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Abstract

Parametric response mapping (PRM) is a computed tomography (CT) based method to phenotype COPD patients. It is capable of differentiating emphysema related air trapping with non-emphysematous air trapping (small airway disease), which helps to identify the extent and localization of the disease. Most studies evaluating the gene expression in smokers and COPD patients related this to spirometric measurements, but none have investigated the relationship with CT-based measurements of lung structure. The current study aimed to examine gene expression profiles of brushed bronchial epithelial cells in association with the PRM-defined CT based measurements of emphysema (PRM^{Emph}) and small airway disease (PRM^{fSAD}). Using the TIP study cohort (COPD = 12 and asymptomatic smokers = 32), we identified a gene expression signature of bronchial brushings, which was associated with PRM^{Emph} in the lungs. One hundred thirty-three genes were identified to be associated with PRM^{Emph}. Among the most significantly associated genes, *CXCL11* is a potent chemokine involved with $CD8^+$ T cell activation during inflammation in COPD, indicating that it may play an essential role in the development of emphysema. The PRM^{Emph} signature was then replicated in two independent datasets. Pathway analysis showed that the PRM^{Emph} signature is associated with proinflammatory and notch signaling pathways. Together these findings indicate that airway epithelium may play a role in the development of emphysema and/or may act as a biomarker for the presence of emphysema. In contrast, its role in relation to functional small airways disease is less clear.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is considered as one of the major non-communicable diseases in the world (20). The persistent airflow limitation is associated with inflammatory responses, which are initially to noxious particles (22). These factors together result in an accelerated decline in lung function (16). COPD is a heterogeneous disease in which fibrosis and loss of small airways and emphysema are two major pathological characteristics of the disease (17).

Current theories behind the development of the emphysematous phenotype of COPD include protease antiprotease imbalance, chronic airway inflammation, and dysregulation of oxidative stress (9, 35). These mechanisms are thought to cause the characteristic symptoms of emphysema, including abnormal inflammatory responses together with alveolar destruction, which leads to a reduction of the alveolar-capillary exchange area (29).

Parametric response mapping (PRM) is a novel computed tomography (CT) based method to phenotype lung diseases (23). Application of PRM to paired inhaled/exhaled CT scans is capable of differentiating emphysema from non-emphysematous air trapping due to functional small airway disease (14, 23, 24).

Gene expression signatures have been studied in different diseases to identify the underlying mechanisms and biological pathways associated with the disease of interest (25, 28). These gene expression profiles of bronchial brushes provide a global picture of the airways, and they can help understand the mechanisms involved in the development of emphysema (18, 29).

Several studies have assessed gene expression in smokers and COPD patients and related this to spirometric measurements (18, 29-31). However, none have investigated the relationship with CT-based measurements of lung structure. In the present study, gene expression profiles of bronchial epithelial cells were investigated in association with the severity of PRM-defined emphysema (PRM^{Emph}) and functional small airway disease (PRM^{fSAD}).

Methods

Study population

The study population was a subset of subjects included in the Top Institute Pharma (TIP) study (3) who underwent bronchoscopy. The TIP study was approved by the ethics committee of UMCG and registered under the National Clinical Trial (NCT) identifier: NCT00850863. All these selected subjects were >35 years of age and current or ex-smokers consist of 12 COPD subjects and 32 asymptomatic smokers who had provided written informed consent. The spirometric measurements were collected according to the international guidelines described in (21) and (37). The clinical characteristics of the current study population are described in table 1.

Bronchial brushes sample collection and processing

Bronchoscopically derived bronchial brushings were collected from the first, and second subsegmental branches of the left lower lobe and total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Valencia, CA). From each sample, 100–200 ng total RNA was processed and examined with Affymetrix Gene Chip Human Gene 1.0 ST, as previously described (GSE97010) (3).

CT images acquisition for PRM

The inspiratory and expiratory low dose chest CT scans were taken using multi-detector CT scanners at full inspiration, and normal expiration. Then the CT image processing was done using PRM. Detail protocols used for CT scan acquisition and PRM processing were previously described in (15). PRM scores are presented as the percent volume of the total lung. PRM processing for inhaled and exhaled CT images of a single patient is illustrated in figure 1A.

Bioinformatic Analysis

Microarray data analyses were done using the Bioconductor-limma package in R software version 3.5.1. Gene expression of the bronchial brushings were correlated to different CT scan variables (PRM^{Emph} and PRM^{fSAD} scores) and Forced Expiratory Volume in one second (FEV1) %predicted using the R package Limma (V3.38.3). Linear models were applied after corrected for gender and packyears. The False Discovery Rate (FDR) less than 0.05 considered as statistical significance.

Gene Set Enrichment Analysis (GSEA)

GSEA gives the quantification of the association of gene sets with the differential expression changes. In this study, GSEA was done using GSEA V.2.0.14 to compare the PRM^{Emph} signature to the difference in bronchial brush gene expression between COPD and non-COPD individuals, using two previously published publicly available independent datasets. These datasets are accessible through following GEO Series accession numbers in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO). (cohort one and cohort two as described below). Cohort one consists of current and ex-smokers with and without COPD (COPD=87, non-COPD =151) (GSE37147) (31). The cohort 2 composed of COPD and non-COPD subjects (COPD= 8, non-COPD =14) (GSE56342) (36).

Gene Set Variation Analysis (GSVA)

GSVA analysis allows us to explore the effect of genes associated with PRM^{Emph} signature on each patient. The GSVA analysis was done using GSVA (1.34.0) package in R software version 3.5.1, by looking at the genes that were positively and negatively associated with PRM^{Emph} signature separately.

Pathway analysis

Pathway analysis was done to identify the pathways related to significant genes associated with PRM^{Emph} score. This analysis was done using the g: Profiler web base tool (26).

Results

Association of bronchial brush gene expression with PRM scores and FEV1 %predicted

Initially, we investigated the gene expression profiles of bronchial epithelial cells in relation to PRM^{Emph}, PRM^{fSAD}, and the FEV1 % predicted. A total number of 133 genes were associated with PRM^{Emph} scores, with 82 genes (61.65%) positively associated and 51 (38.35%) genes negatively associated (FDR < 0.05). In contrast, no genes were significantly associated with PRM^{fSAD}, and FEV1% predicted. The top 20 genes associated with PRM^{Emph} were tabulated in table 2. A volcano plot present in figure 1B represents the differentially expressed genes in bronchial brushings related to emphysema (PRM^{Emph} score), and the heatmap in figure 1C represents the significant genes associated with PRM^{Emph} score, respectively.

Association of identified PRM^{Emph} signature with other clinical parameters and independent datasets

We next compared the overlap between the identified signatures using GSEA and GSVA analysis. The GSEA results for genes significantly associated with FEV1 % predicted, and PRM^{fSAD} are illustrated in Figure 1D & E. These results show a high overlap between genes associated with PRM^{Emph} score, FEV1% predicted and PRM^{fSAD}. This is reflected with the high correlation between FEV1% predicted with PRM^{Emph} scores ($r = -0.508$, p-value = 0.000507, n= 44), and PRM^{Emph} with PRM^{fSAD} scores ($r = 0.852$, p-value = 2.2e-16, n= 44).

We then compared the gene expression signature of PRM^{Emph} with an independent dataset of COPD status signature (GSE37147). Those genes positively associated with PRM^{Emph} scores were enriched among genes expressed in bronchial brushings of the COPD cohort (Figure 1F). The PRM^{Emph} signature was then compared with another independent dataset, consisting of gene expression profiles of COPD status in small airway epithelium (GSE56342). The resulted GSEA plot in Figure 1G shows a similar pattern as in our dataset, further confirming the identified gene expression signature of PRM^{Emph} replicated in different independent cohorts**.** The GSVA results further confirm that there is a continuous relationship in the change of gene expression patterns associated with PRM^{Emph} scores (figure 2A and B).

Pathways associated with PRM^{Emph} signature

Pathway analysis shows that the PRM^{Emph} score is associated with cytokine-mediated signalling pathways, interferon pathways and NOTCH signalling pathways. Both cytokine-

mediated signalling and interferon signalling pathways got increased. In contrast, extracellular metric, collagen and NOTCH signalling related pathways got decreased associated with PRM^{Emph} signature (FDR<0.05) (table 3).

Discussion

The current study examines gene expression profiles of bronchial brushings in association with PRM-defined CT measurements of emphysema and small airway disease. The *CXCL11* gene which produced by the airway epithelium (13), and it is known for its role as a prominent chemokine in $CD8^+$ T cell activation during inflammation in COPD was found as one of the most significantly associated genes with PRM^{Emph} scores, indicating that *CXCL11* may play an essential role in the development of emphysema. The identified PRM^{Emph} signature was then replicated in two independent datasets, providing evidence that the airway epithelium may play a role in the development of emphysema and/or may act as a biomarker for the presence of emphysema.

The top five genes differentially expressed in bronchial brushes related to PRM^{Emph} scores include *SLCO1B3, SPRR1A, FKBP5, CXCL11*, and *CLEC4E*. *CXCL11* is a T-cell chemoattractant and one of the most effective ligands of CXCR3 on $CD8^+$ T cell and $CD4^+$ T cells (5). $CD8^+$ T cell activation has previously been associated with the development of emphysema by inducing alveolar cell apoptosis (2) via producing perforins and granzyme B (6, 12). In addition, the *CXCL11* gene was previously identified as a highly expressed gene in the sputum of COPD patients (8). *FKBP5* is a negative regulator of the glucocorticoid receptor and therefore regulates corticosteroid anti-inflammatory functions (11, 27). This gene has previously been found as corticosteroid sensitive gene, and its upregulation with PRM^{Emph} may be due to a higher dose of corticosteroid use in patients with a high level of emphysema; thus, it could be more of a treatment effect rather than disease effect (27). The *SLCO1B3* gene, which encodes a transmembrane receptor that mediates the sodium-independent uptake of endogenous and xenobiotic compounds, mainly in the liver (32), while the *CLEC4E* gene encodes a protein which belongs to C-type lectin domain family 4 (7), but for these two genes roles related to COPD, is yet to be explained.

The GSEA results, which show the association of PRM^{Emph} gene expression signature with FEV1% predicted and PRM^{fSAD} on the gene set level, show a similar overlapping pattern with the PRM^{Emph} signature, indicating possible similar mechanisms associated with these measurements of the lung (18, 29, 31).

The PRM^{Emph} associated signature was shown to be associated with COPD in two independent datasets from the upper and lower airways. This result follows the theory of

"united airway field of injury," providing evidence that this signature may common throughout the compartments of the lung (4, 31).

The pathway analysis revealed top pathways associated with PRM^{Emph} score include cytokine-mediated signalling pathways and NOTCH signalling pathways which are well known for their role in COPD (2). Cytokine-mediated signalling pathways are responsible for the increased inflammation in COPD. In contrast, NOTCH signalling pathway plays a significant role in lung epithelial morphogenesis, and it is found to be downregulated in COPD patients and cause the lung epithelial metaplasia which leads to mucosal hyperplasia (1, 2, 10, 19, 33, 34).

The limitation of this study is the small number of patients tested in the discovery cohort, however despite these low number of patients the identified signature was able to be observed in two independent datasets of bronchial brushes from COPD, indicating the robustness of the PRM^{Emph} signature. The lack of significance in PRM^{fSAD} may be due to its variability within the GOLD status of COPD and possible multifactorial causes for the development of small airways disease. In addition, the bronchial brushes were collected from the 1st and second subsegmental branches of the left lower lobe of the lung which may not accurately reflect the transcriptomic changes occurring in the peripheral small airways, which are inaccessible to bronchoscopy. Furthermore, our replication study was conducted on COPD status and not PRM, as this data is currently not available for airway gene expression datasets.

In conclusion, we have identified a gene expression signature of bronchial brushings, which is associated with PRM^{Emph} signature in the lungs. In contrast, we did not find gene expression levels to be significantly associated with PRM^{fSAD}. These findings indicate that airway epithelium may play a role in the development of emphysema and/or may act as a biomarker for the presence of emphysema, but not or to a lesser extent for functional small airways disease.

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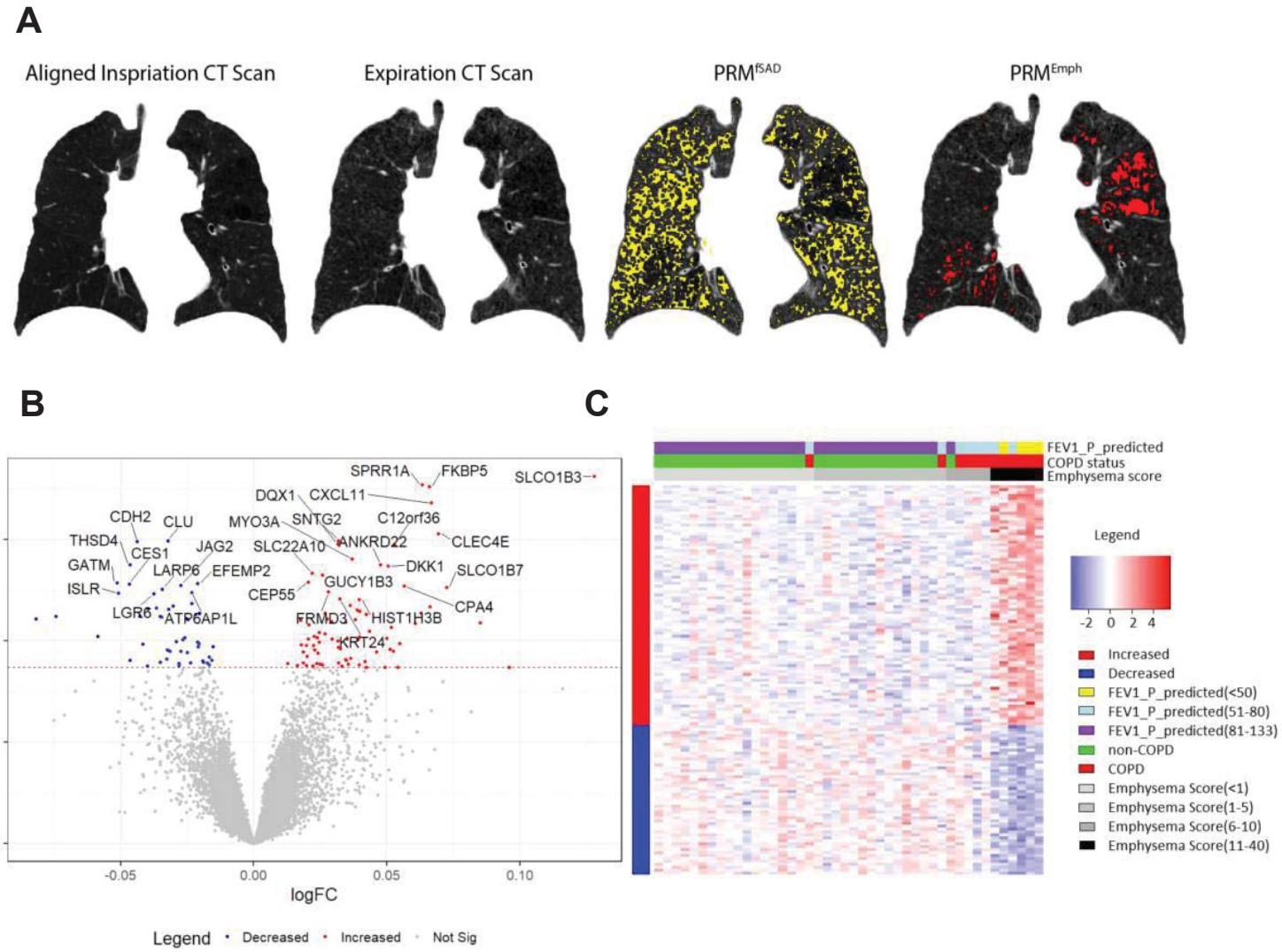
Figure Legends

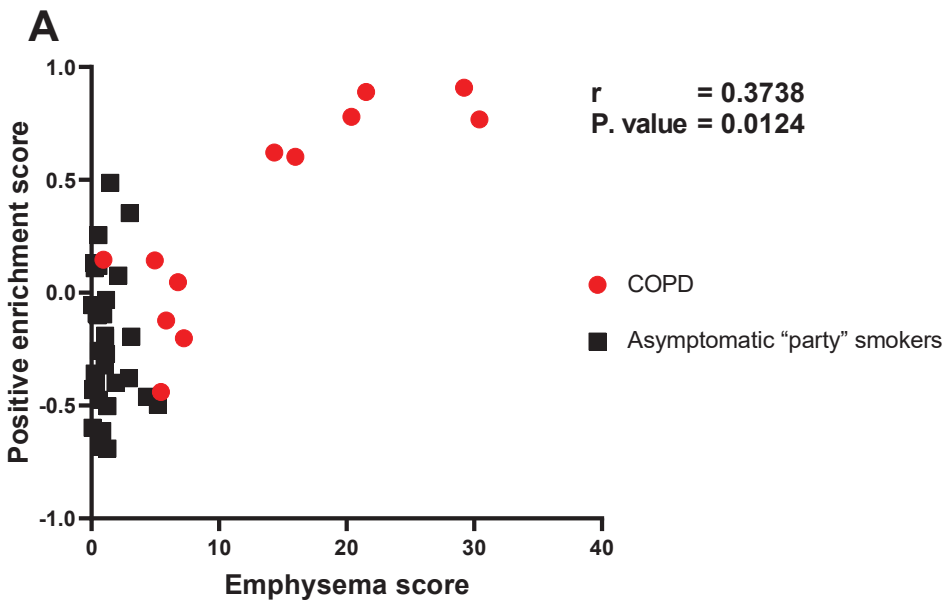
Figure 1: Gene expression and GSEA results of bronchial brushings associated with emphysema score. A) Parametric response mapping of one patient CT scans. Lung tissue Inspiration and expiration CT scans, small airway disease in yellow (PRM^{fSAD}), and emphysematous lung tissue in red (PRM^{Emph}). B) Volcano plot of differential gene expression in bronchial brushings related to emphysema (PRM^{Emph}) score. C) Heatmap shows genes significantly altered associated with the PRM^{Emph} score. The red and blue colours in the heat map representing up and down-regulated gene-expression levels, respectively. Samples with COPD are clustered under red, and non-COPD are under green. Samples grouped related to PRM^{Emph} score range from high to low represented in black to light grey colour gradient, respectively. FEV1 % predicted value less than 50 represented in yellow and FEV1 % predicted value range from 50 to 80 and 80 to133 were grouped under light blue and Purple, respectively. Gene set enrichment analysis (GSEA) of genes significantly associated with PRM^{Emph} score related to D) FEV1% predicted E) PRM^{fSAD} score associated genes in this study, and related to COPD status in F) replicate data set 1(GSE37147) and G) replicate data set 2 (GSE56342). In each GSEA plot, the colored bars represent the ranked t-values of the association of bronchial gene expression. The red colour represents a positive association, whereas blue represents a negative association with the signature. The black vertical lines each represent a significantly differentially expressed gene.

Abbreviations: *logFC -Log2 fold change, n_Emph- normalized emphysema score. FEV1_P_predicted- Forced Expiratory Volume in one-second Percentage predicted,* PRM^{Emph}*- Parametric Response Mapping derived scores of emphysema,* PRM^{fSAD}*- Parametric Response Mapping derived scores of small airway disease.*

Figure 2: GSVA results of the top 10genes associated with $\mathbf{PRM^{Emph}}$ **scores**. A) genes negatively associated with PRM^{Emph} scores B) genes positively associated with PRM^{Emph} score. The samples colored with red and black in the plot represent 32 asymptomatic "party" smokers and 12 COPD patients, respectively.

Abbreviations: r= Spearman correlation value





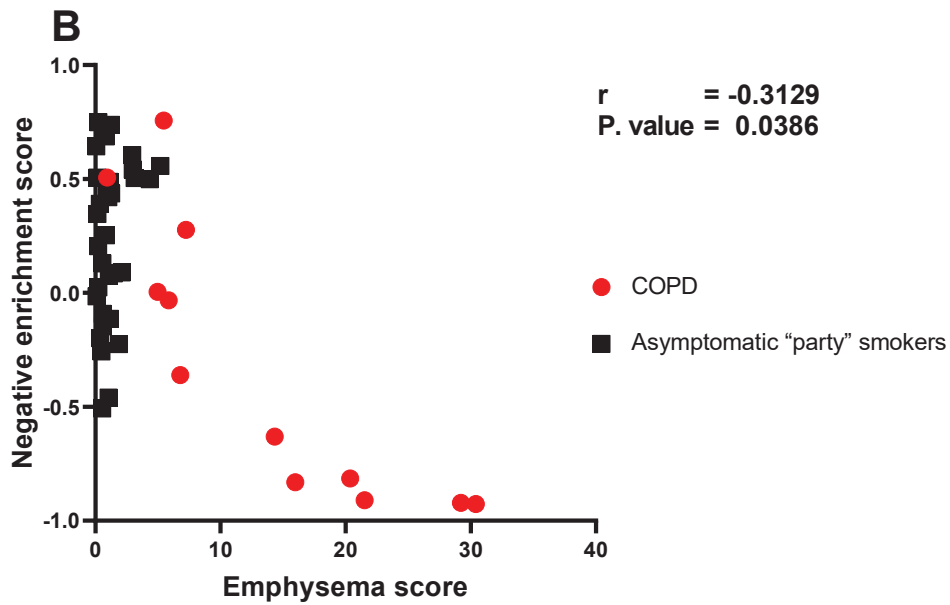


Table 1. Clinical characteristics of the current study population

Character	Asymptomatic smokers	COPD
n	32	12
Male subjects no. (%)	28(87.5)	12(100)
Current smoking, no. (%)	30(93.8)	10(83.3)
Age, mean (SD)	51.28(11)	65.42(7)
PRM^{Emph} score, mean (SD)	1.23(1.25)	13.58(9.95)
FEV1% predicted, mean (SD)	107.94(12.29)	55.29(12.43)
PRM^{fSAD} score, mean (SD)	10.62(10.97)	32.56(6.97)

Abbreviations: SD= standard deviation, PRM^{Emph}- Parametric Response Mapping derived scores of emphysema, FEV1%predicted= Forced Expiratory Volume in one-second percentage predicted, PRM ^{fSAD}- Parametric Response Mapping derived scores of small airway disease

Table 2. Statistical results of top significant genes found in bronchial brushings of party smokers and COPD patients associated with emphysema scores

Gene name	Log FC	t	P.Value	adj.P.Val
SLCO1B3	0.127806024	6.519990726	5.67E-08	5.89E-04
SPRR1A	0.063278543	6.413278613	8.15E-08	5.89E-04
FKBP5	0.06591885	6.3851007	8.96E-08	5.89E-04
CXCL11	0.066597346	6.16666158	1.88E-07	9.28E-04
CLEC4E	0.0693752	5.756387466	7.56E-07	0.002497
CLU	-0.03213479	-5.656230211	1.06E-06	0.002497
SNTG2	0.031901105	5.655277552	1.06E-06	0.002497
CDH2	-0.043848573	-5.644123872	1.11E-06	0.002497
DQX1	0.031684446	5.615244924	1.22E-06	0.002497
C12orf36	0.052959543	5.603764761	1.27E-06	0.002497
MYO3A	0.037012707	5.409883752	2.43E-06	0.004359
ANKRD22	0.047548353	5.334362512	3.13E-06	0.004704
THSD4	-0.046401359	-5.333727566	3.14E-06	0.004704
DKK1	0.050502428	5.315200317	3.34E-06	0.004704
SLC22A10	0.021988434	5.215854455	4.65E-06	0.006119
GUCY1B3	0.025796036	5.192145133	5.04E-06	0.006208
CEP55	0.020556219	5.095856166	6.94E-06	0.007431
GATM	-0.051271784	-5.090310071	7.07E-06	0.007431
EFEMP2	-0.02109123	-5.082125381	7.26E-06	0.007431
CES1	-0.046758077	-5.06965904	7.57E-06	0.007431

Abbreviations: log FC -Log2 fold change, adj.P. Val-Adjusted P-value

Table 3. Top pathways linked with genes significantly associated with PRM^{Emph} signature in bronchial brushings of party smokers and COPD patients

Name of the pathway	Term_id	Adj.P. Val
• **Positively associated pathways**		
Cytokine-mediated signalling pathway	GO:0019221	1.55E-07
Cellular response to cytokine stimulus	GO:0071345	1.53829E-06
Response to cytokine	GO:0034097	7.14025E-06
Defence response to virus	GO:0051607	0.000202424
Response to virus	GO:0009615	0.000341767
Defence response	GO:0006952	0.001182
Immune response	GO:0006955	0.002424
Immune system process	GO:0002376	0.005083
Cellular response to type I interferon	GO:0071357	0.007262
Type I interferon signalling pathway	GO:0060337	0.007262
Response to type I interferon	GO:0034340	0.00924
Negative regulation of multi-organism process	GO:0043901	0.012274
Defence response to another organism	GO:0098542	0.017913
Cornification	GO:0070268	0.018896
Bile acid and bile salt transport	GO:0015721	0.023652
Bile acid and bile salt transport	GO:0015721	0.023652
Response to other organism	GO:0051707	0.034265
Response to external biotic stimulus	GO:0043207	0.034903
Immune effector process	GO:0002252	0.040605
Response to biotic stimulus	GO:0009607	0.043063
• **Negatively associated pathways**		
Extracellular matrix	GO:0031012	0.008048
Collagen-containing extracellular matrix	GO:0062023	0.011341
Constitutive Signalling by NOTCH1 t(7;9) (NOTCH1:M1580_K2555) Translocation Mutant	REAC:R-HSA-2660826	0.039409
Signalling by NOTCH1 t(7;9)(NOTCH1:M1580_K2555) Translocation Mutant	REAC:R-HSA-2660825	0.039409

Abbreviations:adj.P. Val-Adjusted P-value