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MicroRNA Expression Aberration Associated with Bronchopulmonary Dysplasia in Preterm Infants: A Preliminary Study

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Abstract

BACKGROUND: Because environmental insults and genetic factors account for the variance in the risk of bronchopulmonary dysplasia (BPD) in very low birth weight (VLBW; birth weight < 1,500 g) preterm infants, the search for BPD biomarkers has begun to focus on the regulators of non-coding RNA such as microRNAs (miRNAs). Therefore, this study aimed to identify potential miRNAs involved in the pathogenesis of BPD in VLBW preterm infants. **METHODS:** A case-control study (15 BPD cases and 15 sex-matched controls) was conducted to investigate the expression profiles of 365 miRNAs in the peripheral blood of VLBW preterm infants at 36 weeks post-menstrual age (called the older-aged set). The expression levels of identified miRNAs were further evaluated in a subsample of blood collected during the first two weeks postnatal age (called the younger-aged set). Possible biological functions and pathways implicated in the target genes regulated by the miRNAs were explored using database predictions. **RESULTS:** A four-miRNA signature (*miR-152*, *miR-30a-3p*, *miR-133b* and *miR-7*) with aberrant expression levels at 36 weeks derived from a supervised classification with internal cross-validation discriminated the BPD cases from the controls with an accuracy of 0.91. The discriminative accuracy of the four miRNAs was supported by random permutations of either the disease status or the number of miRNAs selected (both $p < 0.0001$). A down-regulation change of *miR-152* and *miR-30a-3p* expression levels and an up-regulation change of *miR-133b* and *miR-7* expression levels were found in the older-aged set compared to the younger-aged set. **CONCLUSIONS:** This is the first study to identify blood-based miRNAs associated with BPD. The findings provide information regarding the roles of these biomarkers in the development of BPD in VLBW preterm infants.

Key words: bronchopulmonary dysplasia; microRNA; peripheral blood; biomarkers; expression profiling

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Introduction

Bronchopulmonary dysplasia (BPD) is an important chronic lung disease in preterm infants with very low birth weight (VLBW; birth weight < 1,500 g).[1, 2] Recent advances in the prevention and management of this respiratory illness such as antenatal steroid treatment, exogenous surfactant therapy and gentle ventilator strategies have changed the underlying pathology of BPD from severe structural disruption to developmental arrest of the neonatal lung.[3-5] The new form of BPD frequently occurs in extremely preterm infants with pathological features including impaired alveolarization, vascular growth abnormalities and mild inflammation.[5, 6]

In addition to environmental insults (e.g., hyperoxia exposure and respiratory therapies) to the immature lung in preterm infants, genetic factors may also account for a portion of the variation in BPD.[7, 8] Although several association studies have examined the roles of genetic variants in inflammatory regulation and surfactant synthesis in BPD, the results have failed to show consistent associations.[9-17] In developmentally appropriate BPD animal models, altered expression levels of certain transcription factors for protein-coding RNA have been linked to alveolarization, angiogenesis, or remodeling of the lung extracellular matrix.[18-22] However, BPD pathogenesis in human infants remains incompletely understood.

Instead of targeting DNA or protein-coding RNA in BPD, recent advances in molecular genetics have provided a new strategy for understanding the post-transcriptional regulation of gene expression via non-coding RNAs, in particular microRNAs (miRNAs), which have a regulatory effect on the translation of messenger RNA (mRNA) and protein production.[23] To date, approximately 1,600 human miRNAs have been identified and sequenced.[24] Approximately 30% of mRNAs are targeted by miRNAs, and aberrant expression of miRNAs can lead to many disease states.[25, 26] Although recent animal studies have identified several miRNAs in the developmental process of the embryonic lung, and alterations in miRNA

expression profiles are associated with BPD,[27-31] information regarding the role of miRNAs in BPD in human infants is limited.

This study, therefore, aimed to investigate the miRNA expression levels in the peripheral bloods of VLBW preterm infants with BPD and sex-matched controls at 36 weeks post-menstrual age (PMA; called the older-aged set). The expression levels of the differentially expressed miRNAs were further examined in a subsample of blood collected during the first two weeks postnatal age (called the younger–aged set) to elucidate the evolution of miRNA expression during BPD development. The possible functions and biological mechanisms implicated by the target genes regulated by these miRNAs were also explored using database prediction.

Methods

Participants

From September 2008 to December 2009, we prospectively enrolled VLBW preterm infants who were admitted to the neonatal intensive care unit (NICU) at the National Taiwan University Hospital and Mackey Memorial Hospital in Taipei, Taiwan, into this study. The recruitment was conducted during the first postnatal week. The inclusion criteria included gestational age (GA) <33 weeks, birth weight <1,500 g, absence of congenital and chromosomal abnormalities, and born in or admitted to the NICU within 7 days of birth. Subjects were excluded if their parents were of non-Taiwanese ethnicity, maternal age <18 years, or maternal history of substance abuse or a psychiatric disorder. BPD was graded for its severity at 36 weeks PMA according to the consensus definition of the National Institute of Health in America.[3]

Of the 54 eligible VLBW preterm infants, 53 (98%) participated, and 1 (2%) refused to participate in this study. After enrollment, 50 infants survived to 36 weeks PMA; 20 had BPD, and 30 did not have BPD. The incidence rate of BPD in our infants (37%) was comparable to

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the figures reported by the participating hospitals (20-40%). A volume of 500 μl of arterial blood was collected from participating infants twice in conjunction with routine daily blood test. The first blood draw was scheduled during the first two weeks of postnatal age (called the younger-aged set), and the second blood draw was conducted at 36 weeks PMA (called the older-aged set).

The perinatal and demographic data of the VLBW preterm infants were obtained from the infants' medical charts or via parental interview during hospitalization. The perinatal data included birth variables, respiratory variables and neonatal morbidities. The birth variables included the child's sex, gestational age, birth weight and birth set. The respiratory variables included the duration of ventilation and continuous positive airway pressure (CPAP) use. The neonatal morbidities included intraventricular hemorrhage, periventricular leukomalacia, patent ductus arteriosus, retinopathy of prematurity, necrotizing enterocolitis and sepsis. The demographic data included infant's sex and maternal education and occupation. This study was approved by the Ethics Committees of the participating hospitals. Written informed consent was obtained from the father or mother after a complete description of the study.

RNA Extraction and miRNA Quantification

Approximately 2-4 μg of RNA from mononuclear leukocyte was extracted from each blood sample using the RiboPure™-Blood Kit (Applied Biosystems, CA, USA). The RNAs of the older-aged set were assayed for the expression of 365 human miRNAs using the Multiplex RT and TaqMan® Low Density Array Human MicroRNA Panel v1.0 and 7900 real-time RT-PCR system (Applied Biosystems), using RNU48 as an endogenous control. The RNAs of the younger-aged set were quantified using the qRT-PCR for the expression levels of the individual miRNAs identified from the older-aged set.

The expression level of each miRNA from the array-based profiling was quantified through the normalized threshold cycle number (ΔC_t), in which $\Delta C_t = [C_t\,(\text{miRNA})] - [C_t\,(\text{U48})]$ and the

relative expression level was calculated as $2^{-(\Delta Ct)}$, which is commonly used in genome-wide miRNA profiling studies.[32] The fold-change in the expression of miRNA between two groups was calculated as $2^{-(\Delta\Delta Ct)}$.

Statistical Analyses

For group comparisons of perinatal and demographic variables between the VLBW preterm infants with BPD and those without BPD, the Fisher's exact test was used for categorical variables, and the Wilcoxon signed rank test was used for continuous variables. Significant variables were considered as potential confounders for subsequent analyses of the relations of miRNA expression levels to BPD occurrence.

To select differentially expressed miRNAs from the older-aged set, the Wilcoxon rank-sum test was used to compare the continuous data of expression levels and Fisher's exact test to compare the binary data of detectability between groups based on a liberal threshold *P* value of 0.2. The markers identified from univariate analyses were then subjected to the stepwise logistic regression analysis to select a cluster of miRNAs that could discriminate the cases from the controls. Furthermore, the leave-one-out cross-validation and two types of permutations (permutation of disease status and number of markers selected) were conducted to evaluate the robustness of discrimination by the identified miRNA signature.[33] Subsequently, the fold-change in the relative expression of miRNA and the direction of fold-change from the younger-aged set to the older-aged set were examined in the subsample with RNAs available at both time points.

The potential target genes regulated by the miRNAs were assessed using miRWALK algorithm.[34] The software Ingenuity Pathways Analysis (IPA; Ingenuity® Systems), which contains global molecular network data, was used to explore the biological and functional pathway for the target genes. All statistical analyses in this study were performed using the Statistical Analysis Software program (version 9.1, SAS Institute, Cary, NC), and a *P* value of <

0.05 was considered significant.

Results

Sample Characteristics

Of the 50 participating infants, the first blood draw was successfully conducted in 21 infants, 11 cases (10 with moderate BPD and one with severe BPD) and 10 controls, within the first two postnatal weeks to form the younger-aged set (Table 1). The second blood draw was successfully performed in 44 infants at 36 weeks PMA; 15 cases (14 with moderate BPD and one with severe BPD) and 15 sex-matched controls were selected to form the older-aged set. In both sets, the cases had younger gestational ages and lower birth weights and required a longer ventilation and CPAP use than the controls (all $p < 0.05$). Furthermore, the cases were more likely to have patent ductus arteriosus than the controls (both $p < 0.05$). Analysis of the respiratory data between the cases and a subgroup of controls (n = 10) with comparable gestational ages (<31 weeks) indicated a longer use of ventilation and CPAP in the former than in the latter (all $p < 0.05$; data not shown).

Insert Table 1 about here

miRNAs Identified from the Older-Aged Set

For the older-aged set, the blood samples tended to be collected earlier for the cases than for the controls (postnatal age 37 ± 11 days vs. 47 ± 12 days), although the difference did not reach significance ($p = 0.09$). Out of the 365 miRNAs examined in the older-aged set, 210 (58%) were detected in the infants, and 155 (42%) were undetectable. The heatmaps of 365 miRNAs in terms of the proportion of infants in each group with detectable expression are illustrated in Figure 1 using the Generalized Association Plots program.[35]

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Insert Figure 1 about here

For 210 detectable miRNAs, the expression levels did not differ between the cases and controls. When miRNA expression was dichotomized as detectable or not, differential detectability between groups was found in four miRNAs. The stepwise logistic regression analysis revealed that *miR-133b* and *miR-7* had significantly higher detectability in the cases than in the controls, whereas *miR-152* and *miR-30a-3p* had significantly lower detectability in the cases than in the controls (all $p < 0.05$; Table 2).

Insert Table 2 about here

The utility of these four miRNAs in discriminating the cases from the controls was evaluated using multivariable logistic regression analysis with a leave-one-out cross-validation procedure. The four-miRNA signature was associated with the greatest accuracy value, with an area under the curve (AUC) of receiver operating characteristics of 0.91 (Figure 2). The sensitivity and specificity for the optimal combination of the four miRNAs (expression of *miR-133b* and *miR-7* together with non-expression of *miR-152* and *miR-30a-3p*) were 0.87 and 0.80, respectively. Because there were high correlations among potential confounding perinatal variables, only some were included as covariates in the analysis. After adjustment for the effect of birth weight and patent ductus arteriosus, the specificity values slightly increased to 0.87, and the sensitivity remained unchanged.

Insert Figure 2 about here

The robustness of BPD discrimination by the four-miRNA signature was further

evaluated using permutations in two ways. First, when the disease status of the subjects was permuted 100,000 times, none of the AUCs derived from the permuted samples (mean = 0.63, SD = 0.07) exceeded the observed value of 0.91 ($p < 0.00001$). Second, when a certain number of miRNAs (ranging from 3 to 9) were randomly selected, the average of AUCs (range, 0.44-0.73) was found to be smaller than those observed for the four-miRNA signature.

Expression of Four miRNAs in the Younger- and Older-Aged Sets

For the 16 infants (seven cases and nine controls) whose RNA expression levels were available in both younger- and older-aged sets, the fold change in the expression levels of the four miRNAs were compared (Figure 3). For the younger-aged set, the fold change in the expression levels between the BPD and the non-BPD group was positive for *miR-152* and negative for *miR-30a-3p*, *miR-133b,* and *miR-7*. For the older-aged set, the fold change in the expression levels was negative for *miR-152* and *miR-30a-3p* and positive for *miR-133b* and *miR-7*. Analysis of the fold change from the younger-aged set to the older-aged set revealed a down-regulation change in the expression of *miR-152* (from 1.2 to -4.8) and *miR-30a-3p* (from -2.1 to -2.7) and an up-regulation change in the expression of *miR-133b* (from -4.9 to 1.2) and *miR-7* (from -2.3 to 1.6).

Insert Figure 3 about here

Target Genes and Functional Relevance of the Four-miRNA Signature

Of the four miRNAs, *miR-152*, *miR-30a-3p* and *miR-7* are included in the human database miRNAMap with detectable expression in lung tissue.[36] The miRWALK algorithm indicated that 7239 target genes were relevant to the four miRNAs, with 66 being shared target genes. The functional pathway analysis showed that the shared target genes may be related to organ and embryonic development as well as developmental disorders (all $P < 0.001$; Table 3).

Among them, *GLI3* and *NFIB* were the most significant target genes involved in lung development (both $P < 0.001$). Canonical biological pathway analysis revealed that the pathways with the most significant changes were the Wnt/β-catenin and axon guidance signaling pathways (both $P = 0.04$).

Insert Table 3 about here

Discussion

To our knowledge, this is the first study to investigate the peripheral blood of VLBW preterm infants in early postnatal life for differentially expressed miRNAs that have an association with BPD. We identified four miRNAs with expression levels at 36 weeks PMA that differed between VLBW preterm infants with BPD and those without BPD. Two time-point comparisons of the expression levels of these miRNAs at 36 weeks PMA with those at the first two weeks postnatal age further revealed a down-regulation change in *miR-152* and *miR-30a-3p* expression together with an up-regulation change in *miR-133b* and *miR-7* expression during the early stage of BPD. Our results provide a preliminary understanding of the roles of miRNAs in the development of BPD in VLBW preterm infants.

The four-miRNA signature had acceptable discriminative value for the detection of BPD in VLBW preterm infants. Our observation concerning the higher discriminative accuracy of the combined altered expressions of these four miRNAs than that of single miRNA was consistent with the findings of a previous study.[33] Because miRNAs are regulators closer in temporal sequence to outcome compared with the genetic information encoded in the genome, the aberrantly expressed miRNAs may be useful biomarkers for BPD. Among the four differentially expressed miRNAs, *miR-30a-3p* and *miR-30* were found to be highly up-regulated in the mouse lung during the period of lung septation.[27, 31] In humans,

miR-30a-3p, *miR-133b*, and *miR-7* are associated with adult lung diseases, with *miR-30a-3p* and *miR-133b* being down-regulated and *miR-7* being up-regulated in the lung tumor tissue of adults with adenocarcinoma.[37-39] Furthermore, *miR-152* is associated with childhood asthma.[40] Thus, these four miRNAs appear to be associated with a variety of lung diseases.

BPD in preterm infants has been postulated to result from lung injuries related to prolonged use of ventilation and oxygen therapy in the neonatal period.[3, 5, 6] In this study, the preterm infants with BPD were subjected to a longer ventilation duration (a median of 7 days vs. 0 days) or CPAP use (a median of 34 days vs. 4 days) than those without BPD. Furthermore, the four miRNAs showed differential expression levels in the peripheral blood of preterm infants with BPD at 36 weeks PMA when respiratory support was in use. A study in humans has shown that hypoxic events induced altered expression levels of several miRNAs (e.g., *miR-152*) in colonic cells.[41] An animal study screening for lung miRNA profiles in rats after chronic hypoxia showed aberrant expression levels of several miRNA markers including *miR-30*.[42] It is possible that the aberrant expression levels of miRNAs might relate to hypoxic or hyperoxic insults from ventilator and oxygen use.

It is noteworthy that our preliminary results suggest a developmental shift in the expression pattern of these four miRNAs (i.e., a down-regulation change of *miR-152* and *miR-30a-3p* expression and an up-regulation change of *miR-133b* and *miR-7* expression) during the neonatal period. A genome-wide profiling study in mice found 21 differentially expressed miRNAs in hyperoxia-exposed BPD mice. Both *miR-30* and *miR-133* showed a more than 2-fold increase in BPD mice than in control mice, and the expression levels increased with advancing age at 2, 7 and 21 post-natal days.[31] An increase of oxygen exposure with the ages might be one possible factor relating to the change in miRNA expression. Our finding concerning *miR-30a-3p* down-regulation in preterm infants with BPD was in contrast to the up-regulation observed in the mouse BPD model.[31] Future investigation of the evolution

of miRNA expression at various time points after oxygen use may elucidate how oxygen therapy might alter the expression levels of the four miRNAs in preterm infants with BPD.

In addition to the lung injury caused by oxidative stress, developmental disorders of alveolarization and dysmorphic pulmonary vasculature have recently been recognized as pathological hallmarks of the new form of BPD.[6] While the lung tissue is not readily accessible for investigation of miRNAs in the surviving preterm infants, blood-based expression profiling is increasingly being undertaken as a non-invasive method to investigate BPD biomarkers. It is plausible that these BPD-related expression aberrations of miRNAs in the peripheral mononuclear leukocyte might relate to the pathologic changes of fewer alveolar capillary and dysmorphic pulmonary vasculature in the preterm infants with BPD. The insult of the air-exchanging function may exert its influence on the gene expression of peripheral lymphocytes via cytokines, growth factors or transmembrane molecules.[43, 44]

An intriguing finding from the functional pathway analyses among database-derived target genes for the four miRNAs indicated that the two most significant target genes, *NFIB* and *GLI3*, are involved in lung development such as alveolarization and pulmonary vasculature, consistent with the pathological hallmarks of the new form of BPD.[6, 45] For example, the transcription factors encoded by *NFIB* genes were shown to be highly expressed in the embryonic mouse lung and might be involved in fetal lung maturation,[46, 47] and *GLI3*, a transcription factor of the Sonic hedgehog cascade, is critical for the patterning of early lung morphogenesis in mice.[48, 49] Moreover, *NFIB*-deficient mice die shortly after birth, and their severe lung hypoplasia resembles BPD .[47, 50] In future work, the miRNA-target genes must be validated using either exogenous (e.g., over-expression of certain miRNAs in lung cell lines to examine the protein expression level of the target genes) or endogenous (e.g., manipulation of miRNAs under physiological conditions) miRNA experiments.[51]

The Wnt/β-catenin signaling pathway identified from the canonical biological pathway

analysis among the miRNA-targeted genes can regulate airway epithelial differentiation and vascular smooth muscle proliferation in the developing lung.[52] Disruption of the Wnt/β-catenin signaling pathway in the primordial mouse lung results in a failure to form lung buds and thereby arrests proliferation in the vascular smooth muscle of the lungs.[53, 54] Further investigation should examine how the target genes and the four-miRNA signature modulate the Wnt/β-catenin signaling pathway in an animal or cell line model of BPD.

The results of this study should be interpreted with some limitations in mind. First, because the number of human miRNAs (n = 365) examined in this study is much less than what is currently available (more than 1,600 to date), further assessment of the miRNAs not included in our experiment is warranted. Second, despite the acceptable discriminative accuracy of the blood-based miRNAs for BPD, these biomarkers might not represent the entire spectrum of regulatory changes in BPD pathophysiology. Direct examination of the lung tissue is needed to clarify the underlying mechanisms. Third, VLBW preterm infants without BPD were treated as the controls in this study. Nevertheless, the individual differences among the cases and the controls in terms of the growth and nutrition status, neonatal care or other medical treatments during the neonatal period were not accounted for in this study. Experimental factors such as the miRNA assay conditions may further influence the miRNA expression levels and, hence, the fold-change in relative expression of miRNA when using the data of controls as the reference. Finally, the sample size in this study was relatively small; therefore, future validation of our results in a larger sample is necessary.

In summary, this study demonstrates the potential utility of a mononuclear leukocyte-based miRNA signature as biomarkers for BPD in VLBW preterm infants. As the pathological features of the new form of BPD have been changed to a developmental disorder of the neonatal lung, the identified miRNAs and related target genes in this study provide insightful information for future investigation of the pathogenesis of BPD in VLBW preterm

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Figure Legends

Figure 1. miRNA expression profiling of 365 miRNAs in VLBW preterm infants of the older-aged set with and without BPD. The heatmaps of individual miRNAs for each group are presented as the proportion of infants with detectable miRNA expression, with higher proportions (more red in color) indicating more infants showing expression. The four differentially expressed miRNAs are marked by an asterisk; two miRNAs (*miR-7* and *miR-133b*) showed higher expression levels (more red in color), and the others (*miR-152* and *miR-30a-3p*) showed lower expression levels (more green in color) in the BPD group.

Figure 2. Receiver operating characteristics (ROC) curve for the discrimination of BPD by the four-miRNA signature model.

Figure 3. Expression pattern of the four-miRNA signature in the younger- and older-aged sets in a subsample of 16 infants (seven cases and nine controls) with their RNA expression levels examined at both time points. *miR-152* and *miR-30a-3p* showed a down-regulation change (A), and *miR-133b* and *miR-7* showed an up-regulation change (B) in the relative expression levels of the BPD group versus the non-BPD group in the older-aged set compared to the younger-aged set.

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Table 1. Perinatal and Demographic Characteristics of VLBW Preterm Infants with and without BPD.

Characteristics	Older-Aged Set		Younger-Aged Set	
	BPD (N=15)	No BPD (N=15)	BPD (N=11)	No BPD (N=10)
Perinatal				
Gestational age (wk)	28 (24-30)*	31 (27-32)*	28 (24-30)†	31 (27-32)†
Birth weight (g)	1060 (506-1440)*	1314 (1040-1478)*	1020 (628-1340)†	1296 (1000-1478)†
Twin or multiple births	5 (33%)	6 (40%)	5 (45%)	6 (60%)
Duration of ventilation (days)	7 (0-71)*	0 (0-3)*	7 (0-71)†	0 (0-2)†
Duration of CPAP (days)	34 (0-58)*	4 (0-11)*	32 (0-56)†	4 (0-11)†
Intraventricular hemorrhage				
Normal	10 (67%)	9 (60%)	8 (72%)	6 (60%)
Grade I-II	4 (26%)	6 (40%)	1 (9%)	4 (40%)
Grade III-IV	1 (7%)	0 (0%)	1 (9%)	0 (0%)
Periventricular leukomalacia	5 (33%)	2 (13%)	1 (9%)	1 (10%)
Patent ductus arteriosus	11 (73%)*	1 (7%)*	7 (64%)†	1 (10%)†
Necrotizing enterocolitis	1 (7%)	0 (0%)	0 (0%)	0 (0%)
Sepsis	1 (7%)	2 (13%)	1 (9%)	1 (10%)
Demographic				
Male gender	6 (40%)	6 (40%)	4 (36%)	5 (50%)
Maternal education (years)	14 (12-16)	16 (12-18)	16 (12-18)	16 (12-18)
Maternal occupation				
Housewife or unskilled labor	8 (53%)	7 (47%)	2 (18%)	7 (70%)
Technician	4 (27%)	6 (40%)	5 (45%)	3 (30%)
Professional	3 (20%)	2 (13%)	4 (36%)	0 (0%)

The data are presented as the median (range) or N (%).
VLBW, very low birth weight; BPD, bronchopulmonary dysplasia; CPAP, continuous positive airway pressure.
*, †, $P < 0.05$ in comparing the two groups in each set.

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Table 2. The Four miRNAs with Aberrant Expressions at 36 Weeks Post-menstrual Age Derived from a Stepwise Logistic Regression Analysis in a Sample of 15 Cases (VLBW preterm infants with BPD) and 15 Sex-matched Controls (VLBW preterm infants without BPD).

miRNA	Chromosomal Region*	Location*	Detectability of miRNA Expression		*P*-value†
			BPD (n=15) N (%)	No BPD (n=15) N (%)	
miR-152	17q21.32	Intron	1 (7)	8 (53)	0.005
miR-30a-3p	6q13	Intron	0 (0)	5 (33)	0.03
miR-133b	6p12.2	Intron	12 (80)	7 (47)	0.04
miR-7	9q21.32	Intron	10 (67)	5 (33)	0.04

VLBW, very low birth weight; BPD, bronchopulmonary dysplasia.

* Information regarding the four miRNAs and the expression levels in the lung was drawn from the human database of miRNAMAP (http://mirnamap.mbc.nctu.edu.tw/).

† Based on the stepwise logistic regression analysis.

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Table 3. Biological Functions and Diseases Related to the Target Genes in the Four-miRNA Signature

Category	Function and Disease*	Involved Target Gene†
Organ development	Organ development	*APTA7A, BCL11B, GLI3, NFIB, SLC1A2*
	Lung development	*GLI3, NFIB*
	Forebrain development	*GLI3, NFIB*
	Brain development	*GLI3, NFIB, SLC1A2*
Embryonic development	Neural tube development	*ENAH, GLI3*
	Embryonic tissue development	*ENAH, GLI3*
	Cell death of embryonic cell lines	*AAK1, MTF1*
	Neurological process of embryonic tissue	*ENAH, GLI3*
Developmental disorder	Developmental disorder	*ATP7A, GLI3, NFIB, SLC1A2, VANGL1*

* The listed functions and diseases were determined through the Ingenuity Pathways Analysis.

† The target genes of the four-miRNA signature involved in the biological function or disease.

All $P < 0.001$.

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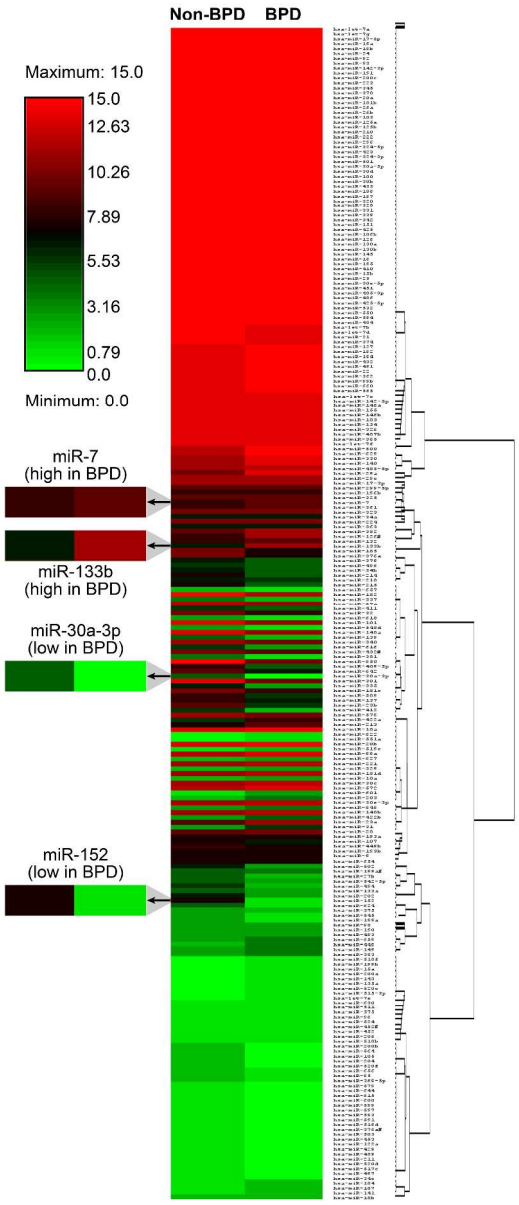

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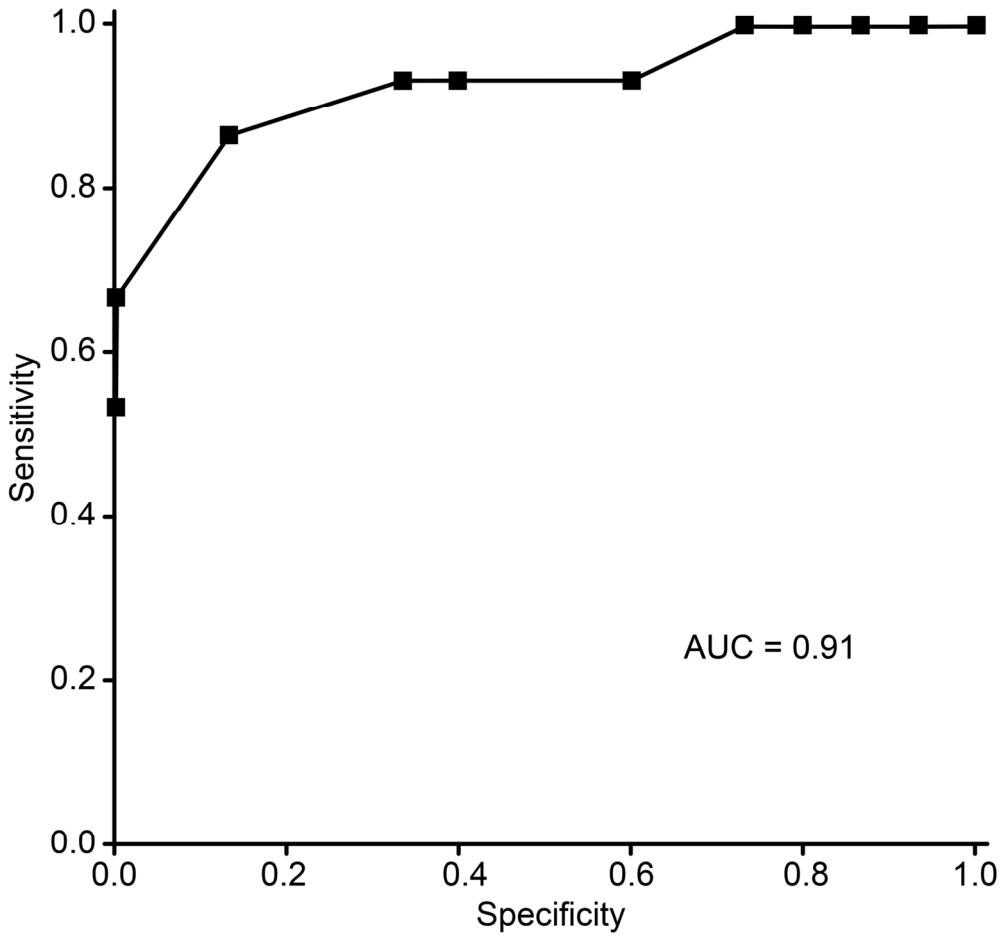

Figure 2. Receiver operating characteristics (ROC) curve for the discrimination of BPD by the four-miRNA signature model.
79x73mm (600 x 600 DPI)

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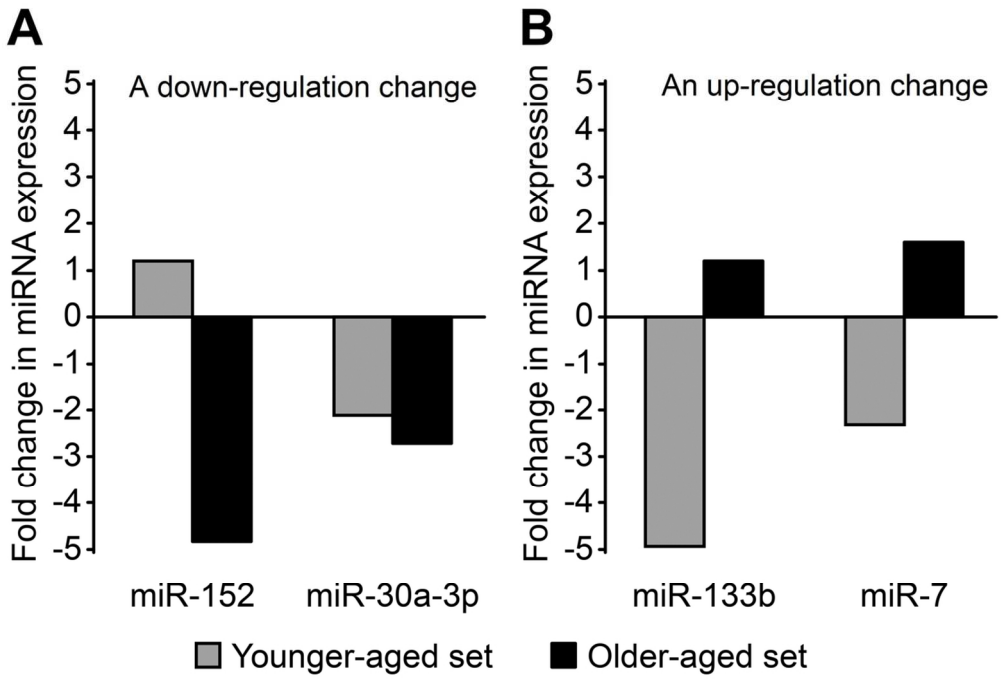

Figure 3. Expression pattern of the four-miRNA signature in the younger- and older-aged sets in a subsample of 16 infants (seven cases and nine controls) with their RNA expression levels examined at both time points. miR-152 and miR-30a-3p showed a down-regulation change (A), and miR-133b and miR-7 showed an up-regulation change (B) in the relative expression levels of the BPD group versus the non-BPD group from the younger-aged set to the older-aged set.
57x38mm (600 x 600 DPI)