

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Circulating microRNA-15b-5p as a biomarker for asthma-COPD overlap

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Abstract

Background: It remains unclear how to characterize different subtypes of asthma and chronic obstructive pulmonary disease (COPD). We previously described serum periostin and chitinase-3-like protein 1 (YKL-40) as useful markers for asthma-COPD overlap (ACO). MicroRNAs (miRNAs) are now recognized as markers for identifying the pathophysiological features in several diseases. This study aimed to identify circulating miRNAs that could discriminate patients with ACO from patients with asthma or COPD.

Methods: This study included two independent cohorts. First, we screened 84 miRNAs for expression levels in patients with ACO (n = 6) or asthma (n = 6) using a quantitative real-time PCR array. The miRNAs showing at least a 2-fold difference in the discovery phase were analyzed in 30 patients each with asthma, COPD, or ACO in the replication phase. The diagnostic accuracy was evaluated using the area under the receiver operating characteristic curve (AUROC).

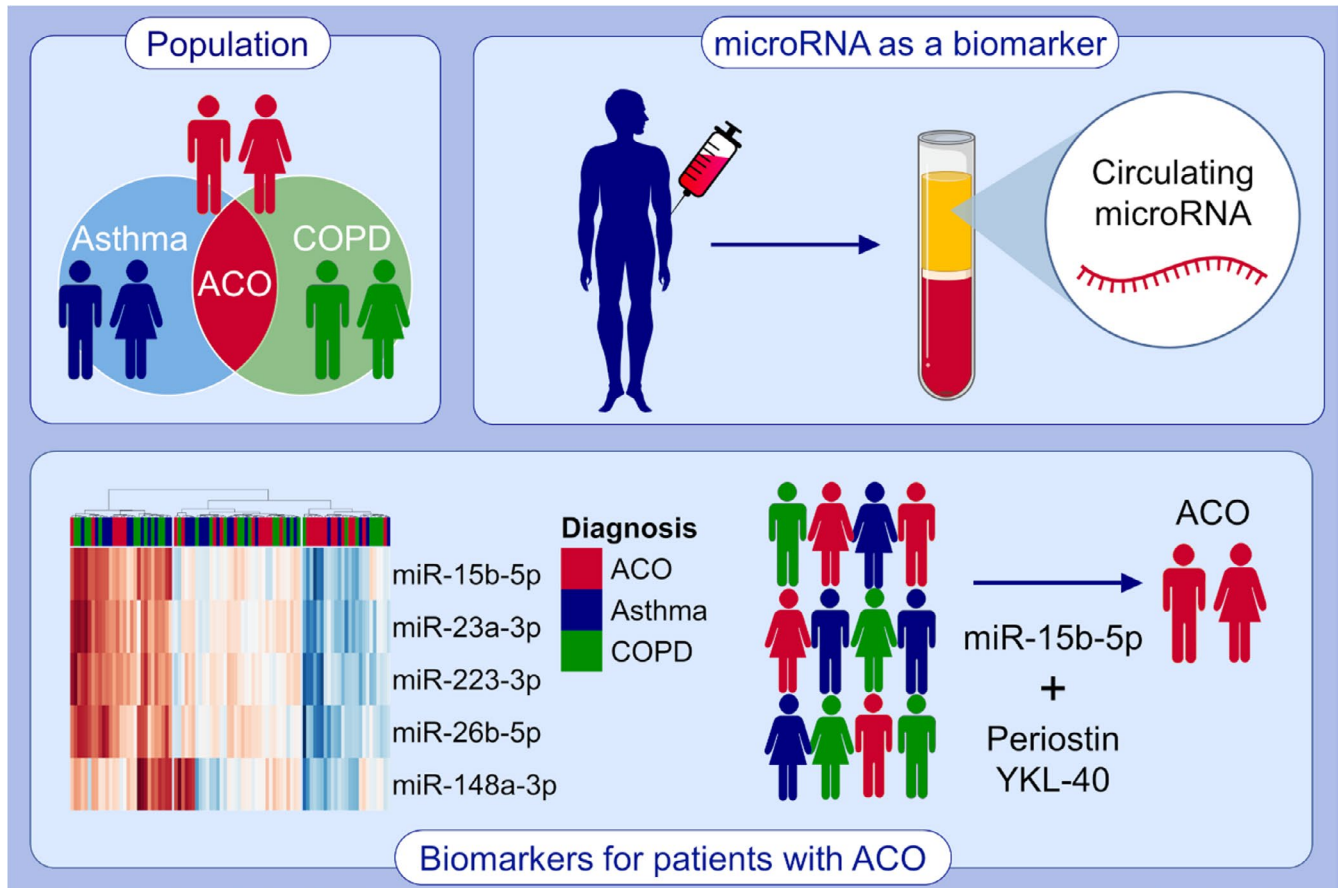
Results: Nine miRNAs were identified in the discovery phase. Five of these miRNAs (miR-148a-3p, miR-15b-5p, miR-223-3p, miR-23a-3p, and miR-26b-5p) had lower levels in ACO patients and could discriminate between ACO patients and patients with either asthma or COPD. miR-15b-5p was the most accurate miRNA for the discrimination of patients with ACO (AUROC, 0.71). Moreover, the combined assessment of miR-15b-5p, serum periostin, and YKL-40 (AUROC, 0.80) improved diagnostic accuracy for ACO compared with the combined model of periostin and YKL-40 (AUROC, 0.69).

Conclusions: Circulating miR-15b-5p is a potential marker for identifying patients with ACO. By elucidating the molecular pathways controlled by miRNAs, we may better understand the pathophysiology of ACO.

KEYWORDS

asthma, asthma-COPD overlap, chronic obstructive pulmonary disease, microRNA, miR-15b-5p

Abbreviations: ACO, asthma-COPD overlap; AUROC, area under the receiver operating characteristic curve; CI, confidence interval; COPD, chronic obstructive pulmonary disease; FeNO, fractional exhaled nitric oxide; ICS, inhaled corticosteroid; KEGG, Kyoto encyclopedia of genes and genomes; miRNA, microRNA; OR, odds ratio; ROC, receiver operating characteristic; YKL-40, chitinase-3-like protein 1.



GRAPHICAL ABSTRACT

Patients with ACO could be identified from patients with asthma and COPD by the expression profiles of five microRNAs in plasma, especially miR-15b-5p. The combined assessment of miR-15b-5p, periostin, and YKL-40 is valuable for characterizing ACO. MiR-15b-5p can serve as a potential biomarker for identifying patients with asthma-COPD overlap, while also furthering our understanding of the pathophysiology of ACO.

1 | INTRODUCTION

Patients with asthma and chronic obstructive pulmonary disease (COPD) can be classified into several subgroups with different clinical and pathophysiological features.^{1,2} It is essential to elucidate the phenotypes and endotypes of these subgroups for the realization of precision medicine.¹ Although several studies have been designed to evaluate potential biomarkers for phenotypes of asthma and COPD, asthma-COPD overlap (ACO) has not been sufficiently characterized. We have attempted to distinguish ACO patients from patients with asthma or COPD using clinical and gene expression profiles.^{3,4} Moreover, we recently investigated whether the combined assessment of serum concentrations of periostin and chitinase-3-like protein 1 (YKL-40) could identify patients with ACO.⁵ Periostin and YKL-40 are known to be biomarkers for type 2 inflammation⁶ and COPD,⁷ respectively; however, few studies have identified a specific biomarker for ACO. Although it is unclear whether specific mechanisms are involved in the pathophysiology of ACO, we hypothesized that some serum/plasma markers might reflect the phenotype of ACO.

MicroRNAs (miRNAs) are short non-coding RNA molecules of approximately 22 nucleotides that negatively regulate gene expression

at the post-transcriptional level by degrading or binding to target mRNAs.⁸ It is known that a single miRNA is involved in the regulation of hundreds of mRNA molecules⁹; therefore, a miRNA is a key molecule for understanding diverse biological processes. Additionally, miRNAs are stable in plasma and other biological fluids; thus, the expression profile of circulating miRNAs can be exploited to identify potential biomarkers for the detection of various diseases.

We hypothesized that patients with ACO have a different expression profile of plasma miRNAs than patients with asthma or COPD. This study aimed to recognize circulating miRNAs as an accurate biomarker for identifying patients with ACO.

2 | METHODS

2.1 | Study subjects

This study included two independent cohorts who were randomly selected from a previous study performed at Shizuoka General Hospital.^{4,5} These cohorts were comprised as follows: (a) a discovery cohort to detect plasma miRNA difference between patients with ACO and asthma

TABLE 1 Characteristics of the subjects in the replication cohort

Variables	ACO (n = 30)	Asthma (n = 30)	COPD (n = 30)	P-value
Age (y)	71 (66, 78)***	43 (40, 50) ^{†††}	74 (70, 78)	<.001
BMI (kg/m ²)	22.7 (21.5, 25.0)	24.1 (20.8, 25.8) ^{††}	20.9 (18.4, 22.3) ^{‡‡}	.002
Male sex, n (%)	28 (93)***	8 (27) ^{†††}	27 (90)	<.001
Pack-years	29 (16, 40)***	0 (0, 5) ^{†††}	46 (25, 71) [‡]	<.001
Past smoking history, n (%)	27 (90.0)***	7 (23.3) ^{†††}	30 (100)	<.001
FEV ₁ , %predicted	61.6 (48.2, 77.3)***	92.8 (82.7, 99.7) ^{†††}	64.2 (42.8, 82.4)	<.001
FEV ₁ /FVC (%)	57.5 (46.0, 65.1)***	76.3 (73.0, 84.4) ^{†††}	52.7 (42.0, 59.5)	<.001
FeNO (ppb)	34.4 (16.5, 58.9)	32.1 (20.6, 55.0) ^{†††}	10.1 (4.4, 16.0) ^{‡‡‡}	<.001
Blood eosinophil count (cells/ μ L)	415 (318, 582)	347 (236, 499) ^{†††}	161 (92, 205) ^{‡‡‡}	<.001
Serum total IgE (IU/mL)	631 (337, 1480)**	228 (89, 459) ^{†††}	48 (17, 84) ^{‡‡‡}	<.001
Serum periostin (ng/mL)	56.4 (49.5, 78.1)	53.1 (48.1, 60.5)	45.8 (41.6, 60.0) ^{‡‡}	.009
Serum YKL-40 (ng/mL)	82.9 (26.7, 186.7)***	21.7 (16.9, 41.3) ^{†††}	90.5 (41.1, 208.2)	<.001
ICS dose (μ g/day)	500 (400, 688)	500 (500, 500) ^{†††}	0 (0, 0) ^{‡‡‡}	<.001
ICS use, n (%)	29 (97)	30 (100) ^{†††}	2 (7) ^{‡‡‡}	<.001
LABA, n (%)	24 (80)	28 (93)	25 (83)	.413
LAMA, n (%)	8 (27)**	0 (0) ^{†††}	24 (80) ^{‡‡‡}	<.001
LTRA, n (%)	8 (27)	10 (33) ^{††}	0 (0) ^{‡‡}	<.001
Theophylline, n (%)	6 (20)	3 (10)	4 (13)	.654

Note: Data are shown as median (interquartile range) or frequency (percentage). Continuous variables are compared with the Kruskal-Wallis test (among three groups) and the Mann-Whitney Utest (between two groups), and categorical variables are compared with Fisher's exact test.

Abbreviations: ACO, asthma-COPD overlap; BMI, body mass index; COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; FeNO, fractional exhaled nitric oxide; ICS, inhaled corticosteroids; LABA, long-acting β_2 agonists; LAMA, long-acting muscarinic antagonists; LTRA, leukotriene receptor antagonists; YKL-40, Chitinase-3-like protein 1.

Asterisks indicate significant difference between ACO and asthma;

* $P < .05$,

** $P < .01$,

*** $P < .001$.

Daggers indicate significant difference between asthma and COPD;

[†] $P < .05$,

^{††} $P < .01$,

^{†††} $P < .001$.

Doddle daggers indicate significant difference between ACO and COPD;

[‡] $P < .05$,

^{‡‡} $P < .01$,

^{‡‡‡} $P < .001$.

and (b) a replication cohort to evaluate the plasma levels of miRNAs found in the discovery cohort in patients with ACO, asthma, or COPD. The discovery cohort included patients with ACO (n = 6) and patients with asthma (n = 6). The replication cohort included patients with ACO (n = 30), patients with asthma (n = 30), and patients with COPD (n = 30). Diagnoses of ACO, asthma, and COPD were performed as described in a previous report.⁵ The diagnostic criteria for ACO are enlisted in Table S1. The Shizuoka General Hospital ethics committee approved this study (SGH 15-01-55). We have previously described the inclusion criteria and methods of measurement for lung function test, fractional exhaled nitric oxide (FeNO), serum periostin, and serum YKL-40.⁵

2.2 | Plasma miRNA extraction

Plasma samples were obtained from patients cross-sectionally using a blood collection tube containing EDTA and stored at -80°C until used. Total RNA, including miRNAs, was extracted from 200 μL plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen) with the addition of the *C. elegans* miR-39 (cel-miR-39) miRNA mimic (Qiagen) as a spike-in control according to the manufacture's protocol. Total RNA was reverse transcribed into cDNA using the miScript II RT Kit with miScript HiSpec buffer (Qiagen).

2.3 | miRNA measurements

A quantitative PCR (qPCR) array was used to detect plasma miRNAs that were more than 2-fold different between ACO and asthma patients in the discovery cohort. We performed qPCR array using pre-designed miScript miRNA PCR Array (Qiagen, MIHS-106Z). This array can profile the expression of 84 miRNAs detectable in plasma. In the replication cohort, we measured the expression levels of the cel-miR-39 control and nine miRNAs found in the discovery cohort. The specific primer of each mature miRNA was purchased from Qiagen (miScript primer assays). The qPCR array and targeted qPCR were performed using the miScript SYBR Green PCR Kit (Qiagen) in accordance with the manufacturer's instructions with the 7500 Fast Real-Time PCR System (Applied Biosystems). Relative plasma miRNA levels were normalized to those of cel-miR-39 expression levels in both the discovery and the replication cohorts.

2.4 | Statistical analysis

Comparisons of plasma miRNA levels were performed using the Kruskal-Wallis test (among three groups) and the Mann-Whitney *U*-test (between two groups). The Spearman rank correlation coefficient was calculated to evaluate the relationship between plasma levels of miRNAs. We evaluated the predictability of each variable for patients with ACO using the multinomial logistic regression model with diagnoses (ACO, asthma, or COPD) as the dependent variable. The odds ratios (OR) and 95% confidence intervals (CI) were estimated for the discrimination of ACO patients from patients with asthma (ACO vs asthma) and patients with COPD (ACO vs COPD) from a model of multinomial logistic regression. We determined that a miRNA that had significant OR for ACO patients, distinct from patients with asthma and patients with COPD, was a biomarker for the discrimination of ACO. The area under the receiver operating characteristics

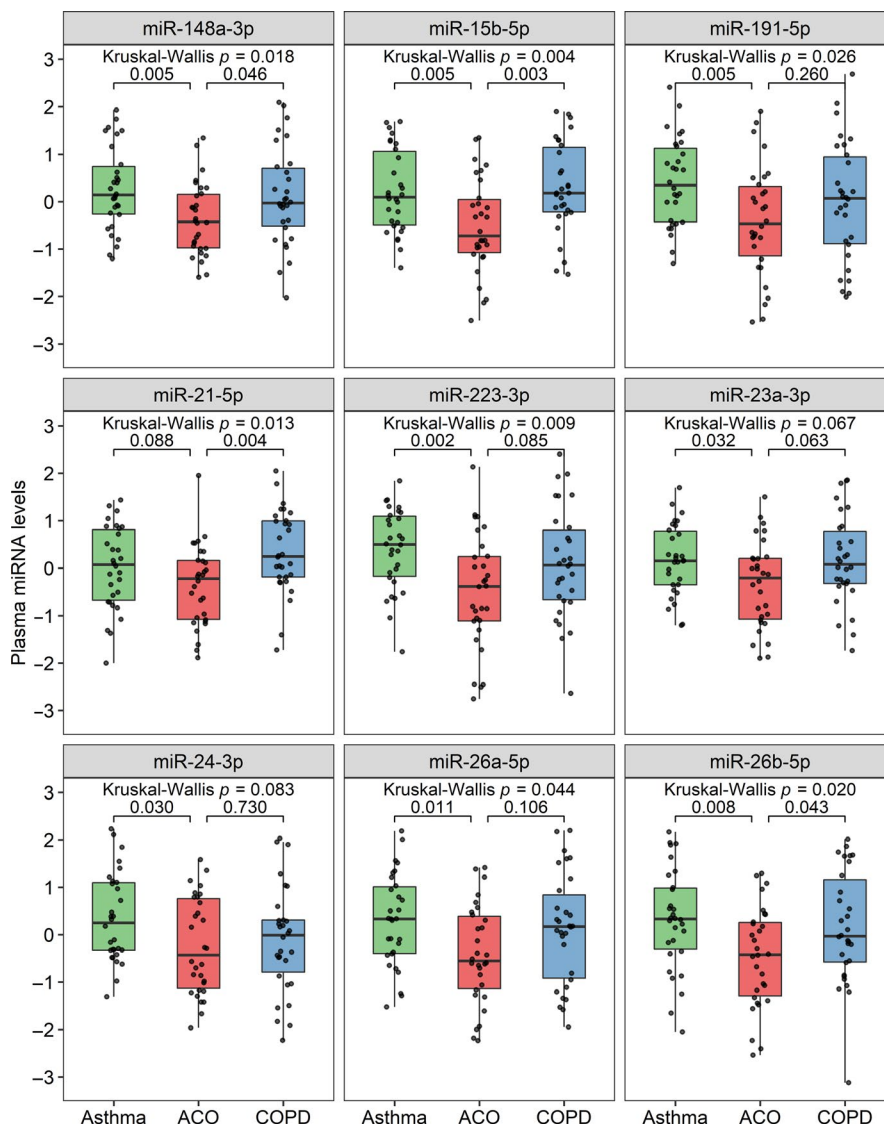


FIGURE 1 Comparison of relative levels of nine plasma microRNAs (miRNAs) among patients with asthma-COPD overlap (ACO), asthma, and chronic obstructive pulmonary disease (COPD). The relative levels of each miRNA were expressed as log₂ transformed values and standardized to an overall mean to zero. Boxes and thick lines in the box indicate the interquartile range and median value, respectively. Differences were assessed using the Kruskal-Wallis test (among the three groups) and Mann-Whitney *U*-test, and *P*-values are indicated in the figure

TABLE 2 The odds ratio for patients with ACO using the multinomial logistic regression model

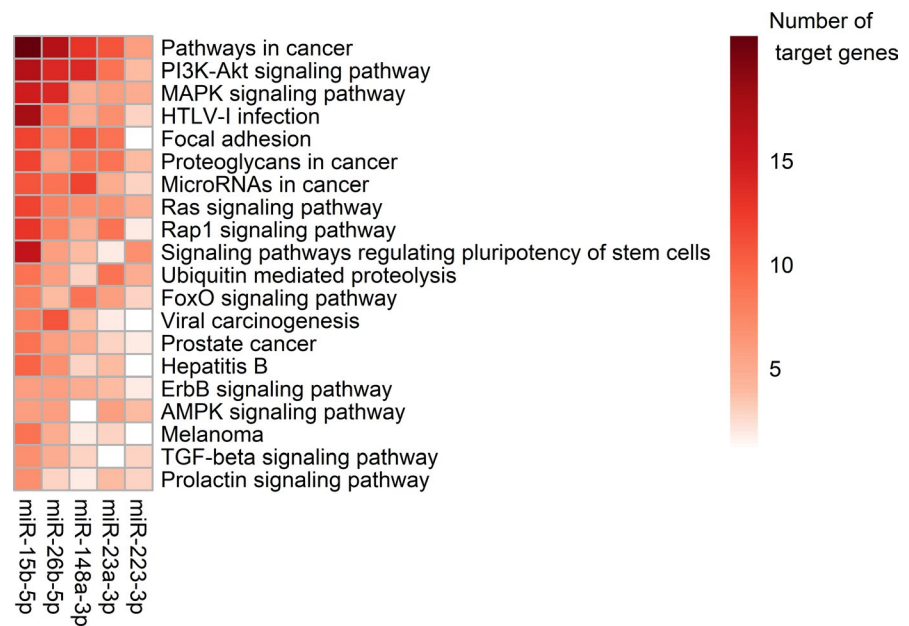
Diagnostic variables [*]	ACO vs Asthma		ACO vs COPD		ACO vs others
	OR [†] (95% CI)	P-value	OR [†] (95% CI)	P-value	AUROC (95% CI)
miR-148a-3p	2.32 (1.23, 4.35)	.009	1.92 (1.03, 3.57)	.040	0.68 (0.56, 0.79)
miR-15b-5p	2.29 (1.26, 4.14)	.006	2.52 (1.38, 4.61)	.003	0.71 (0.60, 0.83)
miR-191-5p	1.93 (1.18, 3.14)	.009	1.37 (0.87, 2.18)	.178	0.65 (0.52, 0.77)
miR-21-5p	1.64 (0.90, 2.99)	.109	2.61 (1.37, 4.99)	.004	0.67 (0.56, 0.79)
miR-223-3p	2.15 (1.28, 3.62)	.004	1.63 (1.00, 2.64)	.049	0.68 (0.56, 0.80)
miR-23a-3p	1.97 (1.07, 3.66)	.031	1.99 (1.07, 3.69)	.029	0.65 (0.53, 0.77)
miR-24-3p	1.75 (1.04, 2.95)	.034	1.14 (0.69, 1.88)	.622	0.60 (0.46, 0.73)
miR-26a-5p	1.93 (1.15, 3.22)	.012	1.64 (0.99, 2.70)	.054	0.66 (0.54, 0.78)
miR-26b-5p	1.92 (1.17, 3.16)	.010	1.68 (1.04, 2.73)	.035	0.68 (0.56, 0.79)

Abbreviations: ACO, asthma-COPD overlap; AUROC, area under the receiver operating characteristics curve; CI, confidence interval; COPD, chronic obstructive pulmonary disease; OR, odds ratio.

^{*}Significant variables were considered as a *P*-value less than .05 in both discriminations of patients with ACO from patients with asthma or COPD and is depicted in bold.

[†]The odds ratio for a log-unit decrease in the plasma miRNA levels.

FIGURE 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of five microRNAs (miRNAs) as asthma-COPD overlap (ACO) signature. The heatmap indicates the number of miRNA target genes. The top 20 significantly enriched KEGG pathways (*P* < .05) are listed in rows and ranked on the basis of the number of target genes



(ROC) curve (AUROC) was estimated from the multinomial logistic regression model. The comparisons of AUROCs were conducted using DeLong's test. A random forest classification was performed to assess the variable importance ranking of variables for the discrimination of patients with ACO, asthma, and COPD.¹⁰

We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using DIANA-mirPath v 3.0.¹¹ The miRNA target genes were predicted using the microT-CDS algorithm with a prediction threshold of 0.9. Significantly enriched KEGG pathways were selected.

All analyses were performed using R version 3.6.2 (R foundation for Statistical Computing, Vienna, Austria). A *P*-value of less than .05 was considered statistically significant.

3 | RESULTS

3.1 | Candidate miRNAs for ACO

The qPCR array detected 52 of the 84 miRNAs in at least one patient of the discovery cohort. The volcano plot shows the differences in the expression levels of each miRNA between ACO and asthma patients (Figure S1). This analysis indicated that the relative plasma levels of nine miRNAs were 2-fold lower in ACO patients compared with asthma patients. None of the miRNAs were 2-fold greater in ACO patients. Therefore, we next measured the nine identified miRNAs (miR-148a-3p, miR-15b-5p, miR-191-5p, miR-21-5p, miR-223-3p, miR-23a-3p, miR-24-3p, miR-26a-5p, and miR-26b-5p) in the replication cohort.

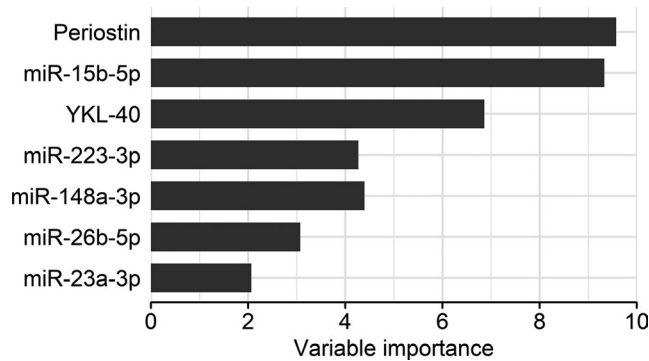


FIGURE 3 Variable importance of relative levels of each plasma microRNAs (miRNAs) and serum levels of periostin and chitinase-3-like protein 1 (YKL-40) for the classification of asthma-COPD overlap (ACO), asthma, and chronic obstructive pulmonary disease (COPD). Variable importance rankings were obtained using a random forest algorithm

3.2 | Five miRNAs as ACO signature

The clinical characteristics of patients in the replication cohort are presented in Table 1. The median values of the nine miRNA expression levels were lower in patients with ACO than in patients with asthma or COPD (Figure 1). The multinomial logistic regression analysis demonstrated that the following five miRNAs significantly characterized patients with ACO: miR-148a-3p, miR-15b-5p, miR-223-3p, miR-23a-3p, and miR-26b-5p (Table 2). The other miRNAs could not statistically distinguish ACO patients from patients with asthma or COPD. Using multinomial logistic models of the five miRNAs, we estimated the probability of patients with ACO (Figure S2). These results demonstrated that miR-15b-5p had a high predictive performance for patients with ACO, which was confirmed with a higher AUROC score (0.71; 95% CI 0.60-0.83, Table 2) than that of the other miRNAs.

3.3 | Pathway analysis

Five miRNAs were identified as potential biomarkers for the discrimination of patients with ACO. The heatmap indicates the top 20 KEGG pathways (based on the number of target genes) associated with miR-148a-3p, miR-15b-5p, miR-223-3p, miR-23a-3p, and miR-26b-5p (Figure 2). The top 3 KEGG pathways were "Pathway in cancer," "PI3K-Akt signaling pathway," and "MAPK signaling pathway." The complete list of pathways and the associated target genes is provided in Table S2.

3.4 | Predictive models for ACO using miR-15b-5p, serum periostin, and YKL-40

We previously reported that serum periostin and YKL-40 are useful biomarkers for the discrimination of patients with ACO from patients with asthma or COPD.⁵ Here, we attempted to determine

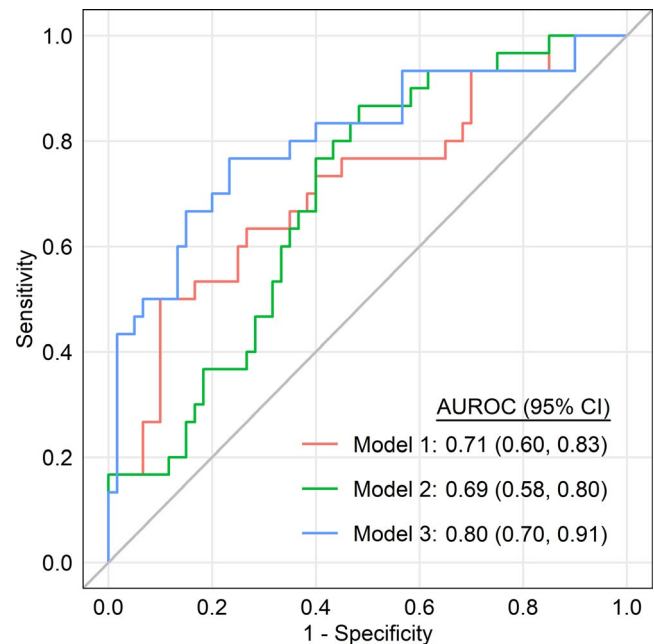


FIGURE 4 Receiver operating characteristic (ROC) curve for identifying patients with ACO. Red line indicates ROC curve for plasma levels of miR-15b-5p (model 1); green line indicates ROC curve for a combination of serum periostin and chitinase-3-like protein 1 (YKL-40) (model 2); and blue line indicates ROC curve for a combination of miR-15b-5p, periostin, and YKL-40 (model 3). The area under the ROC curve (AUROC) of each model is indicated in the figure

whether the five miRNAs found in this study could improve the predictive accuracy of ACO. First, we determined the correlation coefficient between relative individual miRNA levels and serum levels of periostin or YKL-40, and we confirmed that serum periostin and YKL-40 were not correlated with five miRNAs (Figure S3). Second, we performed a random forest analysis to compare the variable importance for the discrimination of patients with ACO (Figure 3). MiR-15b-5p had the highest variable importance score among the five miRNAs, and variable importance was higher in the order of serum periostin, miR-15p-5p, and YKL-40. Next, we developed a predictive model for patients with ACO using plasma miR-15b-5p levels and serum concentrations of periostin and YKL-40 by using multinomial logistic regression analysis (Table S3). The AUROCs estimated from these multinomial logistic regression models are shown in Figure 4. The AUROCs were 0.71 (95% CI, 0.60, 0.83) for miR-15b-5p (model 1), 0.69 (95% CI, 0.58, 0.80) for the combination of serum periostin and YKL-40 (model 2), and 0.80 (0.70, 0.91) for the combination of all three markers (model 3). Model 3 tended to have a higher AUROC when compared with model 1 ($P = .060$) and significantly higher when compared with model 2 ($P = .017$).

4 | DISCUSSION

In the present study, we found that the expression profiles of five plasma miRNAs were different in patients with ACO compared with

patients with asthma or COPD. Among these miRNAs, miR-15b-5p could more accurately identify ACO patients. Moreover, the combined assessment of miR-15b-5p, periostin, and YKL-40 was valuable for characterizing ACO. These findings provide not only a potential biomarker for ACO but also a foothold for further investigation of pathophysiological features of ACO.

This study focused on investigating circulating miRNAs to improve our understanding of ACO phenotypes and endotypes. From the results of the miRNA PCR array and replication cohort, we successfully identified miR-15b-5p as a novel biomarker for ACO. Several studies have described the difference in miRNA levels between patients with asthma or COPD and healthy controls.¹²⁻¹⁵ However, few studies have examined the difference in miRNA expression levels between ACO, asthma, and COPD. Lacedonia et al found that miR-338 in sputum and serum was different between patients with ACO and healthy controls; however, they did not show significant differences in miRNA levels between ACO patients and patients with asthma or COPD.¹⁶ Moreover, although a recent study found miR-320a, miR-185-5p, and miR-1246 in serum were differentially expressed in ACO and asthmatic patients, none of the miRNA could discriminate ACO patients from patients with COPD.¹⁷ The two aforementioned studies performed targeted miRNA measurements and miR-15b-5p was not revealed as a target. A more recent study divided ACO patients into smoking-asthma and COPD with eosinophilia groups and performed a comprehensive analysis of miRNA expression. The study results indicated that serum levels of miR-619-5p and miR-4486 were markers for discrimination of COPD with eosinophilia from patients with asthma and COPD.¹⁸ Several lines of evidence indicate that there is heterogeneity in ACO, as well as in asthma and COPD.^{19,20} Therefore, our results and previous study reporting the association of miRNAs in patients with ACO are important findings to understand the pathophysiology of ACO. However, these findings should be validated in other cohorts to better understand and predict ACO.

We demonstrated the possibility of a relationship between pathophysiological features of ACO and miRNAs identified in the present study by pathway analysis. It is notable that these miRNAs are related to some intracellular signaling pathways. One of these, the mitogen-activated protein kinase (MAPK) pathway, is activated by cellular stress and plays a role in the expression of inflammatory genes. The MAPK pathway has been reported to be involved in the inflammatory response in both asthma and COPD, and MAPK inhibition has been shown to have an effect in the animal model of asthma and COPD.²¹⁻²³ The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway regulates cell proliferation, differentiation, and apoptosis. Increased activity of PI3K was reported in patients with COPD and the model mouse of asthma.^{24,25} PI3K activations are also known to associate with steroid-insensitivity in patients with COPD and severe asthma through a reduction in nuclear levels of histone deacetylase 2, which plays an important role in the steroid response.²⁵⁻²⁷ Among other pathways, the transforming growth factor- β (TGF- β) signaling pathway is involved in airway inflammation and tissue remodeling.^{28,29} Moreover, among the target genes of miR-15p-5p, AKT3,

E2F3, MAP2K1, MAPK8, PIK3R1, RAF1, and VEGFA are associated with pathways implicated in both asthma and COPD. However, there is scarce information available concerning the relationship between these pathways and ACO.

A few studies have reported the association of miR-15b levels with COPD; expression levels of miR-15b were up-regulated in lung tissue of smokers with COPD compared with smokers without COPD.³⁰ It is also reported that patients with COPD had higher levels of miR-15b in the bronchoalveolar lavage fluid than found in healthy controls.³¹ Recently, the effect of inhaled corticosteroid (ICS) treatment on miRNA expression levels was reported among patients with COPD.³² Owing to the small cohort of COPD patients receiving ICS treatment, we could not evaluate this association; however, no correlation was observed between ICS dose and miR-15b-5p levels (Spearman's $r = .02$; $P = .87$) among all subjects receiving ICS treatment. Although, to our knowledge, no study has described the association between miR-15b and asthma, miR-15b is reported to contribute to immune function. A previous study described an essential role for the miR-15/16 family in T cell differentiation and survival.³³ Moreover, overexpression of miR-15b/16 increases regulatory T cell (Treg) induction in vitro and in vivo.³⁴ This report suggested that miR-15b/16 regulated Treg induction through the suppression of the mammalian target of the rapamycin (mTOR) signaling pathway, downstream of the PI3K pathway.³⁴ This study and our results suggest that lower levels of miR-15b may affect the low frequencies of Treg in patients with ACO. In a previous study, we measured mRNA levels of the Treg transcription factor FOXP3 as well as other T helper cell-specific transcription factors, TBX21 (Th1), GATA3 (Th2), and RORC (Th17), in the same subjects as the present study.⁴ When we reanalyzed this cohort (Figure S4), patients with ACO tended to have lower levels of FOXP3 expression in peripheral blood mononuclear cells compared with patients with asthma ($P = .061$) or COPD ($P = .061$). This finding is interesting in terms of understanding the endotype of ACO; however, further studies are needed to fully appreciate its significance.

In clinical practice, on diagnosing ACO, several medical examinations should be performed, including spirometry, computed tomography, and the diffusing capacity test. General blood tests are the most easily and commonly used diagnostic method. The assessment of serum/plasma biomarkers for chronic respiratory disease including ACO would be more useful for the development of precision medicine strategies. In a previous study, we reported that serum periostin is a marker of type-2 inflammation, and it could discriminate patients with asthma and ACO from COPD patients.⁵ Moreover, serum YKL-40 is a valuable marker for characterizing ACO and COPD, and it may reflect airway remodeling and inflammation.⁵ Here, we showed the diagnostic utility of circulating miR-15p-5p as a single marker of ACO. In addition, miR-15b-5p, periostin, and YKL-40, as a combined marker, improved the prediction of ACO. This result suggests that these three markers are involved in different aspects of pathology in patients with chronic respiratory disease.

The current study has limitations. First, there remains no complete agreement on the diagnostic criteria of ACO. Our criterion is almost according to the definition of ACO by the Japanese Respiratory Society.³⁵ Second, a large sample size is needed to characterize several subgroups of ACO. Third, the miRNA PCR array used in this study did not cover all miRNAs.

In conclusion, this study identified differentially expressed circulating miRNAs in patients with ACO. The utility of miR-15b-5p plasma levels as a potential biomarker for discrimination of patients with ACO from patients with asthma or COPD was demonstrated. It is considered that elucidation of the role of miR-15b-5p in the pathophysiology of ACO will contribute to the development of precision medicine for ACO, asthma, and COPD.

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CONFLICT OF INTEREST

T. Shirai reports personal fees from AstraZeneca Japan outside of the submitted work. Y. Gon reports personal fees from AstraZeneca Japan and personal fees from Boehringer Ingelheim outside of the submitted work. The other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

K. Hirai, T. Shirai, and K. Itoh contributed to conception and design of this study. K. Hirai, T. Shirai, T. Shimoshikiyo, M. Ueda, Y. Gon, and S. Muraoka contributed to data generation. K. Hirai, T. Shirai, and K. Itoh contributed to analysis and interpretation of the data. K. Hirai contributed to preparation of the manuscript.

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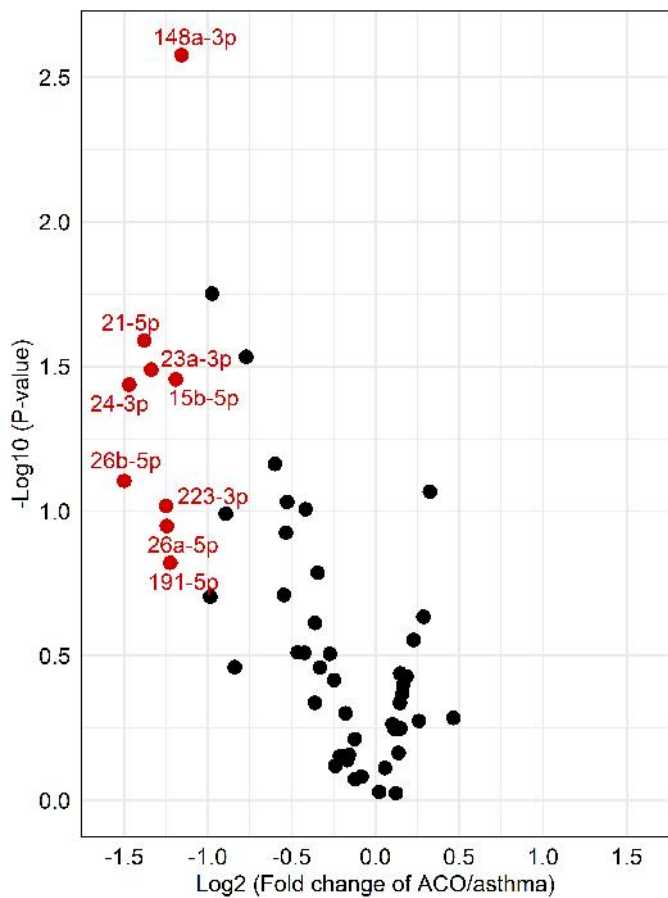
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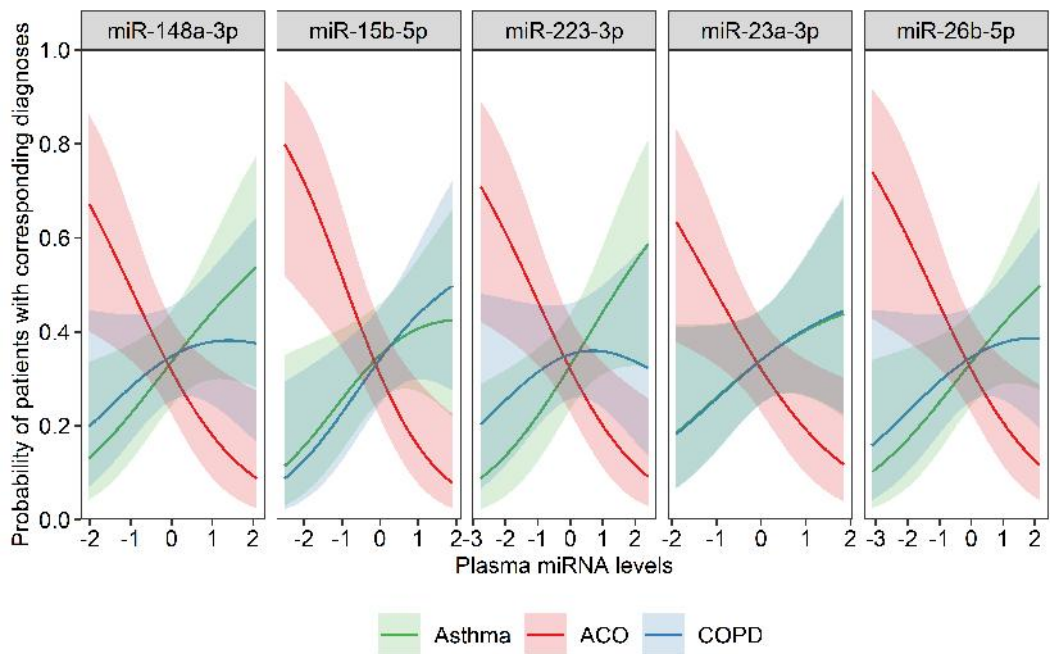
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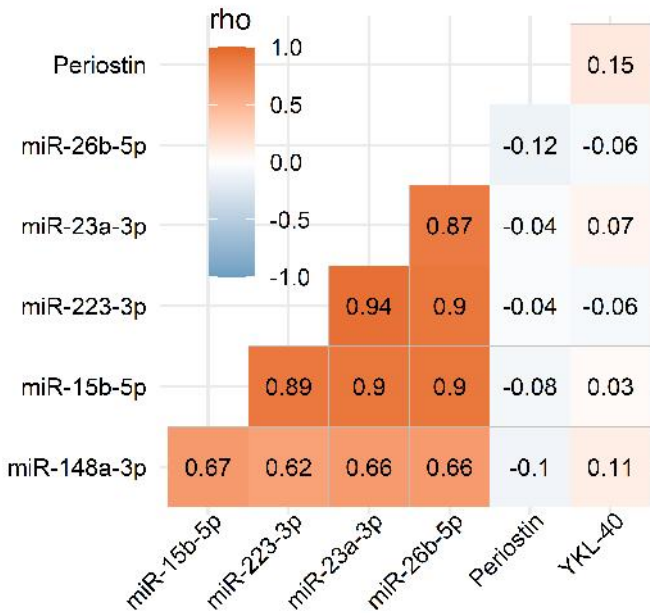
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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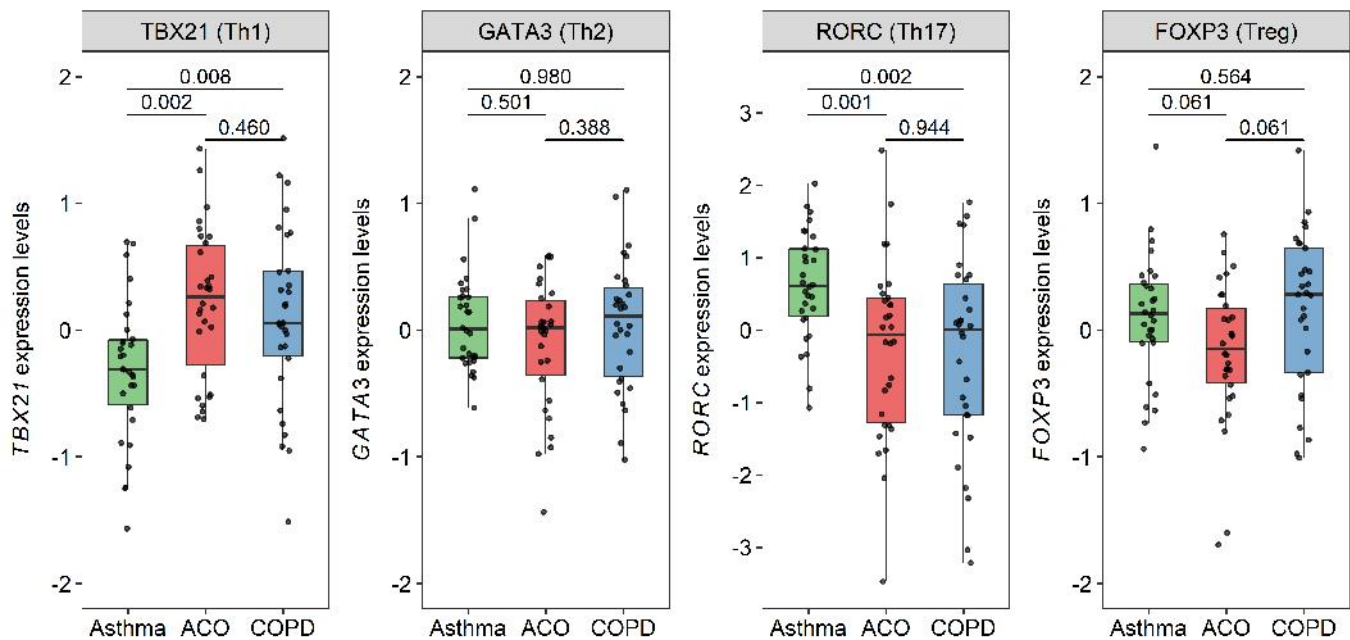


Table S1. Diagnostic criteria for asthma-COPD overlap (ACO)

In patients treated with asthma, fulfilled the following major criteria, and fulfilled at least one of the following minor criteria.	In patients treated with COPD, fulfilled at least 2 of the following criteria.
<u>Major criteria:</u> 1. Age ≥ 40 years old 2. FEV₁/FVC < 0.7 <u>Minor criteria:</u> 1. Smoking pack-years ≥ 10 2. DL_{CO}/VA $< 80\%$ 3. LAA on HRCT	<u>Criteria:</u> 1. History of asthma 2. Peripheral blood eosinophil ≥ 250 cells/μL 3. FeNO > 35 ppb 4. Serum total IgE > 100 IU/mL

ACO, asthma-COPD overlap; COPD, chronic obstructive pulmonary disease; DL_{CO}, diffusing capacity of the lung for carbon monoxide; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; HRCT, high-resolution computed tomography; LAA, low attenuation area; VA, alveolar volume.

Table S2. The potential pathways and target genes.

KEGG pathway miRNA	Target genes
<u>Fatty acid biosynthesis ($P = 8.59 \times 10^{-19}$)</u>	
hsa-miR-15b-5p	ACSL4, FASN
hsa-miR-223-3p	ACSL3
hsa-miR-26b-5p	ACSL3
<u>Prion diseases ($P = 6.12 \times 10^{-18}$)</u>	
hsa-miR-148a-3p	PRNP
hsa-miR-15b-5p	MAP2K1
<u>Signaling pathways regulating pluripotency of stem cells ($P = 4.16 \times 10^{-6}$)</u>	
hsa-miR-148a-3p	APC, IGF1, INHBB, JARID2
hsa-miR-15b-5p	ACVR2A, ACVR2B, AKT3, BMPR1A, FGF2, FGFR1, FZD10, MAP2K1, PCGF5, PIK3R1, RAF1, WNT10B, WNT3A, WNT4, WNT7A, ZFHX3
hsa-miR-223-3p	ACVR2A, IGF1R, IL6ST, KAT6A, LIF, PAX6, SMAD1
hsa-miR-23a-3p	PIK3CB, PIK3R3
hsa-miR-26b-5p	ACVR1C, GSK3B, INHBA, INHBB, REST, SMAD1
<u>Proteoglycans in cancer ($P = 1.69 \times 10^{-4}$)</u>	
hsa-miR-148a-3p	CBLB, IGF1, ITGA5, PPP1CB, ROCK1, SOS1, SOS2, TGFB2, VMP1
hsa-miR-15b-5p	AKT3, FGF2, FGFR1, FZD10, MAP2K1, PIK3R1, RAF1, VEGFA, WNT10B, WNT3A, WNT4, WNT7A
hsa-miR-223-3p	CBLB, IGF1R, RPS6KB1, RRAS2
hsa-miR-23a-3p	ERBB4, GAB1, HOXD10, IL12B, PDPK1, PIK3CB, PIK3R3, PLAUI, PPP1CB
hsa-miR-26b-5p	CBLB, ERBB4, ESR1, HGF, ITGA5, MRAS
<u>Prostate cancer ($P = 2.00 \times 10^{-4}$)</u>	
hsa-miR-148a-3p	CDKN1B, IGF1, SOS1, SOS2, TGFA
hsa-miR-15b-5p	AKT3, BCL2, CCNE1, E2F3, FGFR1, FOXO1, MAP2K1, PIK3R1, RAF1
hsa-miR-223-3p	FOXO1, IGF1R
hsa-miR-23a-3p	PDPK1, PIK3CB, PIK3R3
hsa-miR-26b-5p	CREB1, CREBBP, GSK3B, LEF1, PTEN, RB1
<u>FoxO signaling pathway ($P = 2.60 \times 10^{-4}$)</u>	
hsa-miR-148a-3p	BCL2L11, CDKN1B, GADD45A, IGF1, S1PR1, SOS1, SOS2, TGFB2, USP7
hsa-miR-15b-5p	AKT3, CCND2, FOXO1, INSR, MAP2K1, MAPK8, PIK3R1, RAF1
hsa-miR-223-3p	FOXO1, FOXO3, IGF1R
hsa-miR-23a-3p	FBXO32, PDPK1, PIK3CB, PIK3R3, STK4, TGFB2
hsa-miR-26b-5p	CREBBP, NLK, PTEN, SKP2
<u>Glioma ($P = 4.35 \times 10^{-4}$)</u>	
hsa-miR-148a-3p	IGF1, SOS1, SOS2, TGFA
hsa-miR-15b-5p	AKT3, E2F3, MAP2K1, PIK3R1, RAF1
hsa-miR-223-3p	IGF1R
hsa-miR-23a-3p	PIK3CB, PIK3R3
hsa-miR-26b-5p	CDK6, PTEN, RB1
<u>Renal cell carcinoma ($P = 4.89 \times 10^{-4}$)</u>	
hsa-miR-148a-3p	EPAS1, SOS1, SOS2, TGFA, TGFB2
hsa-miR-15b-5p	AKT3, MAP2K1, PIK3R1, RAF1, VEGFA
hsa-miR-23a-3p	GAB1, MET, PIK3CB, PIK3R3, RAP1A
hsa-miR-26b-5p	CREBBP, HGF, PAK2
<u>Non-small cell lung cancer ($P = 1.56 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	SOS1, SOS2, TGFA
hsa-miR-15b-5p	AKT3, E2F3, MAP2K1, PIK3R1, RAF1
hsa-miR-223-3p	FOXO3
hsa-miR-23a-3p	PDPK1, PIK3CB, PIK3R3, STK4
hsa-miR-26b-5p	CDK6, RB1
<u>PI3K-Akt signaling pathway ($P = 1.65 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	BCL2L11, CDKN1B, COL2A1, COL4A1, CSF1, FLT1, IGF1, ITGA11, ITGA5, ITGA9, SOS1, SOS2, TEK, YWHAB
hsa-miR-15b-5p	AKT3, BCL2, CCND2, CCND3, CCNE1, COL2A1, FGF18, FGF2, FGF7, FGFR1,

hsa-miR-223-3p	GHR, INSR, MAP2K1, MYB, PIK3R1, RAF1, VEGFA
hsa-miR-23a-3p	FOXO3, IGF1R, PRLR, RPS6KB1
hsa-miR-26b-5p	COL4A4, COL4A5, IL6R, MET, PDPK1, PIK3CB, PIK3R3, PPP2R5E, PRLR
	ATF2, CDK6, COL24A1, CREB1, EPHA2, FGF9, GSK3B, HGF, ITGA5, ITGB8, LAMA1, PPP2R3C, PRLR, PTEN
<u>Melanoma ($P = 1.65 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	IGF1, MITF
hsa-miR-15b-5p	AKT3, E2F3, FGF18, FGF2, FGF7, FGFR1, MAP2K1, PIK3R1, RAF1
hsa-miR-223-3p	IGF1R
hsa-miR-23a-3p	MET, PIK3CB, PIK3R3
hsa-miR-26b-5p	CDK6, FGF9, HGF, PTEN, RB1
<u>Ras signaling pathway ($P = 3.64 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	CSF1, FLT1, IGF1, RASAL1, SOS1, SOS2, TEK
hsa-miR-15b-5p	ABL2, AKT3, FGF18, FGF2, FGF7, FGFR1, INSR, MAP2K1, MAPK8, PIK3R1, RAF1, VEGFA
hsa-miR-223-3p	IGF1R, RALA, RASA1, RASGRP1, RRAS2
hsa-miR-23a-3p	GAB1, GRIN2B, MET, PIK3CB, PIK3R3, RAP1A, STK4
hsa-miR-26b-5p	ABL2, EPHA2, FGF9, GRIN2A, GRIN2B, HGF, MRAS, PAK2
<u>Prolactin signaling pathway ($P = 3.64 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	SOS1, SOS2
hsa-miR-15b-5p	AKT3, CCND2, MAP2K1, MAPK8, PIK3R1, RAF1, SOCS6
hsa-miR-223-3p	FOXO3, PRLR, STAT1
hsa-miR-23a-3p	PIK3CB, PIK3R3, PRLR, SOCS6
hsa-miR-26b-5p	ESR1, GSK3B, PRLR
<u>Pathways in cancer ($P = 3.64 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	APC, CBLB, CCDC6, CDKN1B, COL4A1, EPAS1, IGF1, MITF, ROCK1, SOS1, SOS2, TGFA, TGFB2
hsa-miR-15b-5p	AKT3, BCL2, CCDC6, CCNE1, E2F3, FGF18, FGF2, FGF7, FGFR1, FOXO1, FZD10, GNAI3, MAP2K1, MAPK8, PIK3R1, RAF1, VEGFA, WNT10B, WNT3A, WNT4, WNT7A
hsa-miR-223-3p	CBLB, FOXO1, IGF1R, RALA, RASGRP1, STAT1
hsa-miR-23a-3p	ADCY1, COL4A4, COL4A5, CXCL12, MET, PIK3CB, PIK3R3, PTGER4, STK4, TGFB2, XIAP
hsa-miR-26b-5p	BCR, BID, CBLB, CCDC6, CDK6, CREBBP, FGF9, GSK3B, HGF, LAMA1, LEF1, PLCB1, PTEN, PTGS2, RB1, SHH, SKP2
<u>Pancreatic cancer ($P = 3.64 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	TGFA, TGFB2
hsa-miR-15b-5p	AKT3, E2F3, MAP2K1, MAPK8, PIK3R1, RAF1, VEGFA
hsa-miR-223-3p	RALA, STAT1
hsa-miR-23a-3p	PIK3CB, PIK3R3, TGFB2
hsa-miR-26b-5p	CDK6, RB1
<u>MicroRNAs in cancer ($P = 3.98 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	APC, BCL2L1, CDKN1B, DNMT1, DNMT3A, ITGA5, ROCK1, SOS1, SOS2, TGFB2, VMP1, ZEB1
hsa-miR-15b-5p	BCL2, BCL2L2, CCND2, CCNE1, E2F3, MAP2K1, PDCD4, RAF1, RECK, VEGFA, WNT3A
hsa-miR-223-3p	FOXP1, HMGA2, MMP16
hsa-miR-23a-3p	HOXD10, MET, PLA2, SLC7A1, SPRY2
hsa-miR-26b-5p	BAK1, CDK6, CREBBP, EZH2, ITGA5, MMP16, PIM1, PTEN, PTGS2
<u>Focal adhesion ($P = 4.29 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	COL2A1, COL4A1, FLT1, IGF1, ITGA11, ITGA5, ITGA9, PPP1CB, ROCK1, SOS1, SOS2
hsa-miR-15b-5p	AKT3, BCL2, CCND2, CCND3, COL24A1, MAP2K1, MAPK8, MYLK, MYLK4, PIK3R1, RAF1, VEGFA
hsa-miR-223-3p	IGF1R
hsa-miR-23a-3p	COL4A4, COL4A5, MET, PDPK1, PIK3CB, PIK3R3, PPP1CB, RAP1A, XIAP
hsa-miR-26b-5p	COL24A1, GSK3B, HGF, ITGA5, ITGB8, LAMA1, PAK2, PTEN
<u>MAPK signaling pathway ($P = 4.43 \times 10^{-3}$)</u>	

hsa-miR-148a-3p	GADD45A, MAP3K4, SOS1, SOS2, TGFB2
hsa-miR-15b-5p	AKT3, CACNA1E, CACNA2D1, FGF18, FGF2, FGF7, FGFR1, MAP2K1, MAP3K4, MAPK8, MKNK1, NFATC3, PTPRR, RAF1, RPS6KA3
hsa-miR-223-3p	DUSP10, ELK4, RASA1, RASGRP1, RRAS2
hsa-miR-23a-3p	DUSP5, MAP3K1, PPM1A, RAP1A, STK4, TGFB2
hsa-miR-26b-5p	ATF2, CACNB2, CACNB4, DUSP5, FGF9, HSPA8, MAP3K2, MKNK2, MRAS, NLK, PAK2, PPP3CB, RPS6KA6, TAOK1
<u>Estrogen signaling pathway ($P = 4.88 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	SOS1, SOS2
hsa-miR-15b-5p	AKT3, GNAI3, MAP2K1, PIK3R1, RAF1
hsa-miR-23a-3p	ADCY1, PIK3CB, PIK3R3
hsa-miR-26b-5p	ATF2, CREB1, ESR1, HSPA8, PLCB1, PRKCD
<u>p53 signaling pathway ($P = 4.88 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	GADD45A, IGF1
hsa-miR-15b-5p	CCND2, CCND3, CCNE1, CHEK1, PPM1D, RFWD2, SESN1
hsa-miR-223-3p	SIAH1
hsa-miR-23a-3p	APAF1, SESN3
hsa-miR-26b-5p	BID, CDK6, PMAIP1, PTEN
<u>Glycosphingolipid biosynthesis - lacto and neolacto series ($P = 7.84 \times 10^{-3}$)</u>	
hsa-miR-23a-3p	FUT4, FUT9, ST8SIA1
hsa-miR-26b-5p	B3GNT5
<u>Ubiquitin mediated proteolysis ($P = 8.62 \times 10^{-3}$)</u>	
hsa-miR-148a-3p	CBLB, CUL5, TRIP12
hsa-miR-15b-5p	BTRC, CDC27, FBXW7, RFWD2, SMURF1, SMURF2, UBE2K, UBE2Q1, UBE4B
hsa-miR-223-3p	CBLB, FBXW7, SIAH1, UBE2W, UBR5
hsa-miR-23a-3p	CUL3, MAP3K1, NEDD4L, TRIM37, UBE2D1, UBE2D3, UBE2O, UBE2R2, XIAP
hsa-miR-26b-5p	CBLB, SKP2, TRIM37, UBE2G1, UBE2H, UBE4B
<u>Rap1 signaling pathway ($P = 1.16 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	CSF1, FLT1, IGF1, LCP2, TEK
hsa-miR-15b-5p	AKT3, FGF18, FGF2, FGF7, FGFR1, GNAI3, INSR, MAP2K1, PARD6B, PIK3R1, RAF1, SIPA1L2, VEGFA
hsa-miR-223-3p	IGF1R, RALA
hsa-miR-23a-3p	ADCY1, FYB, GRIN2B, MAGI3, MET, PIK3CB, PIK3R3, RAP1A, SIPA1L1
hsa-miR-26b-5p	DOCK4, EPHA2, FGF9, GRIN2A, GRIN2B, HGF, MRAS, PLCB1
<u>Small cell lung cancer ($P = 1.16 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	CDKN1B, COL4A1
hsa-miR-15b-5p	AKT3, BCL2, CCNE1, E2F3, PIK3R1
hsa-miR-23a-3p	APAF1, COL4A4, COL4A5, PIK3CB, PIK3R3, XIAP
hsa-miR-26b-5p	CDK6, LAMA1, PTEN, PTGS2, RB1, SKP2
<u>ErbB signaling pathway ($P = 1.16 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	CBLB, CDKN1B, SOS1, SOS2, TGFA
hsa-miR-15b-5p	ABL2, AKT3, MAP2K1, MAPK8, PIK3R1, RAF1
hsa-miR-223-3p	CBLB, RPS6KB1
hsa-miR-23a-3p	ERBB4, GAB1, PIK3CB, PIK3R3
hsa-miR-26b-5p	ABL2, CBLB, ERBB4, EREG, GSK3B, PAK2
<u>mTOR signaling pathway ($P = 1.16 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	IGF1, RICTOR
hsa-miR-15b-5p	AKT3, PIK3R1, RPS6KA3, VEGFA
hsa-miR-223-3p	RPS6KB1, ULK2
hsa-miR-23a-3p	CAB39, PDPK1, PIK3CB, PIK3R3
hsa-miR-26b-5p	PTEN, RPS6KA6, ULK1, ULK2
<u>Endometrial cancer ($P = 1.16 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	APC, SOS1, SOS2
hsa-miR-15b-5p	AKT3, MAP2K1, PIK3R1, RAF1
hsa-miR-223-3p	FOXO3
hsa-miR-23a-3p	PDPK1, PIK3CB, PIK3R3
hsa-miR-26b-5p	GSK3B, LEF1, PTEN
<u>AMPK signaling pathway ($P = 1.65 \times 10^{-2}$)</u>	

hsa-miR-148a-3p	IGF1
hsa-miR-15b-5p	AKT3, FASN, FOXO1, INSR, PIK3R1, RAB10
hsa-miR-223-3p	FOXO1, FOXO3, IGF1R, RPS6KB1
hsa-miR-23a-3p	CAB39, PDPK1, PIK3CB, PIK3R3, PPARGC1A, PPP2R5E
hsa-miR-26b-5p	CREB1, PFKFB2, PFKFB3, PPP2R3C, STRADB, ULK1
<u>Viral carcinogenesis ($P = 1.72 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	CDKN1B, GTF2H1, USP7, YWHAB
hsa-miR-15b-5p	CCND2, CCND3, CCNE1, CHEK1, DDX3X, HLA-F, PIK3R1, TBPL1
hsa-miR-223-3p	IL6ST
hsa-miR-23a-3p	PIK3CB, PIK3R3
hsa-miR-26b-5p	ATF2, BAK1, CDK6, CREB1, CREBBP, DDX3X, HDAC9, HLA-F, PMAIP1, RB1, SKP2
<u>Colorectal cancer ($P = 2.16 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	APC, TGFB2
hsa-miR-15b-5p	AKT3, BCL2, MAP2K1, MAPK8, PIK3R1, RAF1
hsa-miR-23a-3p	PIK3CB, PIK3R3, TGFB2
hsa-miR-26b-5p	GSK3B, LEF1
<u>HTLV-I infection ($P = 2.19 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	APC, CANX, IL15, NRP1, TGFB2
hsa-miR-15b-5p	AKT3, CCND2, CCND3, CDC27, CHEK1, E2F3, FZD10, HLA-F, MAPK8, MYB, MYBL1, NFATC3, PIK3R1, TBPL1, WNT10B, WNT3A, WNT4, WNT7A
hsa-miR-223-3p	ELK4, RRAS2, XPO1
hsa-miR-23a-3p	ADCY1, MAP3K1, PIK3CB, PIK3R3, TGFB2, XIAP, XPO1
hsa-miR-26b-5p	ATF2, CREB1, CREBBP, GSK3B, HLA-F, MRAS, PPP3CB, RB1, TP53INP1
<u>Hepatitis B ($P = 2.63 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	CDKN1B, TGFB2, YWHAB
hsa-miR-15b-5p	AKT3, BCL2, CCNE1, DDX3X, E2F3, MAP2K1, MAPK8, NFATC3, PIK3R1, RAF1
hsa-miR-223-3p	STAT1
hsa-miR-23a-3p	APAF1, MAP3K1, PIK3CB, PIK3R3
hsa-miR-26b-5p	ATF2, CDK6, CREB1, CREBBP, DDX3X, PTEN, RB1
<u>TGF-beta signaling pathway ($P = 2.84 \times 10^{-2}$)</u>	
hsa-miR-148a-3p	INHBB, ROCK1, TGFB2
hsa-miR-15b-5p	ACVR2A, ACVR2B, BMPR1A, CHRD, SMAD7, SMURF1, SMURF2
hsa-miR-223-3p	ACVR2A, RPS6KB1, SMAD1
hsa-miR-23a-3p	TGFB2
hsa-miR-26b-5p	ACVR1C, CREBBP, INHBA, INHBB, SMAD1

Pathway analysis was performed using DIANA-mirPath (v 3.0) software based on the prediction of the target genes by microT-CDS (v 5.0).

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table S3. The area under the receiver operating characteristics curve and odds ratio for patients with asthma-COPD overlap (ACO)

	ACO vs. Asthma		ACO vs. COPD	
	OR (95% CI)	P value	OR (95% CI)	P value
<u>Model 1</u>				
AUROC (95% CI): 0.71 (0.60, 0.83)				
miR-15b-5p	2.29 (1.26, 4.14)	0.006	2.52 (1.38, 4.61)	0.003
<u>Model 2</u>				
AUROC (95% CI): 0.69 (0.58, 0.80)				
Periostin	1.65 (0.36, 7.67)	0.520	14.46 (2.90, 72.14)	<0.001
YKL-40	2.31 (1.44, 3.72)	0.001	0.67 (0.43, 1.03)	0.067
<u>Model 3</u>				
AUROC (95% CI): 0.80 (0.70, 0.91)				
miR-15b-5p	2.93 (1.41, 6.05)	0.004	3.02 (1.50, 6.05)	0.002
Periostin	1.88 (0.37, 9.62)	0.451	19.76 (3.62, 107.84)	<0.001
YKL-40	2.50 (1.47, 4.24)	< 0.001	0.68 (0.42, 1.11)	0.121

† The odds ratio for a log-unit decrease in the plasma miRNA levels and a log-unit increase in the serum concentrations of periostin and YKL-40.

ACO, asthma-COPD overlap; AUROC, area under the receiver operating characteristics curve; CI, confidence interval; COPD, chronic obstructive pulmonary disease; OR, odds ratio; YKL-40, Chitinase-3-like protein 1.