Original Research

Identifying a novel serum microRNA biomarker panel for the diagnosis of childhood asthma

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Impact Statement

We screened differential serum miRNAs in asthmatic children using the Illumina sequencing method. Then, four differential miRNAs between asthmatic children and healthy children were validated by qRT-PCR. The classification tree model constructed by the four miRNAs showed a good diagnostic ability. These four miRNAs could be used as a potential biomarker panel of childhood asthma, whose main mechanisms may be related to inflammation, immunity, and transcriptional efficiency.

Abstract

The pathological mechanism of childhood asthma is complex, and timely diagnosis is the key to effective prevention and control of childhood asthma. We collected 170 serum samples from 95 children with asthma and 75 healthy children. Serum miRNA biomarkers were analyzed by Illumina sequencing for childhood asthma. Differentially serum miRNAs were confirmed with quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. The Illumina sequencing data showed the differential expression of 111 serum miRNAs among asthmatic and healthy children. After confirmation of miRNAs expression through qRT-PCR, four of them (namely hsa-miR-106a-5p, hsa-miR-18a-5p, hsa-miR-144-3p, and hsa-miR-375) manifested significant differential expression between asthmatic children and healthy controls. The biomarkers classification tree model created with these four miRNAs using the Biomarker Patterns Software could effectively separate childhood asthma and healthy children, with a specificity of 88.3%, a sensitivity of 95.0%, and an area under the curve (AUC) value of 0.942. The regulatory networks containing miRNAs and their gene targets suggested that the four miRNAs might have gene targets implicated in inflammation, immunity, and transcriptional efficiency. Taken together, this four-serum-miRNA panel is a promising biomarker to diagnose childhood asthma noninvasively.

Keywords: Asthma, microRNA, biomarkers, genomics, models, bioinformatics

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Introduction

Bronchial asthma, a chronic respiratory disease with high incidence, has characteristic pathological changes of chronic airway inflammation, irreversible airway remodeling, and airway hyperresponsiveness, and involves various types of inflammatory cells, airway structural cells, and cytokines.[1,2] It is caused by various factors, such as environmental factors, viral infections, and allergens.[3,4] Asthma is diagnosed in approximately 300 million individuals around the world.[5] In recent decades, the prevalence of asthma symptom in Latin America, Eastern Europe, Asia, and Africa has continued to rise.[6] The incidence of childhood asthma is increasing year by year. Asthma brings a heavy burden to children, families, and society. If children's asthma is not diagnosed and treated in time, it may develop into adult asthma. Timely diagnosis of asthma is the key to effective prevention and control of childhood asthma.

Childhood asthma is mainly diagnosed by clinical symptoms, pulmonary function measurement, and treatment effect. The clinical manifestations of asthma in children, especially preschool infants, are not typical. The cooperation degree of young children affects the reliability of pulmonary function indicators. Some children with asthma are not sensitive to corticosteroids, and the effect of asthma treatment is affected by children's medication compliance. Therefore, it is necessary to further explore more specific and sensitive objective indicators to diagnose childhood asthma early.

MiRNAs refer to non-coding RNAs with small lengths that can regulate gene expression to modulate cellular processes including apoptosis, proliferation, and differentiation.[7,8] MiRNAs are associated with the pathogenesis of cancer, pulmonary tuberculosis, Alzheimer's disease and other diseases.[9–12] Panganiban *et al.*[13] utilized several plasma miRNAs, namely miR-206, miR-125b, miR-16, miR-133b, and miR-299, to distinguish asthmatics from healthy individuals.

Table 1. Characteristics of children with asthma and healthy children.

	Illumina sequencing training set	Validation set	Total
Asthma			
Total number of patients	15	80	95
Age (year, average; range)	5 (4–11)	6 (4–11)	6 (4–11)
Gender (female: male)	5:10	28:52	33:62
Healthy controls			
Total number of control subjects	15	60	75
Age (year, average; range)	6 (4–10)	6 (4–10)	6 (4–10)
Gender (female: male)	6:9	25:35	31:44
Total	30	140	170

Childhood asthma deterioration is accompanied by markedly less expression of miR-29c and increased expression of B7 homologue 3 (B7 H3). Hsa-miR-10b-5p, hsa-miR-151a-3p, and hsa-miR-615-3p were negatively associated with the percentage of BALF eosinophils, and the proportion of neutrophils was inversely correlated with hsa-miR-9-5p, hsa-miR-151a-5p, hsa-miR-224-5p, and hsa-miR-581. It was speculated that these miRNAs might be evaluated as biomarkers of severe asthma.

We aimed to analyze serum miRNAs in childhood asthma by Illumina sequencing, along with qRT-PCR verification, to identify those with significant differential expression to guide the diagnosis of the disease.

Materials and methods

Study population

We acquired 170 serum samples from 95 childhood asthma and 75 healthy children from the pediatric clinic and the child health clinic of Zhejiang Provincial Hospital of Chinese Medicine (Hangzhou, China), respectively. Children with asthma fulfilled the criteria for asthma of the Global Initiative for Asthma (GINA) and had no other systemic diseases. Patients with respiratory tract infection in the recent 4 weeks, such as pharyngitis, bronchitis, and pneumonia, were excluded. All healthy children had no history of asthma, allergic diseases, or other systemic diseases. The age and gender were not significantly different between children with asthma and healthy children ($P < 0.05$). All the participants were of Han ethnicity. Detailed information of the participants is summarized in Table 1. The ethical committee of Affiliated Hospital of Hangzhou Normal University (Hangzhou, China) granted approval for this research; all the participants (or their guardians) provided informed consent.

Serum preparation and RNA extraction

Fasting early morning peripheral venous blood samples of 3 mL were obtained and subjected to centrifugation at 4000 g, 4°C for 10 min within 2 hours to collect serum samples, which were randomized into an Illumina sequencing set and a validation set (Table 1). In Illumina sequencing set, two sample pools (asthma group and healthy controls group) were created by combining 15 samples and mixing by inversion. Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Germany) from 200 μL serum from each sample pool. The purity and quantity of RNA samples were tested with a Nanodrop-2000 ultraviolet spectrophotometer (Thermo Fisher Scientific, USA).

Illumina sequencing

Libraries for small RNAs were generated by the TruSeq Small RNA Sample Prep Kits (Illumina, USA) for sequencing with Illumina Hiseq 2000/2500. The raw 50 bp single-ended reads were analyzed by ACGT 101-miR (LC Sciences, USA). Afterwards, 18-26-nt-long unique sequences were mapped to miRBase 21.0 through BLAST. With the thresholds of fold change in miRNA copy number > 1.5 or < 0.5 and $P < 0.05$, differentially expressed known miRNAs were identified by Fisher's exact and chi-square (2*2) tests.

qRT-PCR assay

Serum samples of 80 asthmatic and 60 healthy children constituted the validation set. These samples were subjected to miRNA isolation with the miRcute Serum/plasma miRNA isolation kit (Tiangen, China). Then miRNAs were converted into cDNA by reverse transcriptase using the miRcute Plus miRNA First-Strand cDNA Kit (Tiangen, China), whereas the MiRcute Plus miRNA qPCR Detection Kit (SYBR Green) (Tiangen, China) was utilized for miRNA quantification in serum samples. The universal reverse primer sequence for miRNAs was 5'-AGTGCAGGGTCCGAGGTATT-3,' while the sequences of primers specifically targeting hsa-miR-18a-5p, hsa-miR-106a-5p, hsa-miR-144-3p, and hsa-miR-375 are shown in Table S1. The 20-μL qPCR mixture was composed of 7.2 μL of RNase-free ddH_2O, 10 μL of 2×miRcute Plus miRNA Premix containing Rox and SYBR dyes, 0.4 μL each of sense and antisense primers (10 μM), and 2 μL cDNA. All reactions were run on the Bio-Rad CFX96 system (Bio-Rad, USA) with the following PCR program: initial denaturation for 15 min at 95°C and 40 cycles of 95°C for 20 s and 60°C for 34 s. The tests were performed in triplicate.

Statistical analysis and decision tree modeling

Raw threshold cycle values were normalized with that of miR-16, which was considered as an internal reference gene.[14] Relative expression of miRNAs was determined by the $2^{-\Delta\Delta Ct}$ algorithm: $\Delta\Delta Ct$ = (Ct value of target miRNA—Ct value of miR-16 in childhood asthma samples)—(Ct value of target miRNA- Ct value of miR-16 in healthy control samples). The experiment included a negative control with RNase-free ddH_2O as the template. We utilized GraphPad

Table 2. The number of miRNAs in serum samples from asthmatic children and healthy controls by sequencing analysis.

	Healthy controls		Childhood asthma	
	Known miRNA in miRBase	Novel miRNA	Known miRNA in miRBase	Novel miRNA
Number of pre-miRNA	928	19	1065	18
Number of unique-miRNA	1169	20	1378	17

miRNA: MicroRNA.

Prism 5.0 (Graphpad Software Inc., USA) to perform statistical analyses. Nonparametric Wilcoxon rank-sum test was implemented to determine differences in expression of miRNAs comparing asthmatic and healthy children. ROC curves for miRNAs and logistic regression models were established by MedCalc (Belgium).

A classification tree for childhood asthma was generated by Biomarker Pattern Software (BPS, Ciphergen Biosystems) with the relative expression levels of target miRNAs. Briefly, relative expression levels of target miRNAs were imported as a "root node" into BPS, after which two child nodes were generated from the "root node" based on a cut-off expression level of miRNA defined by BPS: Samples with relative miRNA expression levels lower than or equal to the cut-off level were grouped into the "left-side child node"; the rest were grouped into the "right-side child node." The validation set was subjected to several decision-making rounds and demonstrated to be discriminatory with minimal error. The childhood asthma and healthy control samples could be differentiated according to the relative expression of miRNA levels.

Bioinformatics analysis

TargetScan (http://genes.mit.edu/targetscan/index.html) and MiRanda (http://www.microrna.org/miranda.html) were employed to predict miRNA target(s). Gene targets implicated in childhood asthma were identified based on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Cytoscape (Cytoscape Software, USA). Functional enrichment of targets of the miRNAs was carried out using Gene Ontology (http://www.geneontology.org/). Pathway enrichment of the target genes of the miRNAs was implemented with Kyoto Encyclopedia of Genes and Genomes (KEGG). An miRNA-gene-network depicting the interactions of miRNAs and their targets was established with Cytoscape.

Results

Detection of the serum miRNAs by Illumina sequencing

The expression levels of serum miRNAs from 15 childhood asthma and 15 healthy children were appraised by Illumina sequencing. Altogether, 1395 miRNAs were detected in the serum of asthma children, 1189 miRNAs were detected in healthy controls, and 1046 miRNAs were detected in both. Among them, 20 new miRNAs in healthy controls and 17 new miRNAs in children with asthma were detected (Table 2). There were 111 serum miRNAs displaying significant differential expression between healthy controls and children with asthma, 90 being up-regulated (Table S2) and 21 being down-regulated in children with asthma (Table S3).

Table 3. Differentially expressed serum miRNAs in asthmatic children compared to the healthy controls by qRT-PCR analysis.

miR-names	Fold-change	*P* value
hsa-miR-18a-5p	2.645279	<0.0001***
hsa-miR-106a-5p	1.612864	<0.0001***
hsa-miR-144-3p	1.724157	<0.0001***
hsa-miR-375	1.809566	0.0003***

miRNA: MicroRNA; qRT-PCR: quantitative reverse transcription polymerase chain reaction.
***$P < 0.001$.

Differentially expressed serum miRNAs that met the criteria of ① exhibiting ⩾10 counts in Illumina sequencing in both childhood asthma and control samples; ② having a mean fold change >1.5 or <0.5, with a high percentage; ③ having a significant difference in *P* values; ④ being documented childhood asthma-associated miRNAs were further analyzed. A total of 12 candidate serum miRNAs were subjected to qRT-PCR verification (Table S4).

Identification of differentially expressed miRNAs between childhood asthma and control children by qRT-PCR

In our study, 12 candidate miRNAs were validated in 80 children with asthma and 60 healthy controls by qRT-PCR. The screening criteria of differentially expressed miRNAs were as follows: ① having a mean fold change (childhood asthma/healthy children) >1.5 and $P < 0.05$; ② having a detection rate <75% and a Ct value >35. Hsa-miR-18a-5p ($P < 0.0001$), hsa-miR-106a-5p ($P < 0.0001$), hsa-miR-144-3p ($P < 0.0001$) and hsa-miR-375 ($P = 0.0003$) showed differential expressions between childhood asthma and healthy controls. The comparisons of their expression levels between asthmatic and healthy children revealed values of 2.645279, 1.612864, 1.724157, and 1.809566, respectively (Table 3). Figure 1 displays the different expression levels of the four miRNAs in 80 childhood asthma and 60 control individuals by qRT-PCR.

ROC curve analysis

In differentiating asthmatic children from normal controls, the area under curve (AUC) value of hsa-miR-18a-5p was 0.799 (95% CI: 0.723–0.862, $P < 0.0001$), the specificity was 61.7%, the sensitivity was 98.7% (Figure 2(a)); the AUC of hsa-miR-106a-5p was 0.774 (95% CI: 0.696–0.840, $P < 0.0001$), the

Table 4. Logistic regression analyses of 4 candidate miRNAs on asthmatic children serum samples.

Coefficients and standard errors			
Variable	Coefficient	Std. error	*P* value
hsa-miR-18a-5p	44.543	0.0408	<0.0001
hsa-miR-106a-5p	17.969	0.0428	<0.0001
hsa-miR-144-3p	15.187	0.0414	<0.0001
hsa-miR-375	92.421	0.0455	0.0001
Constant	–5.049		
Logit (p) = -5.049 + 44.543* (hsa-miR-18a-5p) + 17.969*(hsa-miR-106a-5p) + 15.187*(hsa-miR-144-3p) + 92.421*(hsa-miR-375)			
Odds ratios and 95% confidence intervals			
Variable	Odds ratios	95% CI	
hsa-miR-18a-5p	23.510	0.723–0.862	
hsa-miR-106a-5p	22.364	0.696–0.840	
hsa-miR-144-3p	23.155	0.676–0.824	
hsa-miR-375	20.978	0.594–0.755	

miRNA: MicroRNA; CI: confidence interval.

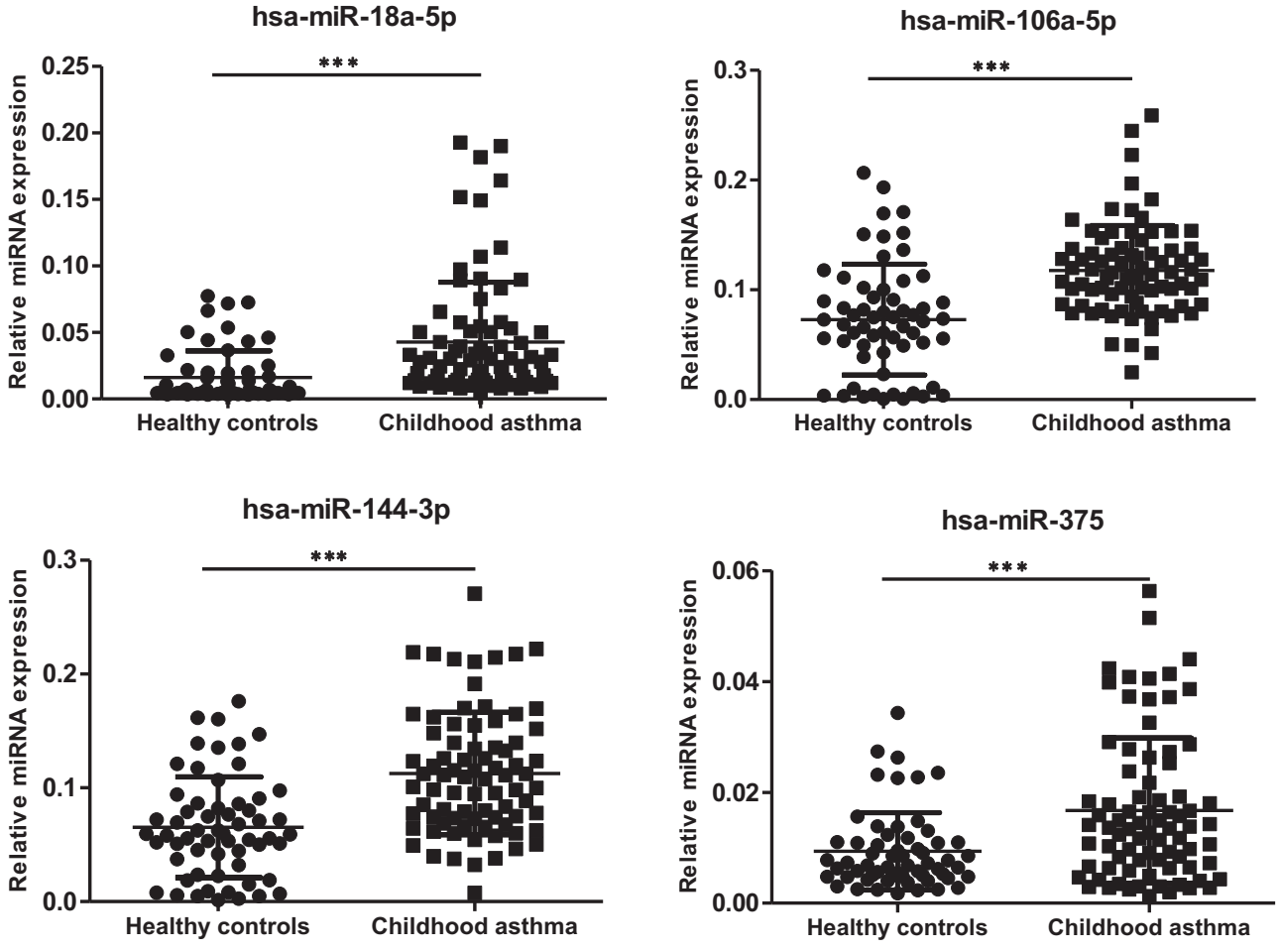

Figure 1. qRT-PCR verification of miRNA levels in childhood asthma and healthy children. Serum miRNA expression of the four miRNAs (hsa-miR-144-3p, hsa-miR-106a-5p, hsa-miR-375 and hsa-miR-18a-5p) was measured in 80 childhood asthma and 60 healthy children by qRT-PCR and relatively quantified by the $2^{-\Delta\Delta Ct}$ algorithm. Nonparametric Mann–Whitney test was adopted for data comparison. ***$P < 0.001$.

specificity was 75.0%, the sensitivity was 73.7% (Figure 2(b)); the AUC value of hsa-miR-144-3p was 0.756 (95% CI: 0.676–0.824, $P < 0.0001$), the specificity was 65.0%, the sensitivity was 76.2% (Figure 2(c)); the AUC value of hsa-miR-375 was 0.678 (95% CI: 0.594–0.755, $P < 0.0001$), the specificity was 76.7%, the sensitivity was 58.7% (Figure 2(d)), respectively.

These four miRNAs constituted a diagnostic model according to multivariate logistic regression analysis.

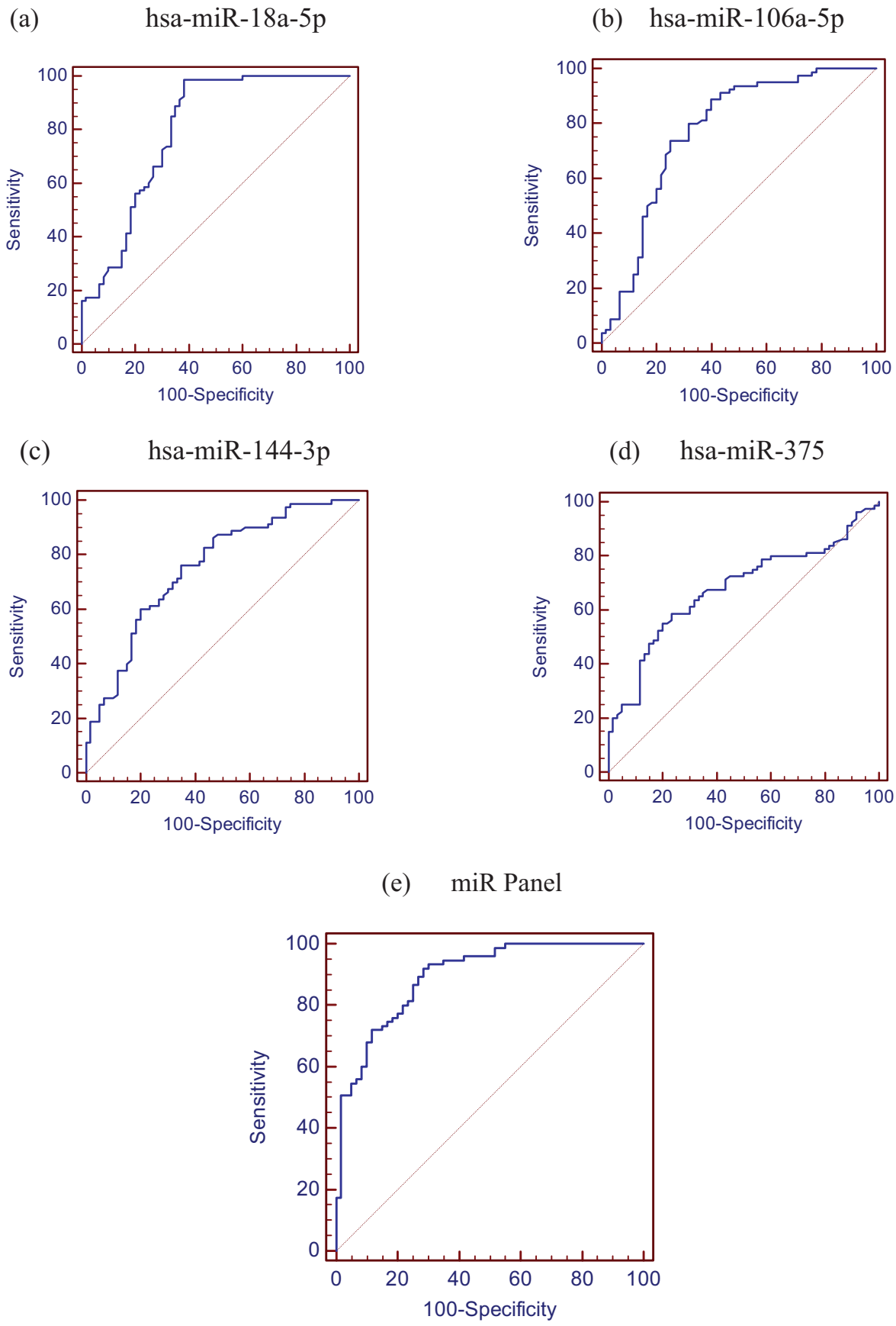

Figure 2. ROC curve analysis of hsa-miR-18a-5p (a), hsa-miR-106a-5p (b), hsa-miR-144-3p (c), hsa-miR-375 (d), and the combination of four miRNAs (e). ROC curve was generated to differentiate childhood asthma ($n = 80$) from healthy controls ($n = 60$). The combination of four miRNAs constituted a diagnostic model by multivariate logistic regression analysis. (A color version of this figure is available in the online journal.)

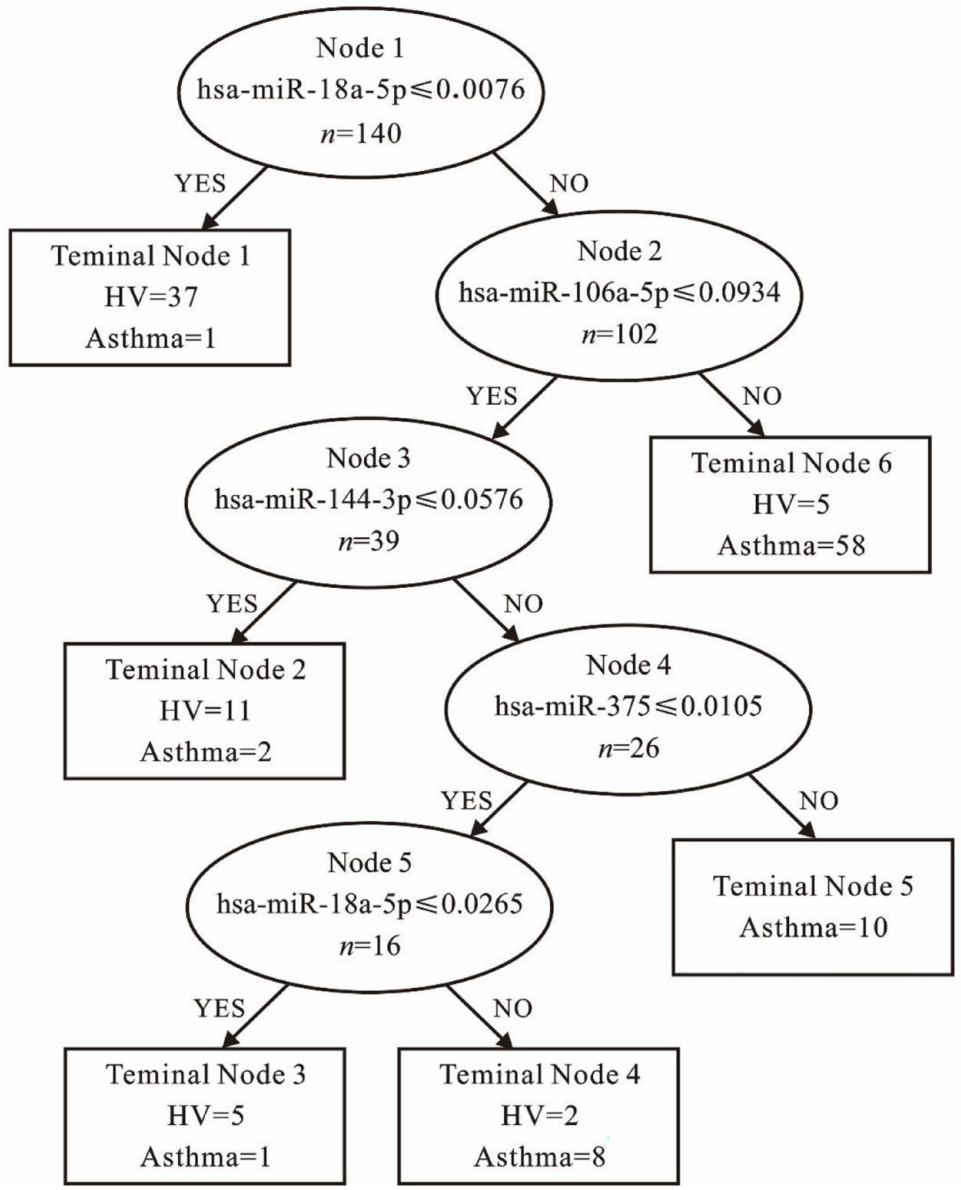

Figure 3. Diagnostic tree model for childhood asthma. The classification tree model using the four miRNAs identified 80 cases of asthmatic children and 60 cases of healthy children. Based on the relative expression level, a cut-off value was defined to group the "root node" into a "left-side child node" and a "right-side child node." Following multiple decision-making rounds, the subjects were classified with the least error.

The model's AUC was 0.894 (95% CI: 0.829–0.940, $P < 0.0001$), the specificity was 71.7%, and the sensitivity was 92.0% (Figure 2(e)). The regression equation was as follows: Logit(p) =-5.049 + 92.421*(hsa-miR-375) + 17.969*(hsa-miR-106a-5p) + 15.187*(hsa-miR-144-3p) + 44.543*(hsa-miR-18a-5p) (Table 4).

Establishment of a classification tree model

The four miRNAs were then used to establish a classification tree model (Figure 3). The model identified 80 cases of asthmatic children and 60 cases of healthy controls with a calculated specificity of 88.3% (53/60) and a sensitivity of 95.0% (76/80), and the AUC value was 0.942. The results were superior to the diagnostic model provided by multivariate logistic regression analysis.

Bioinformatics analysis

Four miRNAs regulated 10953 target genes. GO annotation was performed to reveal the function of the target gene, with biological processes (BP), cellular components (CC), and molecular functions (MF) categories of GO terms being enriched. Related target genes were mainly involved in BP such as gene expression, signal transduction, and protein transport. They were mainly involved in MF such as DNA binding, metal ion binding, protein binding, and protein tyrosine kinase activity. They were mainly located in the CC of the nucleus, cytoplasm, plasma membrane, and cytosol (Figure 4(a)). The KEGG pathway terms associated with the target genes were Wnt signaling pathway, MAPK signaling pathway, TGF-β signaling pathway, endocytosis, etc (Figure 4(b)).

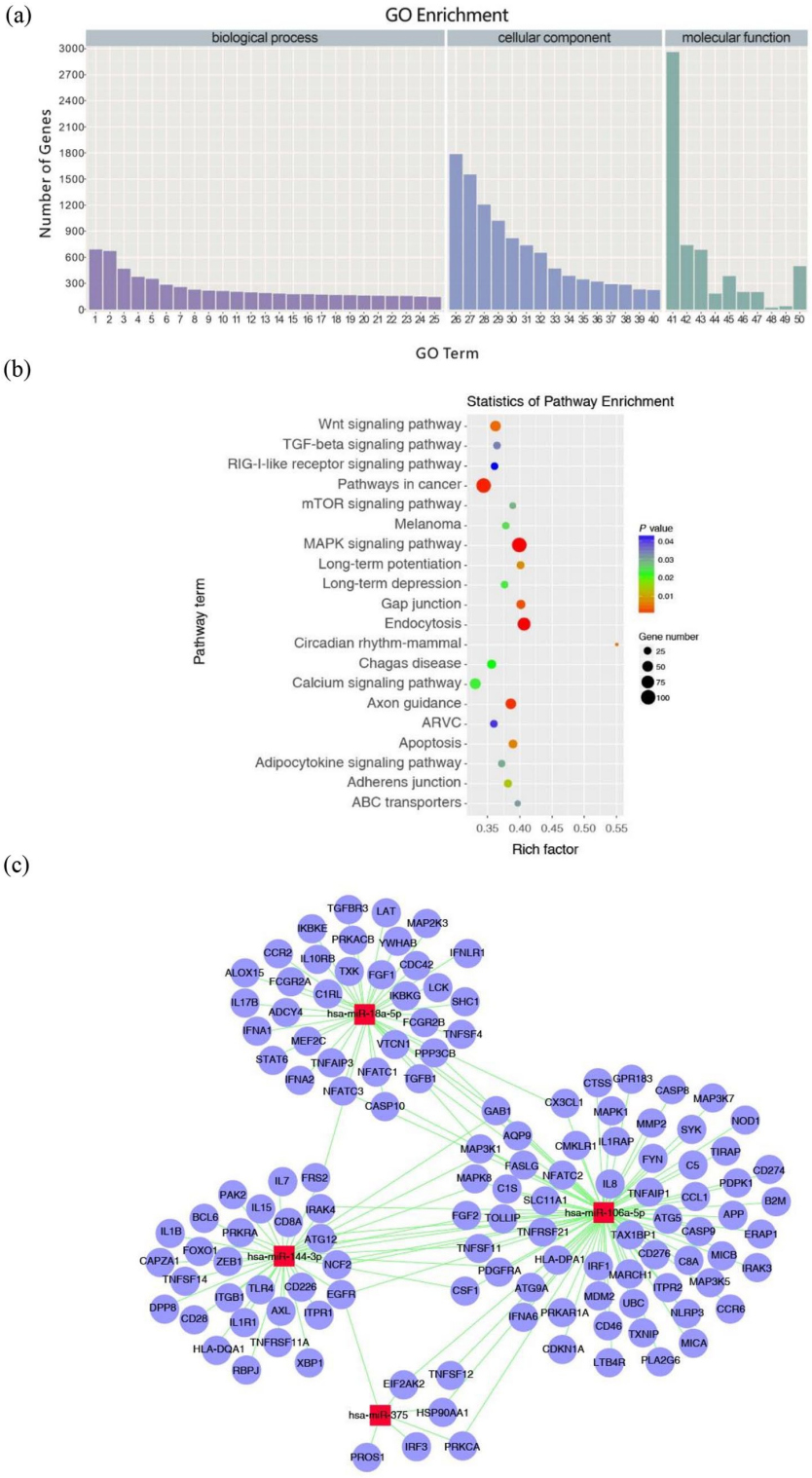

Figure 4. Bioinformatics analysis. GO enrichment analysis (a): the 10953 target genes were predicted gene functions for MFs, BPs, and CCs. 1: transcription, DNA-templated; 2: regulation of transcription, DNA-templated; 3: signal transduction; 4: small molecule metabolic process; 5: metabolic process; 6: positive regulation of transcription from RNA polymerase II promoter; 7: transmembrane transport; 8: protein phosphorylation; 9: positive regulation of transcription, DNA-templated; 10: negative regulation of transcription from RNA polymerase II promoter; 11: gene expression; 12: innate immune response; 13: apoptotic process; 14: protein transport; 15: negative regulation of transcription, DNA-templated; 16: oxidation-reduction process; 17: biological process; 18: transcription from RNA polymerase II promoter; 19: transport; 20: G-protein coupled receptor signaling pathway; 21: multicellular organismal development; 22: intracellular signal transduction; 23: blood coagulation; 24: cell adhesion; 25: viral process; 26: nucleus; 27: cytoplasm; 28: integral component of membrane; 29: plasma membrane; 30: cytosol; 31: membrane; 32: extracellular vesicular exosome; 33: intracellular; 34: mitochondrion; 35: nucleoplasm; 36: integral component of plasma membrane; 37: extracellular region; 38: Golgi apparatus; 39: endoplasmic reticulum; 40: extracellular space; 41: protein binding; 42: metal ion binding; 43: DNA binding; 44: protein tyrosine kinase activity; 45: sequence-specific DNA binding transcription factor activity; 46: protein kinase activity; 47: transferase activity, transferring phosphorus-containing groups; 48: MAP kinase activity; 49: ubiquitin-specific protease activity; 50: ATP binding. KEGG analysis (b): the top 20 KEGG pathway terms associated with the target genes. Size means counts of genes, and color means *P* value in different terms. ARVC: Arrhythmogenic right ventricular cardiomyopathy. The miRNA-gene-network depicting the interactions between miRNAs and their gene targets associated with childhood asthma (c). The online paper includes a color version of this figure.

An miRNA-gene-network was generated (Figure 4(c)). Hsa-miR-106a-5p regulated 74 target genes, hsa-miR-18a-5p regulated 37 genes, hsa-miR-144-3p regulated 35 genes, and hsa-miR-375 regulated five genes. Also, 128 target genes were related with the pathological mechanism of asthma, such as IL7, CD28, MAPK8, and STAT6. The proteins they encode are mainly involved in inflammation, immunity, and transcription efficiency. The MAP3K1, FRS2, and GAB1 genes were co-regulated by hsa-miR-18a-5p, hsa-miR-144-3p, and hsa-miR-106a-5p. The EGFR gene was co-regulated by hsa-miR-144-3p, hsa-miR-375, and hsa-miR-106a-5p.

Discussion

MiRNAs often target one or more genes and regulate gene expression. MiR-18a is a mature miRNA transcribed from the miR-17-92 cluster, regulating survival, activation, differentiation, and cytokine production in T cells.[15,16] Xiao *et al.*[17] reported that the up-regulation of miR-18a-5p exhibited a positive association with Th2 inflammatory factors that promote asthma in the BALF, and miR-18a-5p had a direct correlation with the degree of asthma inflammation and the asthma response severity. The data of this study revealed significantly enhanced expression of hsa-miR-18a-5p in the childhood asthma group relative to the healthy control group, which was consistent with the results reported in the literature.

IL-10 is a class of anti-inflammatory cytokine secreted by various cell types,[18] which plays a key role in many inflammatory disorders, including arthritis, SLE, and asthma.[19,20] MiR-106a expression is mainly detected in B cells, T cells, macrophages, and EC cells.[21] Sharma *et al.*[22] found that hsa-miR-106a regulated IL-10 expression via binding to the gene's 3′UTR. Inhibition of IL-10 by elevated miR-106a might be a key mechanism regulating the balance of pro- and anti-inflammatory responses. Sharma *et al.*[23] demonstrated that mouse allergic airway inflammation was related to decreased IL-10 and increased mmu-miR-106a levels resulted from silencing mmu-miR-106a in ovalbumin-sensitized mice. In our research, the expression of hsa-miR-106a-5p was significantly increased in the childhood asthma group relative to the healthy control group, consistent with the literature.

Th2 cells are essential for the pathogenesis of allergic asthma by secreting Th2 cytokines.[24] Bartel *et al.*[25] identified that Creb1 and Crtc1-3 could be targets of miR-144. IL-13 is a classical Th2 cytokine, which can be expressed in macrophages, B cells, DC and airway epithelial cells.[26,27] IL-13 activates IgE-mediated allergic diseases, including asthma, by activating its receptor and its associated STAT6.[26] IL-13 can promote eosinophil recruitment, and airway hyperreactivity in the airway.[28] Bartel *et al.*[25] found that in allergic airway inflammation, mmu-miR-144 was up-regulated while Creb1/Crtc1-3 was down-regulated, and IL-13 treatment of primary bronchial epithelial cells found that CREB1/CRTC was significantly down-regulated. Overexpression of miR-144 reduced the level of CREB-regulated transcription co-activators 3. Consistent with the literature reports, hsa-miR-144-3p displayed enhanced expression in the childhood asthma group in our study.

TLR7 is critical to the response of single-stranded RNA viruses. TLR7 is obviously absent in severe asthma alveolar macrophages (SA-AM), and the defect of TLR7 is associated with the severity of asthma. Interferon (IFN) is an antiviral cytokine triggered when the viral genome is activated. The acute exacerbation of asthma is mainly virus-related and is associated with IFN deficiency in the airway.[29] Rupani *et al.* revealed up-regulation of miR-375 in SA-AM. The expression of TLR7 was decreased.[3] The deficiency of TLR7 expression results in the decrease of IFN expression of SA-AM after virus infection. Blocking the miR-375, the expression of TLR7 was restored, and IFN response to rhinovirus was enhanced. Studies have shown that human bronchial epithelial cells stimulated by diesel exhaust particles (DEP) or ambient particulate matters could release chemokine CCL20, which is associated with the recruitment of immature DC cells.[30] Bleck *et al.*[31] exhibited that hsa-miR-375 was up-regulated in humans in particulate matter and DEP, and these two pollutants can stimulate the up-regulation of TSLP mRNA and protein and promote Th2 inflammation.

In our study, the classification tree model generated a specificity of 88.3% and a sensitivity of 95.0%. The diagnostic ability of childhood asthma was better than that of single miRNA and the diagnostic model by multivariate logistic regression analysis, suggesting that the four miRNAs have the potential to serve as new serum diagnostic biomarkers for childhood asthma. In a follow-up study, a larger sample size is necessary, and the differential diagnosis groups, such as children with pneumonia, should also be added.

In conclusion, hsa-miR-375, hsa-miR-18a-5p, hsa-miR-144-3p, and hsa-miR-106a-5p all displayed high expression in the serum of childhood asthma patients. Therefore, they might be used as a potential biomarker panel for childhood asthma, whose main mechanisms may be related to inflammation, immunity and transcriptional efficiency. In a further study, analyzing the changes of these four differential miRNAs in different clinical stages of childhood asthma will provide more meaningful information.

AUTHORS' CONTRIBUTIONS

All authors designed the research, analyzed and interpreted the data, and reviewed the manuscript. JL and LH designed the research; JL, LH and XK conducted the experiments; XW performed the statistical analysis; YW and JZ analyzed and interpreted the data; LH drafted the article; JL made revisions on crucial intellectual content in the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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