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Stat3 Downstream Genes Serve as Biomarkers in Human Lung Carcinomas and Chronic Obstructive Pulmonary Disease

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Abstract

Smoking causes lung cancer and chronic obstructive pulmonary disease (COPD) that impose severe health problem to humans. Both diseases are related to each other and can be induced by chronic inflammation in the lung. To identify the molecular mechanism for lung cancer formation, a CCSP-rtTA/(Teto)₇Stat3C bitransgenic model was generated recently. In this model, persistent activation of the Stat3 signaling pathway induced pulmonary inflammation and adenocarcinoma formation in the lung. A group of Stat3 downstream genes were identified by Affymetrix GeneChip microarray analysis that can be used as biomarkers for lung cancer diagnosis and prognosis. To determine which human lung cancers are related to the Stat3 pathway, multiple Stat3 downstream genes were screened in human lung cancers (adenocarcinomas and squamous cell carcinomas) and lung tissue with COPD. In both cancer and COPD, the *Stat3* gene was up-regulated. A panel of Stat3-up-regulated downstream genes in mice was up-regulated in human adenocarcinomas, but not in human squamous cell carcinomas. This panel of genes was also modestly up-regulated in lung tissue with COPD from patients with a history of smoking and not up-regulated in those without histories of smoking. Several Stat3-down-regulated downstream genes also showed differential expression patterns in carcinoma and COPD. These studies support a concept that Stat3 is a potent oncogenic molecule that plays a role in formation of lung adenocarcinomas in both mice and humans. The carcinogenesis of adenocarcinoma and squamous cell carcinoma is mediated by different molecular mechanisms and pathways *in vivo*. *Stat3* and its downstream genes can serve as biomarkers for lung adenocarcinoma and COPD diagnosis and prognosis in mice and humans.

Keywords

Stat3; Adenocarcinoma; Squamous Cell Carcinoma; COPD; Biomarkers; Real-Time qRT-PCR

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Conflict of interest

The authors have no potential conflicts of interest.

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Introduction

Lung cancer is one of the biggest public health challenges facing the United States and many other countries. It accounts for 28% of all cancer deaths (more than colonic, mammary, prostatic and pancreatic cancers combined). Although incidence rates have stabilized, more than 173,000 new cases of lung cancer are diagnosed each year, and it continues to be the most common cause of cancer death among men and women, with more than 163,000 people succumbing each year

(http://www.cancer.gov/ncicancerbulletin/NCI_Cancer_Bulletin_052306/page3). Lung cancer is difficult disease to detect in its early stages. In most cases, the tumors are detected at advanced stages and the overall 5-year survival rate is ~15 percent. Thus, it is essential to better understand the early events that initiate lung carcinogenesis and to find biomarkers for early lung cancer detection. Approximately 80% of human lung cancers are adenocarcinomas and squamous cell carcinomas. Human chronic obstructive pulmonary disease (COPD) (especially with smoking history) is a pre-cancer disease and is the fourth leading cause for human death in the United States. Importantly, both COPD and lung cancer can be induced by repeated exposure to smoking. It is well-known that smokers with COPD are elevated at risk to develop lung cancer. Both lung diseases are associated with inflammation in the lung. Nevertheless, the molecular mechanisms connecting inflammation and COPD and lung cancer are poorly understood.

Inflammation is a protective process to facilitate pathogen clearance and to repair tissue injury in the lung. The magnitudes of inflammatory responses are precisely regulated by pro- and anti-inflammatory molecules. Exuberant inflammation can cause severe consequences and lead to carcinogenesis. One commonly induced pro-inflammatory gene group during pulmonary inflammation is the interleukin 6 (IL-6) family of cytokines. Pro-inflammatory IL-6 cytokines share the common gp130 receptor subunit [1,2]. Upon binding to their cell-surface receptors, IL-6 family members trigger Stat3 phosphorylation by Janus-activated kinases (JAKs). As we demonstrated previously, IL-6, JAKs and Stat3 are co-expressed in alveolar type II (AT II) epithelial cells in the lung [3–5]. Over-expression of dominant negative Stat3 to subvert endogenous Stat3 activity in respiratory epithelial cells causes alveolar destruction, suggesting its role in maintaining alveolar structure and function [4]. On the other hand, persistent activation of Stat3 signaling has the potential to induce lung cancer [6]. Carcinogenesis is a complex process that is influenced by both genetics and the tissue microenvironment. Based on our studies in the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model, Stat3 induces lung adenocarcinoma formation in two steps. The first step involves aberrant expression of cytokines/chemokines and abnormal inflammatory cell infiltration, which hijack the immune system to inhibit immune surveillance and promote neoplasia. The second step involves reactivation of developmental genes that stimulate epithelial cell over-growth. These observations are in agreement with previous findings that *Stat3* is an oncogene [7].

Although extensive surveys of primary tumors and cell lines derived from tumors indicate that inappropriate activation of Stat3 occurs with surprisingly high frequency (50 to 90%) in a wide variety of human cancers, including lung cancers [8,9] [10–13], no Stat3 downstream gene has been used to link the Stat3 pathway to lung cancer formation. During characterization of the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model, multiple Stat3 up or down-regulated downstream genes were identified by Affymetrix GeneChip Microarray analysis, which have been confirmed by Real-Time PCR and immunohistochemistry analyses [6]. These genes can potentially serve as molecular biomarkers for cancer patient diagnosis or prognosis.

In this report, a panel of Stat3-most-up-regulated and down-regulated downstream genes in the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model was studied in human lung carcinomas and COPD tissues to assess if they can be used as biomarkers for diagnostic or

prognostic purposes. Although expression of the *Stat3* gene was up-regulated in human carcinomas and tissues with COPD, its downstream genes were differentially expressed in adenocarcinomas and squamous cell carcinomas and in tissues with COPD from smokers and non-smokers and therefore can be used to distinguish them. These studies also suggest that *Stat3* and its downstream genes more actively participate in the carcinogenesis of adenocarcinomas than in squamous cell carcinomas in the lungs. This is in agreement with observations made in the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model in which only adenocarcinomas were observed. In COPD, smokers seem more skewed towards the COPD-adenocarcinoma transition based on Stat3-up-regulated gene analysis.

Materials and Methods

Human Tissue

Human lung tissues were obtained from the Indiana University Simon Cancer Center Tissue Procurement Lab and the IU/Lilly Clinically-Annotated Tissue Databank. The protocol for an exempt study using these human tissues has been approved by the IUPUI/Clarian Institutional Review Committee. All samples are surgically collected from human tissues. Normal samples represent lung tissues without cancer and COPD.

Isolation of total RNAs and Real-Time PCR

Total RNA from whole lung tissues was purified using the Qiagen total RNA purification kit as recommended by the manufacturer (Qiagen, Valencia, CA). All reverse transcription reactions were set up using the TaqMan[®] Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A total of 2 µg RNA from human whole lung tissues were used in a 100 µl reaction. The cDNA synthesis reactions were carried out using a GeneAmp[®] 9700 Thermocycler (Applied Biosystems) with a suggested cycling protocol of 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. For Real-Time PCR, a total of 2 µl of cDNA were amplified by a pair of sequence-specific DNA oligonucleotide primers for each molecule in the RT reaction in a 50 µl reaction containing SYBR[®] Green PCR Master Mix (Applied Biosystems). The quality of each primer pairs was checked by dissociation curves in a pre-run study. GAPDH primers were used as an endogenous control and normalizer for all cDNA samples. Reactions were analyzed using the Relative Quantification Assay of 7500 System Sequence Detection Software for the 7500 Real-Time PCR System (Applied Biosystems). The default cycling protocol for this software was 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Statistical differences among different human samples were measured by ANOVA. *P* < 0.05 was considered significant.

Human Real-Time PCR Primers

Cbln1:

Upstream: 5'-AAT CGC ACC ATG ATC ATC TAC TTC-3'

Downstream: 5'-TGC GTT CTG AAT CAA AGT TGT TC-3'

Cldn2:

Upstream: 5'- GAA ACT GAT TGG CCC TGG AA -3'

Down Stream: 5'- AAT TAG TCT GGA CAC TGT GGA GTC AT-3'

Fgl1:

Upstream: 5'- AGG GAA CTG CGC AGA AGA AG-3'

Down Stream: 5'- AGG TTT GCA GAG TGA CAC CTG TT -3'

Foxa3:

Upstream: 5'- AGG GAA CTG CGC AGA AGA AG -3'

Downstream: 5'- CCA AAC CCC ACG TCC AGT T-3'

Gjb1:

Upstream: 5'- GTG ACA GGG AGG TGT GAA TGA G-3'

Downstream: 5'- ACG CCA CTG AGC AAG GTG TAC-3'

HNF4a:

Upstream: 5'- TCT TAC GAC ACG TCC CCA TCA-3'

Downstream: 5'- GCT GAC ACC CAG GCT GTT G-3'

Krt18:

Upstream: 5'- AAAGGACTGAGGAGCGCAACT- 3'

Downstream: 5'- CGCTAGCCCCCTCTTCCA- 3'

Lor:

Upstream: 5'- AATAGATCCCCCAGGGTACCA- 3'

Downstream: 5'- CGGTGCCCCTGGAAAAC- 3'

Rdhe2:

Upstream: 5'- AGC CAA AAG GAA GGA GTG TAT AGA GT -3'

Downstream: 5'- TGG AAA CAT CGC CGA CTT CT-3'

Stat3:

Upstream: 5'- GGCCCCCTCGTCATCAAGA- 3'

Downstream: 5'- TTTGACCAGCAACCTGACTTTAGT- 3'

Tmem27:

Upstream: 5'- GGC TGC TCT TTT TTC TGG TGA CT -3'

Downstream: 5'- GCA TTT TCT GCA CCT GGT TGA -3'

Ttr:

Upstream: 5'- CTA CGG GCA CCG GTG AAT C-3'

Downstream: 5'- GCC TCG GAC AGC ATC TAG AAC T-3'

Results

Pre-screening of Human samples

Samples from 20 normal human lung tissues, 30 human pulmonary adenocarcinomas, 29 human pulmonary squamous cell carcinomas, 29 human lung tissues with COPD from non-smokers and 33 human lung tissues with COPD from smokers were obtained. The quality of all human samples was pre-screened by two standards. First, the total RNA was purified. Only samples with high yields of RNA were selected for the Real-Time PCR study. Second, the selected samples were subject to initial Real-Time PCR analyses for GAPDH mRNA expression by using sequence – specific primers. The samples with low expression levels of GAPDH mRNA were disqualified for further analysis. Based upon this screening, 7 of the

normal samples, 4 of the adenocarcinomas, 3 of the squamous cell carcinomas, 9 COPD lung samples from non-smokers and 3 from smokers were eliminated from the study.

Up-regulation of the *Stat3* gene

Since in the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model, Stat3C over-expression caused lung tumor formation, it is important to evaluate if up-regulation of Stat3 is associated with lung cancers and COPD in humans. The expression levels of Stat3 mRNA were examined in human adenocarcinomas and squamous cell carcinomas vs normal human samples by quantitative Real-Time PCR, Figure 1. In comparison with normal human lungs, the average of Stat3 mRNA expression levels was 6.59-fold higher in adenocarcinomas and 9.25-fold higher in squamous cell carcinomas. The average of Stat3 mRNA expression levels was 3.34-fold higher in lung tissues with COPD from non-smokers and 3.68-fold higher in lung tissues with COPD from smokers. A more detailed comparison of Stat3 mRNA expression between different tumor stages, differentiation grades of the tumor and the smoking history is presented in Table 1.

Changes of the Stat3 up-regulated genes in human adenocarcinomas

To further evaluate Stat3 signaling in pulmonary adenocarcinoma and squamous cell carcinoma, the expression levels of Stat3 downstream genes was studied. After gene search and sequence comparison, 8 Stat3 most up-regulated downstream genes in CCSP-rtTA/(teto)₇Stat3C bitransgenic mice were selected for testing. These genes were *Cbln1* (*Cerebellin 1 precursor*), *Cldn2* (*Claudin 2*), *Fgl1* (*Fibrinogen-like protein 1 precursor*), *Foxa3* (*forkhead box A3*), *Gjb1* (*Gap junction beta 1 protein*), *HNF4a* (*hepatic nuclear factor 4 alpha*), *Tmem27* (*Transmembrane protein 27*) and *Ttr* (*Transthyretin precursor*). We first tested the expression levels of these Stat3-up-regulated downstream genes in adenocarcinomas by quantitative Real-Time PCR. In comparison with normal human lungs, the averages of Stat3-up-regulated gene expression levels were all significantly higher in human adenocarcinomas, ranging from 2-fold to 8-fold higher, Figure 2. In contrast to adenocarcinomas, the average expression levels of most Stat3-up-regulated genes in squamous cell carcinomas were significantly reduced (*Cbln1*, *Cldn2*, *Fgl1*, *Gjb1*, *HNF4a* and *Tmem27*). Only *Foxa3* and *Ttr* showed modest average increases compared to normal human lungs, Figure 3.

Changes of the Stat3-up-regulated genes in lung tissue with COPD

Stat3 up-regulation was observed in lung tissues with COPD from both smokers and non-smokers, as demonstrated in Figure 1. To see if the average expression levels of Stat3-up-regulated genes were altered in lung tissues with COPD, the samples were examined by quantitative Real-Time PCR. In lung tissue with COPD from non-smokers, the average expression levels of most Stat3-up-regulated genes remained unchanged (*Cbln1*, *Cldn2*, *Fgl1*, *Gjb1* and *Ttr*). The average expression levels of *Foxa3*, *HNF4a* and *Tmem27* genes were modestly increased compared to normal human lungs, Figure 4. In contrast, the average expression levels of Stat3-up-regulated genes were modestly increased in lung tissues with COPD from smokers (*Cbln1*, *Cldn2*, *Fgl1*, *Foxa3*, *Gjb1*, *Tmem27* and *Ttr*). Only the expression of the *HNF4* gene remained unchanged, Figure 5.

Changes of the Stat3-down-regulated genes in the lung with COPD and in adenocarcinoma and squamous cell carcinoma

In addition to Stat3-up-regulated genes, three Stat3-down-regulated genes in CCSP-rtTA/(teto)₇Stat3C bitransgenic mice were also tested in the adenocarcinomas, squamous cell carcinomas and lung tissue with COPD by quantitative Real-Time PCR. These were *Krt14* (*Keratinocyte differentiation associated protein*), *Lor* (*Loricrin*) and *Rdhe2* (*Retinal short chain dehydrogenase reductase 2*). The average expression levels of the *Krt14* (Figure 6A)

and *Lor* (Figure 6B) genes were significantly decreased in all samples. The average expression level of the *Rdhe2* gene was decreased only in adenocarcinomas and squamous cell carcinomas (Figure 6C).

Discussion

In this study, *Stat3* gene up-regulation was observed in human pulmonary adenocarcinomas and squamous cell carcinomas (Figure 1), suggesting that Stat3 plays a role in initiation and progression of these cancers. This is in agreement with our previous findings in animals. Up-regulation of Stat3 in the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model induced lung adenocarcinomas [6]. The Stat3 process of carcinogenesis is initiated by increased expression of a subset of pro-inflammatory cytokines, chemokines and their receptors. These molecules recruit inflammatory cells into the lung and change the local microenvironment in favor of tumor growth [6]. Since Stat3 signaling can be activated by upstream pro-inflammatory cytokines (e.g. IL-6 family members) and growth factors (e.g. epithelial growth factor or EGF), which are often up-regulated in various pulmonary diseases (e.g. bacterial infection, chronic inflammation), our results in humans and mice provide an insight into the mechanism whereby lung cancers are influenced and induced by other forms of diseases.

In the late stage of Stat3-induced lung adenocarcinomas, multiple Stat3 downstream genes were either up-regulated or down-regulated in the CCSP-rtTA/(teto)₇Stat3C bitransgenic mouse model. To investigate whether or not these genes can be used as biomarkers in human pulmonary adenocarcinomas and squamous cell carcinoma, several Stat3-most-up-regulated or down-regulated genes were chosen for investigation. Expression of Stat3-up-regulated genes (*Cb1n1*, *Cldn2*, *Fgl1*, *Foxa3*, *Gjb1*, *HNF4a*, *Tmem27* and *Ttr*) agreed well between mouse and human adenocarcinomas (Figure 2). On the other hand, most of these genes (*Cb1n1*, *Cldn2*, *Fgl1*, *Gjb1*, *HNF4a* and *Tmem27*) were significantly down-regulated in human squamous cell carcinomas (Figure 3). From among the Stat3-down-regulated genes, *Krt14*, *Lor* and *Rdhe2* were all down-regulated in both adenocarcinomas and squamous cell carcinomas (Figure 6). Therefore, some common molecular pathways under the control of Stat3 contribute to carcinogenesis of both adenocarcinomas and squamous cell carcinoma in the lung. In a combined panel, these Stat3 downstream genes could serve as biomarkers for differentiating between pulmonary adenocarcinoma and squamous cell carcinoma in humans.

Lung cancer and COPD are closely related in humans, especially among smokers. The connection between the two diseases needs to be studied in order to elucidate COPD-cancer transition. Since both diseases are highly associated with inflammation, *Stat3* and its downstream genes may serve as a common mechanism to connect both. Indeed, Stat3 up-regulation was observed in lung tissue with COPD from both smokers and non-smokers (Figure 1). In non-smokers, expression of most of the Stat3-up-regulated genes remained unchanged, (Figure 4). In contrast, most of these genes showed modest increases in lung tissues with COPD from smokers (Figure 5), although to less extent compared to adenocarcinomas. It appears that lung tissues with COPD from smokers is more skewed toward a COPD-adenocarcinoma transition. The degree of up-regulation of *Stat3* and its downstream genes may serve as a useful parameter to predict the COPD-adenocarcinoma transition in human patients. Stat3 up-regulation may serve as an inflammatory warning sign for a pre-cancerous condition in the lung. The transition between COPD and adenocarcinomas may depend on the levels of Stat3 downstream gene expression. Among the Stat3-down-regulated genes, *Krt14* and *Lor* genes were down-regulated, whereas *Rdhe2* was modestly up-regulated in lung tissues with COPD. These parameters may also help to predict the COPD-adenocarcinoma transition.

Although Stat3 has been well studied in various forms of cancer, the functional roles of its downstream genes are poorly understood in cancers, including lung cancer. *Foxa3* and

HNF4 are developmental genes. Up-regulation of these genes reinforced a concept that cancer cell ontogeny is determined by gene expression that recapitulates events important in embryonic development [14]. One highly induced protein in human adenocarcinomas is Ttr. Ttr is a beta-sheet rich protein with a plasma half life of 1.9 days. It behaves as a tetramer and binds to retinol binding protein (RBP) and thyroxine in plasma. Ttr is an anti-acute phase protein, and its concentration is influenced by various conditions of inflammation and infection [15]. Cb1n1 belongs to the C1q/tumor necrosis factor superfamily of secreted proteins. Its derivative hexadecapeptide cerebellin has features of a neuropeptide and has been reported to be present in the brains of many vertebrate species [16]. Cldn2 is a tetraspan transmembrane protein of tight junctions. They determine the barrier properties of cell-cell contact existing between the plasma membranes of two neighboring cells, such as occur in endothelia or epithelia [17]. Another gap junction protein Gjb1 was also up-regulated in human adenocarcinomas. Fgl1 has been implicated as a mitogen for liver cell proliferation. IL-6 induces FGL1 expression in a pattern similar to those of acute phase reactants. Following induction of acute inflammation, serum FGL1 levels are enhanced. Although FGL1 associates with the fibrin matrix, approximately 20% of the total plasma FGL1 remains free. Its presence in bound and free forms in the blood implies biological roles that extend beyond the liver regeneration [18,19]. Tmem27 (collectrin) is a key regulator of renal amino acid uptake and involved in the process of progressive renal failure and renal organogenesis [20,21]. Among Stat3-down-regulated genes, *Krt19* and *Lor* are epithelial cell differentiation markers. It has been reported that tobacco smoke down-regulates *Lor* in epithelial cell hyperplasia and squamous metaplasia [22]. Rdh2f is a sterol regulatory element-binding protein (SREBPs) induced membrane-bound enzyme required for the cellular synthesis and uptake of cholesterol and unsaturated fatty acids. The enzyme specifically uses NADPH to reduce a variety of short-chain aldehydes and retinaldehydes [23].

Although this group of genes has various functions in cells, in a combinatory way, they define a specific pathway for formation of adenocarcinoma and COPD in the lung. Based on our studies, even though the average expression levels of these biomarker genes are higher or lower in human adenocarcinoma and squamous cell carcinoma and COPD samples, the individual expression level of each biomarker gene varied among the conditions. For more accurate prediction of diagnosis and prognosis, it is recommended that a panel of biomarker genes is used. Biomarker is only one valuable parameter to assist diagnosis and prognosis. It has to be used in combination with other means to accurately predict lung cancer formation.

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Figure 1. Stat3 mRNA expression in adenocarcinoma, squamous cell carcinoma and COPD
 Expression of Stat3 mRNA was measured by quantitative Real-Time PCR and normalized by GAPDH mRNA expression. Numbers in each column represent average ΔC_t . Average fold changes were determined by $2^{(\Delta\Delta C_t)}$, in which $\Delta\Delta C_t = \Delta C_t(\text{normal human samples}) - \Delta C_t(\text{cancer or COPD samples})$. Numbers on the bottom represent average fold changes compared to the normal human samples. Normal human samples were set up as one. Nor: normal samples. COPD: COPD samples from smokers. Benign: COPD samples from non-smokers. Adeno: adenocarcinoma. Squa: squamous cell carcinoma.

Figure 2. Expression of Stat3-up-regulated genes in adenocarcinoma

Expression of Stat3-up-regulated genes was measured by quantitative Real-Time PCR and normalized by GAPDH mRNA expression. Numbers in each column represent average ΔC_t . Average fold changes were determined by $2^{(\Delta\Delta C_t)}$, in which $\Delta\Delta C_t = \Delta C_t(\text{normal human samples}) - \Delta C_t(\text{adenocarcinoma samples})$. Numbers on the bottom represent average fold changes compared to the normal human samples. Normal human samples were set up as one. Nor: normal samples. Adeno: adenocarcinoma samples.

Figure 3. Expression of Stat3-up-regulated genes in squamous cell carcinoma

Expression of Stat3 up-regulated genes was measured by quantitative Real-Time PCR and normalized by GAPDH mRNA expression. Numbers in each column represent average ΔC_t . Average fold changes were determined by $2^{(\Delta\Delta C_t)}$, in which $\Delta\Delta C_t = \Delta C_t(\text{normal human samples}) - \Delta C_t(\text{squamous cell carcinoma samples})$. Numbers on the bottom represent average fold changes compared to the normal human samples. Normal human samples were set up as one. Nor: normal samples. Squa: squamous cell carcinoma samples.

Figure 4. Expression of Stat3-up-regulated genes in COPD from non-smokers

Expression of Stat3 up-regulated genes was measured by quantitative Real-Time PCR and normalized by GAPDH mRNA expression. Numbers in each column represent average ΔC_t . Average fold changes were determined by $2^{(\Delta\Delta C_t)}$, in which $\Delta\Delta C_t = \Delta C_t(\text{normal human samples}) - \Delta C_t(\text{benign COPD samples})$. Numbers on the bottom represent average fold changes compared to the normal human samples. Normal human samples were set up as one. Nor: normal samples. Benign: COPD from non-smoker samples.

Figure 5. Expression of Stat3-up-regulated genes in COPD from smokers

Expression of Stat3 up-regulated genes was measured by quantitative Real-Time PCR and normalized by GAPDH mRNA expression. Numbers in each column represent average ΔC_t . Average fold changes were determined by $2^{(\Delta\Delta C_t)}$, in which $\Delta\Delta C_t = \Delta C_t(\text{normal human samples}) - \Delta C_t(\text{COPD from smokers})$. Numbers on the bottom represent average fold changes compared to the normal human samples. Normal human samples were set up as one. Nor: normal samples. COPD: COPD samples from smokers.

Figure 6A

Figure 6B

Figure 6C

Figure 6. Expression of Stat3-down-regulated genes in adenocarcinomas, squamous cell carcinoma and COPD

Expression of Stat3-down-regulated *Krt19* (A), *Lor* (B) and *Rdhe2* (C) genes was measured by quantitative Real-Time PCR and normalized by GAPDH mRNA expression. Numbers in each column represent average ΔCt . Average fold changes were determined by $2^{(\Delta\Delta Ct)}$, in which $\Delta\Delta Ct = \Delta Ct(\text{normal human samples}) - \Delta Ct(\text{cancer or COPD samples})$. Numbers on the bottom represent average fold changes compared to the normal human samples. Normal human samples were set up as one. Nor: normal samples. COPD: COPD samples from smokers. Benign: Benign COPD samples from non-smokers. Adeno: adenocarcinoma. Squa: squamous cell carcinoma.

Table 1
Stat3 mRNA expression in different tumor stages, differentiation grades and smoking history

Diagnosis	Stat3 Expression	
Adenocarcinoma	Average Δ Ct	Average Fold Change
<u>Tumor Stage</u>		
I = 12	4.36 $\pm$ 1.09	5.06
II = 2	3.71 $\pm$ 2.06	4.96
III = 5	4.39 $\pm$ 0.94	7.94
<u>Nuclear Grade</u>		
1/3 = 4	4.40 $\pm$ 0.56	4.92
2/3 = 13	4.28 $\pm$ 1.12	5.36
3/3 = 10	4.11 $\pm$ 1.40	6.02
<u>Smoking History</u>		
Yes = 20	4.43 $\pm$ 0.89	4.82
No = 0	n/a	n/a
Not provided = 8	3.51 $\pm$ 1.28	9.13
Squamous Cell Carcinoma		
<u>Tumor Stage</u>		
I = 7	5.16 $\pm$ 0.67	2.91
II = 2	5.69 $\pm$ 1.57	2.01
III = 1	6.26 $\pm$ 0.57	1.36
<u>Nuclear Grade</u>		
1/3 = 0	n/a	n/a
2/3 = 10	4.66 $\pm$ 1.16	4.11
3/3 = 8	5.01 $\pm$ 0.81	3.23
<u>Smoking History</u>		
Yes = 8	4.92 $\pm$ 1.01	3.43
No = 0	n/a	n/a
Not provided = 11	4.91 $\pm$ 1.01	3.46
COPD	4.96 $\pm$ 1.09	3.34
COPD w/o Smoking	4.82 $\pm$ 1.09	3.68