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Regular paper

Identification of AHNAK expression associated with the pathogenesis of chronic obstructive pulmonary disease by bioinformatic analysis

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Background: Chronic obstructive pulmonary disease (COPD) was a risk factor for lung cancer tumorigenesis. This study aimed to discover novel diagnostic biomarkers for COPD patients and determine their underlying pathogenetic mechanisms. Materials and methods: Differentially expressed genes (DEGs) in COPD samples and normal controls were analyzed and utilized to construct a network associated with a high risk for COPD occurrence. Enrichment analysis was applied on the strength of Gene Ontology (GO) annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The RT-qPCR analysis was performed to determine 10 hub genes in COPD. ELISA assay was utilized to measure IL-1β, IL-6, and IL-10 levels. Spearman's correlation analysis was conducted to detect the correlation between inflammatory cytokines and AHNAK expression. Cell proliferation and apoptosis were evaluated by CCK-8 and flow cytometry assays. Results: AHNAK was significantly increased in COPD serum samples compared with non-COPD smokers and strongly correlated with inflammation. AHNAK level could also discriminate COPD from non-COPD with high accuracy. Conclusion: AHNAK may be a feasible biomarker playing crucial functions in the diagnosis and progression of COPD.

Keywords: AHNAK, chronic obstructive pulmonary disease, differentially expressed genes, enrichment analysis, PPI network

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Abbreviations: COPD, Chronic obstructive pulmonary disease; DEGs, Dif_ferentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclo_pedia of Genes and Genomes; PPI, protein-protein interaction

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) was a classic chronic respiratory disease with a global average prevalence of 13.1% (Blanco *et al*., 2019). In 2015, about 3.2 million people died of COPD (Jolliffe *et al*., 2019); by 2030, COPD is expected to be the fourth most common cause of death and the fifth most common cause of disability worldwide (Barnes, 2007). Its main features included persistent airflow limitation and recurrent airway inflammation, including emphysema, parenchymal emphysema destruction, and mucus overproduction due to capillary bronchitis (Hogg *et al*., 2009). However, the molecular pathogenesis of COPD remains unclear, and effective prevention and treatment methods were lacking. Therefore, further research on the molecular mechanisms of COPD was needed to identify new drug targets.

Patients with COPD frequently presented with abnormal lung inflammation characterized by increased numbers of inflammatory cells (neutrophils, macrophages, and t-lymphocytes) and the release of multiple inflammatory mediators (lipids, chemokines, cytokines, and growth factors) (Guiedem *et al*., 2018; Barnes, 2016). The main cause of COPD is exposure to noxious gases or particles, with smoking being one of the major risk factors (Salvi, 2014). The increase in inflammatory response cells and subsequent release of mediators further amplified the normal inflammatory response to smoking in COPD disease (Barnes, 2017). Up to now, nearly 1 billion people worldwide were smokers (Propper, 2020). However, only a minority of smokers eventually developed COPD, and the exact molecular and cellular pathogenesis of this complex process was not fully understood.

In this study, we used differential analysis from the NCBI Gene Expression Omnibus (GEO) database to screen for AHNAK, a critical gene that was highly expressed in COPD. Furthermore, bioinformatics analysis was utilized to explore its potential pathogenesis. Collectively, these results suggested that AHNAK may be a useful biomarker for COPD.

MATERIALS AND METHODS

Subject enrollment

68 healthy non-COPD smokers who underwent physical examination, 79 stable-COPD patients, and 83 acute exacerbation COPD (AECOPD) patients admitted to Fujian Geriatric Hospital between August 2017 and May 2020 were enrolled in our study. There was no significant difference in gender and age between the two groups (P>0.05). COPD was diagnosed according to the standard of the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The specific manifestations were: pulmonary function first-second force exhalation volume (FEV1) to force spirometry (FVC) ratio less than 0.7 after an inhaled bronchodilator (salbutamol 200 ug), combined with clinical symptoms and signs, excluding irreversible obstructive pulmonary ventilation dysfunction caused by other factors. Exclusion criteria: (1) cases with bronchial asthma, bronchiectasis, interstitial pneumonia, lung cancer, and other definite severe lung diseases were excluded from this study; (2) cardiovascular diseases, metabolic diseases, rheumatic immune diseases, and acute and chronic inflammatory diseases in

Table 1. Clinical information on COPD patients and healthy controls

	Control (n=68)	Stable COPD (n=79)	acute exacerbation COPD (n=83)	*P* value
Age (years)	65.11±7.83	64.84±6.98	65.25±7.92	0.9412
Gender (Male/Female)	38/30	46/33	51/32	0.7835
BMI (kg/m2)	21.62±3.51	21.75±2.99	21.06±2.37	0.2914
FEV1/FVC (%)	83.59±9.22	60.33±6.41	53.47±7.25	<0.0001
CRP (mg/L)	5.19±1.67	25.71±13.36	51.28±27.49	<0.0001
Smoking history(Yes/No)	45/23	75/4	80/3	<0.0001

other parts of the body were excluded; (3) those who had been treated with antibiotics, systemic corticosteroid therapy, or immunosuppressive drugs within 3 months.

All included subjects were drawn 10 ml of venous blood after 8 h fasting, and all samples were sent to the laboratory department for centrifugation. The upper serum layer was taken, and the samples were stored at –80°C until use. All the subjects' information was shown in Table 1. This study was approved by the Ethics Committee of Fujian Geriatric Hospital and followed the Declaration of Helsinki. All participants were informed of the study plan and signed a written informed consent form.

Data download and differentially expressed genes (DEGs) analysis

We used the 'chronic obstructive pulmonary disease' search term as the keyword and limited the search scope to 'expression profile by array' to search the GEO database. In our study, our dataset included 10 samples from COPD patients and 10 control samples. All the data were downloaded and analyzed by R and GEOquery. After analysis, 122 up-regulated DEGs were found in COPD, which was used for subsequent analysis.

Gene Ontology (GO) annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

GO annotations and the Database conducted KEGG analysis of the integrated DEGs in COPD for Annotation, Visualization, and Integrated Discovery (DAVID; v 6.7).

Identification of protein-protein interaction (PPI) network

A hub gene was considered a gene with an absolute value of clinical trait relatio0.8ip of more than 0.2 and an absolute value of Spearman's correlation value of more than 0.8. The interaction of the hub gene and the DEGs were deemed to be key genes.

Real-time quantitative polymerase chain reaction analysis

Total RNAs were extracted from serum samples and cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Then, reverse transcription was conducted to synthesize cDNA from RNA using a PrimeScript RT reagent kit (Takara, Japan). Finally, RT-qPCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Beijing, China) using SYBR Premix EX Taq reagent (Takara, Japan) following the protocol. U6 functioned as an internal control, and data were analyzed relative to GAPDH based on the $2^{-\Delta\Delta CT}$ aquation. The primers sequences were as follows: AHNAK forward, 5'-AGCGTCTGTAGCTTCCTTGT-3', and reverse 5'-GGCAGCCTCAGTCGTGTATT-3'; GAPDH forward, 5'-TGGCACCGTCAAGGCTGAGA-3', and reverse, 5'-TGGTGAAGACGCCAGTGGACTC-3'.

ELISA assay

IL-1β and IL-6 levels in serum samples were determined by corresponding ELISA kits obtained from Solarbio Life Sciences (Beijing, China). The detailed infor-

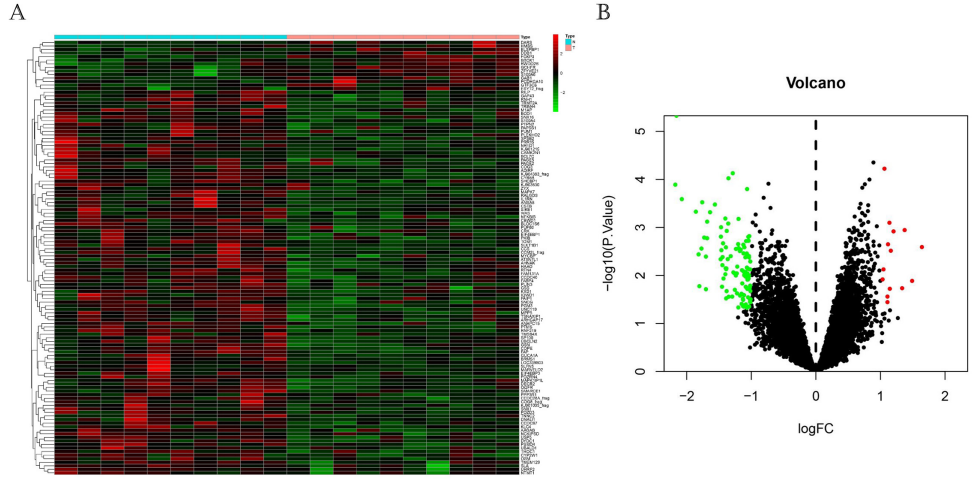

Figure 1. DEGs in COPD.
(**A**) Heatmap results of DEGs in COPD samples and healthy controls. (**B**) Volcano result of DEGs in COPD. COPD, chronic obstructive pulmonary disease; DEG, differentially expressed genes.

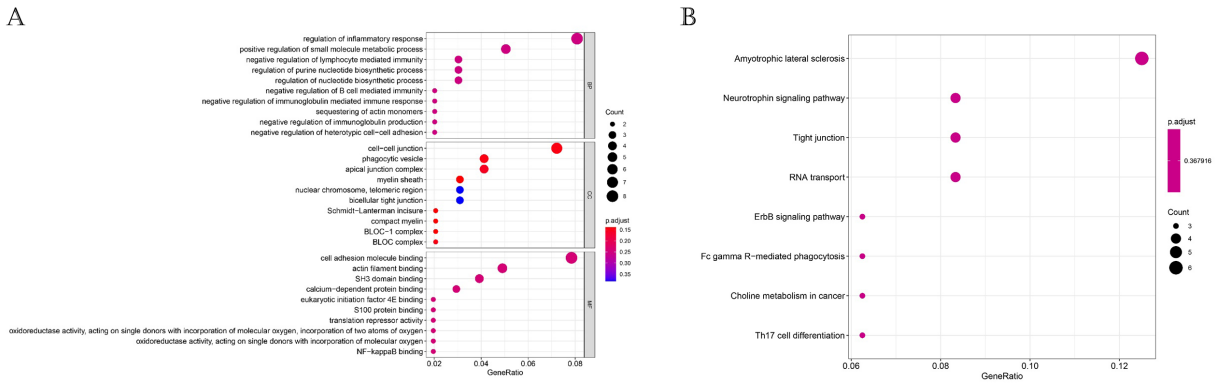

Figure 2. The GO of BP, CC and MF, and KEGG pathways after GEOs analysis.
(**A**) GO results. (**B**) KEGG results. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto encyclopedia of genes and genomes; DEG, differentially expressed genes.

mation of all ELISA kits was as follows: IL-1β (SEKH-0002), IL-6 (SEKH-0013).

Statistical analysis

GraphPad Prism 6.0 and R version 3.5.3 were applied to analyze the data. All the data were presented as mean ± standard deviation (S.D.). Differences between the two groups were compared and analyzed using Student's *t*-test followed by Tukey's posthoc test. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

DEGs of COPD

122 up-regulated genes were identified in our study and presented as a Volcano map (Fig. 1A). Figure 1B displayed the heatmaps of the 122 DEGs in our dataset.

GO functional analysis and pathway-enrichment KEGG analysis of DEGs

Three functional groups of GO analysis consisted of biological processes (BP), cell composition (CC), and molecular function (MF). As shown in Fig. 2A, with the BP results, the up-regulated DEGs were enriched in the categories of regulation of inflammatory response, positive regulation of the small molecular, metabolic process, negative regulation of lymphocyte-mediated immunity, regulation of purine nucleotide biosynthetic process, and regulation of nucleotide biosynthetic process. As for the CC functional groups, the up-regulated DEGs were concentrated at the cell-cell junction, phagocytic vesicle, apical junction complex, myelin sheath, nuclear chromosome, telomeric region, and bicellular tight junction. Finally, with the MF groups, the up-regulated DEGs were enriched at cell adhesion molecule binding, actin filament binding, SH3 domain binding, and calcium-dependent protein binding.

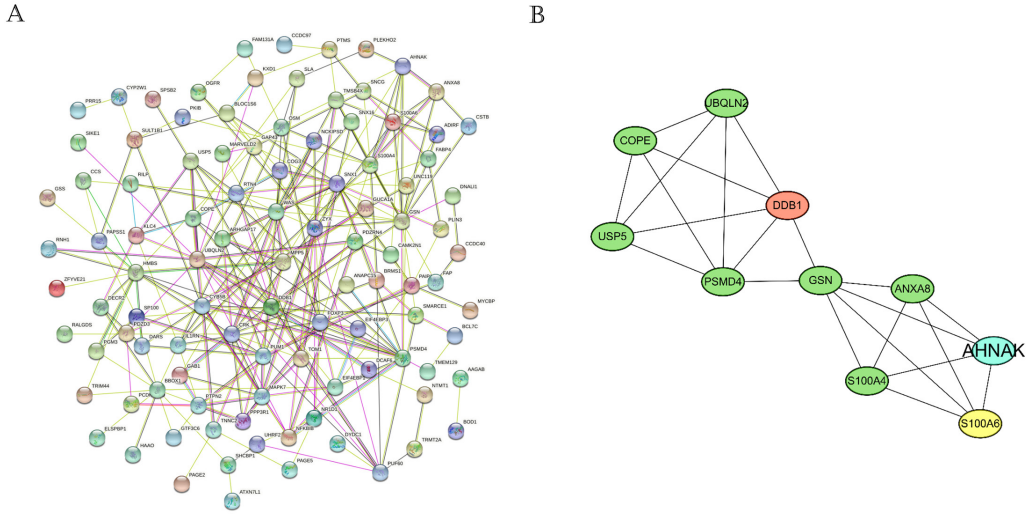

Figure 3. PPI network of DEGs.
(**A**) PPI network results of DEGs. (**B**) The 10 top hub genes in the PPI network. PPI, protein-protein interaction; DEG, differentially expressed genes.

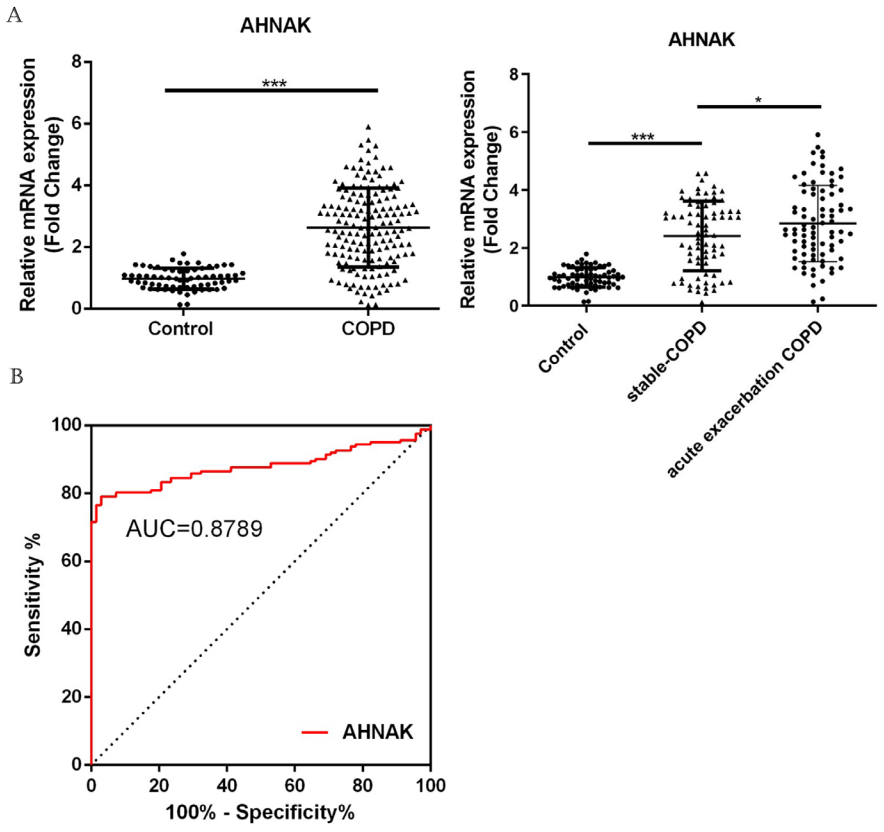

Figure 4. The AHNAK expression in COPD.
(**A**) Expression of AHNAK in COPD serum samples. (**B**) ROC analysis of AHNAK in COPD. COPD, chronic obstructive pulmonary disease

In addition, as shown in Fig. 2B, the KEGG pathways analysis of the DEGs in COPD unveiled that they were mostly concentrated in the categories of amyotrophic lateral sclerosis, neurotrophin signaling pathway, tight junction, and RNA transport.

PPI results of DEGs

The PPI network for integrated DEGs was shown in Fig. 3A and Fig. 3B. The 10 hub genes that extensively interacted with other genes in COPD were AHNAK, S100A6, S100A4, ANXA8, GSN, DDB1, PSMD4, USP5, COPE, and UBQLN2.

Up-regulation in COPD patients

Furthermore, patients were divided into stable COPD and acute exacerbation COPD groups, and the clinical information of the patients and healthy controls were shown in Table 1. Next, after collecting COPD and non-COPD serum samples, we found that AHNAK expression was significantly increased in COPD patients (Fig. 4A, $P<0.001$); moreover, compared with stable-COPD patients, AHNAK was up-regulated in serum samples of acute exacerbation COPD patients ($P<0.05$). the area under the curve (AUC) of AHNAK concerning discriminating COPD from non-COPD smokers was 0.8789 (Fig. 4B, cut-off value >1.454, sensitivity 75.95, specificity 92.65).

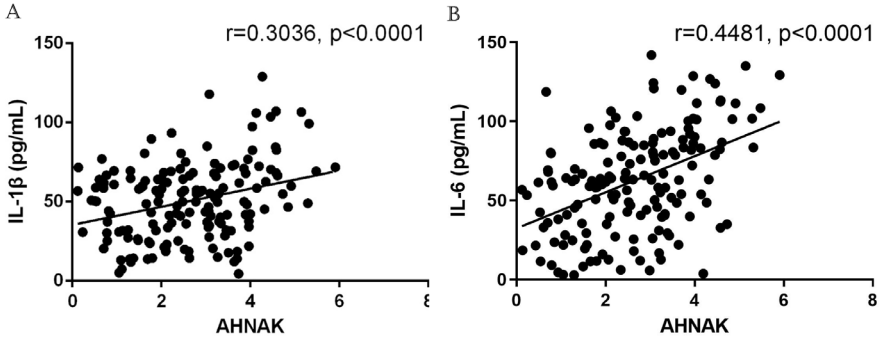

Figure 5. Correlation between AHNANK expression and inflammatory cytokines.
Correlation with AHNAK expression and IL-1β (**A**) and IL-6 (**B**) levels in COPD.

Association between AHNAK and inflammation in COPD

To determine the correlation between AHNAK and inflammation in COPD, we measured inflammatory cytokines levels and performed Spearman's correlation analysis accordingly. As shown in Figs 5A and B, AHNAK was strongly correlated with IL-1β and IL-6 levels in COPD.

DISCUSSION

High-throughput sequencing made rapid progress in the past decade, and advances in bioinformatics deepened our understanding of disease mechanisms and facilitated drug target development, including COPD (Scherer *et al*., 2017; Dorn *et al*., 2014; Regan *et al*., 2019; Matsson *et al*., 2016). Several studies have identified hundreds of DEGs based on mRNA gene expression profiles in COPD (Huang *et al*., 2019; Zhu *et al*., 2020; Morrow *et al*., 2017). A hub gene was a gene playing a critical role in biological processes, and the regulation of other genes in a related pathway was often influenced by this gene. Therefore, the hub gene was often an important target and a hot spot for research. In this study, we screened 122 up-regulated DEGs and analyzed the expression profiles using WGCNA. Among them, we identified 10 hub genes, of which AHNAK was the shared central gene and DEG. These results suggested that AHNAK may play an important role in the progression of COPD.

AHNAK was a large structural scaffold protein with 700 kDa, which was implicated in blood-brain barrier formation, cardiac calcium channel regulation, and tumor metastasis (Alvarez *et al*., 2010; Gentil *et al*., 2005; Haase *et al*., 2005; Sohn *et al*., 2018). What we all know is that AHNAK has been proved to be implicated in various diseases, functioning as a potential biomarker for disease detection or predicting the prognosis. For instance, Zhao and others (Zhao *et al*., 2017) reported that AHNAK was down-regulated in glioma cell lines, inhibiting glioma tumor growth and associated with poor prognosis. Lee and others (Lee *et al*., 2018) revealed that AHNAK was a crucial candidate for bladder urothelial carcinoma diagnosis based on in-depth proteomics. Dumitru and others (Dumitru *et al*., 2013) discovered that AHNAK was dysregulated in laryngeal carcinoma and significantly correlated with poor clinical outcomes in laryngeal carcinoma patients. Sudo and others (Sudo *et al*., 2014) proposed that AHNAK was highly expressed in several mesothelioma cell lines, playing a key role in regulating mesothelioma cell migration and invasion. Zhang and others (Zhang *et al*., 2019) suggested that high AHNAK expression was strongly correlated with short disease-free survival and poor prognosis, exerting as an oncogene in pancreatic ductal adenocarcinoma. More importantly, Nedeljkovic and others (Nedeljkovic *et al*., 2018) implied that AHNAK abnormal expression could be novel candidate variants in COPD. The data we obtained from clinical samples also confirmed the specific high expression of AHNAK in COPD patients, which was consistent with the comprehensive bioinformatic analysis. Therefore, this study provided new insights into the complex pathogenesis of COPD.

In order to explore the mechanisms involved in disease progression, we performed a PPI dynamic analysis. Based on KEGG and GO's enrichment results, we identified 38 pathways enriched in the dynamic PPI network. The top 12 processes were inflammation response, small molecular, metabolic process, cell-cell junction, phagocytic vesicle, apical junction complex, cell adhesion molecular binding, actin filament binding, SH3 domain binding, amyotrophic lateral sclerosis, neurotrophin, tight junction, and RNA transport. Thus, we verified the relationship between AHNAK and inflammatory factors in COPD, and the outcome demonstrated that AHNAK was closely associated with IL-1β and IL-6 levels in COPD patients. These results support that AHNAK represents a sensitive biomarker for the diagnosis of COPD.

Nevertheless, this study maintains some limitations. First, the specific mechanism of action of AHNAK in COPD requires further in vivo studies. In addition, the sample size in this study was relatively small, and the DEGs between the different stages of COPD could not be determined due to the sample size. In future studies, we will use a larger sample size consisting of samples from patients with different stages of COPD to avoid accidental errors. Finally, long-term follow-up investigations of COPD patients are recommended to determine the impact of abnormal AHNAK expression on COPD prognosis.

CONCLUSION

AHNAK was significantly elevated in COPD, functioning as a potential biomarker for COPD detection and treatment.

Declarations

Acknowledgment. This study protocol was approved by the Ethics Committee of Fujian Geriatric Hospital. Written informed consent was provided before the study. No data were used to support this study. The authors declare that they have no competing interests.

Ethics approval. The study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights. The study protocol was approved by the Ethics Committee of Fujian Geriatric Hospital.

REFERENCES

Alvarez JL, Petzhold D, Pankonien I, Behlke J, Kouno M, Vassort G, Morano I, Haase H (2010) Ahnak1 modulates L-type Ca (2+) channel inactivation of rodent cardiomyocytes. *Pflugers Arch* **460**: 719–730. https://doi.org/10.1007/s00424-010-0853-x

Barnes PJ (2007) Chronic obstructive pulmonary disease: a growing but neglected global epidemic. *PLoS Med* **4**: e112. https://doi.org/10.1371/journal.pmed.0040112

Barnes PJ (2016) Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol* **138**: 16–27. https://doi.org/10.1016/j.jaci.2016.05.011

Barnes PJ (2017) Cellular and molecular mechanisms of asthma and COPD. *Clin Sci (Lond)* **131**: 1541–1558. https://doi.org/10.1042/CS20160487

Blanco I, Diego I, Bueno P, Casas-Maldonado F, Miravitlles M (2019) Geographic distribution of COPD prevalence in the world displayed by Geographic Information System maps. *Eur Respir J* **54**: 1900610. https://doi.org/10.1183/13993003.00610-2019

Dorn C, Grunert M, Sperling SR (2014) Application of high-throughput sequencing for studying genomic variations in congenital heart disease. *Brief Funt Genomics* **13**: 51–65. https://doi.org/10.1093/bfgp/elt040

Dumitru CA, Bankfalvi A, Gu X, Zeidler R, Brandau S, Lang S (2013) AHNAK and inflammatory markers predict poor survival in laryngeal carcinoma. *PLoS One* **8**: e56420. https://doi.org/10.1371/journal.pone.0056420

Gentil BJ, Benaud C, Delphin C, Remy C, Berezowski V, Cecchelli R, Feraud O, Vittet D, Baudier J (2005) Specific AHNAK expression in brain endothelial cells with barrier properties. *J Cell Physiol* **203**: 362–371. https://doi.org/10.1002/jcp.20232

Guiedem E, Ikomey GM, Nkenfou C, Walter PE, Mesembe M, Chegou NN, Jacobs GB, Okomo Assoumou MC (2018) Chronic obstructive pulmonary disease (COPD): neutrophils, macrophages

and lymphocytes in patients with anterior tuberculosis compared to tobacco-related COPD. *BMC Res Notes* **11**: 192. https://doi.org/10.1186/s13104-018-3309-6

Haase H, Alvarez J, Petzhold D, Doller A, Behlke J, Erdmann J, Hetzer R, Regitz-Zagrosek V, Vassort G, Morano I (2005) Ahnak is critical for cardiac Ca (V)1.2 calcium channel function and its beta-adrenergic regulation. *FASEB J* **19**: 1969–1977. https://doi.org/10.1096/fj.05-3997com

Hogg JC, Timens W (2009) The pathology of chronic obstructive pulmonary disease. *Annu Rev Pathol* **4**: 435–459. https://doi.org/10.1146/annurev.pathol.4.110807.092145

Huang XW, Li YW, Guo XR, Zhu Z, Kong X, Yu F, Wang Q (2019) Identification of differentially expressed genes and signaling pathways in chronic obstructive pulmonary disease *via* bioinformatic analysis. *FEBS Open Bio* **9**: 1880–1899. https://doi.org/10.1002/2211-5463.12719

Jolliffe DA, Greenberg L, Hooper RL, Mathyssen C, Rafiq R, de Jongh RT, Camargo CA, Griffiths CJ, Janssens W, Martineau AR (2019) Vitamin D to prevent exacerbations of COPD: systematic review and meta-analysis of individual participant data from randomised controlled trials. *Thorax* **74**: 337–345. https://doi.org/10.1136/thoraxjnl-2018-212092

Lee H, Kim K, Woo J, Park J, Kim H, Lee KE, Kim H, Kim Y, Moon KC, Kim JY, Park IA, Shim BB, Moon JH, Han D, Ryu HS (2018) Quantitative proteomic analysis identifies AHNAK (neuroblast differentiation-associated protein AHNAK) as a novel candidate biomarker for bladder urothelial carcinoma diagnosis by liquid-based cytology. *Mol Cell Proteomics* **17**: 1788–1802. https://doi.org/10.1074/mcp.RA118.000562

Matsson H, Söderhäll C, Einarsdottir E, Lamontagne M, Gudmundsson S, Backman H, Lindberg A, Rönmark E, Kere J, Sin D, Postma DS, Boss, BosLundbäck B, Klar J (2016) Targeted high-throughput sequencing of candidate genes for chronic obstructive pulmonary disease. *BMC Pulm Med* **16**: 146. https://doi.org/10.1186/s12890-016-0309-y

Morrow JD, Zhou XB, Lao TT, Jiang Z, DeMeo DL, Cho MH, Qiu W, Cloonan S, Pinto-Plata V, Celli B, Marchetti N, Criner GJ, Bueno R, Washko GR, Glass K, Quackenbush J, Choi AM, Silverman EK, Hersh CP (2017) Functional interactors of three genome-wide association study genes are differentially expressed in severe chronic obstructive pulmonary disease lung tissue. *Sci Rep* **7**: 44232. https://doi.org/10.1038/srep44232

Nedeljkovic I, Terzikhan N, Vonk JM, van der Plaat DA, Lahousse L, van Diemen CC, Hobbs BD, Qiao D, Cho MH, Brusselle GG, Postma DS, Boezen HM, van Duijn CM, Amin N (2018) A Genome-wide linkage study for chronic obstructive pulmonary disease in a Dutch genetic isolate identifies novel rare candidate variants. *Front Genet* **9**: 133. https://doi.org/10.3389/fgene.2018.00133

Propper RE (2020) Does cigarette smoking protect against SARS-CoV-2 infection? *Nicotine Tob Res* **22**: 1666. https://doi.org/10.1093/ntr/ntaa073

Regan EA, Hersh CP, Castaldi PJ, DeMeo DL, Silverman EK, Crapo JD, Bowler RP (2019) Omics and the search for blood biomarkers in chronic obstructive pulmonary disease. Insights from COPDGene. *Am J Respir Cell Mol Biol* **61**: 143–149. https://doi.org/10.1165/rcmb.2018-0245PS

Salvi S (2014) Tobacco smoking and environmental risk factors for chronic obstructive pulmonary disease. *Clin Chest Med* **35**: 17–27. https://doi.org/10.1016/j.ccm.2013.09.011

Scherer F, Kurtz DM, Diehn M, Alizadeh AA (2017) High-throughput sequencing for noninvasive disease detection in hematologic malignancies. *Blood* **130**: 440–452. https://doi.org/10.1182/blood-2017-03-735639

Sohn M, Shin S, Yoo JY, Goh Y, Lee IH, Bae YS (2018) Ahnak promotes tumor metastasis through transforming growth factor-β-mediated epithelial-mesenchymal transition. *Sci Rep* **8**: 14379. https://doi.org/10.1038/s41598-018-32796-2

Sudo H, Tsuji AB, Sugyo A, Abe M, Hino O, Saga T (2014) AHNAK is highly expressed and plays a key role in cell migration and invasion in mesothelioma. *Int J Oncol* **44**: 530–538. https://doi.org/10.3892/ijo.2013.2183

Zhang ZW, Liu XD, Huang R, Liu XG, Liang ZY, Liu TH (2019) Up-regulation of nucleoprotein AHNAK is associated with poor outcome of pancreatic ductal adenocarcinoma prognosis *via* mediating epithelial-mesenchymal transition. *J Cancer* **10**: 3860–3870. https://doi.org/10.7150/jca.31291

Zhao ZJ, Xiao SH, Yuan XR, Yuan J, Zhang C, Li H, Su J, Wang X, Liu Q (2017) AHNAK as a prognosis factor suppresses the tumor progression in glioma. *J Cancer* **8**: 2924–2932. https://doi.org/10.7150/jca.20277

Zhu YZ, Zhou AY, Li QY (2020) Whole transcriptome analyis of human lung tissue to identify COPD-associated genes. *Genomics* **112**: 3135–3141. https://doi.org/10.1016/j.ygeno.2020.05.025