Chronic mucus hypersecretion in COPD and asthma

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CHAPTER 3

MicroRNA-mRNA regulatory networks underlying chronic mucus hypersecretion in COPD

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ABSTRACT

Chronic mucus hypersecretion (CMH) is a common feature in COPD and associated with worse prognosis and quality of life. This study aimed to identify microRNA (miRNA)-mRNA regulatory networks underlying CMH.

miRNA and mRNA expression profiles in bronchial biopsies from 63 COPD patients were associated with CMH using linear regression. Potential mRNA targets of each CMH-associated miRNA were identified using Pearson correlations. GSEA and STRING analyses were used to identify key genes and pathways.

Twenty miRNAs and 539 mRNAs were differentially expressed with CMH in COPD. The expression of 10 miRNAs was significantly correlated with the expression of one or more mRNAs. Of these, miR-134-5p, miR146a-5p and the let-7 family had the highest representation of CMH-associated mRNAs among their negatively correlated predicted targets. KRAS and EDN1 were identified as key regulators of CMH and were negatively correlated predicted targets of miR-134-5p and the let-7a/d/f-5p, respectively. GSEA suggested involvement of MUC5AC-related genes and several other relevant gene sets in CMH. The lower expression of miR-134-5p was confirmed in primary airway fibroblasts from COPD patients with CMH.

We identified miR-134-5p, miR-146a-5p and let-7 family, along with their potential target genes including KRAS and EDN1, as potential key miRNA-mRNA networks regulating CMH in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a frequently occurring lung disease, associated with an abnormal inflammatory response to inhaled noxious particles and gases, including cigarette smoke. A substantial proportion of patients experience chronic cough with sputum production [1, 2] termed chronic bronchitis or chronic mucus hypersecretion (CMH) [3]. CMH in COPD is associated with lower quality of life, accelerated lung function decline, increased risk of exacerbations, and higher mortality [1, 2, 4]. Therefore, there is an urgent need for improved treatment of CMH in COPD patients. Unfortunately, our current understanding of the regulatory mechanisms that drive CMH is still limited.

Goblet cells within the airway epithelial layer together with mucous glands in the airway submucosa are responsible for the secretion of mucins, the principal components of mucus [3]. The most abundant gel-forming mucins found in human airways are MUC5AC and MUC5B [5], which are both increased in COPD [6]. Interestingly, recent *in vitro* studies suggest that fibroblasts play a role in the regulation of airway epithelial mucociliary differentiation and mucus production [7, 8].

microRNAs (miRNA) are small non-coding RNA molecules that target messenger RNA (mRNA), causing mRNA degradation or inhibition of protein translation [9]. Thus far, no studies have reported on differential miRNA expression in CMH, although several miRNAs have been implicated in the response to smoking and COPD [10], including miR-146a-5p [11]. The aim of this study was to identify key miRNA-mRNA interactions underlying CMH in bronchial biopsies from a well-defined COPD cohort.

METHODS

Patient characteristics

Baseline miRNA and mRNA expression was studied in bronchial biopsies from 63 COPD patients who participated in the Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease (GLUCOLD) study (clinicalTrials.gov NCT00158847). Details of the study including patient characteristics were previously described [12, 13]. Briefly, all patients had irreversible airflow limitation (post-bronchodilator forced expiratory volume in 1 second (FEV1) and FEV1/inspiratory vital capacity (IVC)<90% confidence interval (CI) of the predicted value) and chronic respiratory symptoms. All patients were stable, either current or ex-smokers, and not on corticosteroid therapy. The local medical ethics committee approved the study and all patients gave their written informed consent.

Definitions of chronic mucus hypersecretion (CMH)

In our dataset, clinical questionnaires were used, providing information comparable to the most commonly used definition of CMH: symptoms of cough and phlegm on most days for more than three months during at least two consecutive years [4]. Thus, CMH was defined based on patient responses to the question: A) "How often did you cough up sputum during the last three months?" Since a patient's response may vary over time, we decided to include another question B) "How often did you cough up sputum during the last week?", to cover both the longer time frame (3 months) and shorter, more recent time frame (1 week), henceforth referred to as definition A and B, respectively. A full description of the response options to these questions is presented in the online supplement. For each question, patients were divided in three groups: no CMH, mild CMH, and moderate/severe CMH.

microRNAs and mRNA expression profiling

The methods for mRNA and miRNA extraction from bronchial biopsies, for gene expression profiling using Affymetrix arrays, and for the RNA-sequencing are described in the online supplement.

Statistical analysis

mRNA and miRNA analyses on bronchial biopsies were performed using R software (v3.2.5). A linear regression model was used to identify miRNAs and mRNAs that were differentially expressed in patients with mild or with moderate/severe CMH compared to those with no CMH. The model was corrected for age, gender, smoking

history and RNA integrity number (RIN). Multiple testing correction was performed using Benjamini and Hochberg's method. Definition A was applied to acquire the primary lists of candidate miRNAs and mRNAs that were associated with CMH using a false discovery rate adjusted p-value (FDR) cut-off<0.25. Definition B was then applied to further strengthen the primary findings using a nominal p-value cut-off<0.05. Subsequently, the final lists consisted of candidate miRNAs and mRNAs that were associated with CMH according to both definitions. Other statistical tests were performed using GraphPad Prism (v6). Differences in patient characteristics were compared using ANOVA. The correlation of the two CMH definitions was assessed using Spearman's rank correlation coefficient. Mann-Whitney U test was performed to determine significant differences in immunohistochemistry markers between CMH status and miRNA expression *in vitro*. The methods for miRNA-mRNA co-expression network analysis, Gene Set Enrichment Analysis (GSEA), and STRING interaction network analysis are described in the online supplement.

microRNA expression in human primary airway epithelial cells and fibroblasts

The expression of candidate miRNAs was evaluated in air-liquid interface (ALI)-differentiated primary airway epithelial cells (PAECs) and primary airway fibroblasts (PAFs) obtained during lung transplantation procedures. PAECs were obtained from six stage IV COPD (table S1) explanted lungs and six non-COPD donor lungs as described before [14], of which the majority did not have CMH. PAFs were isolated from stage IV COPD patients (table S2) as previously described [15], of which eight had clinical CMH symptoms and eight had no clinical CMH symptoms. Cell culture procedures and RT-qPCR details are described in the online supplement.

RESULTS

Patient characteristics

Microarray data of sufficient quality was obtained from 63 patients for miRNA expression profiles and from 57 patients for mRNA expression profiles. Patient characteristics are shown in table 1. CMH definitions A and B were significantly correlated (r=0.651, p<0.0001, figure S1). There was no significant difference in smoking status, pack-years, age, BMI, or lung function among these three groups (table 1). See figure 1 for the study approach and flow diagram of the main findings.

Table 1. Patient characteristics

	CMH Definition A			CMH Definition B		
	No CMH (n=8)	Mild (n=22)	Moderate/ severe (n=33)	No CMH (n=8)	Mild (n=25)	Moderate/ severe (n=30)
Gender (male), n(%)	8(100.0)	18(81.8)	28(84.8)	8(100.0)	22(88.0)	24(80.0)
Current smokers, n(%)	4(50.0)	14(63.6)	23(69.7)	4(50.0)	17(68.0)	20(67.0)
Pack-years*	41.7 (23.6-50.3)	44.1 (31.9-54.4)	41.5 (35.9-53.5)	40.9 (24.4-51.2)	45.0 (31.2-55.8)	41.5 (36.5-53.5)
Age, years*	64 (57-69)	59 (53-64)	60 (57-66)	56 (50-60)	59 (55-63)	62 (57-69)
BMI, kg/m2*	25.8 (23.2-27.8)	24.2 (22.8-27.2)	24.0 (22.0-28.0)	25.1 (23.8-27.1)	24.4 (22.3-29.4)	24.4 (21.6-28.0)
FEV1, %predicted*	69.9 (48.5-71.8)	63.3 (58.5-67.4)	64.8 (58.2-69.8)	66.6 (61.4-70.5)	65.6 (58.5-71.9)	63.9 (56.3-67.2)
FEV1/FVC*	0.54 (0.42-0.60)	0.49 (0.44-0.54)	0.50 (0.44-0.56)	0.56 (0.49-0.63)	0.50 (0.44-0.56)	0.49 (0.420.54)
RIN*	2.6 (2.5-3.2)	2.7 (2.3-4.7)	2.6 (2.4-3.7)	2.7 (2.5-4.4)	2.7 (2.4-4.0)	2.6 (2.4-3.4)

*Data are presented as median (interquartile range); BMI is body mass index; FEV1 is forced expiratory volume in 1 second; FVC is forced vital capacity; RIN is RNA integrity number.

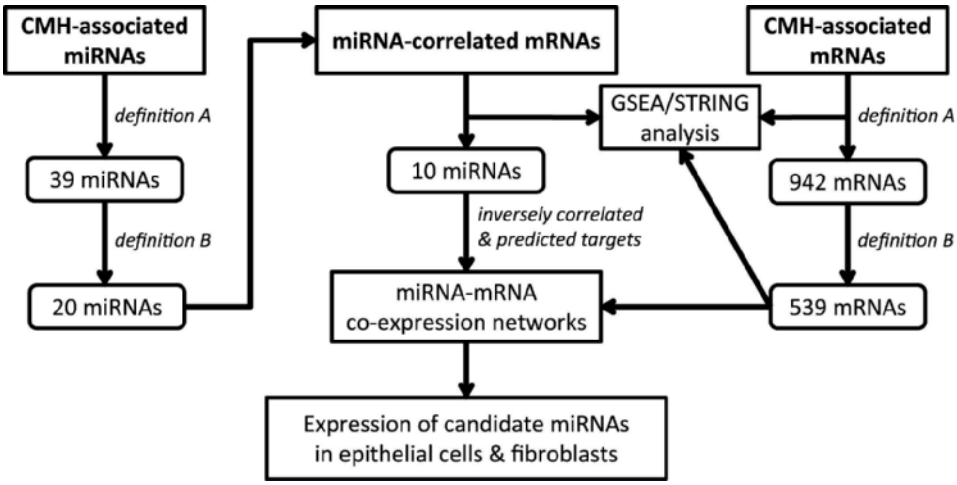


FIGURE 1. Flow diagram of the study approach. A linear regression model was used to identify chronic mucus hypersecretion (CMH) associated microRNAs (miRNAs) and CMH-associated mRNAs based on two CMH definitions. Pearson correlation was used to identify miRNA-correlated mRNAs. Gene set enrichment analysis (GSEA) was performed using gene sets of interest and ranked lists of miRNA-correlated and CMH-associated mRNAs. Interaction network analysis using STRING (search tool for the retrieval of interacting genes/proteins) was performed on the list of CMH-associated mRNAs correlated with at least one CMH-associated miRNA. The list of CMH-associated mRNAs and the lists of negatively correlated predicted targets of the candidate miRNAs were used to create miRNA–mRNA co-expression networks. Finally, the expression of candidate miRNAs was assessed in primary airway epithelial cells and primary airway fibroblasts.

CMH-associated miRNAs

To identify miRNAs associated with CMH, we compared expression profiles of 230 miRNAs in patients with 1) mild and no CMH and 2) moderate/severe and no CMH. According to definition A, three miRNAs (miR-34b-3p, miR-92b-3p and miR-449b-5p) were higher expressed with mild CMH, while miR-664a-5p was lower expressed. With moderate/severe CMH, we found 39 differentially expressed miRNAs, including the four that were associated with mild CMH (FDR<0.25, figure 2a and 2b). Twenty out of the 39 miRNAs were also associated with moderate/severe CMH and in the same direction according to definition B ($p<0.05$). Among these, miR-708-5p had the highest fold increase (2.06) and miR-134-5p had the strongest fold decrease (-2.15) (table S3). No miRNA was differentially expressed in mild CMH according to definition B.

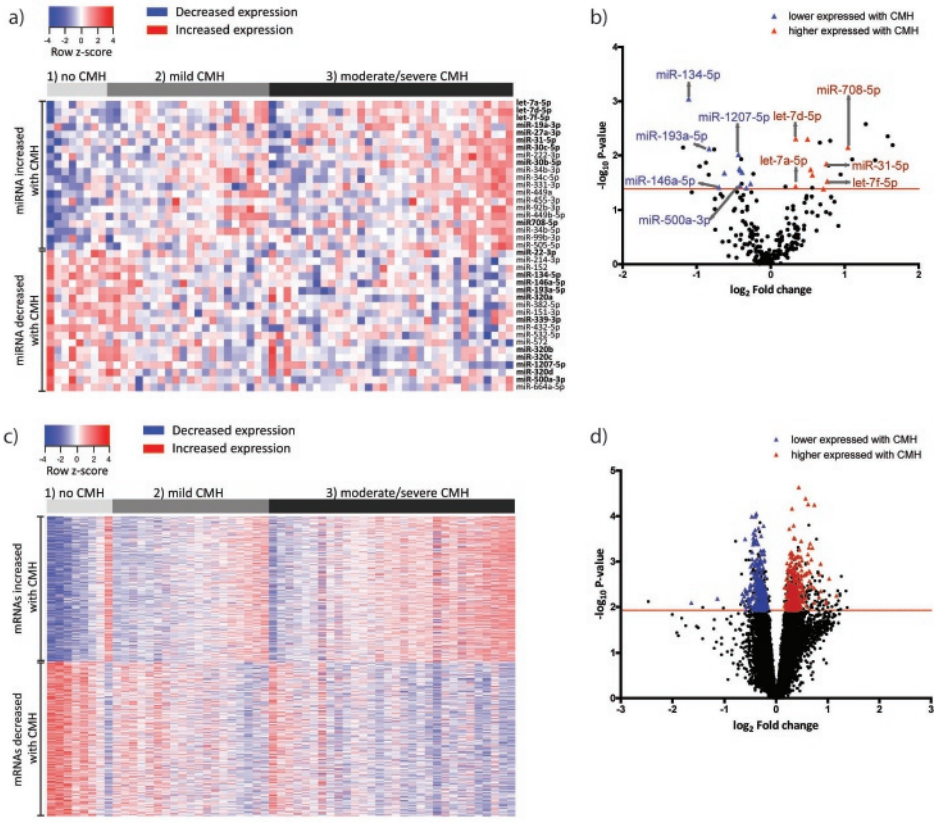


FIGURE 2. MicroRNAs (miRNAs) and mRNAs differentially expressed with chronic mucus hypersecretion (CMH). Patients were classified into three groups depending on their CMH status, as defined by two definitions, with miRNA profiles of patients with moderate/severe CMH being compared to those of patients with no CMH. Heat map [a] shows expression changes of 39 miRNAs in the patients with moderate/severe CMH (n=33) compared to those with no CMH (n=8) according to definition A. Bold labels indicate the 20 miRNAs whose expression was significant based on both definition A and definition B. Volcano plot [b] shows expression changes of 230 miRNAs in the patients with moderate/severe CMH compared to those with no CMH according to definition A. Heat map [c] shows expression changes of 942 mRNAs in the patients with severe CMH (n=30) compared to those with no CMH (n=8) according to definition A. Volcano plot [d] shows expression changes of 19793 mRNAs in the patients with severe CMH compared to those with no CMH according to definition A. Blue indicates significant miRNAs/mRNAs lower expressed with CMH while red represents significant miRNAs/mRNAs higher expressed with CMH. Triangles represent miRNAs/mRNAs of which differential expression was significant based on both definitions. A false-discovery rate (FDR) <0.25 was a cut-off for definition A and a nominal p-value <0.05 was a cut-off for definition B.

CMH-associated mRNAs

To identify mRNAs associated with CMH, we compared expression profiles of 19,793 mRNAs in patients with 1) mild and no CMH and 2) moderate/severe and no CMH. According to CMH definition A, no differences in mRNA expression were found with mild CMH. The expression of 942 mRNAs differed with moderate/severe (FDR<0.25, figure 2c and 2d). Further, 539 out of 942 mRNAs were also associated with CMH in the same direction according to definition B ($p<0.05$, figure 2c and 2d). Among these, 264 mRNAs were higher and 275 mRNAs were lower expressed with moderate/severe CMH (table S4). The top 20 significant mRNAs are shown in table 2.

To validate the findings from the microarray dataset, RNA-sequencing was performed on bronchial biopsies from a subset of the patients in GLUCOLD cohort, with moderate/severe CMH (n=21) and without CMH (n=5). GSEA was performed to assess if the CMH-associated genes in our microarray dataset were enriched among the CMH-associated genes in our RNA-sequencing dataset. Indeed, we found a significant enrichment (figure S2, table S7).

To ensure that the observed differences in miRNA and mRNA expression were not due to changes in cellular composition, we analysed available immunohistochemical data on the numbers of neutrophils, macrophages, eosinophils, CD3+/CD4+/CD8+ lymphocytes, mast cells, and epithelial cells in the bronchial biopsies previously described [13]. We observed that only CD8+ lymphocytes differed with CMH. When accounting for the CD8+ cell number in our linear regression model, all 20 CMH-associated miRNAs and 539 CMH-associated mRNAs remained significant.

Gene Set Enrichment Analysis (GSEA) of CMH-associated mRNAs

To identify pathways, biological processes, and molecular functions in which the CMH-associated mRNAs may be involved, GSEA was performed. The top 20 significant gene sets enriched among mRNAs differentially expressed with CMH according to both definitions are shown in table 3. Among these were gene sets related to cilium development and function, neurohormonal signalling, ion channel activities, and extracellular matrix (ECM) structure. The complete list of significant gene sets (FDR<0.01) is reported in the online supplement (table S5). Furthermore, we investigated whether our CMH-associated mRNAs are involved in the mechanisms relevant to MUC5AC expression. Since MUC5AC was not expressed above background levels in our microarrays, we built further on the findings of Wang et al. [16] who previously identified 73 MUC5AC-associated core genes which were higher expressed in small airway epithelium from individuals with high versus those with low MUC5AC gene expression. Using this list, we found a strong enrichment of MUC5AC-associated genes among the genes higher expressed with CMH according to both definition A (enrichment score (ES)=0.40, $p<0.001$) and B (ES=0.44, $p<0.001$) (figure S3).

Table 2. Top 20 significant mRNAs differently expressed with CMH and their statistics

Gene	Moderate/severe CMH vs No CMH (definition A)				Moderate/severe CMH vs No CMH (definition B)		
	t-value	FC	p-value	FDR	t-value	FC	p-value
AIG1	4.667	1.355	2.32E-05	0.225	2.548	1.213	1.40E-02
LRRC8B	4.498	1.487	4.10E-05	0.225	3.117	1.359	3.03E-03
ROD1	4.410	1.538	5.49E-05	0.225	3.515	1.462	9.43E-04
OSBPL3	4.402	1.671	5.64E-05	0.225	3.130	1.522	2.91E-03
RC3H1	4.345	1.238	6.82E-05	0.225	2.760	1.173	8.07E-03
RQCD1	4.082	1.264	1.61E-04	0.231	4.512	1.316	3.91E-05
LIAS	4.030	1.194	1.90E-04	0.231	2.254	1.124	2.86E-02
USP46	3.884	1.271	3.02E-04	0.231	2.608	1.194	1.20E-02
MTF1	3.873	1.289	3.13E-04	0.231	2.842	1.227	6.47E-03
LOC100133388	-4.265	-1.306	8.86E-05	0.225	-2.072	-1.173	4.34E-02
NKD1	-4.249	-1.312	9.36E-05	0.225	-2.552	-1.198	1.38E-02
FAM115A	-4.223	-1.386	1.02E-04	0.225	-2.518	-1.266	1.50E-02
C1QTNF1	-4.222	-1.328	1.02E-04	0.225	-3.233	-1.270	2.17E-03
RPL23AP64	-4.075	-1.183	1.65E-04	0.231	-2.915	-1.142	5.30E-03
WTIP	-4.039	-1.266	1.84E-04	0.231	-2.435	-1.167	1.85E-02
LIMS2	-4.010	-1.326	2.02E-04	0.231	-4.282	-1.342	8.38E-05
BTN2A3	-3.961	-1.355	2.37E-04	0.231	-2.889	-1.296	5.70E-03
PDGFB	-3.929	-1.321	2.62E-04	0.231	-3.030	-1.262	3.87E-03
C15orf60	-3.916	-1.223	2.73E-04	0.231	-2.902	-1.180	5.50E-03
EDN1	-3.884	-1.357	3.02E-04	0.231	-2.098	-1.207	4.10E-02

FC is fold change, FDR is false discovery rate.

Identification of miRNA-mRNA co-expression networks contributing to CMH in COPD

To identify mRNAs that are regulated by the CMH-associated miRNAs, we assessed positive and negative correlations between the expression of each miRNA and the mRNA expression profile in the matched biopsies (same patient). The expression levels of 10 out of 20 miRNAs, i.e., let-7a-5p, let-7d-5p, let-7f-5p, miR-31-5p, miR-708-5p, miR-134-5p, miR-146a-5p, miR-193-5p, miR-500a-3p and miR-1207-5p were significantly correlated with at least one mRNA (FDR<0.25, table S6).

To identify potential direct targets, the lists of miRNA-negatively correlated mRNAs were compared to the list of miRNAs' predicted targets. mRNAs that overlapped in both lists were used to generate miRNA-mRNA co-expression networks (figure 3a). let-7a-5p, let-7d-5p, let-7f-5p, miR-31-5p, and miR-708-5p, which were higher expressed with CMH, shared several potential targets, and as expected, the members of let-7 family clustered together through their shared target genes. Similarly, miR-134-5p, miR-146a-5p, miR-500a-3p and miR-1207-5p, which were lower expressed with CMH, shared several potential targets (figure 3a). In these networks, the interactions between miRNAs and negatively correlated predicted targets that were also associated with CMH (indicated in figure 3a) are of special interest as these are the potential key drivers of CMH. Among the higher expressed miRNAs, the let-7 cluster represents a key cluster with negative correlation with 16 CMH-associated potential targets. Among the lower expressed miRNAs, miR-134-5p and miR-146a-5p are key miRNAs negatively correlating with 8 and 10 CMH-associated potential targets, respectively. The percentage of CMH-associated potential targets among the negatively correlated predicted targets was highest for miR-134-5p (figure 3a).

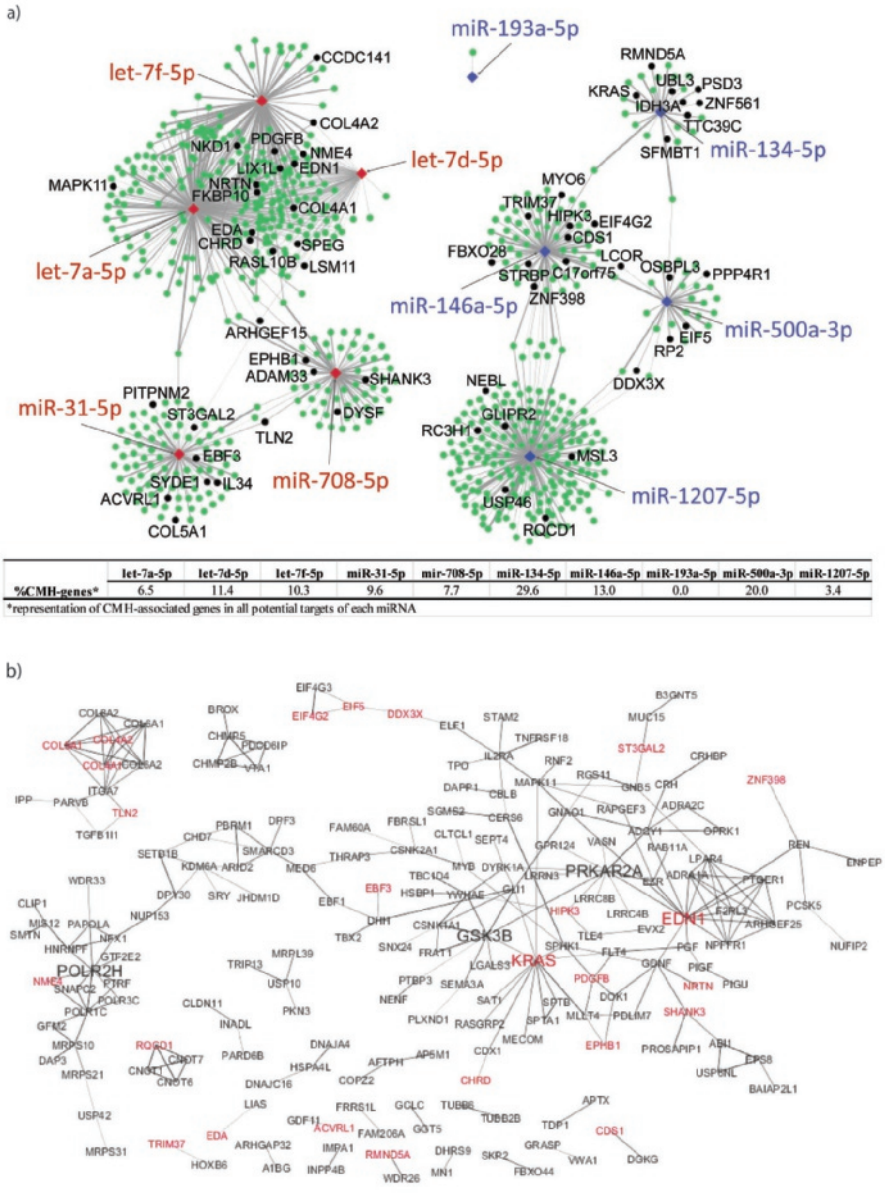
To identify potential interactions among CMH-associated mRNAs, STRING interaction network analysis was performed (figure 3b). The networks point towards KRAS, EDN1, PRKAR2A, GSK3B and POLR2H as hub genes and potential regulators of CMH in our biopsies as they possessed most interactions with other genes. Interestingly, KRAS and EDN1 are potential targets of miR-134-5p and let-7a/d/f-5p, respectively. There was also clear clustering of several collagen and other extracellular matrix (ECM) related genes.

Table 3. Top 20 significant gene sets enriched among CMH-associated mRNAs

Category	Gene sets enriched among genes higher expressed with CMH	NES*	FDR*	#core-enriched genes$^{\Psi}$	core-enriched CMH-associated genes
Cilium/ microtubule development and function	GO Cilium organization	3.80	0.0	117 (171)	SPAG1, TTLL5
	GO Cilium morphogenesis	3.72	0.0	123 (187)	IFT52, SPAG1, TTLL5
	GO Cilium movement	3.24	0.0	30 (33)	-
	GO Axoneme assembly	3.12	0.0	35 (41)	SPAG1, TTLL5
	GO Protein transport along microtubule	2.92	0.0	20 (24)	-
	GO Intraciliary transport	2.90	0.0	20 (24)	-
	GO Axonemal dynein complex assembly	2.90	0.0	19 (19)	SPAG1
	GO Microtubule bundle formation	2.86	0.0	44 (63)	CLIP1, SPAG1, TTLL5
	GO Nonmotile primary cilium assembly	2.71	7.94E-05	16 (22)	-
	GO Microtubule based movement	2.63	6.46E-05	85 (195)	SOD1
Cellular component assembly	GO Cellular component assembly involved in morphogenesis	3.16	0.0	122 (229)	NEBL, TMOD3, SPAG1, TTLL5
	GO Cell projection assembly	2.97	0.0	114 (246)	EZR, SPAG1, TTLL5
	GO Organelle assembly	2.92	0.0	175 (452)	RC3H1, NEBL, WDR45L, CNOT7, MIS12, STAM2, ATXN2, EZR, VTA1, RAB11A, CHMP5, TMOD3, CHMP2B, SPAG1, TTLL5, DDX3X, PDCD6IP
	GO Protein modification by small protein removal	2.90	0.0	69 (112)	USP46, USP42, USP10, USP9Y, USP38, OTUD4
Proteolysis	KEGG Proteasome	2.76	0.0	26 (43)	-
	BIOCARTA Proteasome pathway	2.86	0.0	22 (28)	-
	GO Ubiquitin like protein specific protease activity	2.76	0.0	64 (97)	USP46, USP42, USP10, USP9Y, USP38, OTUD4
	GO Protein polyubiquitination	2.62	6.08E-05	117 (240)	CBFB, CBLB, RNF6, SKP2
Central dogma	GO Ribonucleoprotein complex biogenesis	2.69	7.38E-05	176 (385)	RC3H1, CNOT7, CDH7, RPP40, ATXN2, DDX3X
	GO RNA splicing via transesterification reactions	2.64	6.89E-05	103 (231)	HNRNPF, POLR2H, DHX32, PAPOLA

Category	Gene sets enriched among genes lower expressed with CMH	NES*	FDR*	#core-enriched genes$^{\Psi}$	core-enriched CMH-associated genes
Neuronal and cell signaling	GO Olfactory receptor activity	-2.84	0.0	257 (347)	OR2B11
	GO Hormone activity	-2.82	0.0	81 (113)	EDN1, CRH, NPPB
	GO Neuropeptide signaling pathway	-2.52	0.0	73 (97)	OPRK1, NPFFR1
	GO Adenylate cyclase modulating GPCR signaling pathway	-2.51	0.0	84 (142)	ADRA1A, ADCY1, GNAO1, ADRA2C, OPRK1
	KEGG Neuroactive ligand receptor interaction	-2.48	0.0	183 (269)	F2RL3, ADRA1A, ADRA2C, OPRK1, NPFFR1, LPAR4, PTGER1
	GO GPCR signaling pathway coupled to cyclic nucleotide second messenger	-2.45	0.0	103 (169)	ADRA1A, ADCY1, GNAO1, ADRA2C, OPRK1
	GO Regulation of sensory perception of pain	-2.37	8.00E-05	16 (35)	EDN1, ADRA2C, OPRK1
	GO Neuropeptide hormone activity	-2.36	1.30E-04	25 (29)	CRH
	GO Adenylate cyclase inhibiting GPCR signaling pathway	-2.36	1.40E-04	45 (66)	ADCY1, OPRK1
	GO Growth factor activity	-2.33	2.50E-04	95 (154)	PDGFB, REG1A, IL34, GDF11, CLEC11A, NENF, PGF, GDNF, NRTN
	GO Peptide receptor activity	-2.23	8.40E-04	84 (129)	F2RL3, RAMP1, OPRK1, NPFFR1
Metabolic processes	GO Cyclic nucleotide biosynthetic process	-2.53	0.0	23 (33)	ADCY1, NPPB
	GO Cyclic nucleotide metabolic process	-2.31	2.20E-04	34 (55)	PDE9A, ADCY1, NPPB
	GO Multicellular organismal macromolecule metabolic process	-2.26	5.70E-04	45 (78)	COL5A1, COL6A2, COL8A2, LEPRE1, COL4A2, COL4A1, ENPEP, MRC2, COL6A1
Ion channel	GO Cation channel activity	-2.36	1.50E-04	158 (293)	TRPV2, JPH2, TMEM38A
	GO Extracellular ligand gated ion channel activity	-2.32	2.20E-04	59 (74)	SLC17A7
	GO Potassium channel activity	-2.30	2.60E-04	65 (118)	TMEM38A
	GO Gated channel activity	-2.25	6.30E-04	171 (316)	JPH2, SLC17A7
Others	GO Extracellular matrix structural constituent	-2.31	2.60E-04	46 (75)	COL5A1, COL8A2, COL4A2, COL4A1, EFEMP2
	GO Metanephric nephron development	-2.31	2.30E-04	22 (32)	PDGFB, GDNF

*statistics based on mRNAs associated with CMH definition A. ψ(#total genes). NES is normalized enrichment score, FDR is false discovery rate. GPCR is G-protein coupled receptor.



	let-7a-5p	let-7d-5p	let-7f-5p	miR-31-5p	mir-708-5p	miR-134-5p	miR-146a-5p	miR-193a-5p	miR-500a-3p	miR-1207-5p
%CMH-genes*	6.5	11.4	10.3	9.6	7.7	29.6	13.0	0.0	20.0	3.4

*representation of CMH-associated genes in all potential targets of each miRNA

FIGURE 3. Potential networks underlying chronic mucus hypersecretion (CMH) in chronic obstructive pulmonary disease (COPD). MicroRNA (miRNA)–mRNA co-expression networks are shown in part [a]. Red diamonds represent miRNAs higher expressed with CMH while blue diamonds represent miRNAs lower expressed with CMH. Green circles represent predicted target genes negatively correlated with a particular miRNA while black circles represent negatively correlated predicted targets of the miRNAs whose expression was also associated with CMH. Line width correlates to degree of significance of the miRNA–mRNA correlation. The percentage of CMH-associated genes among the negatively correlated predicted targets of each miRNA is illustrated in the table. Predicted interactions

among CMH-associated targets of CMH-associated miRNAs are shown in part [b]. The networks were created using the STRING (search tool for the retrieval of interacting genes/proteins) database. All mRNAs that were predicted to interact with each other are depicted in the interaction networks while mRNAs that were not predicted to interact with any other mRNA were excluded. Red text represents negatively correlated predicted targets of miRNAs. Large and bold text represents the top five genes with the most interactions to other genes. Line width correlates to interaction scores. The interactions were predicted based on the combined score of all active interaction sources with the minimum required interaction score of 0.700. Only connected nodes are shown.

Enrichment of CMH- and MUC5AC-associated genes among the miRNA-correlated genes

GSEA revealed that genes higher expressed with CMH were enriched among the genes positively correlated with miRNAs higher expressed with CMH and among the genes negatively correlated with miRNAs lower expressed with CMH, and vice versa for the genes that were lower expressed with CMH (figure 4a, 4b and S4).

We found that the MUC5AC-associated gene set [16] was significantly enriched among the genes positively correlated with miRNAs that were higher expressed with CMH and among the genes negatively correlated with miRNAs that were lower expressed with CMH, except for miR-193a-5p (figure 4c and S5).

Expression of candidate miRNAs and mRNAs in human primary airway epithelial cells and fibroblasts

As bronchial biopsy specimens contain various cell types, including mucus producing epithelial cells, we investigated whether the CMH-associated miRNAs identified in biopsies were expressed in PAECs *in vitro*. ALI-differentiated PAECs expressed let-7a-5p (representative of let-7 family), miR-31-5p, miR-708-5p, miR-146a-5p and miR-193-5p (figure 5a), but not miR-134-5p, miR-500a-3p and miR-1207-5p (values>35 Ct). We did not find differences in miRNA expression between COPD and healthy control-derived PAECs (figure 5a), neither did we observe a significant difference in MUC5AC expression between these two groups (figure S6).

As our previous report suggests involvement of fibroblasts in mucus production via crosstalk with epithelial cells [8], we additionally investigated candidate miRNA expression in PAFs derived from COPD patients with or without clinical CMH symptoms. All PAFs expressed let-7a-5p, miR-31-5p, miR-708-5p, miR-134-5p, miR-146a-5p and miR-193a-5p (figure 5b), but not miR-500a-3p and miR-1207-5p. Of interest, the expression of miR-134-5p (figure 5c), but not the other miRNAs, was significantly lower in PAFs derived from the patients with CMH, in line with the findings in our biopsies.

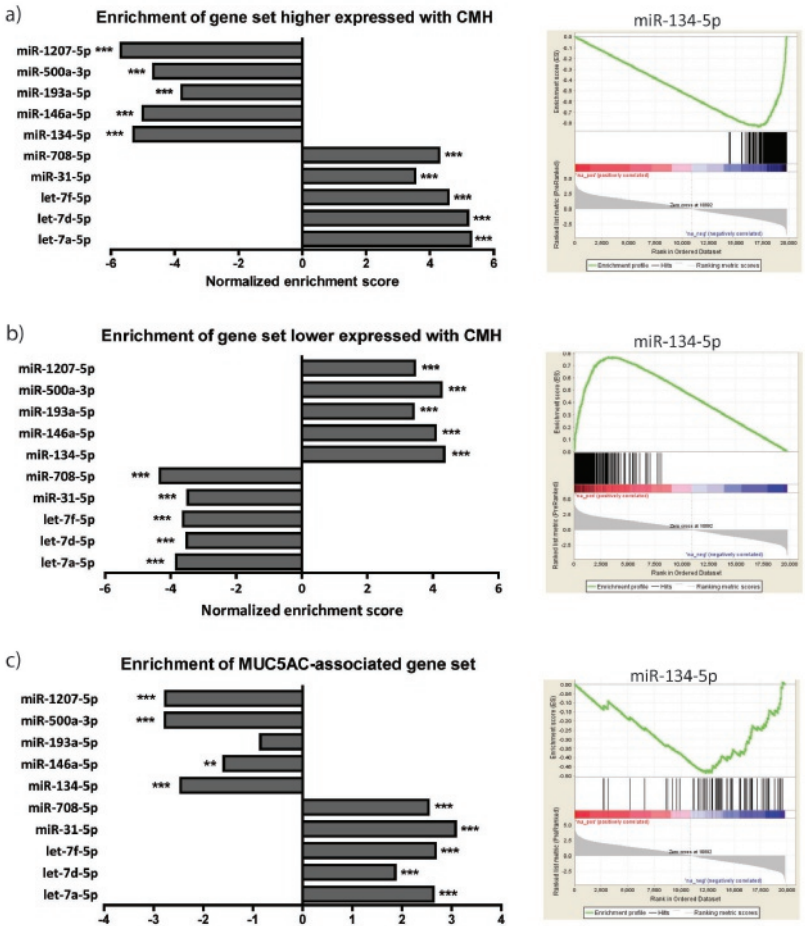


FIGURE 4. Overrepresentation of chronic mucus hypersecretion (CMH) associated genes and MUC5AC-associated genes among microRNA (miRNA) correlated genes. Enrichment of genes higher expressed with CMH among the genes correlated with CMH-associated miRNAs is shown in part [a]. Enrichment of genes lower expressed with CMH among the genes correlated with CMH-associated miRNAs is shown in part [b]. Enrichment of MUC5AC-associated genes among the genes correlated with CMH-associated miRNAs is shown in part [c]. The enrichment plots of miR-134-5p are shown as an example. Asterisks represent significant enrichment. **: $p<0.01$; ***: $p<0.001$.

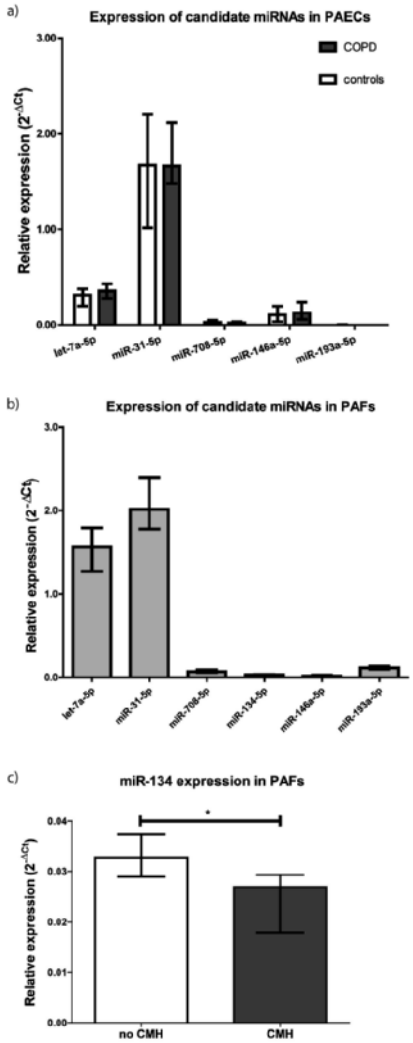


FIGURE 5. Expression of candidate microRNAs (miRNAs) in primary human airway structural cells. miRNAs expressed in primary airway epithelial cells (PAECs) are shown in part [a]. PAECs were derived from chronic obstructive pulmonary disease (COPD) patients (n=6) and healthy controls (n=6) and grown at an air–liquid interface for 14 days. Cells were hormonally deprived overnight and RNA was isolated. miRNAs expressed in primary airway fibroblasts (PAFs) from COPD patients (n=16) are shown in part [b]. The differential expression of miR-134-5p in PAFs from COPD patients with chronic mucus hypersecretion (CMH) compared to those with no CMH is shown in part [c]. PAFs were derived from COPD patients with CMH (n=8) and without CMH (n=8) and grown until confluent. Cells were then serum deprived for 24 h and RNA was isolated. Expression of all miRNAs was normalised to RNU48. Relative expression levels (2-ΔCt, where ΔCt equals the cycle threshold (Ct) of the miRNA of interest minus the Ct of RNU48) are shown as median±interquartile range. *: $p<0.05$ between the indicated values (as assessed by the Mann–Whitney U-test).

To assess whether the top 20 CMH-associated genes were expressed in ALI-differentiated PAECs , we used a publicly available gene expression profiles [17] and found that the majority of these genes were expressed in the ALI-differentiated PAECs (figure S7). LOC100133388, RPL23AP64, C15orf60 were not available in this dataset. Furthermore, using a publicly available gene expression profiles of primary human lung fibroblasts [18], we found that the majority of these genes were also expressed in primary human lung fibroblasts, except NKD1 and PDGFB (figure S8). LOC100133388 was not available in this dataset.

DISCUSSION

We identified 20 miRNAs and 539 mRNAs associated with CMH in bronchial biopsies from COPD patients. Our data suggest that miR-134-5p, miR-146a-5p and the let-7 family may regulate CMH via their potential CMH-associated targets e.g. KRAS and EDN1. The relevance of our CMH signatures was supported by the enrichment of MUC5AC-associated genes [16] among our CMH-associated mRNAs and mRNAs that correlated with the CMH-associated miRNAs. Furthermore, our *in vitro* studies demonstrated that miR134-5p was lower expressed in primary airway fibroblasts from COPD patients with CMH compared to those without CMH, which is in line with the findings in bronchial biopsies and supports a key role for mir134-5p in regulating CMH in COPD.

Since we had both miRNA and mRNA data available from the same bronchial biopsies, we were able to create miRNA-mRNA co-expression networks. This analysis identified the let-7 family, miR-134-5p and miR-146a-5p with their CMH-associated targets to be the key regulators of CMH. miR-134-5p had the highest percentage of CMH-associated genes among its potential targets, of which KRAS was identified as a hub and potential regulatory gene for CMH. KRAS was previously found to be one of the MUC5AC-associated core genes [16]. Interestingly, the expression of miR-134-5p was lower in fibroblasts from COPD patients with CMH than those without, in line with the finding in biopsies. It was not expressed in epithelial cells, suggesting an indirect regulation of CMH via fibroblasts. There are various explanations why the differential expression of miR-134-5p in fibroblasts can affect MUC5AC-associated genes identified in airway epithelium. Firstly, miRNAs can be secreted into exosomes [19] and thus transported from one cell type (e.g. fibroblasts) to the other (e.g. epithelium). Secondly, MUC5AC-associated genes are not only expressed by epithelial cells, but also by fibroblasts. We found that approximately 70% of the MUC5AC-associated genes identified in airway epithelium [16] are also expressed in the lung fibroblasts [18]. Finally, the MUC5AC-associated genes may be secondary targets of miR-134-5p. Our recent studies have shown that CXCL8 produced by lung fibroblasts promotes epithelial mucus production [8] and that the CXCL8 release is increased upon co-culture of fibroblasts and epithelial cells [20]. In line with that report, we observed in this study that CXCL8 is negatively correlated with miR-134-5p (table S6).

Another key miRNA identified in our miRNA-mRNA networks is miR-146a-5p, which was lower expressed with CMH. miR-146a5p was previously reported to be involved in airway inflammation, associated with smoking and COPD [10], and to inhibit MUC5AC production *in vitro* [21]. We recently found miR-146a-5p to be higher expressed in non-COPD lung fibroblasts upon co-culture with bronchial epithelial cells, while there was significantly less increase in COPD fibroblasts,

suggesting a role of miR-146a-5p in disturbed epithelial-fibroblast crosstalk in COPD [22]. Our present findings suggest that miR-146a-5p may also be involved in mucus hypersecretion via epithelial-fibroblast crosstalk, yet it is not clear via which target gene nor in which cell type it has its main effect on CMH. Further mechanistic studies using co-culture or lung organoid models could help elucidating the CMH-related function of miR-146-5p.

The last key miRNA-mRNA cluster identified was the let-7 family, which was higher expressed in association with CMH. The members of the let-7 family shared several potential targets associated with CMH of which EDN1, NKD1, PDGFB, COL4A1 and COL4A2 are of particular interest. EDN1 is localized in submucosal glands and stimulates mucus secretion from serous and mucus cells in mucosal explant culture [23]. NKD1 is an antagonist of the WNT/β-catenin -signalling pathway[24], which plays an important role in respiratory epithelial differentiation [25] and of which specific components, e.g. LEF1, have been reported to regulate MUC5AC production [8, 26]. PDGFB is involved in airway remodelling [27] and epithelial-fibroblast crosstalk [28]. Further, COL4A1 and COL4A2 were also identified in a larger cluster of collagen genes in the CMH interaction networks and are essential components of basement membranes [29]. Basement membrane thickness is associated with an increase of submucosal glands and central airway remodelling in asthma [30], but its role linking airway remodelling with CMH in COPD remains to be investigated. Of note, various CMH-associated targets of let-7a/d/f-5p (EDA, LIX1L, MAPK11, NME4) have been validated with next-generation sequencing [31], supporting our findings.

Apart from that, the CMH-associated genes we identified may be involved in several other biological processes, including cilium development and function, neurohormonal activities, cyclic nucleotide metabolism and signalling, and ion transport. Cilia function and movement is important for mucus clearance [32] and impaired cilia function was observed in COPD airways [33, 34]. The movement of cilia is dependent, at least partially, on cyclic nucleotides, in particular cAMPs [35]. Furthermore, active ion transport, such as that of Ca2+, Na+ and Cl-, plays an important role in regulating mucus viscosity and mucociliary clearance [36, 37], and both Ca2+ and Na+ can be transported through cyclic nucleotide-gated channels [38]. Besides, previous studies suggested that neurohormonal signalling regulates cGMP-induced mucin secretion [39] and the expression of GDNF, the gene encoding for a neurotrophic factor involving in lung development, is associated with CMH in COPD [40]. We also generated gene-interaction networks for CMH and identified KRAS, EDN1, PRKAR2A, GSK3B and POLR2H as hub genes and potential regulators of CMH. Next to KRAS and EDN1, GSK3B is also of interest as it is a pleiotropic signalling molecule that regulates WNT/β-catenin signalling – the pathway that has been shown to induce mucus cell metaplasia in a mouse model [25].

One of the limitations of this study is the relatively small dataset that we used to explore the miRNA and mRNA changes associated with CMH and consequent use of a lenient FDR cut-off. To support our initial findings, several approaches were used. First, we used GSEA to demonstrate the enrichment of MUC5AC-associated genes [16] among the genes higher expressed with CMH. This confirms the relation between our CMH definitions and MUC5AC expression. In addition, we demonstrated significant enrichment of the CMH-associated genes identified in the microarray dataset among the CMH-associated genes identified in the RNA-sequencing dataset. The replication of our findings in a different patient cohort would be informative. However, to the best of our knowledge, there are no other datasets in which both microRNA/mRNA profiles and CMH definitions are available which reflects an urgent need of more studies on this topic.

For the *in vitro* studies, we decided to determine the expression of our candidate miRNAs in PAECs and PAFs as they are present in airway wall biopsies and important cells in the pathogenesis of COPD. As our findings were derived from biopsies, miRNA changes in other cell types may have contributed to our findings; for instance, inflammatory cells or smooth muscle cells. In fact, this could explain the lack of expression of miR-500a-3p and miR-1207-5p in PAECs and PAFs, and validation in other cell types and co-culture studies is warranted in future studies. Furthermore, it will be of interest for future investigations to compare PAECs from patients with CMH and without CMH, which was not possible in the current study. In addition, we focused on the 10 miRNAs that were significantly correlated with mRNA expression in the same subjects in this study. This does not imply that the other 10 miRNAs are not relevant to CMH, since miRNAs not only regulate gene translation by degradation but also by inhibiting the translation [9].

Collectively, we identified three key miRNA-mRNA clusters for CMH: miR-134-5p, miR-146a-5p and the let-7 family, as well as their associated potential target genes. The let-7 and miR-134-5p clusters are connected to the CMH gene expression networks via the potential key regulatory genes KRAS and EDN1. Furthermore, we identified pathways and biological processes, including MUC5AC-associated genes, in which these key miRNA-mRNA clusters are likely to be involved. Future studies involving co-cultures of epithelial cells with fibroblast and other cell types are required to further unravel the functional role of these key genes and miRNAs, and to establish whether they represent potential therapeutic targets for CMH.

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CONTRIBUTIONS

HT, AF, WT, MNH, RG, DSP, MB, IHH, CAB contributed to the study concept and design. PSH, MB and WT coordinated patient inclusion and data collection of GLUCOLD. MB and AS organized and performed the microarrays. GWT and MAG organized and performed the RNA-sequencing. HT and JN conducted *in vitro* laboratory work under supervision of IHH and CAB. HT and AF performed the microarray data analysis under supervision of MB and CAB. HT, AF, MB, IHH and CAB analysed and interpreted data and drafted the manuscript. HT, AF, WT, JN, MNH, RG, PSH, AS, DSP, GWT, MAG, MB, IHH and CAB critically read and revised the manuscript. All authors have read, reviewed and approved the final manuscript.

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SUPPLEMENTARY DATA

Definitions of chronic mucus hypersecretion (CMH)

CMH was defined based on patient's responses to clinical questionnaires including two questions: A) "How often did you cough up sputum during the last three months?" and B) "How often did you cough up sputum during the last week?" The latter was derived from the clinical COPD questionnaire (CCQ) [1]. Question A had four optional answers: i) never, ii) sometimes, iii) almost daily, and iv) only during a cold. Patients who responded i) or iv) were classified as group 1: no CMH. Patients who responded ii) were classified as group 2: mild CMH. Patients who responded iii) were classified as group 3: moderate/severe CMH. Question B had seven optional answers: i) never, ii) sometimes, iii) once in a while, iv) often, v) most of the times, vi) regularly, and vii) always. Patients who responded i) were classified as group 1: no CMH. Patients who responded ii) and iii) were classified as group 2: mild CMH. Patients who responded iv) to vii) were classified as group 3: moderate/severe CMH.

MicroRNA (miRNA) and messenger RNA (mRNA) profiling

Both total mRNA and miRNA was extracted from bronchial biopsies at baseline (n=63) as part of the GLUCOLD study [2, 3]. mRNA profiling was performed at Boston University Microarray Resource Facility as described in GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA, current version available at www.affymetrix.com) as previously described [4]. Microarray hybridization was performed using Affymetrix Human Gene_ST v1.0 Arrays. Isolation of small fractions of RNA for miRNA profiling was done using the miRNeasy mini kit (Qiagen) and RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's protocols. MiRNA profiling was performed at Boston University Microarray Resource Facility using FlashTag™ Biotin HSR Labeling Kit (Affymetrix, Santa Clara, CA, current version available at www.affymetrix.com). miRNAs and mRNAs that were expressed in less than 50% of the patients were excluded. The filtering of miRNAs was done using the standard Affymetrix Expression Console software, and of mRNAs was done using the Robust Multichip Average algorithm and the Entrez Gene Chip Definition File (CDF) v11.0.1[5]. As a result, the expression profiles used for all analyses included 230 miRNAs and 19,793 mRNAs. Both microarray datasets can be accessed at http://www.ncbi.nlm.nih.gov/geo/ (series accession number GSE76774 for miRNA; GSE36221 for mRNA).

To validate the findings from the microarrays, we used the mRNA profiles from the RNA-sequencing (RNA-seq) conducted on libraries belonging to biopsies obtained from 76 GLUCOLD patients. Total mRNA was extracted as described previously [2, 3]. The libraries were prepared using Ribo-Zero Gold kit and

sequenced using 50bp single end read sequencing. The "FastQC" program version 0.11.5 was utilized to perform quality control checks on the raw sequence data from the RNA-Seq (http://www.bioinformatics.babraham.ac.uk). The sequences were trimmed using the java program "trimmomatic 0.33" [6] and the RNA-Seq mapping was conducted using the "Spliced Transcripts Alignment to a Reference (STAR)" program version 2.5.3a, which is an RNA-Seq aligner program [7]. We checked the quality of the libraries by calculating the raw count percentages of the forward reads. Libraries with a percentage lower than 90% were excluded from further analysis. Principal component analysis was also performed (using R) on the libraries in order to detect extreme outlier. After quality check, we decided to keep all samples for further analyses. This dataset can be made available for future potential collaboration upon request. The R package "edgeR_3.16.5" was utilized to perform differential expression analysis on the samples taken from CMH patients at baseline (n=38). We compared mRNA profiles of the patients with moderate/severe CMH (n=22 for definition A and n=21 for definition B) with the patients with no CMH (n=5 for both definitions). These analyses were corrected for age, gender and smoking status.

Identification of CMH-associated miRNAs and mRNAs

A linear regression model was used to identify miRNAs and mRNAs that were differentially expressed in patients with mild CMH or with moderate/severe CMH compared to those without CMH. The model was corrected for age, gender, smoking history and RNA integrity number scores (RIN) as described below, where Mei represents the log2 miRNA/gene expression value for a miRNA/gene in a sample from patient i, εi represents the error that is assumed to be normally distributed.

$$Me_i=\beta_0+\beta_1 X_{RIN\text{-}i}+\beta_2 X_{Smoking\ history\text{-}i}+\beta_3 X_{Age\text{-}i}+\beta_4 X_{Gender\text{-}i}+\beta_5 X_{CMH\ status\text{-}i}+\varepsilon i$$

MiRNA-mRNA co-expression network analysis

Pearson correlation analysis was used to determine the correlation between the expression of each CMH-associated miRNA and all mRNAs in the same patient based on False Discovery Rate (FDR)<0.25. The lists of miRNA's predicted target genes based on TargetScan (v6.2), miRTarBase (v4.5) and miRDB (v5.0) were combined. Those predicted targets that were negatively correlated with the candidate miRNAs were used for creating miRNA-mRNA co-expression networks on Cytoscape (v3.4.0).

Gene Set Enrichment Analysis (GSEA)

GSEA (v2.2.2) was used for the following purposes: 1) to identify enriched pathways (Kegg, v5.2; Biocarta, v5.2), biological processes (Gene Ontology, v5.2), and molecular functions (Gene Ontology, v5.2) in which CMH-associated mRNAs may

be involved, 2) to determine enrichment of a previously published set of MUC5AC-associated genes [8] among mRNAs higher expressed with CMH, 3) to determine enrichment of CMH-associated mRNAs and MUC5AC-associated genes among miRNA-correlated mRNAs, and 4) to determine enrichment of CMH-associated mRNAs identified in the microarray dataset among the CMH-associated mRNAs identified in the RNA-seq dataset. Genes were ranked according to the strength of their t-statistic reflecting their association with CMH or their correlation with the miRNA of interest. Enrichment p-values were calculated after 1000 permutations were performed. Significant enrichment was determined by an FDR<0.01 for the first purpose and by p<0.05 for the second, third and fourth purposes.

STRING interaction network analysis

Of all 539 CMH-associated mRNAs, expression of 538 was significantly correlated with one or more of the 10 CMH-associated miRNAs (table S6). Interactions among these miRNAs' potential target genes were predicted using the STRING database (v10.0) and the interaction networks were created on Cytoscape (v3.4.0) with stringApp (v1.0.5) [9]. Sources of the interactions include text-mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence. Minimum required interaction score is 0.700.

Cell culture and RNA isolation

To compare the expression of candidate miRNAs in primary airway epithelial cells (PAECs) from COPD and non-COPD subjects, PAECs were obtained from 6 GOLD stage IV COPD (table S1) explanted lungs and 6 non-COPD donor lungs as described before [10]. PAECs were used in passage 3, grown to confluence in a transwell system, air-exposed for 14 days in BEGM/DMEM supplemented with 15 ng/ml retinoic acid and hormonally and growth factor-deprived overnight as described previously [10]. To compare the expression of candidate miRNAs in primary airway fibroblasts (PAFs), a separate experiment was performed. PAFs were isolated from GOLD stage IV COPD patients (table S2) with (n=8) and without CMH (n=8) as described before [11] and used in passage 5/6 and cultured in Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS) until confluence. The cells were then serum deprived for 24 hours. The study protocol followed national ethical and professional guidelines ('Code of conduct; Dutch federation of biomedical scientific societies'; http://www.federa.org) for all lung tissues and explant cell culture studies in Groningen.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated using Tri Reagent® according to the manufacturer's protocol. For the miRNAs of interest RNA was converted to cDNA using the TaqMan microRNA

reverse transcription kit (Life Technologies, Bleiswijk, Netherlands) and reverse transcription primers (Life Technologies) for let-7a-5p (assay id: 000377), miR-31-5p (002279), miR-708-5p (002341), miR-134-5p (000459), miR-146a-5p (000468), miR-193a-5p (002281), miR-500a-3p (001046) and miR-1207-5p (241060_mat). As let-7d-5p and let-7f-5p were in the same cluster as let-7a-5p and these three miRNAs shared very similar results in the previous analyses, we only investigated let-7a-5p expression. qPCR was performed using the matched PCR primers (Life Technologies) and Eurogentec qPCR MasterMix Plus (05-QP2X-03; Eurogentech, Maastricht, Netherlands) on the LightCycler 480 II (Roche, Almere, Netherlands). Expression of the miRNAs of interest was normalized to the expression of the small nuclear RNA, RNU48 (001006). To determine MUC5AC expression, RNA was converted to cDNA using iScript™ cDNA Synthesis Kit (BioRad). qPCR was then performed using MUC5AC Taqman® assay (Hs01365616_m1) and the Taqman® Master Mix according to the manufacturer's guidelines (Applied Biosystems, Foster City, CA). MUC5AC expression was normalized to the expression of the reference genes: B2M (Hs99999907_m1) and PPIA (Hs99999904_m1).

Table S1. Characteristics of patients from which primary airway epithelial cells (PAECs) were obtained

	COPD IV patients# (n=6)
Gender (male), n(%)	3(50.0)
Ex-smokers, n(%)	6(100.0)
Pack-year*	41.7 (23.6-50.3)
Age, year*	55 (51-59)
FEV1, %predicted*	18.8 (17.3-19.8)
FEV1/FVC*	0.24 (0.22-0.25)

*Data are presented as median (interquartile range); FEV1 is forced expiratory volume in 1 second; FVC is forced vital capacity; #two patients were CMH-positive.

Table S2. Characteristics of patients from which primary airway fibroblast (PAFs) were obtained

	COPD patients	
	no CMH (n=8)	CMH (n=8)
Gender (male), n(%)	2(25.0)	4(50.0)
Pack-year*	38.0 (30.0-57.0)	30.0 (28.8-36.3)
Age, year*	58 (57-61)	58 (50-59)
FEV1, %predicted*	20.7 (17.3-22.6)	15.2 (14.2-18.5)
FEV1/FVC*	0.26 (0.24-0.31)	0.27 (0.24-0.32)

*Data are presented as median (interquartile range); FEV1 is forced expiratory volume in 1 second; FVC is forced vital capacity.

Table S3. microRNAs differentially expressed with CMH and their statistics

	Severe CMH vs No CMH (definition A)				Severe CMH vs No CMH (definition B)		
miRNA	t-value	FC	p-value	FDR	t-value	FC	p-value
let-7d-5p	2.928	1.263	4.927E-03	0.149	2.453	1.171	1.730E-02
miR-30c-5p	2.922	1.411	5.001E-03	0.149	3.365	1.423	1.400E-03
miR-708-5p	2.794	2.061	7.116E-03	0.149	2.930	2.060	4.900E-03
miR-31-5p	2.528	1.684	1.431E-02	0.182	2.566	1.613	1.300E-02
miR-27-3p	2.433	1.452	1.821E-02	0.182	3.420	1.730	1.200E-03
miR-30b-5p	2.339	1.475	2.291E-02	0.182	3.060	1.475	3.400E-03
let-7f-5p	2.227	1.700	2.996E-02	0.230	2.766	1.789	7.700E-03
let-7a-5p	2.133	1.261	3.731E-02	0.240	2.400	1.223	1.980E-02
miR-19a-3p	2.088	1.638	4.139E-02	0.244	2.463	1.529	1.690E-02
miR-134-5p	-3.507	-2.152	9.002E-04	0.149	-2.433	-1.615	1.820E-02
miR-193a-5p	-2.774	-1.785	7.519E-03	0.149	-2.545	-1.653	1.370E-02
miR-320c	-2.681	-1.355	9.638E-03	0.171	-2.874	-1.316	5.700E-03
miR-339-3p	-2.448	-1.342	1.754E-02	0.182	-2.138	-1.269	3.690E-02
miR-320a	-2.419	-1.334	1.885E-02	0.182	-2.863	-1.320	5.900E-03
miR-320b	-2.384	-1.309	2.054E-02	0.182	-2.873	-1.299	5.700E-03
miR-1207-5p	-2.371	-1.547	2.120E-02	0.182	-3.727	-1.694	5.000E-04
miR-22-3p	-2.183	-1.209	3.326E-02	0.239	-2.250	-1.230	2.840E-02
miR-500a-3p	-2.151	-1.336	3.577E-02	0.240	-2.166	-1.239	3.460E-02
miR-146a-5p	-2.117	-1.626	3.872E-02	0.240	-2.288	-1.569	2.590E-02
miR-320d	-2.106	-1.259	3.969E-02	0.240	-2.288	-1.242	2.590E-02

FC is fold change; FDR is false discovery rate.

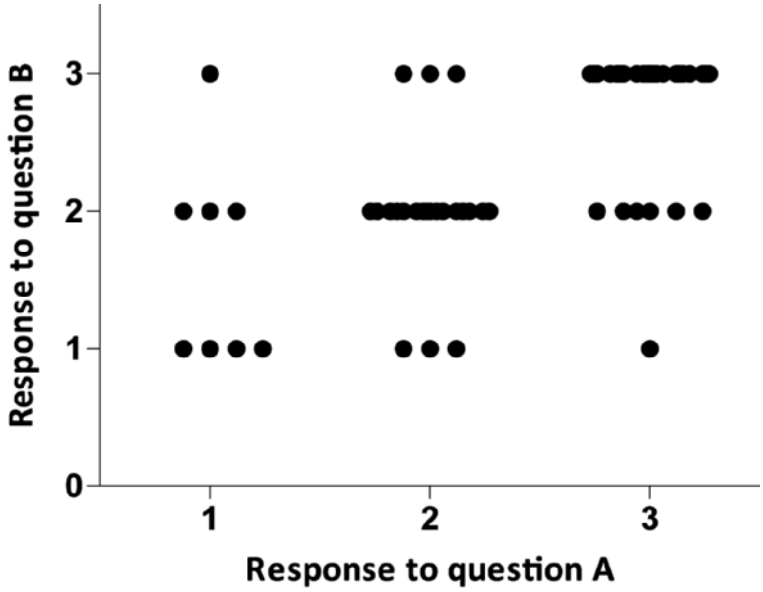

Figure S1. Responses of patients (n=63) to two questions used for defining CMH were strongly correlated. Dot plot showing the patients' answers to question A) "How often did you cough up sputum during the last three months?" and question B) "How often did you cough up sputum during the last week?". 1 represents "never cough" or "only during a cold"; 2 represents "sometimes" or "once in a while"; 3 represents "almost daily" for question A) or "often", "most of the times", "regularly", or "always" for question B). Spearman's rank correlation coefficient was performed. $r=0.651$; $p<0.0001$.

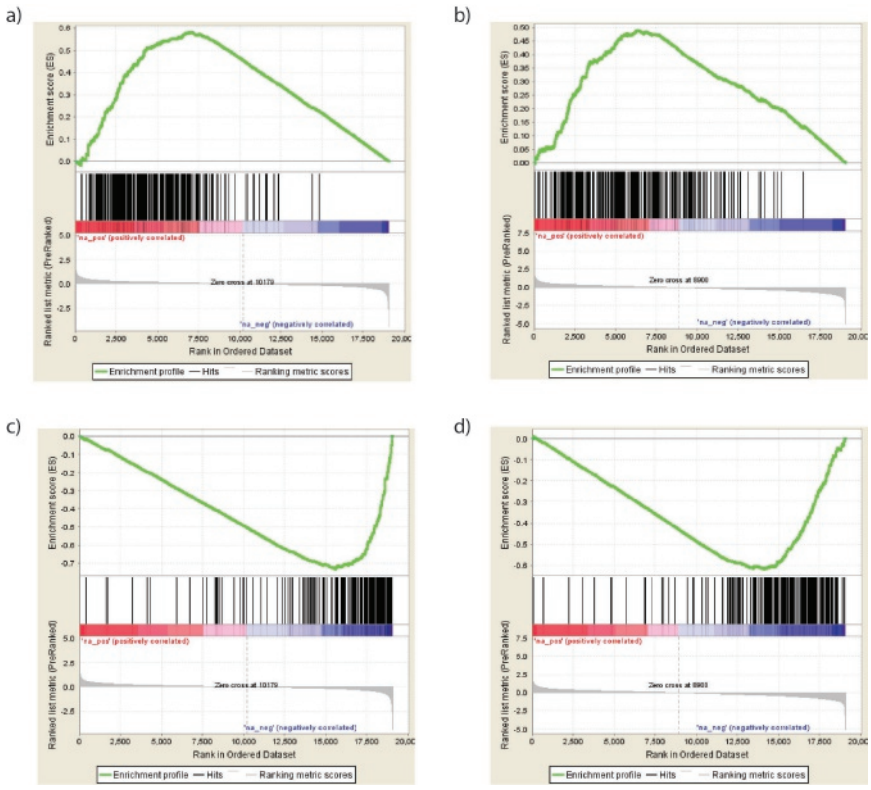

Figure S2. Gene set enrichment analysis (GSEA) on the RNA-seq dataset. Genes higher expressed with CMH in the microarray dataset were positively enriched based on [a] CMH definition A and [b] CMH definition B. Genes lower expressed with CMH in the microarray dataset were negatively enriched based on [c] CMH definition A and [d] CMH definition B. All enrichment was significant ($p<0.001$). Vertical black lines (Hits) represent position of 539 CMH-associated genes identified in the microarray dataset in the ranked gene list. From left to right, 19,075 genes were ranked by t-statistics for association with CMH in the RNA-seq dataset. Red and blue represent positive and negative association, respectively.

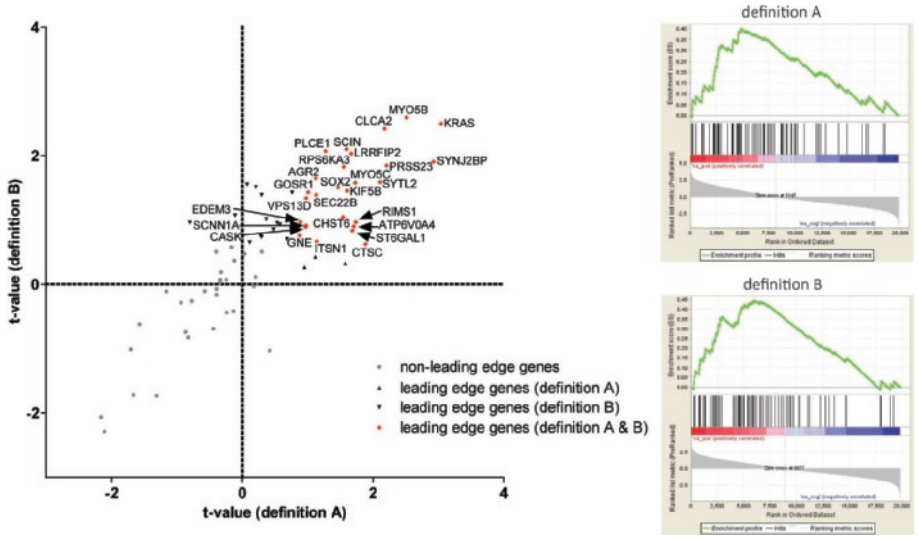

Figure S3. Overrepresentation of MUC5AC-associated genes among CMH-associated mRNAs. Enrichment analysis was performed using the ranked list of CMH-associated mRNAs, based on definition A and B, and the list of 73 MUC5AC- associated genes [8]. Significant enrichment was observed for both definition A (enrichment score (ES)=0.40, $p<0.001$) and B (ES=0.44, $p<0.001$). ES was calculated by walking down the ranked list of genes – a running-sum statistic increases when a gene is in the gene set and decreases when the gene is not. On the left graph, leading edge genes associated with CMH in both definitions are shown in red.

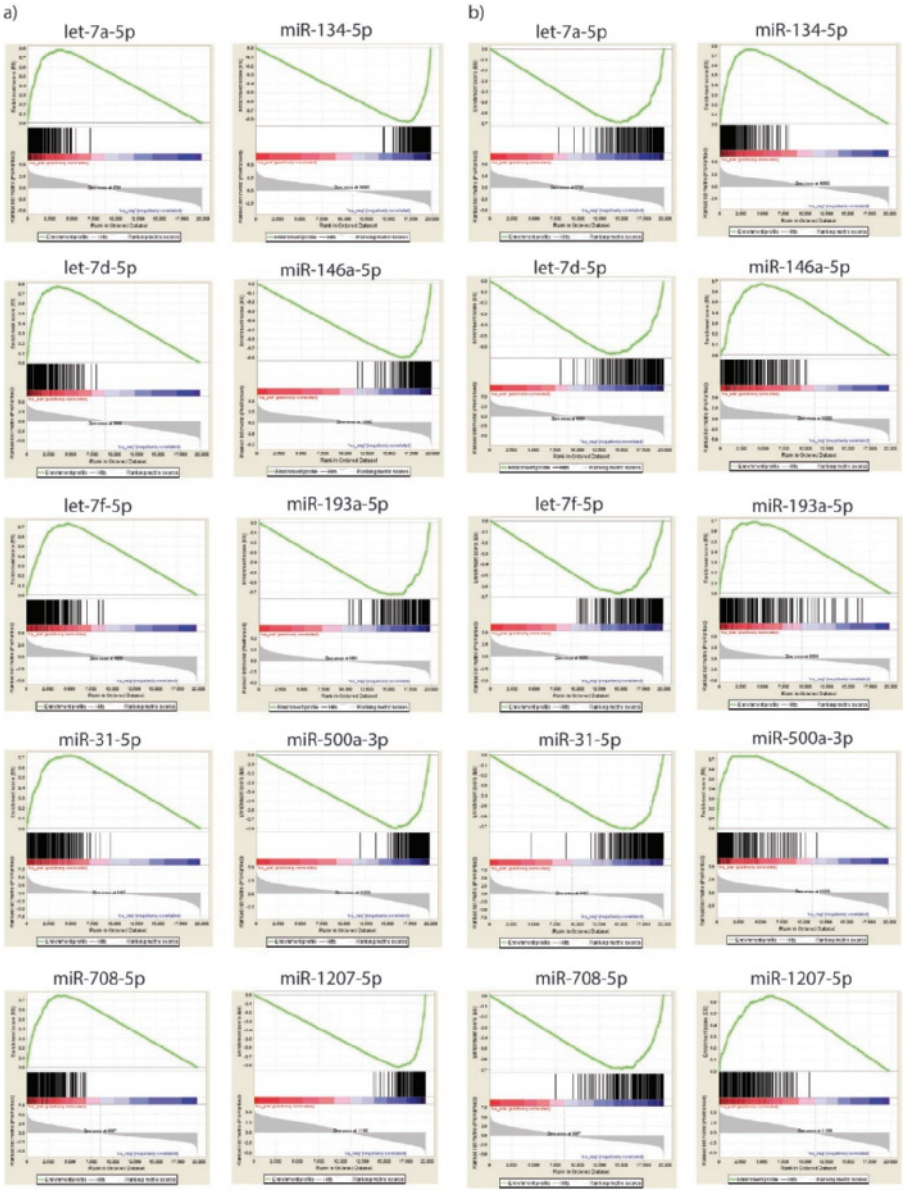

Figure S4. Enrichment of CMH-associated mRNAs among miRNA-correlated mRNAs. a) Enrichment of mRNAs higher expressed with CMH. b) Enrichment of mRNAs lower expressed with CMH. All enrichments were significant ($p<0.001$).

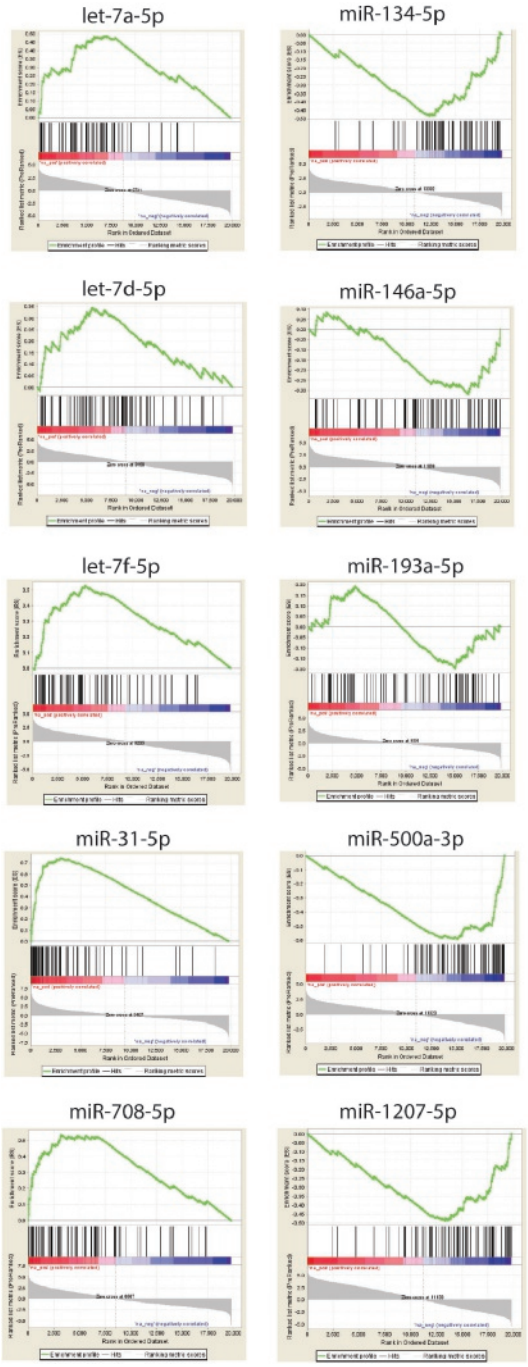

Figure S5. Enrichment of MUC5AC-core genes among miRNA-correlated mRNAs. All enrichments were significant ($p<0.005$) except for miR-193-5p.

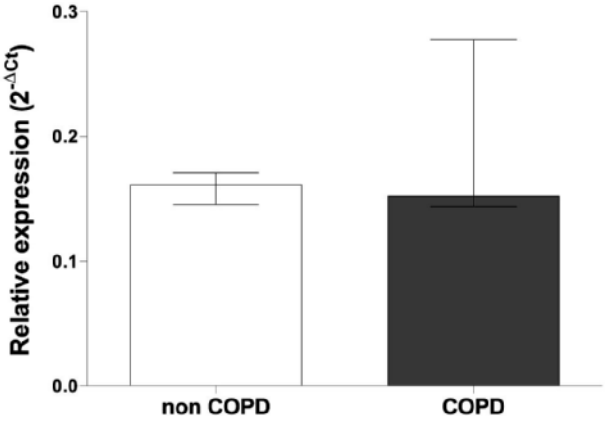

Figure S6. Expression of MUC5AC in primary airway epithelial cells (PAECs). PAECs were derived from COPD patients (n=6) and healthy controls (n=6) and grown at air-liquid interface for 14 days. Cells were hormonally deprived overnight and RNA was isolated. The MUC5AC expression was normalized to B2M and PPIA. Relative expression levels are shown (median±interquartile range).

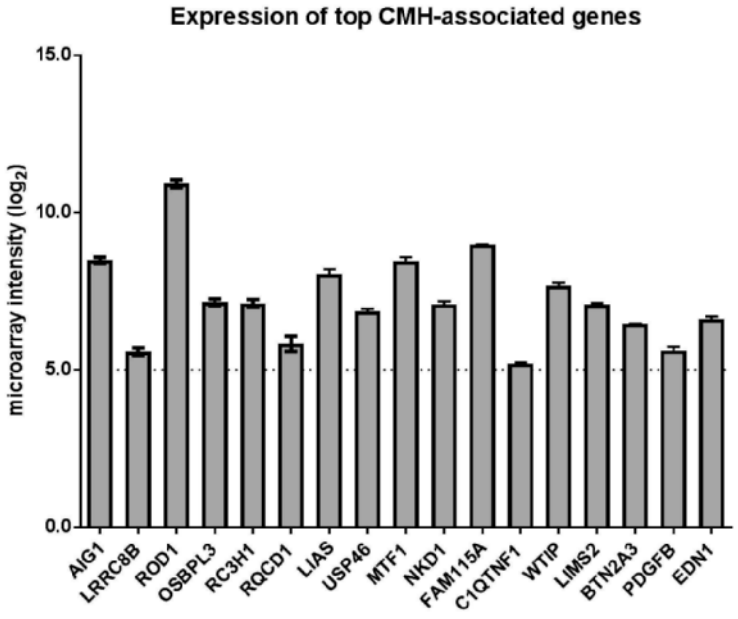

Figure S7. Expression of top CMH-associated genes in ALI-cultured primary airway epithelial cells (day 14)[12]. The microarray dataset is publicly available on www.ncbi.nlm.nih.gov/geo/ (series accession number GSE5264). Genes with microarray intensity above 5.0 were considered expressed.

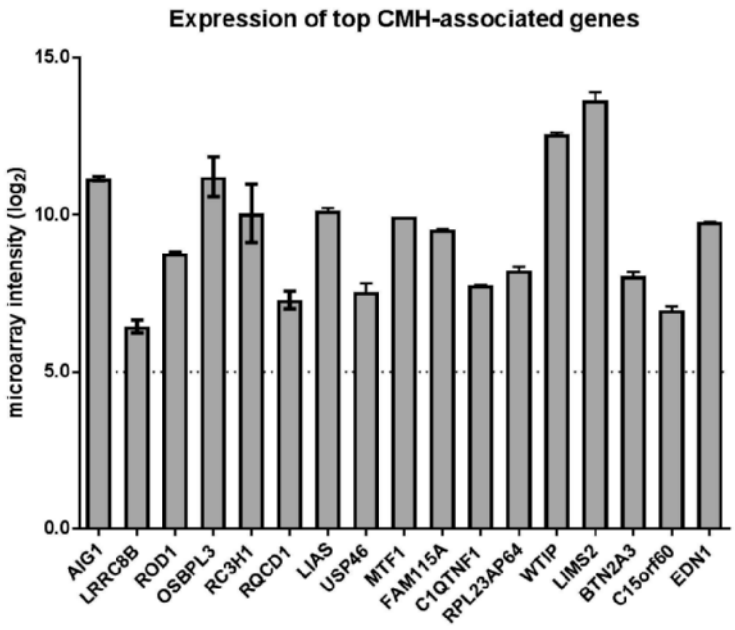

Figure S8. Expression of top CMH-associated genes in primary lung fibroblasts[13]. The microarray dataset is publicly available on www.ncbi.nlm.nih.gov/geo/ (series accession number GSE86183). Genes with microarray intensity above 5.0 were considered expressed.

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Additional supplementary materials can be found at the following link:
https://erj.ersjournals.com/content/52/3/1701556.figures-only