Online Data Supplement:

Hypoxia inducible factor-1 α in human emphysema lung tissue

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Methods

Real-Time Reverse Transcriptase-Polymerase Chain Reaction Analysis of lung tissue

Fresh frozen lung tissue samples were immersed in RNA later ® - ice kit according to the manufacturer's instructions. After overnight soaking, the tissue samples were homogenized by FastPrep-24® (MP biomedicals, Solon, OH). Total tissue RNA was obtained by using RNeasy® Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RT was performed using 0.5 μg of total RNA. cDNA synthesis was performed using the high capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. The temperature profile was comprised of annealing at room temperature for 10 min, extension at 37°C for 120 min, and termination at 85°C for 5 min. Polymerase chain reaction (PCR) was performed with the resulting RT products using specific oligonucleotide primers for HIF-1 α, VEGF, glucose transporter-1 (GLUT-1) and β actin which were designed using the computer software Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). The sequence of these forward and reverse primers is shown in supplemental table 1. All PCR reactions were performed with a LightCycler PCR system (Roche Diagnostics, Meylan, France) using DNA binding SYBR Green dye for the detection of PCR products. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 94°C for 15 s,

annealing at 56°C for 15 s, and extension at 72°C for 15 s. The β actin and GAPDH genes were used as reference. The PCR products were isolated from the LightCycler 480® multiwell plate and visualized by electrophoresis on 1.5% agarose gels with ethidium bromide staining to confirm the products.

Western blot analysis of lung tissue

Fresh frozen human lung tissues were aliquoted as 50mg samples, and the specimens were homogenized on ice using the TissuemiserTM (Fisher Scientific, Waltham, MA). For the extraction of both nuclear and cytoplasmic proteins, we used NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's instructions. Obtained cytoplasmic and nuclear supernatants were analyzed for protein content using a Bradford protein assay [1]. Each sample was quantified, and then 15 μg of nuclear protein and 50 μg of cytoplasmic protein were loaded onto each lane of a 4–12% Bis-Tris Nupage gel with MES SDS running buffer, according to the manufacturer's protocol. The gel was transferred to a PVDF membrane by electrophoresis at 100 V for 1 to 1.5 h. The membrane was blocked in PBS, 0.1% Tween 20 (PBS-T), and 5% nonfat milk at room temperature for 1h. All antibodies were diluted in the same buffer (PBS-T). The membrane was then probed with mouse anti-p53 monoclonal antibody (1: 1000), rabbit anti-

VEGF polyclonal antibody (1: 500), rabbit anti-pAkt polyclonal antibody (1: 1000), mouse anti-HIF-1α monoclonal antibody (1: 400), rabbit anti-HDAC2 polyclonal antibody (1: 1000), mouse anti-Hsp90 monoclonal antibody (1: 1000), rabbit anti-FIH-1 polyclonal antibody (1: 1000), rabbit anti-PHD-2 polyclonal antibody (1:1000), goat anti-Lamin B polyclonal antibody (1: 1000), and mouse anti–ß-actin monoclonal antibody (1:5000), and then incubated for 1h at room temperature. After incubation, the membrane was washed three times with PBS-T and incubated with horseradish peroxidase–conjugated goat anti-rabbit or mouse, and donkey anti-goat IgG (1:2000) for 2h at room temperature. After washing three times with PBS-T, an ECL system was used for detection of the proteins.

Detection of the HIF-1α protein by western blot analysis

We used a mouse monoclonal anti-HIF-1α antibody (Santa Cruz, sc-53546). Since this antibody is mouse monoclonal and shows a few nonspecific signals, we could do a long ECL exposure (3 to 5 minutes, plus-ECL by PerkinElmer Inc.) for the detection of expression of HIF-1α even in normoxia condition. Whole cell lysates of cobalt chloride stimulated HeLa cells were used as a HIF-1α positive control.

HIF-1 α immunohistochemistry

Paraffin embedded formalin-fixed resected lung tissue from patients with or without COPD were randomly sectioned (5 μm). After paraffin removal in xylene, the sections were rehydrated and submitted to microwave treatment (800 W/15 min) in 10 mM citric acid monohydrate solution. After quenching of endogenous peroxidase with 3% H_2O_2 for 15 minutes, the sections were incubated with anti-HIF-1 α mouse monoclonal antibody (sc-53546, 1: 50 dilution, Santa Cruz, CA). After incubation with the primary antibody, immunodetection was performed using the Vectastain® Elite ABC-Peroxidase Kit with diaminobenzidine (DAB) as the substrate completing the immunohistochemistry. For a negative control, the primary antibody was omitted.

Images were acquired with a Carl Zeiss AxioCam color camera (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) and analyzed using AxioVision® Imaging System software (Carl Zeiss Vision GmbH). 10 random lung fields per slides were captured at a 400 x magnification, then AxioVision® Imaging System software was used to measure the total length of alveolar perimeters in pixels per μm^2, and then the number of HIF-1α positive cells was counted by an observer who was blinded to the patients' diagnosis. The total length of alveolar perimeter of each captured pictures were used as the reference of HIF-1α positive cells.

Reference for supplementary method

1. Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976; **72**: 248-254.

Supplementary Table 1. Characteristics of patients

Patients	Sex	Age	Categorization	Pathological diagnosis	CT diagnosis	Smoking (pack-years)	%FEV1
1†	F	62	no COPD	No evidence of emphysema	no evidence of emphysema	0	101
2†	F	80	no COPD	very mild emphysema, centrilobular	no evidence of emphysema	0	151
3†	F	58	no COPD	very mild emphysema, centrilobular	no evidence of emphysema	0	98
4	F	40	no COPD	very mild emphysema, centrilobular	no evidence of emphysema	0	104
5†	F	87	no COPD	very mild emphysema, centrilobular	ND	0	111
6	M	53	no COPD	very mild emphysema, centrilobular	no evidence of emphysema	0	131
7	F	61	no COPD	No evidence of emphysema	no evidence of emphysema	0	NA
8*†	M	62	mild COPD	emphysema, centrilobular	ND	40	87
9†	M	78	mild COPD	emphysema, centrilobular	central distribution of CLE	35	79
10†	M	74	mild COPD	emphysema, centrilobular	ND	60	88
11†	M	63	mild COPD	emphysema, centrilobular	evenly distribution of CLE	25	74
12†	M	81	mild COPD	emphysema, centrilobular	central distribution of CLE	10	67
13*	F	61	mild COPD	emphysema, centrilobular	evenly distribution of CLE	40	83
14	F	55	severe COPD	emphysema, centrilobular	central distribution of CLE	40	31
15	M	58	severe COPD	emphysema, centrilobular	central distribution of CLE	80	37
16†	F	64	severe COPD	emphysema, centrilobular	central distribution of CLE	40	27
17	F	48	severe COPD	emphysema, centrilobular	central distribution of CLE	30	23
18	F	57	severe COPD	emphysema, centrilobular	ND	NA	13
19*	F	55	severe COPD	emphysema, centrilobular	evenly distribution of CLE	20	17

20	M	62	severe COPD	emphysema, centrilobular	central distribution of CLE	60	12
21	M	60	severe COPD	emphysema, centrilobular	central distribution of CLE	90	10
22	F	63	severe COPD	emphysema, centrilobular	central distribution of CLE	66	14
23	M	56	severe COPD	emphysema, centrilobular	evenly distribution of CLE	30	27
24	F	61	severe COPD	emphysema, centrilobular	evenly distribution of CLE	60	29
25	M	71	severe COPD	emphysema, centrilobular	central distribution of CLE	120	16
26	F	63	severe COPD	emphysema, centrilobular	central distribution of CLE	100	19

Definition of abbreviations; ND=no data, NA=not available, CLE=centrilobular emphysema

* ; Patients with history of steroid use.

† ; Tissue associated with lung cancer.

Supplementary Table 2. Primer sequences used for real-time quantitative PCR

gene name	forward primer	reverse primer
HIF-1 α	5' -CCCATTTTCTACTCAGGACACAG- 3'	5'- CTGATCGAAGGAACGTAACTGG -3'
VEGF	5'-TTGCCTTGCTGCTCTACCTC-3'	5'-ATTCTGCCCTCCTCCTTCTG-3'
GLUT-1	5'-GAGCCCAGCAGCAAGAAG-3'	5'-TGATGACTCCAGTGTTGTAGCC-3'
β actin	5'-GCAAGCAGGAGTATGACGAG-3'	5'-CAAATAAAGCCATGCCAATC-3'

Supplementary Table 3. Regression analysis of the relationship between proteins

	all tissue samples (n=26)	samples from patients with normal lung function and patients with severe COPD (n=20)	samples from patients with mild and severe COPD (n=19)
p53 / HDAC2	**R_2=0.20, P<0.05**	R_2=0.18, P=0.06	R_2=0.07, P=0.26
p53 / HIF-1α	R_2=0.02, P=0.46	R_2=0.05, P=0.33	R_2=0.09, P=0.19
Hsp90 / HDAC2	R_2=0.06, P=0.22	R_2=0.03, P=0.44	R_2=0.09, P=0.21
Hsp90 / HIF-1α	R_2=0.01, P=0.57	R_2=0.02, P=0.47	R_2=0.007, P=0.73

Supplementary data figure legends

Figure 1. Western blot analysis of cytoplasmic and nuclear proteins (A, upper; lane from left, Patients #1-6, #8-19, lower; lane from left, Patients #7, #20-26)(B, upper; lane from left, Patients #1-6, #8-19, lower; lane from left, Patients #7, #20-26). VEGF, pAkt, HDAC2, and HIF-1 α were investigated. When the samples were applied to the gel, one protein sample which we had previously used (i.e. the sample from patient #1) was also applied to the other set of the cohort for adjustment of the densitometoric analysis. The bar graph shows the ratios of p53 protein expression relative to ß-actin protein. Data are expressed as mean ± SE. *P < 0.05 versus No COPD.

Figure 2. Western blot analysis of cytoplasmic protein (upper; lane from left, Patients #1-6, #8-19, lower; lane from left, Patients #7, #20-26). P53, Hsp90, PHD-2, and FIH-1 proteins were investigated. A significant increase in lung tissue p53 was observed when samples from patients with severe COPD and normal lung function were included (P<0.05).

Figure 3. Relationship between pack year smoking history and HIF-1α protein in COPD lungs. Open squares=samples from patients with mild COPD; closed diamonds=samples from patients with severe COPD.

Figure 4. Individual FEV1 % predicted was shown.