

# Preliminary evaluation of circulating microRNAs as potential biomarkers in paracoccidioidomycosis

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**Abstract.** MicroRNAs (miRNAs) are small RNAs (length, 19-24 nucleotides) that regulate gene expression by either mRNA degradation or translational inhibition of proteins. Circulating miRNAs, which are extremely stable and protected from RNase-mediated degradation in body fluids, have appeared as candidate biomarkers for numerous diseases. However, little is known about circulating miRNAs in fungal infections. Paracoccidioidomycosis (PCM) is caused by the *Paracoccidioides* species, and is endemic in Central and South America, with predominance in adult male workers from rural areas. The current study aimed to identify a serum miRNA expression profile that could serve as a novel diagnostic biomarker for PCM. Total RNA was isolated and the levels of circulating miRNAs were compared between patients with PCM and healthy control subjects using reverse transcription-quantitative polymerase chain reaction. Bioinformatic analysis was used to evaluate the potential roles of these miRNAs in PCM. Eight miRNAs were differentially expressed in serum samples from patients with PCM. These miRNAs are associated with apoptosis and immune response. The identified miRNAs facilitate with understanding the regulatory mechanisms involved in the host-parasite interaction of PCM. Furthermore, considering that the diagnosis of PCM presents difficulties, these miRNAs may serve as novel biomarkers for this disease.

## Introduction

MicroRNAs (miRNAs) are an abundant class of short regulatory non-coding RNAs (ncRNAs; length, 20-24 nucleotides) that act as post-transcriptional repressors by binding the 3'-untranslated regions (UTRs) of target messenger RNAs (mRNAs). The regulatory activity of miRNAs is exerted at the post-transcriptional level and it is estimated that they act on up to one-third of the protein coding genes. miRNAs have been implicated in various biological and pathological processes, and have emerged as important contributors to cell homeostasis (1).

Cells actively secrete miRNAs, and it is possible to detect those small ncRNAs in all biological fluids. As a consequence, circulating miRNAs have great potential as biomarkers, which has already been reported for various types of human disease, such as cancer (2), metabolic disorders (3) and cardiac conditions (4). In systemic infections, the presence of a pathogenic agent often induces a significant change in the profile of circulating miRNAs that facilitates their use as biomarkers of disease establishment and progression. In certain cases, such as viral infections, these changes in circulating miRNAs are associated with the targeted cell, as demonstrated in the acute and chronic hepatitis C virus infection (5,6). Parasitic flatworms, such as *Schistosoma japonicum* induce a differential expression of circulating miRNAs (7). Furthermore, previous studies have suggested the involvement of circulating miRNAs in response to bacterial infections, including active pulmonary tuberculosis (8). However, the potential use of circulating miRNAs as biomarkers in fungal infections has been investigated comparatively less. In this context, in response to infection with *Candida albicans*, miR-455, miR-125a, miR-146 and miR-155 were upregulated in mouse macrophages and miR-204 and miR-211 were downregulated in renal mice tissues (9,10). In monocytes and dendritic cells infected with *Aspergillus fumigatus*, miR-132 and miR-155 were identified to be differentially expressed (11).

The incidence of fungal infections has increased in recent years and is associated with significant morbidity and mortality (12). Paracoccidioidomycosis (PCM) is a human systemic mycosis that is considered to be clinically important, due to the increase of frequency, the severity of their clinical

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manifestations and the associated mortality rates. PCM is caused by dimorphic fungus of the *Paracoccidioides* species (*Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*) and is endemic in Central and South America, with a predominance of clinical disease in adult men and labourers from rural areas (13). In patients with PCM, the fungus is initially observed in the lungs; however, the yeast spreads to other organs (14).

Considering the central role of miRNAs in infectious diseases and that little is known about the molecular mechanisms underlying PCM, the aim of the present study was to verify the presence of differentially expressed circulating miRNAs in patients with PCM, which could serve as novel biomarkers in the diagnosis of this fungal disease.

## Materials and methods

**Study subjects.** Four male patients diagnosed with chronic PCM, and four male healthy control subjects free of the PCM infection and free of any clinical symptoms of any infectious disease were recruited from the School of Pharmaceutical Sciences, São Paulo State University (UNESP; Araraquara, Brazil) and included in the current study. Venous blood (1 ml) was collected from each participant and samples were centrifuged at 1,800 x g for 10 min at 4°C. The supernatant serum was quickly removed, aliquoted, and frozen at -80°C until the experiments were performed. The present study was approved by the local ethics committee of the School of Pharmaceutical Sciences, São Paulo State University (UNESP) (approval no. 33397114.5.0000.5426) and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained prior to blood sample collection.

**Extraction of RNA.** The extraction of RNA, including miRNAs, from 200 µl serum was performed using miRCURY™ RNA Isolation kit - Biofluids (Exiqon A/S, Vedbaek, Denmark), according to the manufacturer's instructions.

**Synthesis of cDNA and analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** cDNA was synthesized from RNA using a Universal cDNA synthesis kit (Exiqon A/S) according to the manufacturer's instructions. For miRNA screening, a global RT-qPCR low-density panel (microRNA Ready-to-Use PCR, Human panel I+II, V.2 M/R; Exiqon A/S) was used, which contains locked nucleic acid specific primers for 752 unique human miRNAs. The reaction mixture included 40 µl cDNA, 40 ml SYBR-Green PCR Master Mix (Exiqon A/S) and 40 ml RNase-free water. Then, 10 µl of the reaction mixture was added to each well of the panel. Amplification was performed by RT-qPCR (ViiA™ 7 Real-Time PCR System; Thermo Fisher Scientific, Inc., Waltham, MA, USA) followed by the determination of the melting curve according the following conditions: Denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec and 60°C for 60 sec. The experiment was performed in triplicate. The raw Cq values were normalized using the global normalization method in each sample, and the expression levels of all miRNAs were calculated according to the  $\Delta\Delta Cq$  method (15), as implemented in

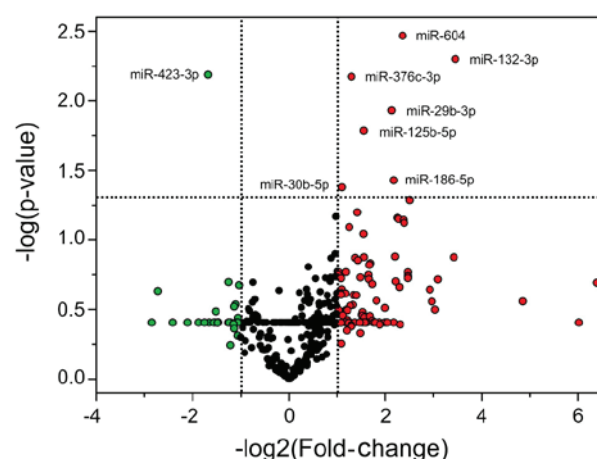


Figure 1. Volcano plot of the detected circulating miRNAs in patients with paracoccidioidomycosis compared with healthy controls. Upregulated and downregulated miRNAs are presented in red and green, respectively. Statistically significant miRNAs are depicted with their miRBase names. miRNA, microRNA.

DataAssist v2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** The statistics toolbox of the DataAssist v2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used and  $P < 0.05$  was considered to indicate a statistically significant difference. A volcano plot was constructed for the analyzed miRNAs and miRBase was used to identify them (16). For bioinformatics analysis, differentially expressed miRNAs were selected and submitted to *in silico* pathways analysis for Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG), using the bioinformatics tool, Database for Annotation, Visualization and Integrated Discovery (DAVID).

## Results

**Circulating miRNA levels in the PCM.** The differentially expressed miRNAs in PCM patients are demonstrated by a non-symmetrical distribution in a volcano plot (Fig. 1). The x-axis represents the  $\log_2$  ratio of the expression difference of miRNA between the groups and the y-axis demonstrates the log of the P-values. From the 752 miRNAs analyzed, seven were overexpressed (miR-132-3p, miR-604, miR-186-5p, miR-29b-3p, miR-125b-5p, miR-376c-3p and miR-30b-5p) and one was underexpressed (miR-423-3p) in the serum of patients with PCM when compared with the healthy control subjects (Table I). The data were regarded as significantly different at  $P < 0.05$  and with a fold-change  $\geq 2$ . Furthermore, the expression profile of the investigated microRNAs was similar among the subjects of each group.

**Pathway analysis of miRNA-controlled pathways.** Based on the prediction of pathways, using the gene annotation tool DAVID (Table II), the differentially expressed miRNAs in PCM participate in the processes of apoptosis, immune response, adhesion and the infection processes of fungus to host cells. The biological processes identified may regulate genes that are included in the mitogen-activated protein kinases

Table I. Differentially expressed miRNAs in patients with paracoccidioidomycosis relative to healthy control subjects.

miRNA	Fold-change	P-value
Upregulated		
hsa-miR-132-3p	10.97	0.0050
hsa-miR-604	5.13	0.0034
hsa-miR-186-5p	4.51	0.0372
hsa-miR-29b-3p	4.38	0.0117
hsa-miR-125b-5p	2.93	0.0164
hsa-miR-376c-3p	2.45	0.0067
hsa-miR-30b-5p	2.14	0.0418
Downregulated		
hsa-miR-423-3p	0.31	0.0065
miRNA, microRNA.		

Table II. Signaling pathways regulated by eight microRNAs identified in patients with paracoccidioidomycosis.

Signaling pathway	P-value
Regulation of apoptosis	1.8E-28
Negative regulation of transcription	3.2E-16
Metabolism regulation of cellular proteins	2.3E-18
Regulation of DNA binding	1.7E-18
Regulation of the cascade of protein kinases	3.8E-12
Regulation of cell migration	8.9E-15
Inflammatory response	2.3E-8
Mitogen activated protein kinases cascade	2.4E-5
Activation of leukocytes	1.1E-9
Activation of T cells	4.5E-6
Activation of B cells	2.7E-3
Regulation of cytokine production	8.5E-10
Regulation of adhesion to the extracellular matrix	8.2E-7
Endocytosis	2.7E-5
Binding to the tumor necrosis factor receptor superfamily	3.6E-6

cascade, inflammatory response, activation of leukocytes, T and B cells, regulation of cytokine production, regulation of adhesion to the extracellular matrix (ECM) and endocytosis.

## Discussion

Previous studies have shown the involvement of circulating miRNAs in certain infectious and non-infectious diseases (2,8,17,18). miRNAs may be used for diagnosis, as well as for monitoring disease progression and treatment efficacy (19). This is a preliminary study that compared the miRNA profile in the serum of patients diagnosed with chronic PCM with healthy control subjects. Eight miRNAs (miR-132-3p, miR-604, miR-186-5p, miR-29b-3p, miR-125b-5p, miR-376c-3p, miR-30b-5p and miR-423-3p) were identified to

be differentially expressed in serum samples obtained from patients with PCM.

The identification of miRNAs differentially expressed in PCM in the present study is considered to be important. It facilitates the understanding of the regulatory mechanisms involved in host-parasite interactions and the infectious process. The *in silico* analysis demonstrated that apoptosis, immune response and adhesion of fungus to host cells are important signaling pathways that are regulated by eight differentially expressed miRNAs in PCM. Following inhalation by the host, *Paracoccidioides* spp. adhere to ECM components, such as collagen, fibronectin and laminin. This process avoids elimination of the fungi by lung ciliary cells and contributes to establishment of the infection (20). Apoptosis is an important mechanism, as described in certain infections, including PCM, and has been implicated in immunosuppressive events, which works in favor of the parasite to limit the exaggerated inflammatory response. The process of apoptosis has been described in PCM patients and in a PCM murine model (21,22). *P. brasiliensis* induces apoptosis in macrophages via caspases (23,24). In addition, the participation of macrophages, cytokines, interleukins (ILs) and tumor necrosis factor (TNF) is observed in PCM (20,25).

In addition, the majority of miRNAs identified in the present study have been previously described in the processes of apoptosis and immune response to other infections. miR-132 and miR-186, for example, have been described to be associated with the promotion of apoptosis, which activates the signaling pathway via caspases (26). Furthermore, the expression of miR-132 was induced in cells of the immune system by the *A. fumigatus* and *Mycobacterium tuberculosis* infection (11,27). Members of the miR-29 family may act in the Wnt signaling pathway, which regulates the activation of macrophages in the inflammatory process (28,29). Furthermore, miR-29 was identified to be overexpressed in the serum and sputum of patients with active pulmonary tuberculosis (8). Comparable with miR-29, miR-376c was recently reported to be differentially expressed in the serum of patients with active tuberculosis (30). In addition, miR-125 may be involved in the immune response to infections. It has been shown that *M. tuberculosis* induces the expression of miR-125b, which binds to the 3'-UTR of the mRNA of TNF in human macrophages and destabilizes the transcript. Blocking biosynthesis of the TNF is an important process in response to bacterial infection (31). miR-30 may also be associated with the immune response process, as it suppresses the polypeptide *N-acetylgalactosaminyltransferase 7* gene, which leads to an increase of IL-10 (32). By contrast, two miRNAs (has-miR-604 and miR-423-3p) were described, but were not associated with infectious disease (33,34).

miRNAs detected in the serum of patients with PCM may serve as biomarkers. The diagnosis of PCM is based on clinical and laboratory findings. Typically, the direct microscopic examination of lesions or tissue samples that are collected is made with 10% potassium hydroxide, 4% sodium hydroxide or calcofluor white to observe the fungi. The culture to observe the thermal dimorphism of *Paracoccidioides* spp. is necessary for a definitive diagnosis; however, the fungus grows slowly. Serologic tests facilitate with diagnosis, however, certain tests for PCM are not well standardized, which may



result in cross-reactions with other infections (35). Thus, the production of antigenic preparations from whole yeast cells and culture filtrate is important to ensure the sensitivity and specificity of the tests. Gp43 has served as the main antigen in the diagnosis of PCM; however, it was demonstrated that high percentages of false negative results for PCM were caused by *P. lutzii* (36). Thus, novel biomarkers along with existing techniques may contribute to improved diagnosis of PCM. In this context, miRNAs have been presented as 'fingerprints' of various pathological conditions (37). These molecules are advantageous due to their stability in body fluids, as they are normally packaged into exosome-like microparticles that protect them against endogenous RNase activity. Circulating miRNAs have been shown to be stable during repeated freezing and thawing, as well as in acidic and alkaline environments (38). Furthermore, detection techniques, including PCR and microarray, are sensitive and rapid (1,39).

In conclusion, eight differentially expressed miRNAs were identified in serum samples from patients with PCM. These miRNAs are associated with apoptosis, immune responses and adhesion processes. Although the small sample size is a limitation, the present study provided, to the best of our knowledge, the first description of circulating miRNAs as potential biomarkers for the diagnosis of PCM. However, further studies are required to extend the identification of miRNAs for a larger number of patients with PCM and to clarify the role of these miRNAs in this mycosis.

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