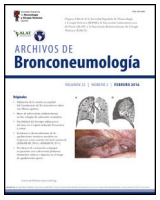
Archivos de Bronconeumología 58 (2022) 177–179

Sociedad Española de Neumología y Cirugía Torácica SEPAR

ARCHIVOS DE
Bronconeumología

www.archbronconeumol.org



Scientific Letter

Specific miRNA Profile in Chronic Obstructive Pulmonary Disease Related to Biomass Smoke Exposure

Perfil específico de miRNAs en enfermedad pulmonar obstructiva crónica asociada a exposición a humo de biomasa

To the Director:

Chronic obstructive pulmonary disease (COPD) is a complex syndrome with an inflammatory component, that emerges from the interaction between the genetic background of the individual and several environmental factors. Cigarette smoking (CS) is the main environmental risk factor for COPD.[1,2] Yet, exposure to smoke coming from biomass burning (biomass smoke, BS) is also recognized as an important environmental COPD risk factor.[3] CS and BS-COPD patients show clinical, functional, imaging, and histopathological differences suggesting that they correspond to two different clinical COPD phenotypes.[4]

Micro-RNAs (miRNAs) are a type of small noncoding RNAs that regulate a wide range of cellular activities. Here, we compared the levels of 2609 circulating miRNAs in patients with COPD associated to cigarette smoking and in never-smoking BS-COPD patients, exploring the hub genes and pathways associated with these differentially expressed miRNAs. 15 never-smoking COPD patients with a history of BS exposure (BS-COPD group) and 15 age and sex-matched ever-smokers with COPD (CS-COPD group, 10 ex-smokers, 5 current smokers) from a rural population in Chile were recruited at the Respiratory Service of the Regional Hospital de Talca, where they attended to undergo diagnostic tests after suspected COPD or for COPD monitoring visits. The diagnosis of COPD followed the GOLD criteria.[5] Medical history was collected using standardized questionnaires including a standardized version of the CanCOLD study questionnaire,[6] adding some questions referring to biomass fuels. Cumulative exposure to BS was calculated as hour-years as previously described[7] and cigarette smoking history was measured by pack-years. This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki, and the Ethics Committees of the Maulean Health Service and Universidad Autónoma de Chile approved the study and all subjects provided written informed consent (approval code: 063-15).

A venous blood sample (5 ml) was obtained from each participant by venipuncture and was stored in a serum Vacutainer tube (BD 366431). Samples were centrifugated at room temperature twice: at $800 \times g$ during 10 min, followed by $10{,}000 \times g$ for 15 min. The supernatant serum was recovered, placed into Eppendorf tubes, and stored at −80 °C until RNA purification. For total RNA extraction, we used phenolchloroform separation and the miRNeasy Serum/Plasma kit (Cat. no 1071073, Qiagen, Venlo, The Netherlands) on a QIAcube (Qiagen). During the RNA extraction step, Glycogen (Cat. no AM9510, Invitrogen, Waltham, Massachusetts, USA) was used as a carrier. Quantification and integrity assessment of RNA was performed by Nanodrop spectrometry and on Agilent's Bioanalyzer. The eluate was concentrated using Ampure beads XP (Agencourt Bioscience, Beverly, Massachusetts, USA). A next-generation high-throughput sequencing platform (HiSeq2500, Illumina, San Diego, California, USA) optimized for low-input RNA (BGI Genomics, Shenzhen, Guangdong, China) was used for generating miRNA profiles. In order to ensure high quality data, we added NEBNext library kits, batch controls and internal small-noncoding-RNA spike-in controls (*C. elegans* miR-39). Sequencing libraries were indexed, and 10 samples were sequenced per lane, yielding 15 million sequences per sample on average. After initially trimming the RNAseq reads for adapters using AdapterRemoval (v2.1.7), we mapped the collapsed reads (generated by FASTX v0.14) to the human genome (hg38) using Bowtie2 (10 alignments per read were allowed). All multi-mapped reads with equivalent mapping score were counted. Finally, we compiled a comprehensive annotation set from miRBase (v21), using SeqBuster (v3.1) to get miRNA profiles and HTSeq (v0.7.2) to count the mapped reads.

Age and sex were similar in CS- and BS-COPD, but BMI was higher and school years significantly lower in the latter group (Table 1). Both smoking history and cumulative exposure to BS were high in the respective groups. Symptoms (mMRC, CAT and BODE), lung function tests and the 6MWT were similar in both groups. BS-COPD patients reported more exacerbations in the previous year than CS-COPD patients, although the difference was not significative. Finally, GOLD group distribution was similar in CS- and BS-COPD.

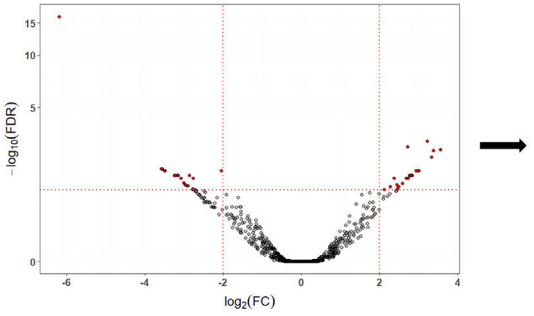
MicroRNAs expression was compared between groups using the R package edgeR. Forty-five miRNAs were differentially expressed in BS- vs. CS-COPD (|log2 fold change (FC)| of >2 and Benjamini–Hochberg false discovery rate (FDR)-correct *P* values <.05) (Fig. 1), 23 up and 22 down-regulated in the BS-COPD group. Among the miRNAs differentially expressed in BS-COPD, those with a false discovery rate (FDR) ≤ 0.01 (9 upregulated and 6 downregulated) were screened with the microT-CDS algorithm (miRNA target genes score ≥ 0.95).[8] The analysis identified 287 genes related to the upregulated miRNAs and 173 genes related to the downregulated miRNAs (Fig. S1). The biological processes related to these genes were identified through Gene Ontology (GO) analysis, using the ClusterProfiler package. Eighteen terms were found to be involved in the differentially expressed miRNAs enrichment analysis (Fig. S2). Overall, these were related to four main processes: cell responses to hypoxia, neuronal development and differentiation, regulation of cell cycle G2/M phase transition, and chromatin and histone modification. In the enrichment map showed in Fig. S3, highly similar terms were gathered into clusters that underpin general trends. Terms related to cell responses to hypoxia and regulation of cell cycle G2/M phase transition had intersecting gene sets with each other. We also used X2K to construct the upstream regulatory network of the differentially expressed miRNAs.[9] This analysis revealed that CREB1, PPARG, YY1, CHD1, UBTF, SPI1, TAF1,

https://doi.org/10.1016/j.arbres.2021.03.020

Table 1
Demographic and Clinical Characteristics of Ever-smokers With COPD (CS) and Never-smokers With COPD Associated to Biomass Smoke (BS).

	CS-COPD $n = 15$	BS-COPD $n = 15$	*P*-Value
Sex, M/F	5/10	5/10	
Age, years	70.93 ± 3.86	70.13 ± 6.17	.67
BMI, kg/m^2	26.94 ± 4.77	30.12 ± 6.92	.15
Scholarship, years	9.87 ± 3.92	4.06 ± 3.31	<.0001
Smoking history, pack-years	60.58 ± 29.73	––	
Biomass smoke exposure, hour-years	––	316.67 ± 223.47	
Exacerbations in the last year	1.73 ± 1.44	2.93 ± 1.69	.52
FEV_1, % pred	49.40 ± 17.62	56.13 ± 13.49	.25
FEV_1/FVC, %	56.40 ± 11.02	58.06 ± 7.58	.63
DL_{CO}, % pred	76.42 ± 34.87	78.25 ± 26.85	.52
Oxygen saturation, %	94.38 ± 3.74	91.42 ± 4.65	.29
6MWT, m	360.75 ± 124.85	250.00 ± 144.39	.25
mMRC	2.87 ± 1.19	2.27 ± 1.09	.25
CAT	15.40 ± 4.79	15.80 ± 7.78	.86
BODE	3.13 ± 2.23	4.14 ± 1.86	.81

Definition of abbreviations: *BMI* body-mass index, *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *DL_{CO}* carbon monoxide diffusing capacity, *6MWT* 6 minutes walking test, *mMRC* modified Medical Research Council scale, *CAT* COPD assessment test, *BODE* body-mass, airflow Obstruction, Dyspnea and Exercise index.
Data presented as mean ± standard deviation, unless otherwise indicated. Pulmonary function was assessed in all participants using standard procedures and equipment (Masterlab; Jaeger, Würzburg, Germany). Oxygen saturation was measured by pulse-oximetry (Ohmeda TuffSat, Soma Technology, Connecticut, USA). Dyspnea was measured using the modified Medical Research Council dyspnea scale (mMRC) and the COPD assessment test (CAT) was used to measure the impact of COPD on patient quality of life. The 6-minute walking test (6MWT) was also performed to each participant, according to European Respiratory Society/American Thoracic Society guidelines. The Body-mass, airflow Obstruction, Dyspnea and Exercise (BODE) score was calculated for each patient. Subjects were excluded if there was history of asthma, rhinitis or any extra-pulmonary disease affecting lung function, positive bronchodilator test, FEV1 increasing by ≥12% and 200 ml and exacerbation or hospitalization record during the previous two months.



Up BS-COPD				Down BS-COPD			
miRNAs	Log Fold Change	pvalue	FDR	miRNAs	Log Fold Change	pvalue	FDR
hsa-miR-6748-3p	3.22	1.56e-06	0.0009	hsa-miR-125b-1-3p	-6.20	5.21e-20	6.01e-17
hsa-miR-1262	2.71	4.25e-06	0.002	hsa-miR-4742-5p	-3.59	7.87e-05	0.01
hsa-miR-6746-5p	3.56	7.33e-06	0.002	hsa-miR-4658	-3.55	7.95e-05	0.01
hsa-miR-1537-3p	3.38	1.02e-05	0.002	hsa-miR-4754	-3.49	0.0001	0.01
hsa-miR-6851-5p	3.33	2.20e-05	0.004	hsa-miR-6855-3p	-3.50	0.0001	0.01
hsa-miR-876-3p	3.01	0.0001	0.01	hsa-miR-3168	-2.06	0.0002	0.01
hsa-miR-548at-3p	2.98	0.0002	0.01	hsa-miR-4477b	-3.25	0.0003	0.02
hsa-miR-6854-3p	2.99	0.0002	0.01	hsa-miR-3685	-3.24	0.0004	0.02
hsa-miR-7153-3p	2.93	0.0002	0.01	hsa-miR-6858-5p	-2.86	0.0004	0.02
hsa-miR-215-3p	2.84	0.0003	0.02	hsa-miR-6718-5p	-3.16	0.0004	0.02
hsa-miR-4477a	2.78	0.0003	0.02	hsa-miR-6132	-3.18	0.0004	0.02
hsa-miR-6085	2.77	0.0004	0.02	hsa-miR-6748-5p	-3.08	0.0006	0.02
hsa-miR-6803-5p	2.80	0.0004	0.02	hsa-let-7g-3p	-2.76	0.0006	0.02
hsa-miR-3650	2.73	0.0005	0.02	hsa-miR-4299	-3.08	0.0006	0.02
hsa-miR-3199	2.37	0.0005	0.02	hsa-miR-6849-5p	-3.01	0.0009	0.03
hsa-miR-6791-3p	2.68	0.0006	0.02	hsa-miR-4463	-2.93	0.001	0.04
hsa-miR-1247-5p	2.59	0.0009	0.03	hsa-miR-6735-3p	-2.90	0.001	0.04
hsa-miR-4785	2.44	0.001	0.04	hsa-miR-6751-5p	-2.97	0.001	0.04
hsa-miR-1237-3p	2.49	0.001	0.04	hsa-miR-7853-5p	-2.78	0.002	0.05
hsa-miR-6839-5p	2.27	0.001	0.04	hsa-miR-570-3p	-2.78	0.002	0.05
hsa-miR-6779-5p	2.27	0.001	0.04	hsa-miR-1193	-2.73	0.002	0.05
hsa-miR-4723-5p	2.46	0.002	0.05	hsa-miR-3139	-2.78	0.002	0.05
hsa-miR-4777-3p	2.11	0.002	0.05				

Fig. 1. Differential miRNA expression in never-smoking COPD patients exposed to biomass smoke versus cigarette smoking COPD patients. (A) Volcano plot showing the differential miRNA expression (in fold change on *x*-axis) and significance level (−log10-FDR value on *y*-axis). To exclude poorly detectable miRNAs, data was pre-processed so that total counts per million (CPM) mapped reads for each miRNA in all samples pertaining to the comparison subset was > 10. After that, the filtered data were normalized using the trimmed mean of M-values (TMM) normalization method in EdgeR. Of the 2609 miRNAs analyzed, 1460 were discarded because they were poorly detectable according to the quality criteria.

and REST were the most important transcription factors (TFs) regulating gene expression, whereas GSK3B, MAPK14, CDK1, HIPK2, CK2ALPHA, ERK2, MAPK3 and ERK1 were the most important kinases controlling the expression of genes (Fig. S4).

A growing body of evidence have shown up/down-regulation of several miRNAs in CS-COPD patients when compared to subjects without COPD.[10–13] Interestingly, none of the miRNA described so far were found to be differentially expressed between CS-COPD and BS-COPD in our study. Moreover, there is a paucity of data regarding the effects of BS on miRNA expression.[14]

Regarding the TFs related to the differentially expressed miRNAs, our analysis identified PPARG, YY1 and CREB1 as those with the highest interactions with other genes. Interestingly, alveolar hypoxia has been shown to lead to selective phosphorylation and activation of CREB1 in the lungs, suggesting an important role of this TF in the pulmonary response to decreased oxygen levels.[15] With respect to the top predicted kinases of the inferred upstream regulatory network, GSK3β was enriched more than the rest. Glycogen synthase kinase-3β (GSK3β) is regulated by oxidative stress and may play a key role in oxidant-mediated signaling during COPD inflammation. Moreover, a recent miRNAs study developed in bronchial biopsies of COPD patients pointed to GSK3β as potential regulator of chronic mucus hypersecretion.[16] Remarkably, BS exposure has been related to cough and mucus overproduction,[17] and BS-COPD patients have more cough and phlegm symptoms than CS-COPD subjects.[18]

Several limitations in our design should be kept in mind, which precludes making solid conclusions. These include that our study lacks of a replication cohort, being a hypothetical work based on in silico data pending of confirmation with functional studies. In addition, the sample size of our cohort was relatively small ($n = 30$) and we did not include a group of control subjects without COPD. Nevertheless, the high number of miRNAs studied support our findings, which may be useful in order to set specific therapeutic targets or prognostic indicators for future investigations on COPD related to BS exposure.

The results of our study show that the expression of circulating miRNAs is different in CS- and BS-COPD patients. Since differentially expressed miRNAs in BS-COPD relate to genes and pathways involved in hypoxemia and mucus hypersecretion, we hypothesize that these mechanisms are more relevant in these patients.

Authors' Contributions

Study concept and design: JO; Data acquisition: RSS, JO; Data analysis: JO, RDP; Data interpretation: JO, RDP, HDH, AA; Funding acquisition: JO; Investigation: JO, RDP; Methodology: JO, RDP;

Supervision: JO; Writing – original draft: JO; Writing – review & editing: JO, RDP, HDH, RSS, AA.

Statement of Ethics

This research complies with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Funding Sources

Funding support for this study was provided by the Chilean National Science and Technology Fund (CONICYT), FONDECYT Project N° 11150022 (JO).

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

Authors thank participants in the study for their willingness to contribute to medical research, Ms. Viviana Parra, Ms. Hanuxa Celedón and Mr. Hans Jürgen Kürsch for their technical assistance and the American Thoracic Society (ATS) Methods in Epidemiologic, Clinical and Operations Research (MECOR) Program for its support with the study's concept.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.arbres.2021.03.020.

References

1. Løkke A, Lange P, Scharling H, Fabricius P, Vestbo J. Developing COPD: a 25 year follow up study of the general population. Thorax. 2006;61:935–9.
2. Olloquequi J, Montes JF, Prats A, Rodríguez E, Montero MA, García-Valero J, et al. Significant increase of CD57+ cells in pulmonary lymphoid follicles of COPD patients. Eur Respir J. 2011;37:289–98.
3. Capistrano SJ, van Reyk D, Chen H, Oliver BG. Evidence of biomass smoke exposure as a causative factor for the development of COPD. Toxics. 2017;5:36.
4. Torres-Duque CA, García-Rodriguez MC, González-García M. Enfermedad pulmonar obstructiva crónica por humo de leña: ¿un fenotipo diferente o una entidad distinta? Arch Bronconeumol. 2016;52:425–31.
5. Halpin DMG, Criner GJ, Papi A, Singh D, Anzueto A, Martinez FJ, et al. Global initiative for the diagnosis, management, and prevention of chronic obstructive lung disease. Am J Respir Crit Care Med. 2021;203:24–36.
6. Tan WC, Sin DD, Bourbeau J, Hernandez P, Chapman KR, Cowie R, et al. Characteristics of COPD in never-smokers and ever-smokers in the general population: results from the CanCOLD study. Thorax. 2015;70:822–9.
7. Olloquequi J, Jaime S, Parra V, Cornejo-Córdova E, Valdivia G, Agustí À, et al. Comparative analysis of COPD associated with tobacco smoking, biomass smoke exposure or both. Respir Res. 2018;19:13.
8. Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 2013;41:W169–73.
9. Clarke DJB, Kuleshov MV, Schilder BM, Torre D, Duffy ME, Keenan AB, et al. EXpression2Kinases (X2K) Web: linking expression signatures to upstream cell signaling networks. Nucleic Acids Res. 2018;46:W171–9.
10. Hassan F, Nuovo GJ, Crawford M, Boyaka PN, Kirkby S, Nana-Sinkam SP, et al. MiR-101 and miR-144 regulate the expression of the CFTR chloride channel in the lung. PLoS ONE. 2012;7:e50837.
11. Mizuno S, Bogaard HJ, Gomez-Arroyo J, Alhussaini A, Kraskauskas D, Cool CD, et al. MicroRNA-199a-5p is associated with hypoxia-inducible factor-1α expression in lungs from patients with COPD. Chest. 2012;142:663–72.
12. Shen W, Liu J, Zhao G, Fan M, Song G, Zhang Y, et al. Repression of toll-like receptor-4 by microRNA-149-3p is associated with smoking-related COPD. Int J COPD. 2017;12:705–15.
13. Savarimuthu Francis SM, Davidson MR, Tan ME, Wright CM, Clarke BE, Duhig EE, et al. MicroRNA-34c is associated with emphysema severity and modulates SERPINE1 expression. BMC Genomics. 2014:15.
14. Velasco-Torres Y, López VR, Pérez-Bautista O, Buendía-Roldan I, Ramírez-Venegas A, Pérez-Ramos J, et al. MiR-34a in serum is involved in mild-to-moderate COPD in women exposed to biomass smoke. BMC Pulmon Med. 2019;19:227.
15. Leonard MO, Howell K, Madden SF, Costello CM, Higgins DG, Taylor CT, et al. Hypoxia selectively activates the CREB family of transcription factors in the In vivo lung. Am J Respir Crit Care Med. 2008;178:977–83.
16. Tasena H, Faiz A, Timens W, Noordhoek J, Hylkema MN, Gosens R, et al. MicroRNA–mRNA regulatory networks underlying chronic mucus hypersecretion in COPD. Eur Respir J. 2018;52:1701556.
17. KC R, Shukla SD, Gautam SS, Hansbro PM, O'Toole RF. The role of environmental exposure to non-cigarette smoke in lung disease. Clin Transl Med. 2018;7:39.
18. Pérez-Padilla R, Ramirez-Venegas A, Sansores-Martinez R. Clinical characteristics of patients with biomass smoke-associated COPD and chronic bronchitis, 2004–2014. Chron Obstruct Pulmon Dis: J COPD Found. 2014;1:23–32.

Roberto Díaz-Peña[a,b], Rafael S. Silva[c], Howard Dean Hosgood III[d], Àlvar Agustí[e], Jordi Olloquequi[a,*]

[a] *Laboratory of Cellular and Molecular Pathology, Facultad de Ciencias de la Salud, Instituto de Ciencias Biomédicas, Universidad Autónoma de Chile, Talca, Chile*
[b] *Liquid Biopsy Analysis Unit, Translational Medical Oncology (Oncomet), Health Research Institute of Santiago (IDIS), 15706 Santiago de Compostela, Spain*
[c] *Unidad Respiratorio, Centro de Diagnóstico Terapéutico, Hospital Regional de Talca, Talca, Chile*
[d] *Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, NY, USA*
[e] *Respiratory Institute, Hospital Clínic, Institut d'Investigacions Biomédiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona. Barcelona, CIBER Enfermedades Respiratorias (CIBERES), Spain*

* Corresponding author.
E-mail address: jolloquequig@uautonoma.cl (J. Olloquequi).