Lung Cancer xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Angiogenic markers in breath condensate identify non-small cell lung cancer

C. Gessner[a], B. Rechner[b], S. Hammerschmidt[a], H. Kuhn[a], G. Hoheisel[a], U. Sack[b], P. Ruschpler[b], H. Wirtz[a,*]

[a] Department of Respiratory Medicine, University of Leipzig, Liebigstrasse 20, 04103 Leipzig, Germany
[b] Institutes of Clinical Immunology and Transfusion Medicine, University of Leipzig, Johannisallee 30, 04103 Leipzig, Germany

ARTICLE INFO

Article history:
Received 26 December 2008
Received in revised form 10 June 2009
Accepted 14 June 2009

Keywords:
Exhaled breath condensate
Multiplex bead based immunoassay
Angiogenic markers
Lung cancer
Biomarkers
VEGF
bFGF
Angiogenin

ABSTRACT

Early recognition of lung cancer is a prerequisite for any strategy to improve lung cancer treatment outcome. Here we report a cross-sectional study intended as a proof of principle investigation using breath based detection (exhaled breath condensate, EBC) of angiogenic markers (VEGF, bFGF, angiogenin), TNF-α and IL-8 to discriminate 74 individuals, with confirmed presence or absence (X-ray, CT) of non-small lung cancer (NSCLC). Levels of angiogenic markers bFGF, angiogenin and VEGF in EBC significantly discriminated between 17 individuals with newly detected NSCLC versus stable and exacerbated chronic obstructive pulmonary disease (COPD) patients as well as healthy volunteers. Levels of IL-8 and TNF-α in EBC indicated acute inflammation, e.g. in acute exacerbated COPD (AECOPD) and were not indicative of lung cancer. In a different group of patients that were already treated with two cycles of chemotherapy and who responded with at least a 25% reduction in primary tumor diameter, levels of angiogenic markers were lower compared to patients with newly diagnosed NSCLC. We suggest that breath based detection of angiogenic markers may help in the early detection of lung cancer.

1. Introduction

Three out of four patients with lung cancer are diagnosed because symptoms of advanced disease are recognized. A practical, non-invasive and relatively inexpensive method for the detection of lung cancer in a population at risk would be a prerequisite to improve the unfavorable outcome of this disease, which is the leading cause of cancer related death in the United States (Cancer facts and Figures 2007, in American Cancer Society, 2007) [1]. Currently early detection by screening for lung cancer is considered by using low dose CT and other means such as fluorescent bronchoscopy, induced sputum or attempts to measure patterns of volatile organic compounds in the exhalate by one of several different methods [2–4] In addition to costs, radiation exposure or invasiveness (in case of bronchoscopy), a significant effect of even the more thoroughly studied methods in improving outcome has not yet been demonstrated. Several large trials investigating the role of low dose CT in lung cancer screening are ongoing throughout the world, but results will have to be awaited.

While looking for tumors in imaging tests may be quite sensitive, it will of cause never be highly specific. In theory, a sensitive test for the presence of lung cancer would be preferred over a sensitive test for the presence of a nodule.

One approach to specific detection of lung cancer in the future may involve molecular markers [1]. Recent progress in understanding tumor biology has facilitated the search for potential markers. Several closely tumor associated markers have been identified and some of these may prove to be suitable for early tumor detection. Molecular abnormalities such as mutations in the tumor suppressor gene p53 [5], deletion or mutation of the retinoblastoma gene [6] were detected and have been suggested to be potential markers for the detection of lung cancer.

An important aspect of tumor biology is angiogenesis [7]. Tumors induce the generation of blood vessels that are necessary for further growth [8]. Angiogenic molecules have therefore been suggested as tumor biomarkers [9]. Increased levels of vascular endothelial growth factor (VEGF) in serum and tumor tissue have already been correlated with poor prognosis in patients with lung cancer [10–12]. Similarly, increased levels of basic fibroblast growth factor (bFGF), another potent angiogenic molecule, have been associated with poor outcome in lung cancer [13]. However, these investigations used angiogenic growth factor levels in lung tissue of lung cancer patients which requires invasive diagnostic procedures not suited for broad application.

Breath based methods may be a novel approach for identifying highly tumor-specific molecules in exhaled breath condensate (EBC) [14] and may be used for selecting patients for further diagnostic work ups. We have previously demonstrated that detection

* Corresponding author at: Department of Respiratory Medicine, University of Leipzig, Liebigstrasse 20, 04103 Leipzig, Germany. Tel.: +49 341 971 2600; fax: +49 341 971 2609.
E-mail address: wirtzh@medizin.uni-leipzig.de (H. Wirtz).

0169-5002/$ – see front matter © 2009 Published by Elsevier Ireland Ltd.
doi:10.1016/j.lungcan.2009.06.010

of p53 mutations in EBC of NSCLC patients is possible although quite time-consuming [15]. In this study we set out to investigate the possibility of detecting more tumor-specific molecules in the exhalate. We chose angiogenic molecules for their well-established association with tumor tissue as well as indicators of inflammation in order to differentiate inflammation from tumor, e.g. in COPD. Detecting more than one marker in the small volume samples of exhaled breath condensate became possible using multiplex bead based immunoassays as previously reported in a study from this group investigating cytokines in EBC of COPD patients [16,17].

In this proof of principle study, we chose to investigate the following set of five markers in a customized array: three markers associated with angiogenesis (VEGF, bFGF, angiogenin) and two associated predominantly with inflammation, e.g. in COPD (TNF-α, IL-8). These markers were tested in a group of individuals, of whom 17 were just previously diagnosed with NSCLC while others included exacerbated and stable COPD patients, and individuals without lung disease.

2. Materials and methods

2.1. Study subjects and clinical scores

EBC was collected from 74 individuals (49 men, 25 women; age: 61 ± 9 years): (a) healthy non-smoking individuals (volunteers, $n = 12$); (b) patients admitted for suspected lung cancer at the time of confirmation of NSCLC (NSCLC; $n = 17$); (c) a different group of NSCLC patients with tumors in at least partial remission (i.e. reduction in primary tumor diameter of at least 25%) following two cycles of a platinum based chemotherapy (NSCLC-PR; $n = 15$); (d) patients with stable COPD (sCOPD; $n = 15$; stable COPD was defined by the lack of symptoms typical for an acute exacerbation and no need for a change in medication for at least eight weeks prior to presentation); (e) patients with exacerbated COPD according to the Anthonisen criteria [18] (AECOPD; $n = 15$).

Patient characteristics are depicted in Table 1. NSCLC was verified in all patients by histological examination of tumor biopsies. EBC of patients with AECOPD was collected as early as possible and within 36 h of hospitalization. All AECOPD patients exhibited one or more criteria for admission to a hospital as suggested by Burge and Wedzicha (respiratory rate > 25/min; pulse rate > 110/min; PaO_2 < 8 kPa; abnormal chest radiograph; serious concomitant disease; altered mental state; living alone [19]). Diagnosis of AECOPD was based on criteria described by Anthonisen (presence of at least one of the following three major symptoms: increase in dyspnoea, sputum volume increase, sputum change to purulence and at least one of the following minor symptoms: cough, wheeze, sore throat, nasal discharge, and fever). All AECOPD patients were treated with oral corticosteroids and i.v. antibiotic therapy. All patients with sCOPD, AECOPD, NSCLC and healthy individuals reported to be non/ex-smokers for at least two years or smokers (current smokers and ex-smokers for up to one year) with comparable rates in all groups.

Lung function (Table 1) was performed the same day as EBC collection in all stable COPD patients, tumor patients and volunteers and within one week in AECOPD patients. Capillary blood gas analysis was performed on all patients within the first couple of hours following admission.

All COPD patients were in GOLD classes III or IV ([20], update 2006). None of the patients in this series was treated with oral steroids prior to admission. Instead all patients were on oral theophylline and inhalation therapy (LABA and/or long acting anticholinergic and/or inhaled corticosteroid) according to GOLD recommendations. All patients were regularly seen by a pulmonary physician. Approval for this investigation was obtained from the ethics committee of the University of Leipzig.

2.2. EBC collection and markers

EBC was collected for 20 min during regular breathing through a mouth piece of the EcoScreen® (Jaeger/Cardinal Health, Hoechberg, Germany) while wearing a nose clip as previously described [21]. All EBC samples were examined for amylase activity in order to exclude contamination by saliva (100 μl of reconstituted EBC; alpha-Amylase ESP1491300 kit; Boehringer Mannheim, Germany). EBC protein concentration was measured using the Micro-BCA-Protein-Assay (Pierce, Rockford USA; detection limit: 0.5 μg/ml).

2.3. Lyophilization of EBC fluid

Immediately upon collection, condensate samples were frozen at −20 °C. A major portion of the samples (2 ml) used in the fluorescent bead array assay, was lyophilized on the evaporator (Uniequip, Martinsried, Germany). The resulting pellet was resuspended in 60 μl of ddH_2O for direct use in the assay. This procedure resulted in a 33-fold concentration.

Table 1
Patient's characteristics.

Patients	VOL	Stable COPD	AECB-GW	NSCLC	NSCLC-PR
Total number	12	15	15	17	15
Age (mean ± S.D.)*	60.3 ± 10.0	58.5 ± 8.5	60.2 ± 10.2	62.7 ± 6.4	63.5 ± 8.1
Sex					
Male	8	10	10	11	10
Female	4	5	5	6	5
Smoking status[a]	Smokers/non-smoker	Smokers/ex-smoker	Smokers/ex-smoker	Smoker/ex-smokers	Smokers/ex-smoker
	5/7	7/8	9/6	7/10	4/11
Pack years	25 ± 5	29 ± 6	27 ± 5	37 ± 6	36 ± 9
FEV1 (L)[b]	3.25 ± 0.66	1.21 ± 0.32	1.04 ± 0.29	1.37 ± 0.39	1.38 ± 0.41
FVC (L)[b]	3.80 ± 0.62	2.09 ± 0.40	1.91 ± 0.40	2.25 ± 0.38	2.21 ± 0.48
FEV1/FEVC (%)[b]	85.1 ± 4.89	57.3 ± 8.53	53.7 ± 5.63	59.9 ± 8.57	61.8 ± 5.94
PaO_2 (kPa)[c]	9.76 ± 0.40	7.44 ± 0.54	6.72 ± 0.19	7.59 ± 0.53	7.69 ± 0.58
$PaCO_2$ (kPa)[c]	5.14 ± 0.23	5.90 ± 0.28	6.49 ± 0.19	5.79 ± 0.33	5.80 ± 0.33
Protein in EBC (μg/ml) (mean ± S.D.)*	13.7 ± 2.7	12.9 ± 2.8	12.1 ± 2.5	13.2 ± 3.2	13.2 ± 3.2

All data are shown as mean ± S.D.
* $p > 0.05$ (no significant difference between investigated groups).
[a] Smoking was defined as current smokers or ex-smokers that discontinued smoking no longer than twelve month, non-smoking as no smoking longer than one year.
[b] Results were measured after AECB.
[c] Results were measured at time of hospitalization.

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010

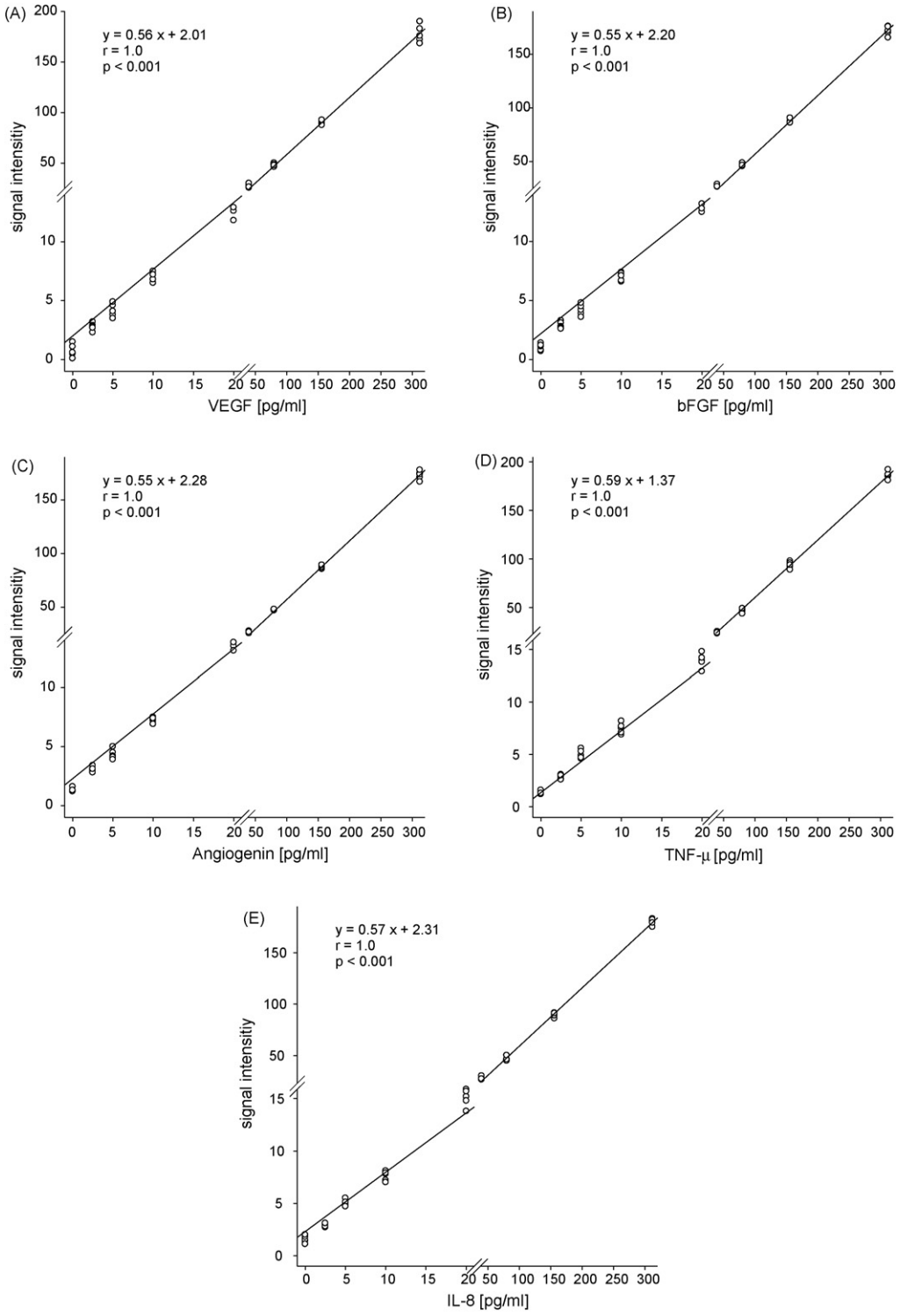

Fig. 1. Calibration curves of VEGF (A), bFGF (B), angiogenin (C), TNF-α (D) and IL-8 (E) for EBC concentration ranges (2.5–312 pg/ml). For improved visualization of lower concentrations, both axes were broken.

2.4. Multiplex bead based immunoassay

A multiplex fluorescent bead immunoassay (cytometric bead array, CBA; Becton-Dickinson, San Jose, CA, USA) was adapted to exhaled breath condensate. A mixture of five bead populations with distinct fluorescence intensities and coated with capture antibodies specific for angiogenin, bFGF, VEGF, IL-8, and TNF-α proteins were incubated with 1 ml of lyophilized breath condensate reconstituted with 60 μl of ddH_2O (duplicate samples were prepared). Angiogenic factors in EBC samples and recombinant standards bound to capture beads were detected by phycoerythrin (PE)-conjugated detection antibodies

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010

Table 2
Recovery and variance of angiogenin, VEGF, bFGF, IL-8, and TNF-α in samples following spiking, lyophilization, reconstitution, and flow cytometric detection.

Cytokine	c (defined) (pg/ml)	Precision					Recovery (%)
		Mean	S.D.	95% CI		Variance	
				Min	Max		
Angiogenin	7.3	6.7	1.0	5.2	8.4	0.9	91.8
	14.7	14.1	1.2	12.2	16.1	1.4	95.9
	29.3	27.1	2.0	24.3	30.5	3.86	92.5
	58.7	56.5	3.2	51.8	60.8	9.0	96.3
	117.3	120.1	7.2	112.7	125.6	17.7	102.4
	234.5	233.2	13.1	219.4	259.6	171.3	99.4
VEGF	7.3	6.6	1.1	5.3	8.5	1.2	90.4
	14.7	14.1	0.9	12.9	15.8	0.9	95.9
	29.3	26.2	2.2	22.4	28.4	5.0	89.4
	58.7	55.5	2.6	51.7	58.9	6.6	94.5
	117.3	115.4	5.8	108.3	124.5	33.1	98.4
	234.5	238.7	13.9	219.4	259.2	192.7	101.8
bFGF	7.3	6.8	1.3	12.5	16.0	1.8	93.2
	14.7	14.3	1.6	5.2	8.3	3.1	97.3
	29.3	27.9	2.4	23.4	31.3	5.8	95.2
	58.7	56.1	3.4	49.2	60.3	11.9	95.6
	117.3	119.3	8.8	104.5	134.8	7.0	101.7
	234.5	228.0	13.0	209.4	246.3	168.2	97.2
IL-8	7.3	6.8	0.8	5.3	7.8	0.8	93.2
	14.7	13.9	1.3	11.9	15.8	1.6	94.6
	29.3	28.6	2.7	24.7	33.5	7.2	97.6
	58.7	60.0	4.6	54.6	69.2	21.0	102.2
	117.3	113.9	5.0	105.3	119.5	25.2	97.1
	234.5	227.1	9.7	213.2	238.5	93.7	96.8
TNF-α	7.3	6.5	1.4	4.9	7.7	1.3	89.0
	14.7	12.7	1.5	10.2	15.1	2.2	86.4
	29.3	29.4	4.6	22.7	35.9	20.8	100.3
	58.7	55.2	2.5	51.4	58.9	6.5	94.0
	117.3	120.0	4.8	113.6	127.5	23.3	102.3
	234.5	244.0	8.4	231.6	253.7	69.7	104.0

c: concentration; S.D.: standard deviation; CI: confidence interval of mean; $n = 3$.

of equal specificity in a flow cytometer (FC500™, Beckman Coulter).

2.5. Low concentration calibration of fluorescent bead array assay

A calibration curve has been devised for the lower range of concentrations of angiogenic markers, IL-8 and TNF-α known from pilot experiments to be expected in exhaled breath condensate (Fig. 1A–E). A threshold concentration of 2.5 pg/ml was observed for reliable measurements. The calibration curve therefore ranged from 2.5 to 312 pg/ml. All data points were repeated five times.

2.6. Statistical analysis

Statistical analysis was performed with the SPSS software program (SPSS Inc., Chicago, USA). Linear regression analysis was applied to investigate the correlation of cytokine levels in EBC. Comparison of patient groups (three or more) was performed using the Kruskal–Wallis and Mann–Whitney *U*-tests. Statistical significance was accepted at the 5% level.

3. Results

3.1. Exhaled breath condensate characteristics

None of the condensate samples exhibited amylase concentrations measurable with the assay used. Even though the detection limit of the amylase assay may be higher than concentrations expected in pure EBC [22,23] a relevant saliva contamination may be excluded since amylase concentration in saliva is 10,000 times higher than that in EBC.

Total EBC protein concentration was measured from 100 μl aliquots in all unprocessed samples. Results are shown in Table 1. There was no significant difference in any of the subgroups analyzed ($p = 0.27$).

3.2. Validation of the multiplex bead based immunoassay

The fluorescent bead array assay was validated in terms of recovery and variance by measuring a known concentration of each of the angiogenic factors in 1 ml samples of a spiked reconstitution buffer following the procedure of freezing, lyophilization and reconstitution. Three separate series of 6 concentrations (7.3, 14.7, 29.3, 58.7, 117.3, and 234.5 pg/ml) were prepared in triplicates and frozen at −20 °C. Test samples were then lyophilized using a vacuum concentrator (Uniequip, Martinsried, Germany) and reconstituted at days one, seven and thirty. Data are shown in Table 2. Recovery of all markers and concentrations ranged from 86.4 to 104%. Previously the effects of lyophilization, reconstitution, and varying buffer composition of a different set of markers have been shown to be negligible [17].

3.3. Angiogenic factors, TNF-α, and IL-8 in EBC

VEGF, angiogenin and bFGF were clearly elevated although to varying degrees in patients with newly diagnosed lung cancer (VEGF: 40 ± 10 pg/ml; angiogenin: 68.8 ± 32.8 pg/ml; bFGF: 82.3 ± 34.4 pg/ml; TNF-α: 0.22 ± 0.24 pg/ml; IL-8: 0.34 ± 0.47 pg/ml) compared to stable COPD patients without lung carcinoma (VEGF: 5.7 ± 5.5 pg/ml; angiogenin: 4.2 ± 3.3 pg/ml; bFGF: 7.0 ± 5.1 pg/ml; TNF-α: 0.16 ± 0.20 pg/ml; IL-8: 0.29 ± 0.40 pg/ml) but also to all other groups (Fig. 2A–C and

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010

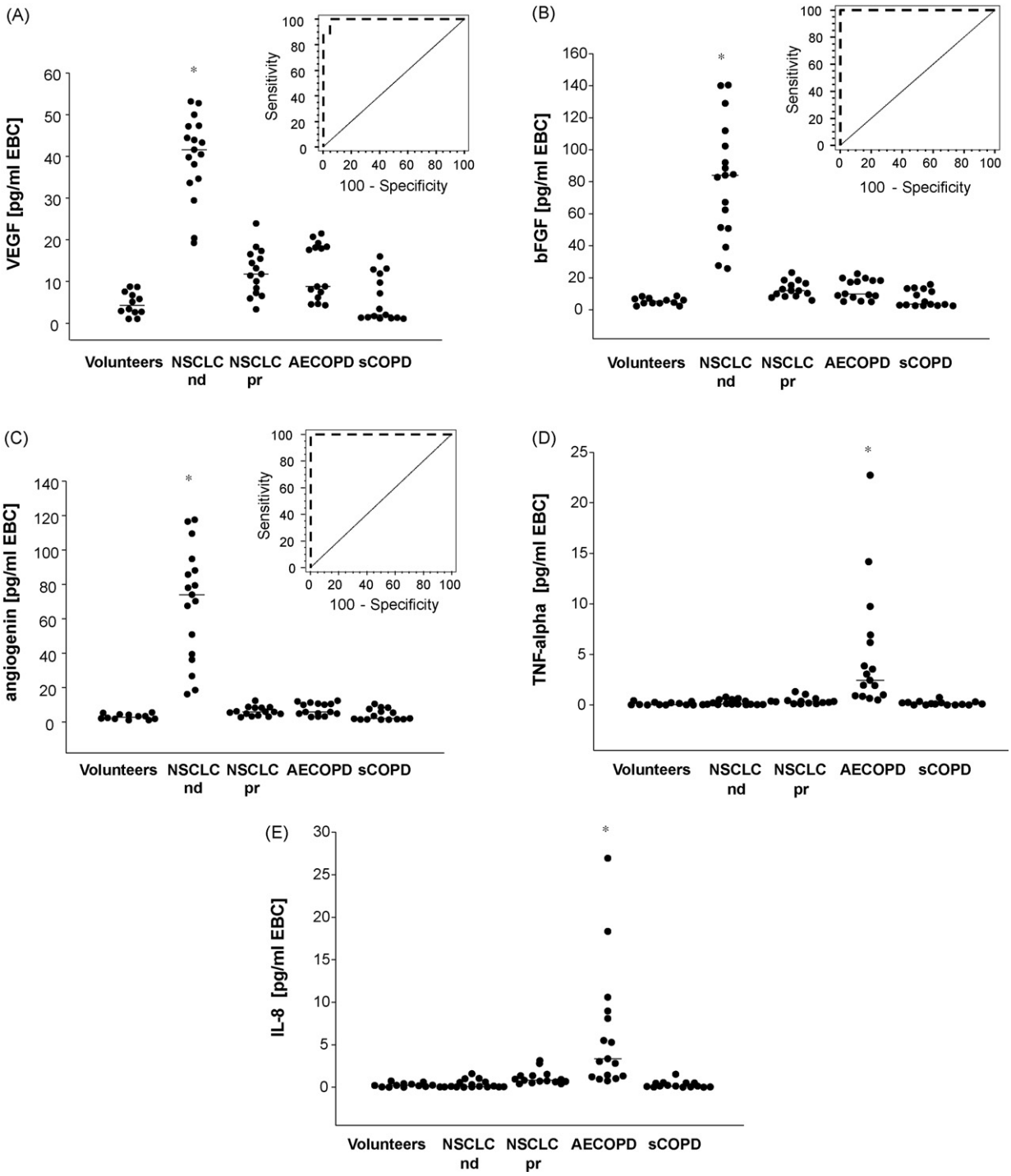

Fig. 2. Concentrations of VEGF (A), bFGF (B), angiogenin (C), TNF-α (D) and IL-8 (E) in volunteers and patients with newly diagnosed NSCLC, NSCLC in partial remission, AECOPD and stable COPD (pg/ml). Means are indicated by horizontal lines. Inserts in the upper right quadrants are receiver operating curves (ROCs) for VEGF (A), bFGF (B) and angiogenin (C). Asterisks demonstrate significant difference of the marked group versus all other groups ($p < 0.05$).

Table 3
Patient's cytokine and angiogenetic levels.

Patients	VOL	Stable COPD	AECB-GW	NSCLC	NSCLC-PR
Total number	12	15	15	17	15
IL-8 (pg/ml; mean ± S.D.)	n.d. (0.26 ± 0.23)	n.d. (0.29 ± 0.40)	6.54 ± 7.41	n.d. (0.34 ± 0.47)	n.d. (1.12 ± 0.82)
TNF-α (pg/ml; mean ± S.D.)	n.d. (0.12 ± 0.15)	n.d. (0.16 ± 0.20)	5.16 ± 6.23	n.d. (0.22 ± 0.24)	n.d. (0.41 ± 0.35)
VEGF (pg/ml; mean ± S.D.)	4.72 ± 2.80	5.71 ± 5.49	12.42 ± 6.65	39.97 ± 9.96	12.27 ± 5.54
bFGF (pg/ml; mean ± S.D.)	5.40 ± 2.12	6.99 ± 5.11	12.92 ± 6.24	82.33 ± 34.37	12.79 ± 4.80
Angiogenin (pg/ml; mean ± S.D.)	3.03 ± 1.56	4.20 ± 3.25	7.51 ± 3.52	68.76 ± 32.76	6.15 ± 2.66

All data are shown as mean ± S.D.

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010

Table 4
Tumor parameters and correlation with VEGF, bFGF, angiogenin, IL-8, and TNF-α (ANOVA rank test [Kruskal–Wallis one-way analysis of variance on ranks] for three or more groups, Mann–Whitney rank sum test for two groups).

		VEGF	bFGF	Angiogenin	IL-8	TNF-α
T-stage of primary tumor						
T1	3 (18%)	$p = 0.71$	$p = 0.72$	$p = 0.56$	$p = 0.33$	$p = 0.16$
T2	7 (41%)	n.s.	n.s.	n.s.	n.s.	n.s.
T3	6 (35%)					
T4	1 (6%)					
Lymph node metastases						
N0	2 (12%)	$p = 0.42$	$p = 0.43$	$p = .45$	$p = 0.78$	$p = 0.93$
N1	4 (23%)	n.s.	n.s.	n.s.	n.s.	n.s.
N2	8 (47%)					
N3	3 (18%)					
Distant metastasis						
M0	10 (59%)	$p = 0.22$	$p = 0.22$	$p = 0.09$	$p = 0.12$	$p = 0.49$
M1	7 (41%)	n.s.	n.s.	n.s.	n.s.	n.s.
Tumor stage groups						
I	2 (12%)	$p = 0.26$	$p = 0.26$	$p = 0.20$	$p = 0.45$	$p = 0.89$
II	4(23.5%)	n.s.	n.s.	n.s.	n.s.	n.s.
III	4(23.5%)					
IV	7 (41%)					
Tumor location						
Central	4 (24%)	$p = 0.19$	$p = 0.23$	$p = 0.61$	$p = 0.50$	$p = 0.36$
Peripheral	13 (76%)	n.s.	n.s.	n.s.	n.s.	n.s.
Histological classification						
Adenocarcinoma	7 (41%)	$p = 0.26$	$p = 0.33$	$p = 0.49$	$p = 0.23$	$p = 0.38$
Squamous cell carcinoma	8 (47%)	n.s.	n.s.	n.s.	n.s.	n.s.
Large cell carcinoma	2 (12%)					

Table 3). There was almost no overlap with any of the other groups except for the two lowest individual values in the NSCLC group which were at a similar level as were the highest individual values in all of the other groups, and the AECOPD group specifically. We observed no influence of age, gender, tumor location or histology, tumor stage or TNM classifiers on the levels of VEGF and other angiogenic factors in this limited number of patients (Table 4).

In a different group of patients with NSCLC following two courses of chemotherapy resulting in at least partial tumor remission (>25% reduction in primary tumor diameter) significantly lower EBC levels of all angiogenic factors were observed—comparable to the levels seen in patients with AECOPD ($p < 0.0001$). However, in these patients pro-inflammatory markers were somewhat elevated ($p < 0.03$). When levels of angiogenic molecules in EBC were analyzed for sensitivity and specificity using receiver operating curves (ROCs) we observed excellent characteristics for all angiogenic molecules, with VEGF being just slightly inferior compared to bFGF and angiogenin (Table 3). The ROC curve analysis of patients with lung cancer compared to healthy controls, stable and exacerbated COPD showed an area under the curve for VEGF of 0.994 (standard error 0.013, $p = 0.0001$, sensitivity 100%, specificity 95.2%), for angiogenin of 1.0 (standard error 0.0, $p < 0.0001$, sensitivity 100%, specificity 100%), and of 1.0 for bFGF (standard error 0.0, $p < 0.0001$, sensitivity 100%, specificity 100%).

4. Discussion

In this study, levels of angiogenic markers in breath condensate clearly differentiated between patients with NSCLC at the time of histological confirmed diagnosis, and patients with either stable or exacerbated COPD, or healthy volunteers. There is no overlap in angiogenic factors between patients with and without NSCLC for angiogenin and bFGF and almost no overlap for VEGF. In contrast, a great increase of inflammatory markers, IL-8 and TNF-α, in EBC was seen in exacerbated COPD albeit not in all cases. The increases of angiogenic markers did not correlate with the central or peripheral localization of the tumor, or the T, N or M classification, or with NSCLC histological subtype in this limited number of patients.

Early diagnosis of NSCLC is highly desirable because early recognition will allow detection of possibly curable disease. We are however aware of the ongoing discussion on the general usefulness of lung cancer screening and the connected arguments both pro and contra [24,25].

A practical way of diagnosing lung cancer in the future might be a two-step process that involves a test that is easily performed and very “cancer specific”. This highly specific test would select patients, in whom CT imaging tests would have a high likelihood to turn out positive. One advantage of this scenario could be a great reduction of unnecessary diagnostic work ups due to “nodules”. Another possible advantage might be improved patient acceptance due to the fact, that a breath based test is easily performed within the doctor’s office, without the need of additional visits at a radiologist’s office.

A breath based test however would have to be of great specificity and sensitivity. This proof of concept study of detection of angiogenic molecules in exhaled breath condensate demonstrates that the method described is indeed highly specific and sensitive in the relevant population (lung cancer versus COPD patients: both current and ex-smokers). For the proof of concept purpose of this study it was necessary to investigate a group of patients with a definite and verified diagnosis of NSCLC as early as possible following verification. This meant examining patients at the time of histological verified diagnosis. Due to this prerequisite we could not at this point test the detection of angiogenic molecules in EBC for the diagnosis of NSCLC at any time point earlier than that of the definite diagnosis of NSCLC as achieved by conventional diagnostic procedures. Our study is therefore limited to demonstrating the feasibility of a sensitive identification of lung cancer patients among a relevant group of individuals with either known disease (NSCLC) or individuals at risk (COPD) using the levels of angiogenic factors from breath condensate. Further validation of this method in the future will have to demonstrate the ability of detecting NSCLC in a previously undiagnosed population at risk. It will also have to be elucidated whether SCLC will also be detectable from angio-

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010

genic molecules in breath condensate. An additional limitation of this study is the limited number of patients involved with very few early cancer stages.

Adding the information provided by increased inflammatory cytokines in EBC was useful for detecting patients with AECOPD and may in rare cases increase the specificity of the test, but does not appear to be necessary for the question of whether or not the patient is at high risk for having lung cancer. AECOPD is usually recognized easily by its clinical features. However a worsened cough due to lung cancer in an outpatient in the pulmonary physician's office may occasionally be mistaken for AECOPD. The EBC based test used in this study may then provide two rather specific indications leading to both directions by suggesting increased angiogenesis and the lack of AECOPD typical inflammation.

Other authors have previously described EBC findings in lung cancer patients. Carpagnano et al. reported increased IL-6 in a study of elderly NSCLC patients versus younger healthy controls [26]. EBC IL-6 is also increased in other clinically relevant situations such as COPD and we and others have demonstrated this recently [16,27]. IL-6 therefore does not seem to qualify as a highly tumor-specific marker in EBC. Similarly, endothelin was reported to be increased considerably in EBC samples of NSCLC patients by the same group and may well be of greater specificity [28]. Several lung diseases however also exhibit increased levels of endothelin in the lung such as pulmonary hypertension and pulmonary fibrosis [29]. It is unclear however, whether endothelin levels are increased in EBC in any of these diseases. DNA alterations measured from minute amounts of DNA in EBC have been demonstrated by our group in form of somatic mutations of p53 in a number of lung cancer patients but not in controls [15]. In addition, the group of Carpagnano et al. has described microsatellite instability and loss of heterozygosity in smokers and in patients with NSCLC [30]. While the presence of NSCLC correlated to increased microsatellite instability, a more severe smoking history did the same. Thus microsatellite instability appears to reflect field cancerization in the respiratory tract, but may not efficiently demarcate NSCLC.

Elevated VEGF in BALF of NSCLC patients compared to controls has previously been reported by Ohta et al. [31]. Dalaveris et al observed increased VEGF levels in serum but not in EBC of lung cancer patients [32]. We cannot completely resolve this incongruency of normal EBC VEGF levels in Dalaveris study and the significantly increased VEGF levels in NSCLC patients of our investigation. However, there are important differences in the two studies: (a) the methods of measuring angiogenic molecules were different; (b) Dalaveris et al. included both SCLC as well as NSCLC patients while our patient group was very homogenic with only NSCLC patients; (c) Dalaveris and coauthors did not state whether all patients included in their study were untreated at the time of breath condensate collection. Since we observed a great difference in EBC levels of VEGF in patients being treated with chemotherapy (and responding), compared to other newly diagnosed NSCLC patients, this might explain much of the difference.

TNF-α was increased in both EBC and serum of lung cancer patients (SCLC and NSCLC) in the study by Dalaveris et al. [32]. Similarly TNF-α in EBC was elevated in NSCLC patients in a study of Carpagnano et al. [33]. However, considerable overlap existed between lung cancer patients and healthy controls for TNF-α levels in both serum and EBC. In our study, TNF-α was generally lower compared to the study of Dalveris et al, and a trend for increased values in tumor patients compared to controls was observed, but this effect was not significant. Again the reason for this difference remains unclear. In our study EBC was concentrated and TNF-α was measured by a different technology (multiplex bead based immunoassay). In AECOPD patients our test kit worked well in picking up greatly elevated EBC TNF-α levels.

5. Conclusion

In summary, EBC has potential to aid in the early diagnosis of lung cancer [14]. A number of divers efforts have been reported to detect lung cancer from exhaled air/breath condensate. They all have their strong points and weaknesses. Sensitivity but also specificity appears to be of greatest importance to avoid anxiety and anger in potential patients. Our investigation demonstrates that highly tumor-specific markers such as angiogenic factors measured by sensitive assays in breath condensate might help to differentiate patients with and without NSCLC. This study will have to be followed by a validation in a larger population.

Conflict of interest

All authors declare that they have no financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

References

[1] Hirsch FR, Merrick DT, Franklin WA. Role of biomarkers for early detection of lung cancer and chemoprevention. Eur Respir J 2002;19:1151–8.
[2] Pastorino U. Early detection of lung cancer. Respiration 2006;73:5–13.
[3] Ravenel JG, Costello P, Silvestri GA. Screening for lung cancer. AJR Am J Roentgenol 2008;190:755–61.
[4] Conrad DH, Goyette J, Thomas PS. Proteomics as a method for early detection of cancer: a review of proteomics, exhaled breath condensate, and lung cancer screening. J Gen Intern Med 2008;23(Suppl. 1):78–84.
[5] Ahrendt SA, Hu Y, Buta M, McDermott MP, Benoit N, Yang SC, et al. p53 mutations and survival in stage I non-small-cell lung cancer: results of a prospective study. J Natl Cancer Inst 2003;95:961–70.
[6] Xu HJ, Quinlan DC, Davidson AG, Hu SX, Summers CL, Li J, et al. Altered retinoblastoma protein expression and prognosis in early-stage non-small-cell lung carcinoma. J Natl Cancer Inst 1994;86:695–9.
[7] Kuhn H, Hammerschmidt S, Wirtz H. Targeting tumorangiogenesis in lung cancer by suppression of VEGF and its receptor—results from clinical trials and novel experimental approaches. Curr Med Chem 2007;14:3157–65.
[8] Folkman J. Is angiogenesis an organizing principle in biology and medicine? J Pediatr Surg 2007;42:1–11.
[9] Herbst RS, Onn A, Sandler A. Angiogenesis and lung cancer: prognostic and therapeutic implications. J Clin Oncol 2005;23:3243–56.
[10] Fontanini G, Vignati S, Boldrini L, Chine S, Silvestri V, Lucchi M, et al. Vascular endothelial growth factor is associated with neovascularization and influences progression of non-small cell lung carcinoma. Clin Cancer Res 1997;3:861–5.
[11] Fontanini G, Faviana P, Lucchi M, Boldrini L, Mussi A, Camacci T, et al. A high vascular count and overexpression of vascular endothelial growth factor are associated with unfavourable prognosis in operated small cell lung carcinoma. Br J Cancer 2002;86:558–63.
[12] Han H, Silverman JF, Santucci TS, Macherey RS, d'Amato TA, Tung MY, et al. Vascular endothelial growth factor expression in stage I non-small cell lung cancer correlates with neoangiogenesis and a poor prognosis. Ann Surg Oncol 2001;8:72–9.
[13] Iwasaki A, Kuwahara M, Yoshinaga Y, Shirakusa T. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) levels, as prognostic indicators in NSCLC. Eur J Cardiothorac Surg 2004;25:443–8.
[14] Chan HP, Lewis C, Thomas PS. Exhaled breath analysis: novel approach for early detection of lung cancer. Lung Cancer 2008.
[15] Gessner C, Kuhn H, Toepfer K, Hammerschmidt S, Schauer J, Wirtz H. Detection of p53 gene mutations in exhaled breath condensate of non-small cell lung cancer patients. Lung Cancer 2004;43:215–22.
[16] Gessner C, Scheibe R, Wotzel M, Hammerschmidt S, Kuhn H, Engelmann L, et al. Exhaled breath condensate cytokine patterns in chronic obstructive pulmonary disease. Respir Med 2005;99:1229–40.
[17] Sack U, Scheibe R, Wotzel M, Hammerschmidt S, Kuhn H, Emmrich F, et al. Multiplex analysis of cytokines in exhaled breath condensate. Cytometry A 2006;69:169–72.
[18] Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. Ann Intern Med 1987;106:196–204.
[19] Burge S, Wedzicha JA. COPD exacerbations: definitions and classifications. Eur Respir J, Suppl 2003;41:46s–53s.
[20] Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. Am J Respir Crit Care Med 2001;163:1256–76.
[21] Gessner C, Kuhn H, Seyfarth HJ, Pankau H, Winkler J, Schauer J, et al. Factors influencing breath condensate volume. Pneumologie 2001;55:414–9.

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010

[22] Huszar E, Vass G, Vizi E, Csoma Z, Barat E, Molnar-Vilagos G, et al. Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. Eur Respir J 2002;20:1393–8.
[23] Effros RM, Hoagland KW, Bosbous M, Castillo D, Foss B, Dunning M, et al. Dilution of respiratory solutes in exhaled condensates. Am J Respir Crit Care Med 2002;165:663–9.
[24] Goodwin M, Gleeson FV. The pitfalls of lung cancer screening. Cancer Imaging 2004;4:52–7.
[25] Gomez M, Silvestri GA. Lung cancer screening. Am J Med Sci 2008;335:46–50.
[26] Carpagnano GE, Resta O, Foschino-Barbaro MP, Gramiccioni E, Carpagnano F. Interleukin-6 is increased in breath condensate of patients with non-small cell lung cancer. Int J Biol Markers 2002;17:141–5.
[27] Bucchioni E, Kharitonov SA, Allegra L, Barnes PJ. High levels of interleukin-6 in the exhaled breath condensate of patients with COPD. Respir Med 2003;97:1299–302.
[28] Carpagnano GE, Foschino-Barbaro MP, Resta O, Gramiccioni E, Carpagnano F. Endothelin-1 is increased in the breath condensate of patients with non-small-cell lung cancer. Oncology 2004;66:180–4.
[29] Bacakoglu F, Atasever A, Ozhan MH, Gurgun C, Ozkilic H, Guzelant A. Plasma and bronchoalveolar lavage fluid levels of endothelin-1 in patients with chronic obstructive pulmonary disease and pulmonary hypertension. Respiration 2003;70:594–9.
[30] Carpagnano GE, Foschino-Barbaro MP, Mule G, Resta O, Tommasi S, Mangia A, et al. 3p microsatellite alterations in exhaled breath condensate from patients with non-small cell lung cancer. Am J Respir Crit Care Med 2005;172:738–44.
[31] Ohta Y, Ohta N, Tamura M, Wu J, Tsunezuka Y, Oda M, et al. Vascular endothelial growth factor expression in airways of patients with lung cancer: a possible diagnostic tool of responsive angiogenic status on the host side. Chest 2002;121:1624–7.
[32] Dalaveris E, Kerenidi T, Katsabeki-Katsafli A, Kiropoulos T, Tanou K, Gourgoulianis KI, et al. VEGF, TNF-alpha and 8-isoprostane levels in exhaled breath condensate and serum of patients with lung cancer. Lung Cancer 2008.
[33] Carpagnano GE, Spanevello A, Curci C, Salerno F, Palladino GP, Resta O, et al. IL-2, TNF-alpha, and leptin: local versus systemic concentrations in NSCLC patients. Oncol Res 2007;16:375–81.

Please cite this article in press as: Gessner C, et al. Angiogenic markers in breath condensate identify non-small cell lung cancer. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.06.010