Research Article

In vivo Observation of Lung Cancer Cells on Endobronchial Lesions using an Endocytoscopy

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Abstract

Objectives: Endocytoscopy is a new endoscopic imaging device for *in vivo* visualizing cellular structures. The aim of this study was to observe lung cancer cells using an endocytoscopy and compare them with the microscopic findings.

Methods: Between July 2009 and April 2011, 12 patients with lung cancer (5 with squamous cell carcinoma, 4 with small cell carcinoma and 3 with adenocarcinoma) and 3 control subjects who had no abnormal findings in large airway were examined. The patients had endobronchial lesions of lung cancer. After conventional bronchoscopy, the lesions were stained with 0.25% methylene blue dye and examined with endocytoscopy. The endocytoscopic images of the lesions were compared with the corresponding microscopic findings of the biopsy and/or brushing specimen. In the same way, normal bronchial mucosa in the control subjects were examined by endocytosocpy and compared with the microscopic findings of the biopsy specimen.

Results: Endocytoscopy showed columnar epithelial cells on the normal bronchial mucosa in the control subjects. These cells were arranged regularly. On the other hand, in patients with lung cancer, polymorphic or oval cells were observed in squamous cell carcinoma, and round or oval cells were observed in both small cell carcinoma and adenocarcinoma. The heterogeneity in the cell distribution was found. In quantitative analysis, the cell area ($p<0.002$) and the nucleus-cytoplasm ratio ($p<0.002$) of tumor cells in the endoscopic images were significantly higher than those of normal bronchial epithelial cells, respectively.

Conclusion: Endocytoscopy was supposed to have the potential to provide *in vivo* microscopic diagnosis during bronchoscopy.

INTRODUCTION

Bronchoscopy is an essential diagnostic tool for abnormal lesions in the respiratory tract. Recently, several diagnostic modalities in endoscopic imaging have been developed for differentiating abnormal mucosal lesions from normal bronchial mucosa, such as narrow band imaging, autofluorescence imaging, optical coherent tomography and high magnification bronchoscopy. Those modalities have been applied for detection of abnormalities in the bronchial mucosa [1-3]. However, direct observation of the cellular structures on the bronchial mucosa or the lesions by bronchoscopy will be helpful for *in vivo* diagnosis.

More recently, two types of endoscopes allowing real-time *in vivo* visualization of cellular images have been developed.

One is an application of laser-scanning confocal technology; confocal microendoscopy [3,4]. Based on tissue illumination after application of fluorescence agents, confocal microendoscopy uses a laser illumination source that is focused into a spot in the tissue. The light emitted from the focal point is imaged to a pinhole to reject out-of-focus emitted light. Tissue can be optimally sectioned to image a single cellular plane. However, currently, the images obtained from the confocal microscopy are not yet adequate for histological diagnosis when compared with conventional histology acquired from microscopy.

The other is an application of contact microscopic technology; endocytoscopy. Based on the principle of contact light microscopy, endocytoscopy allows visualization of the superficial cellular structures on the bronchial mucosa. Several investigators had

reported *in vivo* observation of tumor cells and normal epithelial cells on the mucosa in gastro-intestinal [5-8] and respiratory tract [9-11] by using endocytoscopy.

In this study, we observed endobronchial lesions of lung cancer by using endocytoscopy and compared these images with the corresponding microscopic findings of the specimens obtained by histological and/or cytological examinations.

PATIENTS AND METHODS

Equipment

The integrated type of endocytoscopy (prototype, BF-Y0005, Olympus Medical Systems Co., Tokyo, Japan) was used for this study, which has two systems; conventional imaging and microscopic imaging (Figure 1). The tip of the microscopic imaging system is based on the principle of contact light microscopy, which consists of a fixed-focus and high-power objective lens that provides microscopic images. These images are conducted to a charge-coupled device. Endocytoscopy provides 570-fold magnifying power in a 19-inch video monitor, which has the spatial resolution of 4μm, a field of view of 400μm, and a depth of view of 0-50μm. Conventional fiberscope for usual observation and forceps channel are also installed.

Patients

Between July 2009 and April 2011, 12 patients with endobronchial lesions with lung cancer (5 with squamous cell carcinoma, 4 with small cell carcinoma and 3 with adenocarcinoma) and 3 control subjects with no abnormality in large airway were enrolled into this study. The profiles of the patients and the control subjects were summarized in Table 1.

Procedures

The patients and the control subjects underwent conventional bronchoscopy and subsequent endocytoscopy. First, we observed trachea and extrapulmonary bronchi of the patients and the control subjects under local anesthesia by conventional bronchoscopy. In brief, after muscle injection of atropine sulfate, 0.5mg, and pentazocine, 15mg, and nebulization of 12ml of a 2% lidocaine solution, we orally inserted bronchoscope into the

Figure 1 Endocytoscopy. Integrated type of endocytoscopy has two separated systems. High magnification system is used for microscopic observation, and conventional fiberscopy system used for usual observation. (A: Objective lens for microscopic view, B: Objective lens for conventional view, C: Light guide lens, D: Forceps channel).

Table 1: Profiles of patients with lung cancer and control subjects.

Case	Age/Sex	Site	Dx	Bronchoscope finding
1	81/F	Right main br	Sq	Mucosal nodule
2	64/M	Left main br	Sq	Mucosal invasion with luminal narrowing
3	66/M	RUL br	Sq	Mucosal nodule with necrosis with obstruction
4	73/M	Left main br	Sq	Polypoid tumor
5	57/M	RUL br	Sq	Mucosal nodule with necrosis
6	58/M	Right truncus intermedius	Sm	Mucosal invasion with luminal narrowing
7	52/M	Right B^8 br	Sm	Mucosal nodule
8	76/M	Right main br	Sm	Mucosal invasion with irregularity
9	69/M	RUL br	Sm	Mucosal invasion with irregularity
10	64/F	LLL br	Ad	Mucosal invasion with luminal narrowing
11	54/F	Left upper division br	Ad	Mucosal invasion with irregularity
12	82/M	Right B^5 br	Ad	Mucosal invasion with luminal narrowing
13	63/M	Right B^6 br	control	normal finding
14	64/M	Right B^6 br	control	normal finding
15	70/F	Right B^6 br	control	normal finding

Abbreviations: No: Patient's Number; Dx: Diagnosis; Ad: Adenocarcinoma; Sm: Small Cell Carcinoma; Sq: Squamous Cell Carcinoma; Control: Control Subject; br: Bronchus; RUL: Right Upper Lobe; LLL: Left Lower Lobe

trachea and examined endobronchial lumen. After conventional bronchoscopy, the mucosal lesions on bronchial mucosa were stained with 2ml or less of 0.25% methylene blue dye, which were sprayed onto the surface of mucosa through a spraying catheter. One minute after the dye spraying, we gently touched the tip of endocytoscopy to the mucosal lesions on the mucosal surface. *In vivo* microscopic images of endocytoscopy were obtained after switching to microscopic imaging. The microscopic images were continuously observed as long as the tip of endoscopy was in contact with the mucosal surface.

After observation with endocytoscopy, we performed biopsy for pathological diagnosis and/or blushing for cytological diagnosis of the mucosal lesions in patients with lung cancer. The mucosal lesions included nodular or polypoid lesions, invasive lesions with mucosal irregularity, and invasive lesions with luminal narrowing or obstruction (Table 1). In control subjects, we observed the orifice of right B^6 bronchus by endocytoscopy and obtained the tissue specimen from the spur of the observed area. Then, these microscopic images of endocytoscopy were compared with the corresponding microscopic findings of the specimen obtained by biopsy and/or blushing. In addition, Hematoxylin-eosin staining and Papanicolaou staining of specimens were used for pathological diagnosis and cytological diagnosis, respectively.

All patients and control subjects gave their informed consent before bronchoscopic examination. The Sapporo Medical University Human Ethics committee approved this study.

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Measurement of cell area and nucleus-cytoplasm ratio in endocytoscopic image

Endocytoscopic images of tumor cells on mucosal lesions of patients with primary lung cancer were morphometrically compared with those of normal bronchial epithelial cells of control subjects.

We selected 4 endocytoscopic images from 3 control subjects, 5 images from 4 patients with adenocarcinoma, 5 images from 4 patients with small cell carcinoma, and 6 images from 5 patients with squamous cell carcinoma.

Then from these images, we selected randomly 20 cells in total from each normal epithelial cell, adenocarcinoma, small cell carcinoma, and squamous cell carcinoma groups for measuring the areas of both cell and cell nucleus.

Next, we measured both the cell area and the cell nucleus area of each cell in the endocytoscopic images by the computer software ImageJ [12], using the horizontal length of the endoscopic image is equal to 400μm. The nucleus-cytoplasm ratio was calculated from the formula that the cell nucleus area divided by the cell area.

Statistical analysis

The results of the cell size and the nucleus-cytoplasm ratio were expressed as median with first quartile point (25 percentile) and third quartile point (75 percentile). The differences between the four groups were assessed by using the Kruskal Wallis H-test. When the differences were considered statistically significant, subsequently Mann-Whitney U-test with Bonferroni correction was performed for post hoc test. The differences were considered statistically significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

Endocytoscopy showed cellular structures on the normal bronchial mucosa and the endobronchial tumor lesions invaded by lung cancer. The images were supposed to be similar to horizontal section of the superficial cell layer. By staining with 0.25% methylene blue dye, the nuclei and the cytoplasm were stained dark and pale blue, respectively.

In control subjects with normal bronchoscopic findings, endocytoscopy showed columnar epithelial cells which were approximately equal size and arranged regularly on the normal bronchial mucosa (Figure 2). They were observed as rectangular or round shape. On the other hand, in patients with lung cancer, endocytoscopy showed tumor cells on the endobronchial lesions, which were larger than the normal bronchial epithelial cells. Irregular shaped polymorphic or oval cells were observed on the surface of the tumor with squamous cell carcinoma (Figure 3). Round or oval cells were observed on the surface of the tumor with both small cell carcinoma and adenocarcinoma (Figure 4, 5). There was heterogeneity of the distribution, the size and the shape of tumor cells.

Next, we compared both the cell area and the nucleus-cytoplasm ratio of endocytoscopic images in order to define the difference of the cell size and the nucleus-cytoplasm ratio of tumor cells between lung cancer and control groups. According to our quantitative analysis, there were significant differences in both the cell area and the nucleus-cytoplasm ratio among four groups including control, adenocarcinoma, small cell carcinoma and squamous cell carcinoma (Table 2). As shown in Figure 5, the cell area and the nucleus-cytoplasm ratio of tumor cells of three histological types were significantly higher as compared with those of normal bronchial epithelial cells, respectively. Among three histological types of lung cancer, the cell area of squamous cell carcinoma was significantly higher than that of small cell carcinoma and the nucleus-cytoplasm ratio of small cell carcinoma was significantly higher than that of adenocarcinoma. However, there was no significant difference in the cell area between adenocarcinoma and small cell carcinoma, and between adenocarcinoma and squamous cell carcinoma. There was no significant difference in the nucleus-cytoplasm ratio between adenocarcinoma and squamous cell carcinoma, and between small cell carcinoma and squamous cell carcinoma. These results supported our findings of the endocytoscopic images such as the cell size or the nucleus-cytoplasm ratio. Based on our analysis, we thought that the cell images of endocytoscopy expressed well the characteristics of living cells.

There were several studies evaluating endocytoscopy about diagnosis of lung cancer. Shibuya et al. [9] reported that
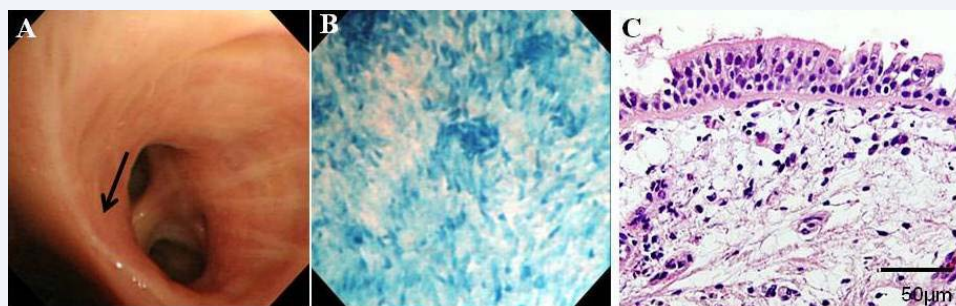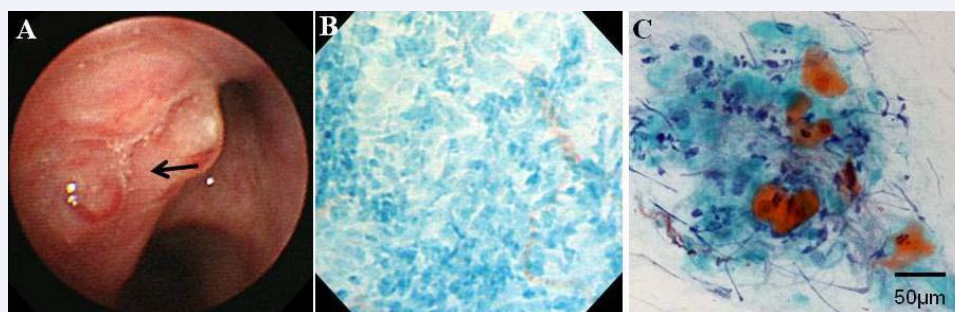

Figure 2 Normal bronchial mucosa.
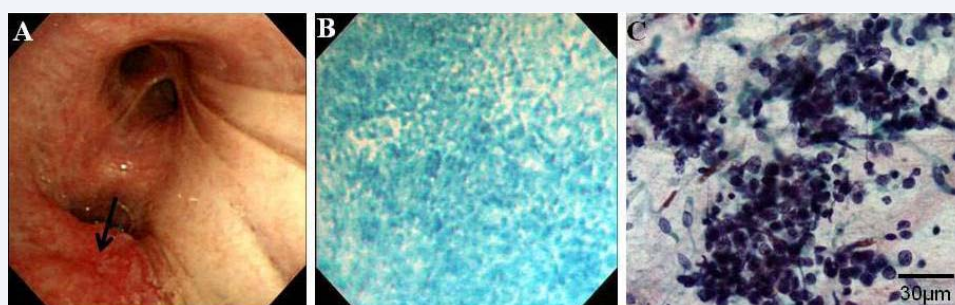(A) The orifice of right B⁶ bronchus is observed. Arrow shows the observed area by endocytoscopy.
B) Endocytoscopy shows columnar epithelial cells on the mucosal surface with 0.25% methylene blue dye staining. The nucleus and cytoplasm are stained with dark and pale blue, respectively. The epithelial cells are regularly arranged and the nucleus-cytoplasm ratio is relatively constant. Cilia of the epithelial cells are not visualized.
(C) Biopsied specimen of the observed site shows normal bronchial epithelial cells. (Hematoxylin-eosin staining)

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Figure 3 Squamous cell carcinoma

(A) Nodular mucosal lesion is observed in the right main bronchus. Arrow shows the observed area by endocytoscopy.

(B) Endocytoscopy shows polygonal or oval cells on the nodular lesion with 0.25% methylene blue dye staining. Heterogeneities in the cell distribution and the cell shape are seen. The nucleus-cytoplasm ratio is high.

(C) Cytological examination reveals squamous cell carcinoma. (Papanicolaou staining)

Figure 4 Small cell carcinoma

(A) Invasive mucosal lesion in right main bronchus and intermediate bronchus is observed. Arrow shows the observed area by endocytoscopy.

(B) Endocytoscopy shows comparatively small round or oval cells on the lesions with 0.25% methylene blue dye staining. Heterogeneities in the cell distribution are seen. The nucleus-cytoplasm ratio is also high. The cell size of tumor cells was larger than that of normal bronchial epithelial cells.

(C) Cytological examination reveals small cell carcinoma. (Papanicolaou staining)

Table 2: Cell area and nucleus-cytoplasm ratio.

|	Control subject	Ad	Sm	Sq	*p value*
Number of cells	20	20	20	20	-
Cell area (μm^2)	102.9 (82.4-128.7)	197.9 (178.3-262.5)	173.9 (144.7-219.6)	255.3 (210.7-295.8)	<0.01
Nucleus-cytoplasm ratio	0.30 (0.28-0.33)	0.40 (0.35-0.46)	0.60 (0.51-0.68)	0.55 (0.41-0.62)	<0.01

The data are expressed as median with first quartile point (25 percentile) and third quartile point (75 percentile).

Abbreviations: Ad: Adenocarcinoma; Sm: Small Cell Carcinoma; Sq: Squamous Cell Carcinoma

endocytoscopy was useful for discrimination between normal bronchial epithelium, dysplastic mucosa and squamous cell carcinoma. Neumann et al. [10] reported that endocytoscopy visualized numerous small blue cells with hyperchromatic nuclei in patients with small cell carcinoma. These reports proved endocytoscopy useful for *in vivo* diagnosis. According to our study, endocytoscopic images were supposed to correspond with microscopic findings of the specimens obtained by biopsy and/or brushing. Endocytoscopy is thought to provide cellular features of endobronchial tumor lesions that are crucial for the pathological diagnosis of lung cancer; the size, the shape and the nucleus-cytoplasm ratio of tumor cells. Therefore, characteristic cellular images of endocytoscopy may be helpful for real-time *in vivo* diagnosis of endobronchial tumors.

However, there were several limitations in endocytoscopic observation. The first, endocytoscopy did not allow the visualization of deeper layers of the bronchial mucosa, because the observation depth is dependent on the absorption spectra and light penetration depth of the tissue. Therefore, it is impossible to visualize the tumor cells invading submucosal part. The second, endobronchial lesions in the lateral wall of the bronchus was difficult to keep contact with the objective lens of the endocytoscopy. Although the observation was relatively easy for the tumors protruding into the bronchial lumen with forming polypoid lesions or the tumor occurring from bronchial bifurcation, it was difficult for flat tumor that occurred on the side wall of bronchus. The third, respiratory movement and transmission of heart beat made it difficult to obtain interpretable microscopic images during endocytoscopic observation. Because of the contact observation method, microscopic images were often blurred by movement of breathing or vibration transmitted from heart beat in large airway.
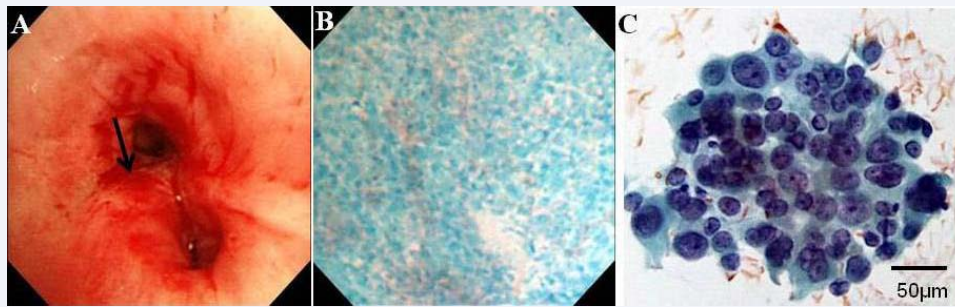
Figure 5 Adenocarcinoma.
(A) Invasive mucosal lesion is observed in left lower lobe bronchus. Arrow shows the observed area by endocytoscopy
(B) Endocytoscopy shows round or oval cells on the lesion with 0.25% methylene blue dye staining. Heterogeneities in the cell distribution are seen. The nucleus-cytoplasm ratio is high. The cell size of tumor cells was larger than that of normal bronchial epithelial cells.
(C) Cytological examination reveals adenocarcinoma. (Papanicolaou staining)
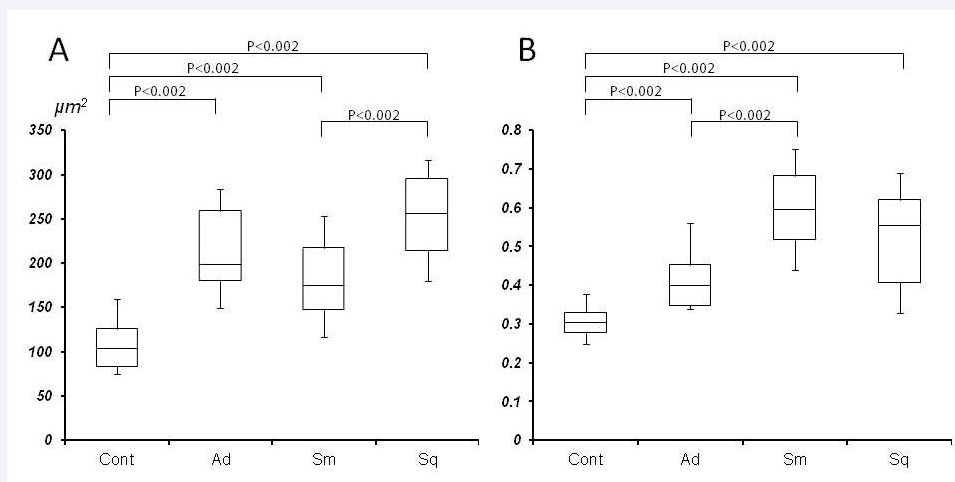
Figure 6 Comparison of cell area (A) and nucleus-cytoplasm ratio (B).
Both the cell area and the nucleus-cytoplasm ratio of lung cancer cells are significantly increased as compared with those of normal bronchial epithelial cells, respectively. Among histological types of lung cancer cells, the cell area of squamous cell carcinoma is significantly higher than that of small cell carcinoma, and the nucleus-cytoplasm ratio of small cell carcinoma is significantly higher than that of adenocarcinoma.

In this study, endocytoscopy with 570-fold magnifying power was used. However, the images were thought to be insufficient to differentiating the histological types of lung cancer as compared to those of microscopy. Development of high quality imaging method of *in vivo* cellular structure might be required for endoscopic diagnosis of atypical or malignant cells. In the future, endobronchial tumor may be judged to be benign or malignant by *in vivo* observation during bronchoscopy.

CONCLUSION

Endocytoscopy showed columnar epithelial cells on normal bronchial mucosa and tumor cells on the endobronchial lesions of lung cancer. The endocytoscopic images of the epithelial and the tumor cells were comparatively corresponded with the cellular features of microscopic findings. Endocytoscopy may have the potential to provide pathologic diagnosis during bronchoscopy. Microscopic visualization of tissue at cellular level may lead to real-time *in vivo* diagnosis in the future.

REFERENCES

1. Thiberville L, Salaun M. Bronchoscopic advances: on the way to the cells. Respiration. 2010; 79: 441-449.

2. Dooms C, Seijo L, Gasparini S, Trisolini R, Ninane V, Tournoy KG. Diagnostic bronchoscopy state of art. Eur Respir Rev. 2010; 19: 229-236.

3. Ohtani K, Lee AMD, Lam S. Frontiers in bronchoscopic imaging. Respirology. 2012; 17: 261-269.

4. Thiberville L, Salaun M, Lachkar S, Dominique S, Moreno-Swirc S, Vever-Bizet C, et al. Confocal fluorescence endomicroscopy of the human airways. Proc Am Thorac Soc. 2009; 6: 444-449.

5. Inoue H, Kudo S, Shiokawa A. Novel endoscopic imaging techniques toward in vivo observation of living cancer cells in the gastrointestinal tract. Dig Dis. 2004; 22: 334-337.

6. Kumagai Y, Monma K, Kawada K. Magnifying chromoendoscopy of the

esophagus: in-vivo pathological diagnosis using an endocytoscopy system. Endoscopy. 2004; 36: 590-594.

7. Kumagai Y, Kawada K, Yamazaki S, Iida M, Momma K, Odajima H, et al. Endocytoscopic observation for esophageal squamous cell carcinoma: can biopsy histology be omitted? Dis Esophagus. 2009; 22: 505-512.

8. Meroni E, Gatteschi B, Fasoli A, Munizzi F, Frascio F, Pugliese V, et al. Detection of tissue abnormalities in normal mucosa surrounding colorectal cancer using an endocytoscopy system. Endoscopy. 2007; 39: 369-370.

9. Shibuya K, Fujiwara T, Yasufuku K, Alaa M, Chiyo M, Nakajima T, et al. In vivo microscopic imaging of the bronchial mucosa using an endo-cytoscopy system. Lung Cancer. 2011; 72: 184-190.

10. Neumann H, Vieth M, Neurath MF, Fuchs FS. In vivo diagnosis of small-cell lung cancer by endocytoscopy. J Clin Oncol. 2011; 29: e131-132.

11. Kitamura Y, Yamada G, Narita Y, Kameda M, Yamada Y, Kitada J, et al. Bronchoscopic observation of endobronchial tumor cells. J Bronchology Interv Pulmonol. 2012; 19: 311-312.

12. Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. Biophotonics International. 2004; 11: 36-42.