Identification of novel target genes in human lung tissue involved in chronic obstructive pulmonary disease

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Introduction: As part of a study aimed at illuminating at least some of the complex molecular events taking place in COPD, we screened tissues by means of transcriptome analyses.

Materials and methods: Tissues were subjected to transcriptome analysis. Candidate genes were identified and validated by immunohistochemistry. Primary human lung cells were subjected to stimulation with cigarette smoke extract for further validation by real-time PCR.

Results: Six candidate genes were selected for further investigations: Aquaporin 3 (AQP3), extracellular matrix protein 1 (ECM1), four and a half LIM domain 1 (FHL1), milk fat globule epidermal growth factor 8 (MFGE8), lactadherin, phosphodiesterase 4D-interacting protein (PDE4DIP), and creatine transporter SLC6A8. All six proteins were allocated to distinct cell types by immunohistochemistry. Upon stimulation with cigarette smoke extract, human type II pneumocytes showed a dose-dependent down-regulation of MFGE8, while ECM1 and FHL1 also tended to be down-regulated. Although present, none of the candidates was regulated by cigarette smoke extract in primary human macrophages.

Discussion: MFGE8 turned out to be an interesting new candidate gene in COPD deserving further studies.

Keywords: COPD, transcriptome, MFGE8, CSE, cigarette smoke extract, ETS2

Introduction
The complex disease mechanisms resulting in COPD are poorly understood. We investigated COPD-related changes of the gene expression in tumor-free lung tissues of COPD patients by transcriptome analysis.

Materials and methods
Lung tissue
Tumor-free lung tissue obtained from lobectomy or pneumectomy specimens, with a minimum distance to the tumor of 10 cm, of 19 lung cancer patients, was fixed using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]-glutamic acid buffer-mediated organic solvent protection effect (HOPE) and embedded in paraffin according to DCS Innovative Diagnostik-Systeme protocol (Hamburg, Germany). Fourteen patients (mean age 66 years; 11 males; eight ex-smokers and six current smokers) had concomitant COPD; five patients served as control (mean age 69.5 years, all female and never-smokers). Detailed information is deposited at Gene Expression Omnibus (GSE106986).

This study was conducted using material from non-small-cell lung cancer resections and performed anonymously. Patient tissue was used and all experimental procedures were performed anonymously. Patient tissue was used and all experimental procedures.
were performed with the permission of the local ethical com-
mittee at the University of Lübeck (AZ12-220).

Transcriptome analysis
Microarray analysis was performed using extracted RNA of
human lung tissues. RNA quality was determined with the
Agilent Bioanalyzer. The Low Input Quick Amp Labeling
Kit (Agilent Technologies, Santa Clara, CA, USA) was used
according to the manufacturer’s protocol for cDNA synthesis,
amplification, and labeling with Cyanine 3-CTP (Cy3) during
reverse transcription by T7 RNA polymerase. Cy3-labeled
cRNA was purified using the RNeasy Mini Kit (Qiagen NV,
Venlo, the Netherlands) and the specific activity was calculated
using a NanoDrop 2000 spectrophotometer (Thermo Fisher
Scientific, Waltham, MA, USA). Labeled cRNA was hybrid-
ized to Agilent Human Whole Genome Gene Expression 4×44
k V2 arrays and scanned with a SureScan microarray scanner
(Agilent Technologies) at a resolution of 5 μm. GeneSpring
software version 13 (one-color gene expression protocol)
was used for quantile normalization and statistical analyses
(one-way ANOVA and Benjamini–Hochberg multiple testing
correction, cutoff p-value <0.05). The complete gene list is
deposited at Gene Expression Omnibus (GSE106986).

Histologic analyses
A tissue microarray of HOPE-fixed, paraffin-embedded tissues
of 20 patients was used for analysis. The samples were dewaxed
in 100% isopropanol for 10 min at 60°C. Sections were air
dried, rehydrated in 70% acetone for 10 min at 4°C, and trans-
ferrred into distilled water for 10 min at 4°C and 5 min at room
temperature. Endogenous peroxidases were blocked by incu-
bation in 3% H2O2 for 10 min. The polymer blocking reagent
(Zytomed, Bargteheide, Germany) was incubated 5 min before
the first antibodies were applied. The first antibodies were used
in concentrations of anti-AQP3 (Abcam), anti-MFGE8 (Abcam);
anti-ECM1 (Abcam); anti-SLC6A8 (for: tgggaggtgaccctttgtc/rev:
cgtaggggaatgtaggt), PDE4DIP (for: agccactgtctttgctcagg/rev:
gcttcaccaaggattcacct), FHL1 (for: tttgagctactgggctgtga/rev:
ggccgtgatctgcttgtc), ECM1 (for: gctggattgctacaagaact/rev:
accacactggagcctttacc), IL8 (for: cgaggatgctaatc/rev: cggagctggaggtt),
penicillin/streptomycin, and L-Glutamine) for 16 h. The eluted CD45-
negative P2 cells were seeded on 48-well plates at low
density (4×104 cells/well) in RPMI (10% fetal calf serum,
penicillin/streptomycin, and L-Glutamine) for 16 h cell
stimulation. Cells were harvested and used for RNA isolation.

Quantitative real-time polymerase chain
reaction of stimulated human primary alveolar
pneumocytes type 2 and macrophages
RNA was isolated using the RNeasy Plus Mini Kit (Qiagen)
and quantified with the Agilent Bioanalyzer. The RevertAid
First Strand cDNA Synthesis Kit (Thermo Fisher Scientific)
was used for cDNA synthesis. Quantitative polymerase chain
reaction was implemented with universal Probe Library
equipment (Roche) and the following primers for AQP3 (for:
eccttttacacgccttgg/rev: caggaggggacacatg), ECM1 (for: gct-
ggagggaggttccag/rev: ggctacagttttgtaaggtact), FHL1 (for:
scccttcagactaagacagat/rev: accacagtggatcccttac), IL8 (for:
ggattgtgccgaaggtgct/rev: atggtttctggaggtact), MFGF8 (for:
ffgtggatgctaatc/rev: cggagctggaggtt), PDE4DIP (for: agccactgtctttgctcagg/rev:
ggtctagttttgtaaggtact), ECM1 (for: gctggattgctacaagaact/rev:
cggagctggaggtt), penicillin/streptomycin, and L-Glutamine) for 16 h cell
stimulation. Cells were harvested and used for RNA isolation.

Results
After quantile normalization and ANOVA, 3,974 genes
were found to be differently regulated in COPD patients
Novel target genes in human lung tissue involved in COPD compared to non-COPD patients (Figure 1A). Six candidate genes, which have not been previously evaluated in COPD, were selected for further investigations. 1) Aquaporin 3 (AQP3), a water/glycerol channel, which is upregulated in murine asthma models and involved in the chemokine production of alveolar macrophages.1 2) Extracellular matrix protein 1 (ECM1), a secreted glycoprotein that is expected to be involved in the development of various epithelial tumors.2 3) Four and a half LIM domain 1 (FHL1), a protein that is highly expressed in muscle disorders with the development of respiratory failure.3 4) Milk fat globule epidermal growth factor 8 (MFGE8, lactadherin), a secreted glycoprotein that opsonizes apoptotic cells but also collagen for phagocytes. Furthermore, it counteracts airway smooth muscle hypercontractility through binding to integrin receptors on the muscles in an ovalbumin challenge model of asthma.4 5) Phosphodiesterase 4D-interacting protein (PDE4DIP), a protein that is a major component of skeletal muscles interacting with phosphodiesterase PDE4D. 6) Creatine transporter SLC6A8, a plasma membrane protein

Figure 1 Transcriptome analyses and selected protein candidates in tissue microarrays.
Notes: (A) Heatmap of significantly regulated genes with 2.5-fold induction in individual tissue samples (one-way analysis of variance with Benjamini–Hochberg correction, cutoff p-value <0.05). (B) Histologic analyses of HOPE-fixed lung tissue of COPD patients. a) AQP3, b) ECM1, c) FHL1, d) MFGE8, e) PDE4DIP, and f) SLC6A8. Magnification ×200. The presence of the respective proteins of the candidate genes in the lung tissue should validate the transcriptome results and reveal the cell types expressing the proteins.
Abbreviations: AQP3, aquaporin 3; ECM1, extracellular matrix protein 1; FHL1, four and a half LIM domain 1; HOPE, HEPES-glutamic acid buffer mediated organic solvent protection effect; GOLD, Global Initiative for Chronic Obstructive Lung Disease; MFGE8, milk fat globule epidermal growth factor 8; PDE4DIP, phosphodiesterase 4D-interacting protein.
that transports creatine required for rapid ATP supply into tissues such as muscle and brain.

The corresponding proteins were assigned to distinct cell types by immunohistochemistry of tissue from COPD patients (Figure 1B). AQP3 was detected in type I pneumocytes (P1), type II pneumocytes (P2), macrophages, and bronchi; ECM1 in P2, macrophages, bronchi, and extracellular region; FHL1 in P2, macrophages, bronchi, and capillaries; MFGE8 was present in P1, P2, and bronchi; PDE4DIP in macrophages, bronchi smooth muscles, and capillaries; SLC6A8 in macrophages, bronchi, and capillaries.

In a Gene Set Enrichment Analysis (MSigDB) after Baran et al, we found a potential regulation of three of the six genes (AQP3, ECM1, and FHL1) by the transcription factor ETS proto-oncogene 2 (ETS2). ETS2 was described to be substantially involved in the pathogenesis of pulmonary fibrosis in its phosphorylated form, suggesting that ETS2 might also have a role in peribronchiolar fibrosis in COPD.

We further investigated the possible regulation of the selected genes in a CSE model of primary human lung cells (Figure 2). After CSE stimulation primary human type II pneumocytes exhibited a significant, dose-dependent regulation on the RNA level for one (MFGE8) of the six studied candidates, while ECM1 and FHL1 showed a tendency to be downregulated. Despite the presence of five candidate proteins in alveolar macrophages of COPD patients (all except MFGE8, Figure 1B), none of them were found to be regulated upon CSE stimulation in human primary macrophages. Thus, the protein expression of the chosen candidates in alveolar macrophages appears to be independent of CSE stimulation.

MFGE8 is described as an inhibitor of the inflammasome-induced interleukin 1β (IL1β) production and thus a potential candidate for reducing the inflammatory cytokines with simultaneous clearance of apoptotic cells. Inflammatory mediators such as IL1β, which are increased in lungs and bronchoalveolar lavage fluids of COPD patients, might be promising targets in therapeutic studies.

Particularly the CSE-inducible genes are valuable subjects for further investigations. In conclusion, we found an interesting protein, MFGE8, which is present in type II cells of human lungs and significantly downregulated after CSE stimulation, a possible contribution to the inflammatory manifestations of COPD. Thus, we will investigate in further studies MFGE8 as a potential inhibitor for CSE-induced inflammatory effects on human lungs.

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The authors report no other conflicts of interest in this work.

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