

Circular RNA Expression of Peripheral Blood Mononuclear Cells Associated with Risk of Acute Exacerbation in Smoking Chronic Obstructive Pulmonary Disease

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Purpose: Circular RNAs (circRNAs) are newly identified endogenous non-coding RNAs that function as crucial gene modulators in the development of several diseases. By assessing the expression levels of circRNAs in peripheral blood mononuclear cells (PBMCs) from patients with chronic obstructive pulmonary disease (COPD), this study attempted to find new biomarkers for COPD screening.

Patients and Methods: We confirmed altered circRNA expression in PBMCs of COPD (n=41) vs controls (n=29). Further analysis focused on the highest and lowest circRNA expression levels. The *T*-test is used to assess the statistical variances in circRNAs among COPD patients in the smoking and non-smoking cohorts. Additionally, among smokers, the Spearman correlation test assesses the association between circRNAs and clinical indicators.

Results: Two circRNAs, hsa_circ_0042590 and hsa_circ_0049875, that were highly upregulated and downregulated in PBMCs from COPD patients were identified and verified. Smokers with COPD had lower hsa_circ_0042590 and higher hsa_circ_0049875, in comparison to non-smokers. There was a significant correlation ($r=0.52$, $P<0.01$) between the number of acute exacerbations (AEs) that smokers with COPD experienced in the previous year and the following year ($r=0.67$, $P<0.001$). Moreover, hsa_circ_0049875 was connected to the quantity of AEs in the year prior ($r=0.68$, $P<0.0001$) as well as the year after ($r=0.72$, $P<0.0001$). AUC: 0.79, 95% CI: 0.1210–0.3209, $P<0.0001$) for hsa_circ_0049875 showed a strong diagnostic value for COPD, according to ROC curve analysis. Hsa_circ_0042590 showed a close second with an AUC of 0.83 and 95% CI: -0.1972 – 0.0739 ($P<0.0001$).

Conclusion: This research identified a strong correlation between smoking and hsa_circ_0049875 and hsa_circ_0042590 in COPD PBMCs. The number of AEs in the preceding and succeeding years was substantially linked with the existence of hsa_circ_0042590 and hsa_circ_0049875 in COPD patients who smoke. Additionally, according to our research, hsa_circ_0049875 and hsa_circ_0042590 may be valuable biomarkers for COPD diagnosis.

Keywords: chronic obstructive pulmonary disease, circular RNAs, smoking, acute exacerbation, biomarker

Introduction

Chronic obstructive pulmonary disease (COPD) is mainly manifested as incompletely reversible airflow limitation in the lungs, and the airflow limitation is usually gradual, moreover, its degree is often closely related to the lung's inflammatory response to harmful particles or gases.¹ At present, COPD is a global public health problem.² According to a systematic analysis, 391.9 million people worldwide had COPD in 2019 at a prevalence of 10.3% according to the

GOLD criteria.³ COPD-related fatalities accounted for 5.7% of all deaths in 2017. The bulk of COPD cases worldwide are concentrated in China, the nation with the largest population. According to estimates, the prevalence of COPD in China was 13.6%, and in 2016,⁴ COPD was the country's fifth most prevalent cause of death. The percentage of COPD patients who have at least one AECOPD episode per year varies from 9.16% to 22.40%.⁵ However, most current COPD treatments focus on relieving symptoms and preventing acute exacerbations. Elucidating the pathophysiology of COPD is crucial in developing targeted interventions for respiratory diseases.

In recent years, the research of non-coding ribonucleic acid (ncRNA) in the field of disease pathogenesis has been heating up, and the research content has become more and more in-depth. Among the various types of non-coding RNA, circRNA has emerged as a particularly noteworthy focus in current research into the molecular mechanisms of diseases due to its distinctive properties and diverse functions.⁶ CircRNAs have emerged as important players in gene regulation by absorbing miRNAs or proteins,⁷ They possess several noteworthy properties that contribute to their vital role in this process. Firstly, circRNAs are widely expressed and produced by a significant proportion of actively transcribed human genes (ranging from 5.8% to 23%).⁸ Secondly, Circular RNAs have a closed-loop structure, which enhances their stability and reduces their susceptibility to exonucleases such as ribonuclease R (RNase R). Thirdly, most circRNAs are evolutionarily conserved, meaning they have been maintained throughout evolution across different species. Fourthly, a high concentration of circRNAs is found in the cytoplasm, with many derived from introns present in the nucleus.⁹ Fifthly, circRNAs exhibit tissue- and cancer-specific expression patterns, rendering them potential biomarkers with high sensitivity and specificity for different tissues and cancer types.⁶ Furthermore, recent research has revealed that certain circular RNAs (circRNAs) can produce functional proteins.⁶ Overall, due to their unique structural conformation, stability, and diverse functional properties, circRNAs, covalently closed transcripts, can serve as biomarkers and regulate genes and biological processes.¹⁰

Multiple studies have demonstrated the importance of circRNAs in PBMCs as pivotal biomolecules for elucidating the pathogenesis of associated diseases. For systemic diseases, many studies on circRNAs in PBMC have focused on diseases such as rheumatoid arthritis,¹¹ cancer,¹² systemic lupus erythematosus,¹³ and coronary artery disease.¹⁴ For respiratory system diseases, emphasis has been placed on lung cancer,¹⁵ pneumonia,¹⁶ and pulmonary tuberculosis.¹⁷ However, no reports have been on the expression of circRNAs in COPD patients' PBMCs and their potential for COPD diagnosis and treatment. The project aims to explore the possible application of circRNAs in PBMCs as a diagnostic tool for COPD.

Materials and Methods

Patients Characteristics

This research was centered on COPD patients at the First Affiliated Hospital of Soochow University from January to December 2022. To be eligible for the study, patients had to meet the following criteria: (1) an FEV₁/FVC ratio of less than 70% after 20 minutes of albuterol administration; (2) aged between 45 and 75 years; and (3) no acute exacerbation within the last 6 months. The control group comprised individuals who received a physical examination at the hospital within the same time frame and met the following criteria: (1) a post-albuterol inhalation spirometry test showing a FEV₁/FVC score of at least 0.7; (2) no history of chronic cough, wheezing, or expectoration; and (3) no COPD or other chronic respiratory diseases. Both the COPD group and control group were ineligible if they exhibited any of the following: (1) cardiovascular diseases, cerebrovascular disease, hepatic insufficiency, kidney insufficiency, diabetes mellitus, or rheumatic disease; (2) malignant tumor; (3) acute infectious disease; or (4) pregnancy. The participants provided informed consent and the study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Soochow University. The study protocols were conducted in accordance with the principles outlined in the Declaration of Helsinki of the World Medical Association.

PBMC Preparation and RNA Extraction

PBMCs were separated from 5 mL of blood using Ficoll-Paque PLUS (1.077 g/mL, GE Healthcare, Uppsala, Sweden) through density centrifugation (400 g, 35 min, 25°C). The cells were then preserved in Trizol (5–7 × 10⁶ mL concentration, Invitrogen, Carlsbad, CA, USA) and stored at –80°C. Total RNA was isolated from PMBCs using Trizol reagent

following the manufacturer's protocol. The amount of RNA was determined using a NanoDrop ND-1000 (Agilent, Santa Clara, CA, USA).

Reverse Transcription and Quantitative Real-Time PCR

1 µg of RNA was reverse-transcribed using the ReverTra Ace[®] qPCR RT Kit (616,700, TOYOBO, Japan). Each sample was repeated 3 times using cDNA as a template to amplify the target gene and used customized qRT-PCR primer sequences (Aksomics, China) for reaction, with GAPDH as an internal reference. Finally, we used the Quant Studio 6 Flex system (442,800, TOYOBO, Japan) and followed the react instructions. The 2- $\Delta\Delta$ Ct method was utilized to determine the level of gene expression.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8.0, a software developed by GraphPad Software, based in San Diego, California, USA. The numerical data were presented as mean \pm SD. To identify differences in circRNA expression between groups, Student's t-tests were employed. A P-value less than 0.05 and fold change (FC) greater than 1.5 were considered indicative of significant gene expression. The Spearman rank correlation was used to evaluate the relationship between circRNA levels and clinical data. ROC curves were used to evaluate the diagnostic potential of dysregulated circRNAs in PBMCs of COPD patients compared to healthy controls.

Results

All research participants' baseline characteristics are shown in Table 1. There were 70 people in all, 41 in the COPD group and 29 in the control group. Age, gender, and BMI did not significantly differ between the two groups. In contrast to the control group, the FEV₁/FVC (%), FEV₁%, and FEV₁ predicted values were considerably lower in the COPD group (P < 0.01). Additionally, CAT and MMRC scores were considerably higher (P < 0.01) in COPD patients. Patients with COPD showed differential expression of circRNAs. The most upregulated circRNA (hsa_circ_0049875) and the

Table 1 Clinical Characters and Laboratory Measures of the Participants

Parameter	COPD Group	Control Group	P-value
Age (year)	66.12 \pm 10.92	64.79 \pm 8.47	0.59
Gender (male/female)	34/7	24/5	0.99
Smoke history(yes/no)	27/14	7/22	<0.01
BMI (kg/m ²)	22.71 \pm 2.7	21.90 \pm 3.42	0.27
FEV ₁ (L)	1.58 \pm 0.77	2.78 \pm 0.41	<0.01
FEV ₁ (% predicted)	58.63 \pm 25.78	99.98 \pm 10.21	<0.01
FEV ₁ /FVC (%)	54.03 \pm 13.25	86.32 \pm 5.79	<0.01
WBC (10 ⁹ /L)	6.75 \pm 1.80	5.99 \pm 1.71	0.08
LYM (10 ⁹ /L)	1.92 \pm 0.61	1.93 \pm 0.63	0.97
NEU (10 ⁹ /L)	4.18 \pm 1.64	3.50 \pm 1.46	0.08
MPV(10 ⁹ /L)	10.78 \pm 1.18	11.05 \pm 0.98	0.32
PDW(10 ⁹ /L)	12.90 \pm 2.79	13.38 \pm 2.35	0.45
CRP (mg/L)	9.78 \pm 5.64	8.12 \pm 4.71	0.20
MMRC	2.14 \pm 1.14	0.31 \pm 0.47	<0.01
CAT	16.38 \pm 9.04	11.46 \pm 7.69	0.02

Note: Data are presented as mean \pm SD.

Abbreviations: COPD, chronic obstructive pulmonary disease; BMI, body mass index; FEV₁, forced expiratory volume in 1 second; FEV₁%predicted, forced expiratory volume in 1 second as a percentage of predicted value; FVC, forced vital capacity; SD, standard deviation; WBC, white blood cell; CRP, C-reactive protein; LYM, Lymphocyte count; NEU, neutrophil count; MPV, Mean platelet volume; PDW, Platelet distribution width; MMRC, modified British medical research council; CAT, COPD assessment test.

most downregulated circRNA (hsa_circ_0042590) were chosen for additional examination to determine the most clinically relevant biomarker.

Comparison of Different Expressions of Hsa_circ_0042590 and Hsa_circ_0049875 in PBMCs in the COPD and Control Group as Well as in Smoking COPD and Non-Smoking COPD Groups

The COPD group exhibited aberrant levels of hsa_circ_0042590 and hsa_circ_0049875 in PBMCs. In the COPD group, The expressions of hsa_circ_0049875 were upregulated by (0.3470 ± 0.2625) in comparison to the control group. (0.1261 ± 0.0714) . Conversely, the expressions of hsa_circ_0042590 were downregulated by (0.04634 ± 0.04436) compared with the control group (0.1820 ± 0.1911) . These differences were found to be statistically significant ($P < 0.05$). We conducted a *T*-test to evaluate the levels of hsa_circ_0042590 and hsa_circ_0049875 in the PBMCs of patients with COPD, with and without a history of smoking. In the COPD group with a smoking history, the levels of hsa_circ_0042590 showed a significant decrease ($P < 0.01$), while hsa_circ_0049875 levels were found to be significantly increased ($P < 0.01$) compared to the non-smoking COPD group (Figure 1).

The levels of expression for two circRNAs were confirmed using qRT-PCR in PBMCs obtained from all the enrolled patients. Student's *t*-test was used to analyze the data obtained from hsa_circ_0042590 and hsa_circ_0049875. Within the COPD group, participants were further divided into smoking and non-smoking subgroups based on their smoking history, and the differences in the levels of expression for hsa_circ_0042590, and hsa_circ_0049875 were compared between these two groups using the *T*-test. The results are reported as means \pm SD.

Spearman Correlation Analysis Was Performed to Assess the Relationship Between Clinical Variables and Confirmed circRNAs in PBMCs Obtained from Both Smoking and Non-Smoking COPD Patients

The aim was to determine whether hsa_circ_0042590 and hsa_circ_0049875 in PBMCs could be biomarkers for COPD severity. According to the findings of the Spearman correlation tests, a significant correlation between the levels of hsa_circ_0042590 in PBMCs from smoking COPD patients and the number of acute exacerbations (AEs) in the previous year ($r = 0.52$, $P < 0.01$) and the following year ($r = 0.67$, $P < 0.001$) were found. Additionally, Furthermore, it was shown that hsa_circ_0049875 was connected to the quantity of AEs in the year prior ($r = 0.68$, $P < 0.0001$) and the year after ($r = 0.72$, $P < 0.0001$), as demonstrated in Table 2 and Figure 2.

ROC Curve Analysis of PMBC circRNAs That Have Been Verified in COPD Patients

We conducted an ROC curve analysis to evaluate the potential use of substantially and differentially expressed circRNAs in COPD diagnosis. The levels of hsa_circ_0049875 and has_circ_0042590 in PBMCs were able to identify COPD

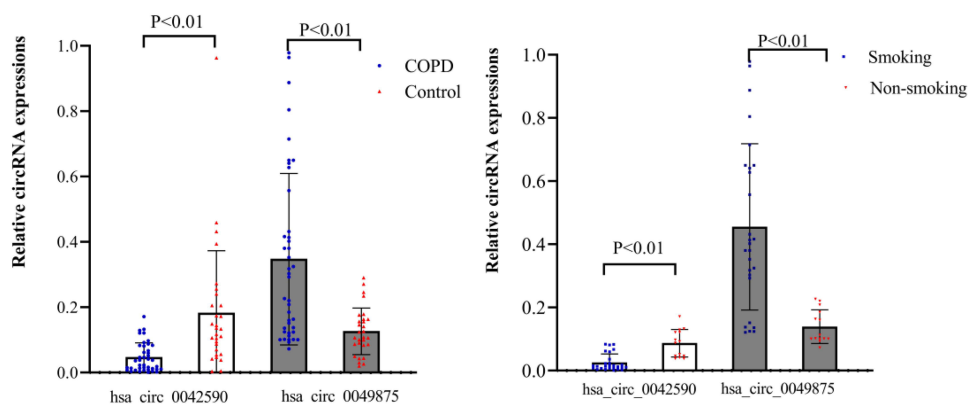


Figure 1 Reverse transcription-quantitative PCR results of the relative expression levels of circRNAs in PBMCs from patients with COPD and control group as well as with smoking-history COPD and non-smoking history COPD.

Table 2 Spearman Rank Correlation Coefficients of Clinical Variables and Quantitative RT-PCR-Confirmed circRNAs in PBMCs from Smoking and Non-Smoking COPD Patients

Index	has_circ_0042590		has_circ_0049875	
	Smoking	Non-Smoking	Smoking	Non-Smoking
BMI (kg/m ²)	-0.30	0.15	-0.15	0.27
FEV ₁ (L)	0.26	-0.05	-0.18	-0.17
FEV ₁ (% predicted)	0.45	-0.03	-0.17	-0.15
FEV ₁ /FVC (%)	-0.05	-0.17	-0.11	-0.05
WBC(10 ⁹ /L)	0.19	0.07	0.11	0.02
LYM (10 ⁹ /L)	-0.34	0.78	0.12	0.39
NEU (10 ⁹ /L)	0.36	-0.33	0.13	-0.23
MPV(10 ⁹ /L)	-0.02	0.54	-0.18	0.16
PDW(10 ⁹ /L)	-0.01	0.52	-0.23	0.21
CRP (mg/L)	-0.15	-0.10	-0.23	0.12
MMRC	-0.31	-0.29	-0.02	0.29
CAT	-0.21	0.07	0.14	0.21
AEs in the previous year	0.52*	0.26	0.68***	0.04
AEs in the following year	0.67**	0.45	0.72***	0.03

Notes: The levels of two circRNAs were found to be associated with the occurrence of adverse events in smoking COPD patients in the preceding and subsequent years, as determined by Spearman's rank correlation test. The specific values of P and r were indicated in each chart. *P<0.01; **P<0.001; ***P<0.0001.

Abbreviations: BMI, body mass index; FEV₁, forced expiratory volume in 1 second; FEV₁%predicted, forced expiratory volume in 1 second as a percentage of predicted value; FVC, forced vital capacity; SD, standard deviation; WBC, white blood cell; CRP, C-reactive protein; LYM, Lymphocyte count; NEU, neutrophil count; MPV, Mean platelet volume; PDW, Platelet distribution width; MMRC, modified British medical research council; CAT, COPD assessment test; AEs, acute exacerbations.

patients from healthy controls, as shown by the ROC curves for validated circRNAs. AUC values for has_circ_0042590 (AUC: 0.83, 95% CI 0.1972–0.0739, P<0.0001) and hsa_circ_0049875 (AUC: 0.79, 95% CI 0.1210–0.3209, P<0.0001) were the highest, respectively, according to Table 3, Figure 3. As a result, has_circ_0042590 and hsa_circ_0049875 may be useful biomarkers for COPD diagnosis.

Discussion

COPD is the result of prolonged exposure to harmful inhaled particles, specifically from tobacco smoke and pollutants.¹⁸ It is distinguished by a progressive and irreversible decline in airflow and deterioration of lung tissue.¹⁹ AECOPD is the main cause of hospitalization and death in people with COPD.²⁰ It is a medical condition marked by an abrupt decline in airway function and respiratory symptoms. CircRNAs are distinct, very conserved molecules that are expressed in several human tissues, including the skin, lung, breast, digestive tract, urinary system, and nervous system.^{21,22} Numerous investigations have shown that circRNAs control many diseases via various pathways. The mononuclear cells in the blood called peripheral blood mononuclear cells (PBMCs) are essential to the immune system of the body.²³ It has been demonstrated that the gene expression signatures found in PBMCs, which represent the body's general immunological function, are important in the development of COPD.²⁴

This paper used circRNA microarray to determine the circRNA expression patterns in PBMCs from COPD patients and healthy controls. Because of their potential role in the development of COPD, circRNAs' unique expression patterns may serve as biomarkers for the identification of the disease. We identified two differentially expressed circRNAs with significant potential as clinically applicable biomarkers. In COPD patients, hsa_circ_0049875 was overexpressed whereas hsa_circ_0042590 demonstrated a substantial decrease when compared to healthy controls. There were statistically significant differences in the expression levels of hsa_circ_0049875 and hsa_circ_0042590 between COPD patients who smoked and those who did not. The expression of hsa_circ_0042590 was correlated with the number of acute exacerbations (AEs) in the prior year (r=0.52, P<0.01) and the subsequent year (r=0.67, P<0.001) in COPD patients with a history of smoking, whereas hsa_circ_0049875 was correlated with the number of AEs in the prior year (r=0.68,

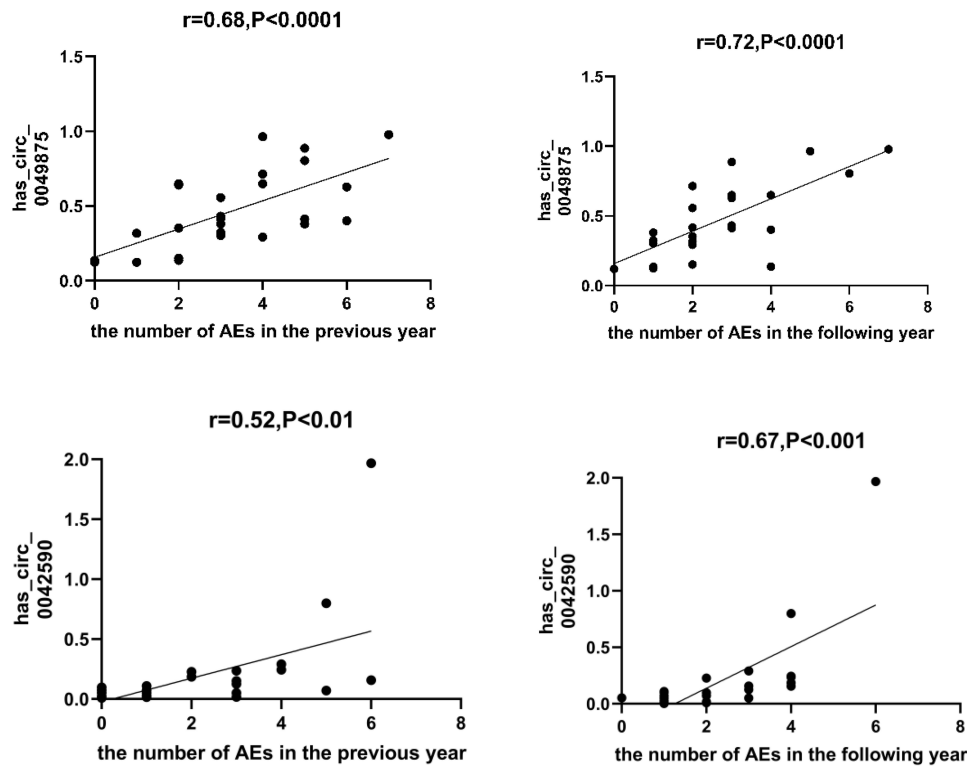


Figure 2 Correlation between the expression levels of confirmed circRNAs and the number of AEs in the previous and following year in smoking COPD patients.

$P < 0.0001$) and the subsequent year ($r = 0.72$, $P < 0.0001$). Furthermore, *hsa_circ_0049875* ($AUC = 0.79$) and *hsa_circ_0042590* ($AUC = 0.83$) have substantial diagnostic values for COPD, according to ROC curve analysis.

Previous reports of circRNA dysregulation in COPD patients are inconsistent due to varying samples and techniques. For example, *Circ_0061041* has been linked to airway remodeling and the CSE-triggered epithelial-mesenchymal switch in COPD.^{25,26} Meanwhile, *hsa_circRNA_0003060* has been shown to significantly down-regulate the primary human small-airway epithelial cell (HSAECs) model of COPD that is induced by.²⁷ Additionally, *CircFOXO3* may be a promising target for the treatment of inflammatory illnesses as it has been found to play a critical role in the pathological remodeling of inflammatory processes brought on by exposure to cigarette smoke.²⁸ Moreover, emphysema development later on has been seen to benefit from the protective effects of CD69. Xue et al discovered that in human pulmonary microvascular endothelial cells, *circ0006872* induces apoptosis, inflammation, and oxidative stress.²⁹ Furthermore, significant differences in the downregulation of *hsa_circ_0022342* in PBMC were observed in Chronic thromboembolic pulmonary hypertension.³⁰ Research on the expression of circRNAs in patient-derived peripheral blood mononuclear cells suggests that circRNA expression may impact immunological homeostasis and contribute to the pathogenesis of COPD.

According to earlier research, RNAs are crucial in the remodeling and inflammation of the airways brought on by smoking.^{31–33} These results imply that circRNAs' role in the pathophysiology of COPD may be connected to their

Table 3 Sensitivity and Specificity of the Candidate Biomarkers in Controls and COPD Patients

Candidate Biomarkers	AUC	95% CI	P value
<i>hsa_circ_0042590</i>	0.83	–0.1972–0.0739	<0.0001
<i>hsa_circ_0049875</i>	0.79	0.1210–0.3209	<0.0001

Abbreviations: AUC, area under the curve; 95% CI, 95% CI Confidence Interval.

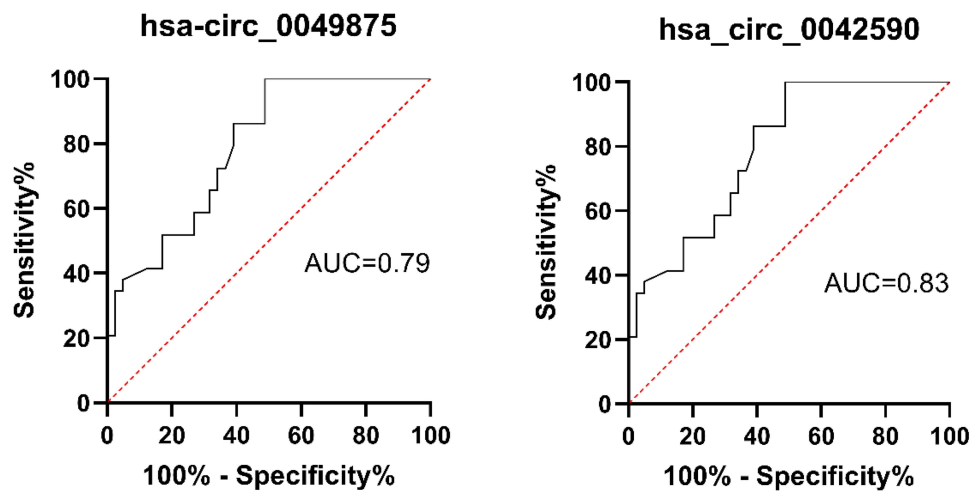


Figure 3 Receiver operating characteristic (ROC) curve analysis of confirmed circRNAs in PBMCs from COPD patients. The AUC values are given on the graphs.

dysregulated expression. Additionally, we found that *hsa_circ_0049875* and *hsa_circ_0042590* levels in PBMCs had potential as COPD diagnostic indicators. The aberrant expression of *hsa_circ_0049875* and *hsa_circ_0042590* in smokers with COPD can forecast the frequency of exacerbations in the preceding and following years. This is especially the case with *hsa_circ_0049875* and *hsa_circ_0042590*, which stood out among the rest in terms of ROC AUC value and so have the potential to be used as diagnostic biomarkers. Furthermore, our extensive review of the literature confirmed that our study was the first to identify abnormalities in *hsa_circ_0049875* and *hsa_circ_0042590*. These results point to a potential function for circRNAs in the chronic inflammatory immune response associated with COPD and imply that the dysregulation of these two circRNAs, as emphasized in our investigation, is implicated in the development of COPD.^{34–36}

In conclusion, we have deduced that *hsa_circ_0049875* and *hsa_circ_0042590* may be important factors in the onset of COPD. A crucial first step in understanding COPD better is the identification of novel circRNAs with differential expression. Future investigations into the pathogenesis of COPD may benefit from these results, which may assist in determining if circRNAs in PBMCs might function as innovative, non-invasive biomarkers for the diagnosis and treatment of COPD. However, further research is required to corroborate our findings because of our inadequate understanding of circRNAs. The tiny sample size in this study is one of its limitations. To support the results of our investigation, we thus plan to collect additional samples.

Conclusion

This study found a strong correlation between smoking and *hsa_circ_0049875* and *hsa_circ_0042590* in COPD PBMCs. The frequency of AEs in the prior and subsequent years was shown to be substantially linked with *hsa_circ_0049875* and *hsa_circ_0042590* in COPD patients with a previous history of smoking. Our findings also suggest that *hsa_circ_0049875* and *hsa_circ_0042590* may also be used as COPD diagnostic biomarkers.

Acknowledgments

Xiu-Qin Zhang and Da-Xiong Zeng are equal corresponding authors.

Funding

There is no funding to report.

Disclosure

The authors had no conflicts of interest.

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