of specific mRNA targets at the post-transcription level, and are thus important in a wide range of physiological and pathological processes (9), including cell differentiation, cell proliferation, cell cycle regulation and organ development. miRNAs are highly conserved between different mammals and target almost 30% of the protein-coding genes in humans, including transcription factors, signaling proteins, receptors and metabolic enzymes (10-13). miRNA expression levels vary markedly among tissues and it has been suggested that dysregulation of miRNAs can contribute to pathogenic diseases (14).

In vivo, there are several resources of miRNAs, including tissue miRNAs and circulating miRNAs. As early as 1948, Mandel and Paris had demonstrated that RNAs are common in serum and plasma (15). Notably, miRNAs are also present in large quantities in the blood. It exhibits high stability to avoid blood damage from RNases following PH change or repeated freezing and thawing (16,17). The source of miRNAs

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remain to be fully elucidated, however they may be from apoptotic or necrotic cells (18). Although there have been no previous reports regarding plasma miRNAs in RDS, those of other diseases can be of assistance. Ai *et al* demonstrated that, in the plasma of patients with myocardial infarction, the expression level of miRNA-1 was at the same high level as in the myocardium (19). Taylor *et al* (12) also reported higher specific cancer-associated miRNAs in the peripheral blood of patients with ovarian cancer, from exosomes, compared with patients with benign diseases. Therefore, theoretically, plasma miRNAs may be a suitable potential biomarker in the diagnosis of clinical diseases. With the assistance of microarrays and subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the present study aimed to identify significantly expressed miRNAs in the plasma of infants with RDS, compared with normal premature infants.

Materials and methods

Ethical statement. The present study was approved by the Medical Ethics Committee of Nanjing Medical University Affiliated Nanjing Children's Hospital (Nanjing, China; permit number 201002008). Written informed consent was obtained from the guardians on behalf of the infants enrolled in the present study. All clinical investigations were performed according to the principles expressed in the Declaration of Helsinki (20). The guardians had the right to withdraw from the study at any time. Initially, 22 infants with RDS were recruited, however, the guardians of two of the infants withdrew from the investigation..

Patients. A total of 20 infants with RDS and 29 infants without RDS (controls) at a GA of 28-34 weeks. were recruited between October 2010 and May 2011 from the Neonatal Intensive Care Unit of Nanjing Children's Hospital. Written, informed consent was obtained from the family of the patient.

Inclusion criteria. In the RDS group the diagnostic criteria for RDS were as follows: GA between 28 and 34 weeks, classic symptoms of progressive dyspnea and corresponding X-ray signs, and required assistant ventilation via continuous positive airway pressure or mechanical ventilation. The control group comprised normal premature infants without breathing difficulty breathing or signs of RDS on X-ray.

Exclusion criterion. The criteria for exclusion in the present study included: The application of cortical hormone prior to or following birth, severe deformity or chromosomal abnormalities, a diagnosis of congenital adrenal hyperplasia, severe perinatal asphyxia or repeated hypoglycemia.

Sample collection. Fasting venous blood samples were collected from the infants in the morning between 7.00 and 9.00 am) on the 1st, 4th and 10th days following birth. Subsequently, the blood samples were transferred into EDTA anticoagulant tubes and centrifuged (CS-15R; Beckman Coulter, Inc., Fullerton, CA, USA) for 5 mins at 1,006.2 x g. The plasma was subsequently maintained in a refrigerator at 4˚C for further experiments.

RNA extraction. The total RNA was isolated from the plasma using TRIzol (Invitrogen Life Technologies, Carslbad, CA, USA) and an miRNeasy Mini kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. The RNA quality and quantity was measured using a nanodrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA) and the RNA integrity was determined using 1% agarose gel electrophoresis.

miRNA microarray. Following RNA isolation, an miRCURY™ Hy3™/Hy5™ Power Labeling kit (Exiqon, Inc., Vedbaek, Denmark) was used, according to the manufacturer's instructions for miRNA labeling. A total of three slides were used between the RDS group and control group. For each slide, 1 μg of each sample was 3'-end-labeled with Hy3™ fluorescent label, using T4 RNA ligase in the following procedure: The RNA, in 2.0 μl water, was combined with 1.0 μl CIP buffer and CIP (Exiqon, Inc.). The mixture was incubated for 30 min at 37˚C, and was terminated by incubation for 5 min at 95˚C. Subsequently, 3.0 μl labeling buffer, 1.5 μl fluorescent label (Hy3™), 2.0 μl dimethyl sulfoxide (DMSO) and2.0 μl labeling enzyme were added to the mixture. The labeling reaction was incubated for 1 h at 16˚C, and terminated by incubation for 15 min at 65˚C.

Following termination of the labeling procedure, the Hy3™-labeled samples were hybridized on the miRCURY™ LNA Array (v.16.0; Exiqon, Inc.), according to manufacturer's instructions. The 25 μl mixture from the Hy3™-labeled samples were added to 25 μl hybridization buffer and were denatured for 2 min at 95˚C, incubated on ice for 2 min and then hybridized to the microarray for 16-20 h at 56˚C in a 12-Bay Hybridization system (Nimblegen Systems, Inc., Madison, WI, USA), which provides an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance signal. Following hybridization, the slides were washed several times using a wash buffer kit (Exiqon, Inc.), and finally dried by centrifugation for 5 min at 134.1 x g. The slides were then scanned using an Axon GenePix 4000B Microarray Scanner (Axon Instruments, Foster City, CA, USA).

The scanned images were imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction. The replicated miRNAs were averaged and miRNAs with intensities >50 in all the samples were selected for calculation of the normalization factor. The data were normalized using the median normalization. Following normalization, differentially expressed miRNAs were identified through Volcano Plot filtering (fold-change≥1.3; P≤0.05). In addition, hierarchical clustering was performed using MEV software (v4.6; TIGR, Boston, MA, USA).

RT-qPCR. Following isolation of the RNA from the plasma using TRIzol reagent, single-strand cDNA was synthesized as follows: The RT mixture contained 1 μg total RNA, 0.3 μl rno-miRNA reverse primer (1 μM), 0.1 μl MMLV Revertase (200 U/μl; Epicentre, Madison, WI, USA), 2 μl 10X RT Buffer, 2 μl dNTP mix (2.5 mM each; HyTest, Ltd., Turku, Finalnd) and 0.3 μl ribonuclease inhibitor (40 U/μl; Epicentre), in a 20 μl total volume. The reaction was performed at 16˚C for 30 min and at 42˚C for 40 min,

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

microRNA	Primer (5'-3')
U6	CGCTTCACGAATTTGCGTGTCAT
hsa-miR-301a	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCTTTGA
hsa-miR-513a-3p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCTTCT
hsa-miR-3679-3p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGATGAA
hsa-miR-103a-2*	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAAGGC

miR, microRNA.

Table II. Comparison of the clinical characteristics between patients in the RDS and control groups.

Group	Number of patients	GA (weeks)	Gender ratio (male/female)	Weight (g)	Apgar score	
					1 min	5 min
RDS	20	31.1±1.6	15/5	1747.5±434.1	8.10±0.64	8.80±0.69
Control	29	29.5±7.5	19/10	1987.2±434.4	8.38±0.90	9.00±0.84
t-value	-	0.092	0.751[a]	1.899	1.190	0.873
P-value	-	0.361	0.386	0.064	0.240	0.387

[a] χ^2-value. RDS, respiratory distress syndrome; GA, gestational age.

followed by heat inactivation at 85˚C for 5 min. For qPCR, 1 μl cDNA was added to 24 μl aster mix containing 2.5 μl dNTP (2.5 mM each), 2.5 μl 10X PCR buffer (Promega Corporation, Madison, WI, USA) and 1 unit Taq polymerase (Promega), final concentration 0.25X Sybergreen1 (Invitrogen Life Technologies) and 2 μl reverse and forward primers (Invitrogen Life Technologies). The cDNA was amplified for 35 cycles on an Applied Rotor-Gene 3000 (Corbett Research, Syndey, Australia) PCR system. The primer sequences used are listed in Table I. RT-qPCR for U6 snRNA were performed in each plate as an endogenous control. The quantity of the PCR products were calculated from the threshold cycle (C_t), and the comparative C_t method was used. The quantity of each miRNA relative to U6 snRNA was calculated using the equation: $2 - (Ct^{microRNA} - Ct^{U6})$.

Statistical analysis. The data were analyzed using the SPSS 13.0 statistical package (SPSS, Inc., Chicago, MO, USA), and the data from the RT-qPCR was assessed using an independent samples t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

General features of the clinical data. The present study comprised 20 infants with RDS and 29 infants in total. The mean GAs and weights in the two groups were 31.1±1.6 weeks; 1747.5±434.1 g and 29.5±7.5 weeks; 1987.2±434.4 g, respectively. The constituent male/female ratios were 15/5 and 19/10, respectively. The mean 1 min Apgar scores were 8.10±0.64 and 8.38±0.90, and the mean 5 min Apgar scores were 8.80±0.69, and 9.00±0.84, respectively. No significant differences were identified in these values between the two groups (Table II).

miRNA expression profile. The sixth generation of the miRCURY™ LNA array (v.16.0; Exiqon), contains >1,891 capture probes, covering all human, mouse and rat miRNAs annotated in miRBase 16.0. As a result, in the three slides analyzed in the present study, 171 differentially expressed miRNAs passed the fold-change filtering, which identifies those with a fold-change >1.3 between the two groups, including 116 upregulated and 55 downregulated miRNAs (Table III). From these differentially expressed miRNAs, specific criterion were used to screen specific miRNAs for further investigation. The miRNAs selected were those that passed through Volcano Plot filtering, with a fold-change ≥1.3 and $P\leq0.05$. Following screening, two significantly upregulated and seven significantly downregulated miRNAs met the filtering requirements. The detailed fold-changes of these nine miRNAs in microarrays were compared using histograms (Figs. 1 and 2), based on the data in Table III. Hierarchical clustering was then performed to highlight distinguishable miRNA expression profiling between the two groups. In the heat map diagram (Fig. 3), each row represents one of the nine miRNAs and each column represents a slide. The miRNA clustering tree is showed on the left and the slide clustering tree is shown at the top.

RT-qPCR. Of the nine miRNAs identified, the four exhibiting the highest fold-changes, regardless of whether upregulated or downregulated, were further selected. These contained two upregulated miRNAs (hsa-miR-513a-3p and hsa-miR-103-2*) and two downregulated miRNAs (hsa-miR-301a and

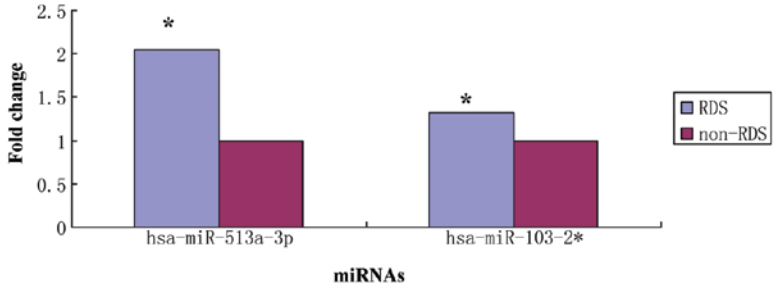

Figure 1. Fold-changes of two upregulated miRNAs between the RDS and non-RDS groups. Higher expression levels were observed in the RDS group (*P<0.05). miRNA, microRNA; RDS, respiratory distress syndrome.

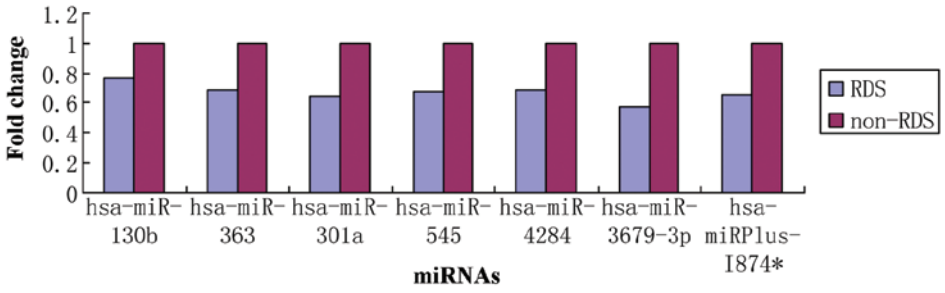

Figure 2. Fold-changes of seven downregulated miRNAs between the RDS and non-RDS groups (*P<0.05). miRNA, microRNA; RDS, respiratory distress syndrome.

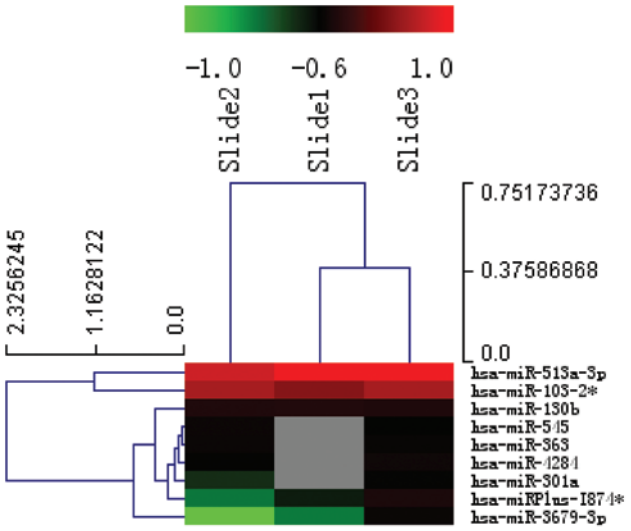

Figure 3. miRNA expression profile. In the heat map diagram, each row represents an miRNA and each column represents a one of the triplicate repeats. The miRNA clustering tree is shown on the left, and the slide clustering tree appears at the top. Red indicates high relative expression, green indicates low relative expression and black represents zero. miRNA, microRNA.

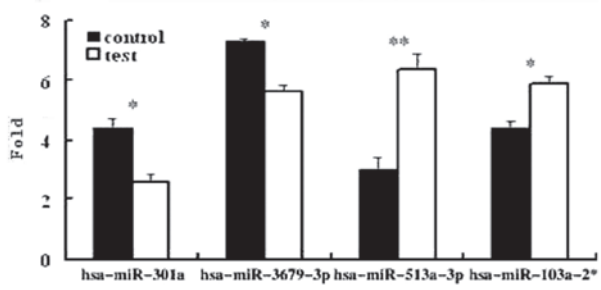

Figure 4. miRNA expression profile of the four selected miRNAs, determined using reverse transcription-quantitative polymerase chain reaction. The test group refers to the respiratory distress syndrome group. (*P<0.05 and **P<0.01, compared with the test group). miRNA, microRNA.

hsa-miR-3679-3p). These four selected miRNAs were subsequently confirmed using RT-qPCR, The relative expression levels of these are shown in Fig. 4.

Discussion

Clinical advancements in neonatal techniques have improved the prognosis of preterm infants, however, the incidences of BPD and RDS have also gradually increased (21,22). Although there are few statistics from large sample studies in China, in Europe, the incidence of RDS in infants of a GA between 23 and 25 weeks has risen to ~91% per year (23). Currently, it is suggested that RDS is a complex network disease caused by several factors. The disease is characterized by immature lung development and a lack of PS. Previously, the let-7 family and miRNA-17-92 cluster have been demonstrated to be important in lung development (5-8). Ventura *et al* (24) demonstrated that mice deficient in the miRNA-17-92 cluster exhibit lung hypoplasia defects, characterized by smaller, hypoplastic lungs, ventricular septal defects and abnormal B-cell development. In our preliminary study (25), several novel differentially expressed miRNAs were identified during normal lung development in rats, including miRNA-296 and miRNA-93.

In the present study, nine novel miRNAs were identified, including two upregulated and seven downregulated miRNAs. Among these, hsa-miR-513a-3p, hsa-miR-103-2*, hsa-miR-301a

Table III. miRNA expression profiles, with fold-changes, of upregulated and downregulated miRNAs between the two groups.

miRNA	Fold-change
Upregulated	
hsa-miR-3171	102.393
hsa-miR-938	33.867
hsa-miR-937	23.561
hsa-miR-513b	19.954
hsa-miR-3681	9.188
hsa-miRPlus-K1303*	8.900
hsa-miR-1275	8.203
hsa-miR-330-3p	5.242
hsa-miR-4275	5.239
hsa-miRPlus-A1072	5.050
hsa-miR-138-2*	4.169
hsa-miR-4288	3.941
hsa-miR-203	3.522
hsa-miR-483-3p	3.487
hsa-miR-589	3.104
hsa-miR-3907	2.989
hsa-miR-132	2.988
hsa-miR-23a	2.942
hsa-miR-183	2.733
hsa-miR-3178	2.690
hsa-miR-214	2.658
hsa-miR-491-3p	2.584
hsa-miRPlus-I382*	2.569
hsa-miR-769-3p	2.562
hsa-miR-640	2.423
hsa-miR-520d-5p	2.371
hsa-miR-720	2.273
hsa-miR-487b	2.268
hsa-miR-3915	2.219
hsa-miR-574-3p	2.196
hsa-miR-1260b	2.185
hsa-miR-1246	2.166
hsa-miR-519e	2.139
hsa-miR-138-1*	2.105
hsa-miR-154	2.062
hsa-miR-513a-3p	2.046
hsa-miR-513a-5p	2.011
hsa-miR-519e*	1.985
hsa-miR-597	1.964
hsa-miR-3685	1.953
hsa-miR-4324	1.922
hsa-miR-23c	1.907
hsa-miR-4291	1.868
hsa-miR-1260	1.839
hsa-miR-642b	1.838
hsa-miR-629*	1.832
hsa-miR-1290	1.816
hsa-miR-16	1.810
hsa-miR-99b*	1.808
hsa-miR-2115*	1.807
hsa-miR-559	1.717
hsa-miR-508-5p	1.712
hsa-miR-631	1.710
hsa-miR-221	1.709
hsa-miR-424	1.703
hsa-miR-891a	1.696
hsa-miR-518a-5p/hsa-miR-527	1.693
hsa-miR-933	1.656
hsa-let-7i*	1.641
hsa-miR-675	1.641
hsa-miR-224*	1.609
hsa-miR-211	1.585
hsa-miRPlus-A1015	1.583
hsa-miR-711	1.574
hsa-miR-3646	1.538
hsa-miR-1284	1.521
hsa-miR-25*	1.519
hsa-miR-345	1.517
hsa-let-7d*	1.515
hsa-miR-373*	1.512
hsa-miR-665	1.506
hsa-miR-492	1.505
hsa-miR-29b	1.496
hsa-miR-144	1.482
hsa-miR-452	1.479
hsa-miR-425*	1.470
hsa-miR-7-2*	1.464
hsa-miR-3615	1.462
hsa-miR-502-5p	1.457
hsa-miR-196a*	1.443
hsa-miR-638	1.443
hsa-miR-3161	1.440
hsa-miR-1273e	1.439
hsa-miR-501-5p	1.438
hsa-miR-525-5p	1.432
hsa-miR-1280	1.430
hsa-let-7e	1.417
hsa-miR-155	1.413
hsa-miR-1274b	1.405
hsa-miR-625	1.404
hsa-miR-3935	1.402
hsa-miR-92a	1.402
hsa-miR-101	1.401
hsa-miR-302c*	1.400
hsa-miR-451	1.396
hsa-miR-181a	1.396
hsa-miR-487a	1.394
hsa-miR-600	1.393
hsa-miR-1914	1.387
hsa-miR-125a-5p	1.384
hsa-miR-146a	1.374
hsa-miR-3686	1.364
hsa-miR-16-1*	1.362
hsa-miR-223	1.352
hsa-miR-187*	1.340

Table III. Continued.

miRNA	Fold-change
hsa-let-7a-2*	1.342
hsa-miR-4325	1.337
hsa-miRPlus-J1011	1.332
hsa-miR-3653	1.329
hsa-miR-1273c	1.328
hsa-miR-3196	1.327
hsa-miR-103-2*	1.319
hsa-miR-625*	1.315
hsa-miR-409-3p	1.314
hsa-miR-3202	1.310
hsa-miR-634	1.309
Downregulated	
hsa-miR-885-5p	0.459
hsa-miRPlus-A1073	0.483
hsa-miR-1281	0.487
hsa-miRPlus-A1087	0.537
hsa-miR-3940	0.556
hsa-miR-337-5p	0.570
hsa-miR-136*	0.571
hsa-miR-3679-3p	0.575
hsa-miR-718	0.578
hsa-miR-376b	0.578
hsa-miR-940	0.590
hsa-miR-432	0.599
hsa-miR-379	0.600
hsa-miR-376a*	0.624
hsa-miR-3201	0.641
hsa-miR-325	0.645
hsa-miR-3620	0.647
hsa-miR-301a	0.648
hsa-miR-2113	0.653
hsa-miRPlus-I874*	0.657
hsa-miR-301b	0.658
hsa-miR-3663-5p	0.666
hsa-miR-519d	0.672
hsa-miR-493	0.673
hsa-miR-620	0.674
hsa-miR-545	0.678
hsa-miR-32	0.682
hsa-miR-4284	0.685
hsa-miR-363	0.687
hsa-miR-4255	0.691
hsa-miR-548e	0.695
hsa-miRPlus-I107*	0.695
hsa-miR-24-2*	0.700
hsa-let-7b*	0.704
hsa-miR-552	0.708
hsa-miR-7	0.717
hsa-miR-431	0.718
hsa-miR-361-3p	0.719
hsa-miR-190	0.724
hsa-miR-126	0.728
hsa-miR-20b	0.729
hsa-miR-299-3p	0.731
hsa-miR-744	0.732
hsa-miR-1470	0.734
hsa-miR-146b-3p	0.743
hsa-miR-374c	0.744
hsa-miRPlus-B1114	0.745
hsa-miR-323-3p	0.746
hsa-miR-381	0.750
hsa-miR-140-5p	0.754
hsa-miR-542-3p	0.757
hsa-miR-377	0.764
hsa-miRPlus-A1086	0.764
hsa-miR-505*	0.768
hsa-miR-130b	0.769

Differentially expressed miRNAs passed fold-change filtering (fold-change >1.3 between the two groups). miRNA/miR, microRNA.

and hsa-miR-3679-3p were further analyzed, as they exhibited higher fold-changes in the microarray. Few previous studies have investigated miR-513a-3p. A study by Zhang *et al* demonstrated that miR-513a-3p can sensitize human lung adenocarcinoma cells to cisplatin by targeting glutathione S-transferase P1, which has been reported to contribute to cisplatin resistance in a number of studies (25-28). miR-103-2*, which has also received less investigation, is generally induced in response to hypoxia (29) and is also involved in pyruvate and lipid metabolism (30). Ellis *et al* reported that miR-103 changes significantly in patients with heart failure (HF), compared with patients with non-HF dyspnoea and healthy controls (31). Further clarification of the development of RDS may be beneficial.

The present study demonstrated for the first time, to the best of our knowledge, that miR-3679-3p is significantly expressed during the pathological process of RDS. Hsa-miR-301a is a newly identified miRNA, of which the majority of associated studies have focussed on cancer. Lu *et al* demonstrated that miR-301a downregulates nuclear factor-κB repressing factor (NKRF) and elevates the activation of NF-κB in cancer cells (32). This is considered to interact with specific negative regulatory elements to mediate the transcriptional activity of NF-κB, which regulates the expression of three NF-κB-responsive genes, interleukin (IL)-8, interferon-b, and nitric oxide synthase 2A. IL-8 is a cytokine with high levels of expression in the lung tissues of patients of model animals with RDS (33,34).

In addition to the four novel miRNAs evaluated, the five remaining downregulated miRNAs identified in the present study may also be involved in RDS due to their significant fold-changes between the RDS and control groups. Takahashi *et al* reported that miRNA-363 is overexpressed in CD4 (+) and CD8 (+) CB cells in human cord blood and adult

peripheral blood cells upon proinflammatory stimulation (35), which suggested its immunomodulatory role in inflammatory diseases, including RDS. As for miRNA-130b, miRNA-545, miRNA-4284 and miRNA-I874*, previous reports have demonstrated their association with cancer, rather than lung diseases and are, therefore, not discussed further.

In conclusion, the present study identified several differentially expressed miRNAs between the plasmaa of RDS and control groups for the first time, to the best of our knowledge. These results support the hypothesis that, to some degree, these novel miRNAs may be involved in the pathogenesis of RDS, and may assist in providing meaningful biomarkers for the diagnosis of RDS.

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