Lung Cancer 72 (2011) 32–38

Contents lists available at ScienceDirect

Lung Cancer

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

Bronchoscopic Microsampling is a Useful Complementary Diagnostic Tool for Detecting Lung Cancer

Hiroyuki Yasuda[a,1], Kenzo Soejima[a,*], Sohei Nakayama[a,1], Ichiro Kawada[a], Ichiro Nakachi[a], Satoshi Yoda[a], Ryosuke Satomi[a], Shinnosuke Ikemura[a], Hideki Terai[a], Takashi Sato[a], Hideo Watanabe[a], Katsuhiko Naoki[a], Yuichiro Hayashi[b], Akitoshi Ishizaka[a]

[a] Department of Pulmonary Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[b] Department of Pathology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

ARTICLE INFO

Article history:
Received 17 May 2010
Received in revised form 24 July 2010
Accepted 30 July 2010

Keywords:
bronchoscopic microsampling
lung cancer
epigenetics
epithelial lining fluid
EGFR mutation
EML4-ALK

SUMMARY

Purpose: Bronchoscopic microsampling (BMS) is a novel and direct method with which to obtain epithelial lining fluid (ELF) from the lungs. Analysis of DNA hypermethylation of tumor suppressor genes (TSGs) is expected to be a sensitive tool for the early detection of lung cancer. It has been reported that the existence of *EGFR* mutations and *EML4-ALK* gene rearrangements are related to the sensitivity of corresponding kinase inhibitors. We aimed to evaluate the suitability of ELF as a sample for analyzing molecular changes specific for lung cancer.
Patients and methods: We collected ELF from 61 lung cancer patients by BMS from the airway close to the peripheral lung nodule and purified the nucleic acids. We performed methylation specific PCR in each ELF as well as matched serum and tumor tissue for TSGs for DNA methylation analysis. We also examined *EGFR* mutations and *EML4-ALK* rearrangement.
Results: The sensitivity for detecting DNA hypermethylation in ELF vs serum was 74.1% vs 18.5%. We found 60.1% of patients had at least one hypermethylation in ELF, while only 27.9% had it in serum. Of note, DNA hypermethylation was detected even in stage I patients (60.0%) and the detection rate was almost the same level in each stage. We also found the sensitivity for detecting *EGFR* mutation in ELF vs serum was 58.3% vs 8.3%. We detected an *EML4-ALK* fusion gene using ELF in one patient.
Conclusions: BMS is an alternative method to detect cancer specific genetic and epigenetic alterations and will be a useful complementary diagnostic tool for lung cancer.
Summary: Investigation of genetic and epigenetic changes associated with lung cancer has clinical importance for its diagnosis and management. The clinical usefulness of bronchoscopic microsampling (BMS) in lung cancer has not yet been evaluated. This study demonstrates that BMS could be useful for detecting lung cancer specific molecular changes and valuable for early diagnosis and determination of treatment options for lung cancer.

1. Introduction

Lung cancer is one of the most common malignancy and a leading cause of cancer-related death in the world [1]. The diagnosis of lung cancer depends on the pathological analysis of tumor samples, which are usually obtained by a bronchoscope. However, the diagnosis of lung cancer is often difficult, which is due to the location of the tumor, the condition of the patient, or the size of the tumor.

On the other hand, many of the molecular characteristics of lung cancer have already been elucidated. Analysis of DNA hypermethylation of tumor suppressor genes (TSGs) is expected to be a sensitive tool for early detection of lung cancer [2–3]. It has also been reported that the existence of *EGFR* mutations and *EML4-ALK* gene rearrangements is closely related to the sensitivity of *EGFR*- and *ALK*-tyrosine kinase inhibitors (TKIs), respectively [4–7]. For a patient-tailored treatment, the analysis of these molecular characteristics is becoming increasingly important. It is, however, often difficult to obtain enough tumor samples for both pathological diagnosis and further molecular analysis using only a traditional bronchoscopic approach. Although alternative surrogate samples other than tissue, such as sputum [8–10], bronchial aspirates [11–12], bronchoalveolar lavage fluid (BALF) [13–14], or serum [15–18] have been proposed for the diagnosis or molecular analysis

* Corresponding author. Department of Pulmonary Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
Tel.: +81 3 3353 1211x61430; fax: +81 3 3353 2502.
E-mail address: ksoejima@cpnet.med.keio.ac.jp (K. Soejima).
[1] These authors contributed equally to this work

0169-5002/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2010.07.016

of lung cancer, the sensitivity of these samples is still low in most cases.

To overcome these problems, we attempted to utilize epithelial lining fluid (ELF) for the molecular analysis of lung cancer patients. We have previously reported that the measurement of tumor markers, such as CYFRA21-1 and CEA in ELF, was a useful diagnostic adjunct in patients with small peripheral lung nodules [19]. We also found that ELF from the airways near tumors contains tumor-derived nucleic acid, both RNA and DNA. It has already been reported that remote media like urine from bladder cancer patients or aspirates from nipples of breast cancer patients contain cancer-derived nucleic acid. We used a bronchoscopic microsampling (BMS) method to obtain ELF [19–21]. This method requires only microsampling probes and the procedure is very easy and safe. The advantages of this method are that ELF can be obtained without dilution by saline like BALF and it is not necessary to reach the tumor directly.

In the present study, we compared the usefulness of ELF with serum for detecting the cancer specific molecular changes, both for genetics and epigenetics, and found ELF can be a reliable sample for molecular analysis of lung cancer and that it can serve as a supplemental diagnostic tool.

2. Patients and methods

2.1. Patients

Sixty-eight patients with an abnormal chest shadow that suggested primary lung cancer were admitted to Keio University Hospital and enrolled in this study. Written informed consent was obtained before the study. The study was approved by the Institutional Review Board of Keio University School of Medicine. Sixty-one out of 68 patients were confirmed to have lung cancer either by transbronchial lung biopsy (TBLB) under bronchoscopy, computed tomography (CT)- or ultrasound (US)-guided needle biopsy, lymph node biopsy or pleurocentesis (Table 1).

2.2. BMS and Collection of Tumor Specimens

The subjects underwent bronchoscopy for the diagnosis of lung cancer. During bronchoscopy, BMS was performed before the TBLB, bronchial curette, and bronchial wash procedures to prevent the contamination of blood cells due to hemorrhage. The BMS probe (Olympus Co. Ltd., Tokyo, Japan) and sampling procedure have already been described in detail [20–21]. In brief, after inserting a bronchoscope, the BMS probe in the outer sheath was inserted through the bronchoscope and the probe was advanced from the outer sheath toward the affected lesion at the subsegmental bronchus or more distal level, and then the probe was placed on the bronchial membrane for 10 seconds to absorb ELF. We also collected ELF from the lower bronchus from the opposite side of the affected lung instead of including patients without disease for the control. This procedure was repeated 3 times at each side of the lungs. In order to ensure a true evaluation of ELF itself, we were extremely careful not to touch the tumors directly with the BMS probe. The probe was cut at a point 3 cm distal from its tip and placed in a tube.

Tumor specimens were collected either by the TBLB, CT- or US-guided needle biopsy, lymph node biopsy or surgical resection.

Table 1
Patient characteristics

Patients, n		61
Age (years)	median	68
	range	48-83
Sex	male	38 (62.3%)
	female	23 (37.7%)
Histology	Ad (incl. BAC)	43 (70.5%)
	Sq	7 (11.5%)
	LCNEC	1 (1.6%)
	NSCLC	7 (11.5%)
	small cell	3 (4.9%)
Stage	I	20 (32.8%)
	II	1 (1.6%)
	III	19 (31.1%)
	IV	21 (34.4%)
Smoking (B.I)	median	700
	range	0-4000
Tumor location	central	8 (13.1%)
	peripheral	53 (86.9%)
Tumor size (mm)	median	27
	range	9-114

Ad: adenocarcinoma, BAC: bronchioloalveolar carcinoma, Sq: squamous cell carcinoma, LCNEC: large cell neuroendocrine carcinoma, NSCLC: non-small cell lung carcinoma, B.I.: Brinkman index.

2.3. Nucleic Acid Extraction

The 3 cut probes were hooked at the edge of a microcentrifuge tube and centrifuged at 2500 rpm for 10 minutes at 4 °C. The centrifuged ELF was resuspended in 200 μl of PBS (phosphate buffered saline). DNA and RNA were each extracted from 50 μl of resuspended PBS according to the manufacturer's protocol using a High Pure Vial nucleic acid kit (Roche Diagnostics, Mannheim, Germany) and an RNeasy blood and tissue kit (QIAGEN Sciences, MD), respectively. DNA and RNA in serum were both extracted by the High Pure Viral nucleic acid kit and those in tissue were extracted using a DNeasy blood & tissue kit (QIAGEN) and RNeasy blood and tissue kit according to the manufacturer's protocol, respectively.

2.4. Bisulfite Treatment and Methylation Specific PCR (MSP)

Bisulfite treatment was performed on DNA from tissue samples, ELF, and serum samples using an EZ DNA Methylation Gold kit (Zymo Research, CA) according to the manufacturer's protocol. After bisulfite treatment, MSP was performed using specific primers to methylated and unmethylated DNA sequences for 4 genes, namely, *APC, ESR1, p16, and RARb* [22–24]. The primer sequences and PCR conditions are presented in supplemental Table 1.

2.5. Detection of EGFR Mutations and EML4-ALK Fusion Gene

EGFR mutations in exons 18 (T719X), 19 (deletion), 21 (L858R), and 20 (T790 M) were analyzed by a PNA-LNA clamp method using DNA as template. The PNA-LNA clamp method used has been reported previously [25]. Twenty-three out of 61 patients were chosen for this *EGFR* mutation analysis based on the existence of one of the following characteristics; female, never or light smoker, or pathological diagnosis of adenocarcinoma.

Detection of *EML4-ALK* fusion gene was performed by multiplex RT-PCR as previously described [26]. The primer sequences and PCR conditions are presented in supplemental Table 1.

2.6. Statistical analysis

In this study we defined sensitivity as analytical sensitivity, which referred the proportion of the number of individuals in the classifications true positive/(true positive + false negative), and specificity as analytical specificity, which referred the proportion of the number of individuals in the classifications true negative/(true negative + false positive). The chi-square test was used to compare the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of DNA hypermethylation between the