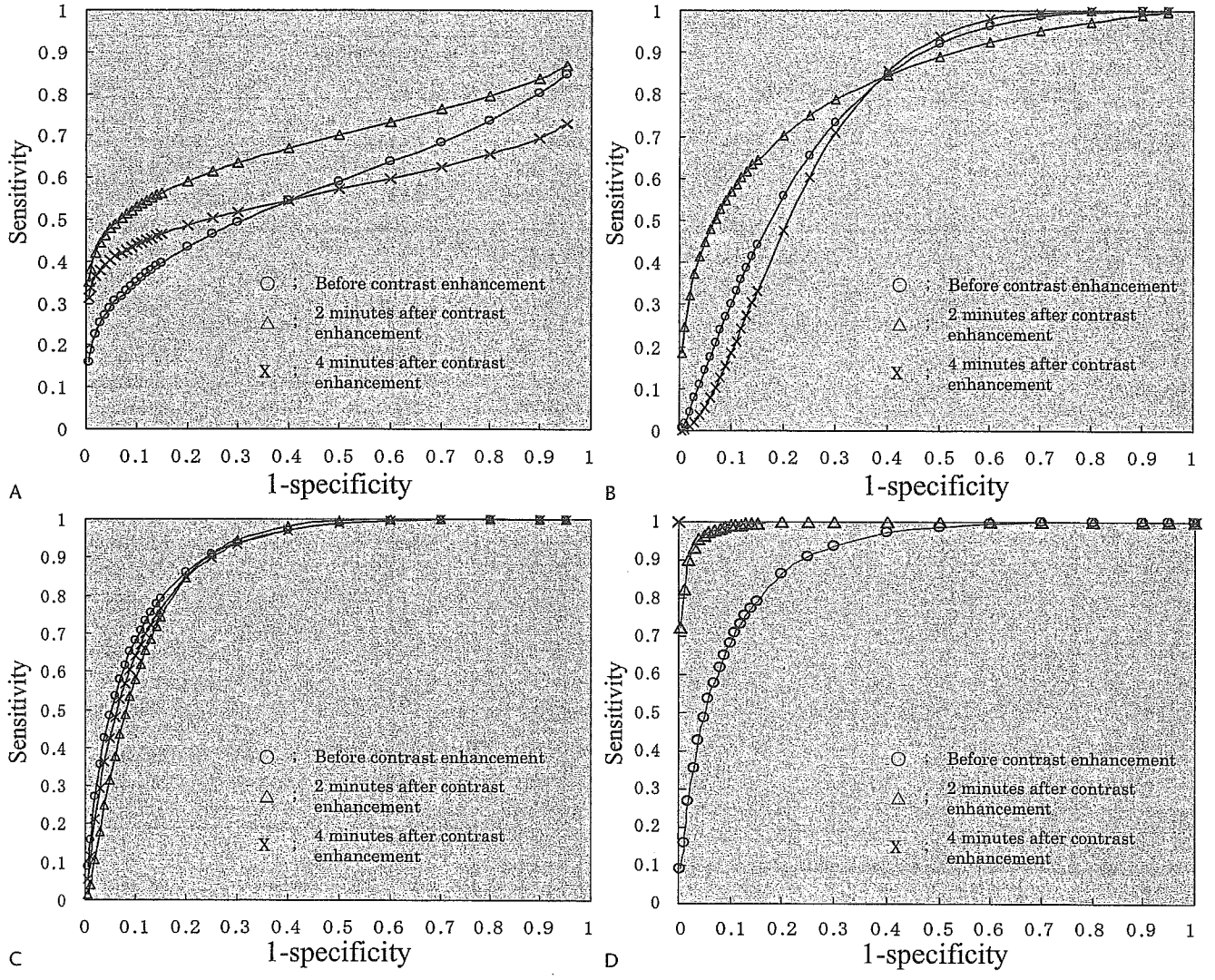


FIGURE 4. Receiver operating characteristic curves for each parameter used to differentiate between malignant and benign nodules. The open circles (○) are before contrast enhancement, and the open triangles (△) and crosses (x) are 2 and 4 minutes after contrast enhancement, respectively. A, Areas under the open circle (○), open triangle (△), and cross (x) curves for attenuation are 0.58 ± 0.07, 0.69 ± 0.07, and 0.57 ± 0.08, respectively. B, Areas under the open circle (○), open triangle (△), and cross (x) curves for curvedness value are 0.78 ± 0.06, 0.83 ± 0.05, and 0.76 ± 0.06, respectively. C, Areas under the open circle (○), open triangle (△), and cross (x) curves for shape index are 0.90 ± 0.04, 0.89 ± 0.05, and 0.90 ± 0.04, respectively. D, Areas under the open circle (○), open triangle (△), and cross (x) curves for the combination of all 3 parameters (attenuation, shape index, and curvedness value) are 0.91 ± 0.04, 0.99 ± 0.01, and 1.00, respectively.

enhancement, 0 FN findings and 3 FP findings (cases 41, 57, and 58) 2 minutes after enhancement, and 0 FN findings and 0 FP findings 4 minutes after enhancement. Sensitivity values were 94%, 100%, and 100%; specificity values were 74%, 89%, and 100%; and accuracy values were 85%, 92%, and 100%, respectively. Positive predictive values were 83%, 92%, and 100%, and negative predictive values were 91%, 100%, 100%, respectively.

DISCUSSION

The usefulness of diagnostic imaging, focusing mainly on CT, for the evaluation of SPNs has been reported by researchers at a number of medical institutions.[1–4] Several of them have also attempted to differentiate between benign and malignant lesions by using contrast medium and evaluating attenuation within nodules over time.[5–8] These studies were based on attenuation and contrast enhancement patterns obtained for only a few slices in which the nodule was demonstrated, however.

In the present study, the entire nodule was scanned using CE dynamic HCT, and changes in the density and characteristic values (attenuation, shape index, and curvedness value) within the nodule were calculated for 3D quantification with a computer to discriminate between benign and malignant lesions. Contrast-enhanced dynamic HCT in combination with

the computer-aided diagnosis may thus improve the differential diagnosis of BNs and MNs.

With regard to the evaluation and interpretation of the CT data on the lesion, conventional studies have focused only on the 2-dimensional assessment of attenuation and the enhancement patterns in a few slices. The results of these studies were simple and practical, identifying the factors effective for the differential diagnosis to be an observed contrast effect of 20 Hounsfield units (HU) or greater[5,6] or 15 HU or greater,[8] enhancement of the entire lesion,[5] and a high CT value ratio between the nodule and arteries.[7] One problem was that the attenuation was strongly affected by the slice selected or the position of the ROI in the lesion, which was set manually. In the present study, this problem was avoided by automatically extracting the lesion as 3D volume data.[9,12] In addition, the nodule was evaluated by calculating the characteristic values within the nodule using a computer and measuring the density using 3 parameters (attenuation, shape index, and curvedness value).[10] The results showed that evaluation based on the combination of all 3 parameters provided the best results. Using this analysis method, each pixel within a tumor is expressed locally using the attenuation and the shape index and curvedness obtained from the 3D curvature, and the entire lesion is then characterized as benign or malignant using the histogram characteristic values. When these 3 histogram characteristic values were compared with each another, the shape histogram characteristic value was found to be superior to the other 2 values. The combination of these 3 characteristic values provided even better results. It is thought that a more detailed characteristic value for the internal structure of a tumor can be obtained by expressing the internal structure as a combination of attenuation and 3D curvatures.

When a linear discriminant function score not less than 0 at 2 and 4 minutes after enhancement was considered to indicate malignancy, the results showed 0 FN findings and 3 FP findings at 2 minutes after enhancement and no FN or FP findings at 4 minutes. When a linear discriminant function score of 0 or higher was considered to indicate malignancy, benign and malignant lesions were distinguished in all the patients using the data obtained 4 minutes after enhancement. It was considered that the values at 2 minutes were affected by the degree of minute blood vessel density within the nodule and that the values at 4 minutes were affected by the rate of contrast medium flowing into the papillary vessels and interstitial tissues or by the volume of the interstitial tissues.[18] In summary, compared with the techniques used in previous studies, the method described in the present study permits lesions to be extracted with fewer manual operations and higher reproducibility and is based on 3D analysis using 3 parameters (attenuation, shape index, and curvedness value).

The limitations of the present study are as follows. Although the objective of this study was to evaluate the entire nodule, it was difficult to visualize the entire nodule over time, even when an HCT scanner was used. As a result, lesions could not be assessed in 10 patients. It is expected that this problem can be overcome by the introduction of multislice HCT scanners in the near future. In this study, the score was assessed at each time point (before contrast enhancement and 2 and 4 minutes after contrast enhancement). In a strict sense, these scores do not represent the changes in the density of the lesion over time. In the assessment of changes over time, it is important to acquire CT images in exactly the same slice at each time point. The changes over time can then be obtained by performing subtraction between the images before and after contrast enhancement. In practice, however, it is difficult to acquire exactly the same slice at each time point because of the patient's respiratory motion. We are currently working to develop a new algorithm to overcome this problem. When this algorithm is complete, we plan to assess the changes in contrast medium density in lesions over time using subtraction.

In the future, CT-based lung cancer screening is expected to become more widely accepted, resulting in the detection of a larger number of SPNs.[19,20] Therefore, it is likely to become increasingly important to determine whether these lesions are benign or malignant based on evaluation of the images obtained.

Contrast-enhanced dynamic HCT was used for the computer-aided diagnosis of SPNs in the present study. The data obtained using this imaging technique permit the internal structure of lesions to be quantified in a 3D manner and evaluated over time. The results showed that this method is effective for differentiating between BNs and MNs. In the future, further prospective studies should be conducted based on the results reported here and standards for the evaluation of lesions using computer-aided analysis should be established.

REFERENCES

1. Mori K, Saitou Y, Tominaga K, et al. Small nodular lesions in the lung periphery: new approach to diagnosis with CT. *Radiology.* 1990;177: 843–849.
2. Kuriyama K, Tateishi R, Doi O, et al. CT-pathologic correlation in small peripheral lung cancers. *AJR Am J Roentgenol.* 1987;149:1139–1143.
3. Zwirewich CV, Vedal S, Miller RR, et al. Solitary pulmonary nodule; high-resolution CT and radiologic pathologic correlation. *Radiology.* 1991;179:469–476.
4. Siegelman SS, Khouri NF, Leo FR, et al. Solitary pulmonary nodules: CT assessment. *Radiology.* 1986;160:307–312.
5. Yamashita K, Matsunobe S, Tsuda T, et al. Solitary pulmonary nodule: preliminary study of evaluation with incremental dynamic CT. *Radiology.* 1995;194:399–405.
6. Swensen SJ, Brown LR, Colby TV, et al. Lung nodule enhancement at CT; prospective findings. *Radiology.* 1996;201:447–455.
7. Zhang M, Kono M. Solitary pulmonary nodules: evaluation of blood flow patterns with dynamic CT. *Radiology.* 1997;205:471–478.
8. Swensen SJ, Viggiano RW, Midthun DE, et al. Lung nodule enhancement at CT: multicenter study. *Radiology.* 2000;214:73–80.
9. Kawata Y, Niki N, Ohmatsu H, et al. Quantitative surface characterization of pulmonary nodules based on thin-section CT images. *IEEE Trans Nucl Sci.* 1998;45:2132–2138.
10. Kawata Y, Niki N, Mori K, et al. Curvature based analysis of internal structure of pulmonary nodules using thin-section CT images. *Proc IEEE Int Conf Image Processing.* 1998;3:851–855.
11. Armato SG, Li F, Giger ML, et al. Lung cancer: performance of automated lung nodule detection applied to cancers missed in a CT screening program. *Radiology.* 2002;225:685–692.
12. Caselles V, Kimmel R, Sapiro G, et al. Minimal surfaces based object segmentation. *IEEE Trans Pattern Anal Mach Intell.* 1997;19:394–398.
13. Koenderink JJ, van Doorn AJ. Surface shape and curvature scales. *Image Vis Comput.* 1992;10:557–565.

14. Dorai C, Jain AK. COSMOS-A representation scheme for 3D free-form objects. *IEEE Trans Pattern Anal Mach Intell.* 1997;19:1115–1130.
15. Duda RO, Hart PE. Pattern classification and scene analysis. In: Rosen CA, ed. *Linear Discriminant Functions.* New York: John Wiley & Sons; 1973:130–188.
16. Jain AK, Duin RPW, Mao J. Statistical pattern recognition: a review. *IEEE Trans Pattern Anal Mach Intell.* 2000;22:4–37.
17. Yankelevitz DF, Henschke CI. Does 2-year stability imply that pulmonary nodules are benign? *AJR Am J Roentgenol.* 1997;168:325–328.
18. Newhouse JH, Murphy RX. Tissue distribution of soluble contrast: effect of dose variation and changes with time. *AJR Am J Roentgenol.* 1981;136: 463–467.
19. Kaneko M, Eguchi K, Ohmatsu H, et al. Peripheral lung cancer: screening and detection with low-dose spiral CT versus radiography. *Radiology.* 1996;201:798–802.
20. Mori K, Tominaga K, Moriyama N, et al. Utility of low-dose helical CT as a second step after plain chest radiography for mass screening for lung cancer. *J Thorac Imaging.* 1997;12:173–180.

Int. J. Cancer: **117**, 460–468 (2005)

Identification of postoperative adjuvant chemotherapy responders in non-small cell lung cancer by novel biomarker

Takashi Hirano[1]*, Harubumi Kato[1], Masahiro Maeda[2], Yunbo Gong[1], Yanning Shou[1], Miho Nakamura[2], Junichi Maeda[1], Koichi Yashima[1], Yasufumi Kato[1], Shingo Akimoto[3], Tatsuo Ohira[1], Masahiro Tsuboi[1] and Norihiko Ikeda[1]

[1]*Department of Surgery, Tokyo Medical University, Shinjuku-ku, Tokyo, Japan*
[2]*IBL, 1091 Naka, Fujioka-City, Gunma, Japan*
[3]*MedicalProteoscope, Shinjuku-sumitomo Bldg. 7F, Nishishinjuku, Shinjuku-ku, Tokyo, Japan*

Cisplatin-based (CDDP-based) adjuvant chemotherapy of non-small cell lung cancer (NSCLC) was reported to yield 5–15% improvement in 5-year survival compared to complete resection alone. The importance of information concerning preselection of good responders has become increasingly evident. The purpose of our study is the establishment of a preselection of good responders for CDDP-based adjuvant chemotherapy. We investigated protein expressions comparing intensity between parent strains (H69 and PC14 lung cancer cultured cells) and resistant strains against CDDP using 2-dimensional polyacrylamide gel electrophoresis (2-DE). Immunohistochemically, we evaluated the relationship between protein expression associated with CDDP-resistance and the clinical effects of platinum-based postoperative adjuvant chemotherapy using 126 surgically-resected NCLC materials. We detected 2 kinds of polypeptides that changed expression levels on 2-DE gels. The analyses of the amino acid sequence showed that these polypeptides were reticulocalbin (RCN) and glutathione-*S*-transferase-π (GST-π). The 2-DE analysis showed decreased expression in RCN and overexpression in GST-π with the acquisition of CDDP-drug resistance. RCN-transfectant of H69 CDDP-resistant strain showed intermediate sensitivity between the parent strain and the CDDP-resistant strain. RCN-positive cases showed a statistically significant better disease-free survival only in the cases receiving postoperative platinum-based adjuvant chemotherapy after curative resection ($p = 0.007$). In addition, cases that were both RCN-positive and GST-π-negative showed a statistically significantly better outcome ($p = 0.0150$). In the cases without postoperative adjuvant chemotherapy no relationship between the outcome and these expressions was seen. The evaluation of RCN and GST-π might provide valuable information concerning postoperatively therapeutic strategy from the standpoint of individualized postoperative therapy.

Key words: reticulocalbin; glutathione-*S*-transferase-π; cisplatin; individual therapy; non-small cell lung cancer

Death due to lung cancer is still increasing in Japan and most Western countries, despite intensive application of various therapeutic strategies, and it is still the leading cause of cancer death in Japan. Though the opportunities of relatively early detection and treatment increase, still more than half of cases of primary lung cancer show advanced stage at the initial definitive diagnoses. Several years ago, we routinely carried out postoperative adjuvant chemotherapy using platinum (cisplatin [CDDP] or carboplatin) for patients with advanced stage non-small cell lung carcinoma (NSCLC) because distant metastasis occurred frequently after surgical treatment only. Clinically favorable outcome was not obtained, however, and the 5-year survival of Stage IIIA was approximately 25%, despite postoperative adjuvant chemotherapy. It had been believed that the efficacy of adjuvant chemotherapy in surgically resected lung cancer was controversial and that the patient's outcome was not attribute to postoperative adjuvant chemotherapy because the 1995 meta-analysis suggested that patients with complete surgical resection who received CDDP-based chemotherapy had only a 5% improvement in 5-year survival compared with those treated only by complete resection.[1]

In 2004 reports demonstrated the benefit of postoperative adjuvant chemotherapy.[2–4] The indication of postoperative chemotherapy is no longer considered controversial. We reported recently that postoperative adjuvant chemotherapy with oral UFT (DPD Inhibitory Fluoropyrimidine, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) provided better survival than surgical treatment alone in patients with Stage I adenocarcinoma of the lung.[5] We believe that the concept of postoperatively adjuvant chemotherapy that micrometastasis will be controlled does not conflict with the improvement of the prognosis in primary lung cancer. Several courses of systemic chemotherapy are usually designed. However, it is often difficult for patients who have undergone surgical resection to receive even a single course of systemic chemotherapy just after surgery. Some patients with advanced lung cancer (where surgical treatment is not indicated) sometimes respond favorably to systemic chemotherapy. It is very important, therefore, that cases that will respond well to adjuvant chemotherapy are selected.

Comprehensive analysis of human proteins (*i.e.*, proteomics) based on the establishment of human genome database has begun. Proteomics is the science by which proteins are investigated with regard to their roles as functional elements. Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a strong tool in proteomics. We have applied this methodology to clinical materials of solid malignant neoplasm since 1992.[6–8] We detected some polypeptides related to drug resistance comparing 2-DE patterns of parent strains of lung cancer cultured cells with the CDDP-resistant strains. We investigated the relationship between the expression of these polypeptides and its clinically postoperative effects of patients with NSCLC.

Material and methods

Cultured cells

Lung cancer cultured cells (H69 and PC14) and their CDDP-resistant strains were kindly provided by Dr. Saijo (National Cancer Center, Tokyo, Japan).

Surgically resected NSCLC materials for 2-DE analysis

Surgically resected materials were cut in the middle and cancerous cells were collected by scraping on the surface of the tumor. After the collection of tumor cells the sample preparation for 2-DE was started as soon as possible.

2-DE polyacrylamide gel electrophoresis

In cultured cells and surgically resected materials of NSCLC we prepared samples for 2-DE using the non-enzymatic sample preparation described previously.[9] Tumor cells were fractured by repeated freezing and thawing, and the soluble fractions were lyophilized after adding DNAase-RNAase. The materials were resolubilized using a sample buffer. Isoelectric focusing (IEF) was

Grant sponsor: Japan Society for the Promotion of Science; Grant number: 10671271; Grant sponsor: Tokyo Medical University.
***Correspondence to:** Department of Surgery, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
Fax: +81-3-3342-6154. E-mail: thirano@tokyo-med.ac.jp
Received 8 November 2004; Accepted after revision 25 February 2005
DOI 10.1002/ijc.21172
Published online 17 May 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – CLINICOPATHOLOGICAL BACKGROUND OF SURGICALLY RESECTED NSCLS CASES FOR EVALUATION OF IMMUNOHISTOCHEMICAL EXPRESSION OF RETICULOCALBIN AND GST-π

	Platinum-based adjuvant chemotherapy	
	(−)	(+)
Number of cases	59 cases	67 cases
Male:Female	42:17	43:24
Age (Mean)	43–79 (65.7)	35–79 (62.6)
Histopathological type		
Adenocarcinoma	43 cases	41 cases
Squamous cell carcinoma	13 cases	21 cases
Large cell carcinoma	2 cases	2 cases
Adenosquamous cell carcinoma	1 cases	3 cases
Stage I A	23 cases	2 cases
Stage I B	17 cases	7 cases
Stage II A	3 cases	7 cases
Stage II B	6 cases	10 cases
Stage III A	8 cases	29 cases
Stage III B	2 cases	12 cases
Reduced expression of RCN	42 cases (71.2 %)	45 cases (67.2 %)
Overexpression of GST-π	29 cases (49.2 %)	33 cases (49.3 %)

used in the first dimension followed by SDS-PAGE. A sample corresponding to 30 μg of protein was applied to IEF tubes and focused for 14.5 hr at 800 V and for 1 hr at 1,000 V. After IEF, IEF gels were set on top of a linear gradient 10–13% of SDS polyacrylamide gel and electrophoresed overnight using 10 mA per gel at 10°C. After protein fixation followed by SDS-PAGE, proteins were visualized by silver staining.

Collection of polypeptides and its analysis of amino acid sequence

Polypeptides were transferred to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) using the Western blotting method and visualized using Coomassie brilliant blue staining. The spots were collected from the dried Immobilon polyvinyl difluoride membrane. Collected polypeptide was applied to a gas-phase protein sequencer (HPG1005A Protein Sequencing System; Hewlett-Packard Co., Palo Alto, CA) to determine the N-terminal amino acid sequence.

Cloning of human reticulocalbin cDNA and expression in Escherichia coli *and mammalian cells*

The full-length cDNA of human reticulocalbin (RCN) was amplified from the first-strand cDNA obtained from H69 cells by using forward primer (5′-GCGGTACCGGGACGATGGCGC-GCGGTGGC-3′: hRCN-1) and reverse primer (5′-GCAAGCTTG-AGTGTCTATCAAAGCTCATC-3′: hRCN-2) synthesized and based on the published human RCN cDNA sequence. The amplified products were digested with *Kpn*I and *Hind*III, ligated into pRSET B vector (Invitrogen, Carlsbad, CA) and used to transform *E. coli* JM109 cells. RCN cDNA cloned by PCR was sequenced completely.

The full-length reticulocalbin cDNA in pRSET B vector was transferred into pcDNA3.1(+) (Invitrogen). RCN cDNA in pcDNA3.1(+) was transfected into CDDP-resistant H69 cells (H69/CDDP) by using Lipofectamine (Invitrogen). Resistant colonies against G418 were selected, cultured and cloned by limiting dilutions. The expression level of RCN protein in each stable transfectant was checked by Western blotting with anti-RCN mAb (see below). These stable transfectants were used for MTT assay against CDDP.

MTT assay of H69/CDDP cells transfected with RCN cDNA

Effect of transfected RCN cDNA on sensitivity to cisplatin in H69/CDDP cells was determined by MTT assay. Briefly, 50 μl aliquots of exponentially growing cell suspension containing 10^4 H69, H69/CDDP and H69/CDDP transfected with RCN cDNA were seeded in 96-well microtiter plates (NUNC, Rochester, NY) with 50 μl aliquots of cisplatin at various concentration (100–1.56 μM), incubated for 4 days. After incubation, 25 μl of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (NTT) (Sigma Chemical, St. Louis, MO) solution (6 mg/mL in PBS) was added to each well and the plates were incubated for 4 hr. One hundred microliters of 10% SDS solution was then added to each well and incubated at 37°C overnight. The optical density of each well was measured at 590 nm. Each experiment included triplicate wells for each cisplatin concentration and 3 independent experiments were carried out. Wells containing only medium and MTT were used as controls. We statistically estimated the difference of cellular viability at 50 μM of cisplatin in NTT assay using the Mann-Whitney *U*-test.

Production and purification of monoclonal antibodies against RCN

The synthetic peptide used for immunization was KPTVRK-ERVVRPDSELG, which corresponds to the K30 to G46 amino acid sequence of RCN. This peptide was coupled with bovine thyroglobulin and mixed with incomplete adjuvant, then injected subcutaneously (s.c.) into mice (BALB/c).

After immunization, spleen cells were collected and polyethylene glycol-mediated cell fusion between spleen cells and X63-Ag8-653 cells was induced.[10] The hybridoma cells were screened with an antigen-coated enzyme-linked immunosorbent assay (ELISA). Positive clones were cultured for production of antibodies in RPMI1640 supplemented with 10% FCS. Monoclonal antibody (mAb) was extracted from the culture medium and purified with an antigen peptide-conjugated affinity column.

Another mAb was produced against recombinant RCN protein expressed as GST fused protein in *E. coli* as follows. The C-terminal region of RCN, which corresponded to the 90 amino acid residues of RCN, was amplified from the full-length RCN cDNA obtained from H69 cells by using forward primer (5′-GCG-TCGACGGGAGCAGTTTAACGAATTCC-3′: hRCN-13) and reverse primer (5′-CTGCGGCCGCGAGTGTCTATCAAAGCT-CAT-3′: hRCN-6). The amplified products were digested with *Sal*I and *Not*I, ligated into pGEX-6P-1 vector (Amersham Bioscience, Piscataway, NJ) and used to transform *E. coli* BL21/pLys cells (RCN-C/GST). RCN-C/GST fusion protein was purified with Glutathione-Sepharose 4B (Amersham Bioscience) and used for production of mAb against C-terminal region of RCN protein as an immunogen.

The hybridoma cells were produced and screened as described above. Positive clones were cultured for production of antibodies in ASF104 media (Ajinomoto, Tokyo, Japan) as a complete medium and TIL-High-glucose (IBL) as a nutrient medium using INTEGRA CELLine culture vessel (INTEGRA Bioscience, Inc.,

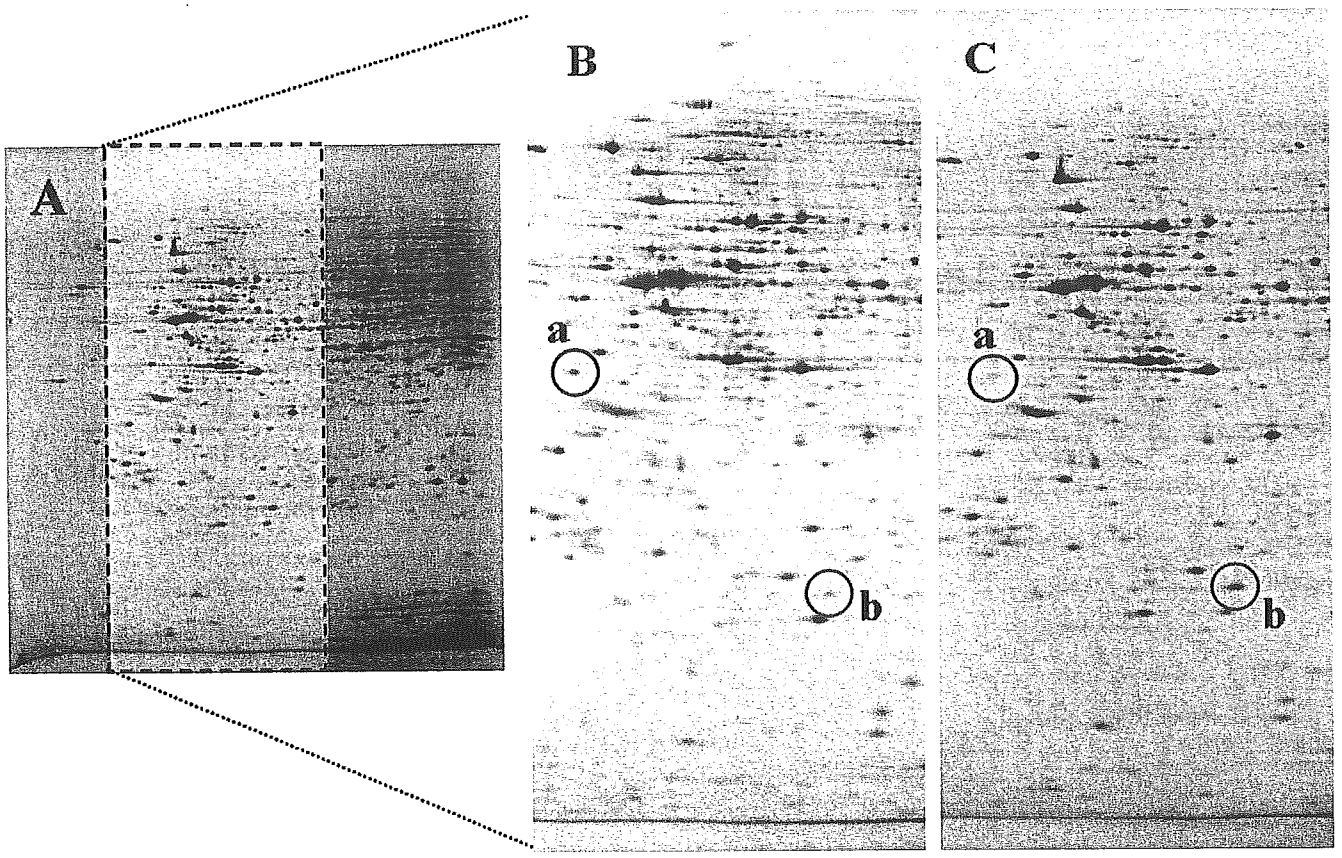


FIGURE 1 – (*a*) Overview in the 2-DE gel of H69 cultured cells. (*b*) Center area in 2-DE gel in parent strain of H69. (*c*) Center area in 2-DE gel in CDDP-resistant strain of H69. The intensity of spot a decreased remarkably in CDDP-resistant strain. The elevation of the intensity of Spot b was observed.

Chur, Switzerland) and purified with Protein A-Sepharose CL-4B affinity column (Amersham Bioscience).

Western blot and immunodetection with mAb against RCN after 2-DE

H69 cells (parent strain) were subjected to 2-DE, and proteins were transferred electrophoretically to Immobilon PVDF membrane (Millipore). The membrane was immunoblotted with anti-RCN mAbs and detection carried out with an enhanced chemiluminescence (ECL) Western detection reagents kit (Amersham Bioscience). Western blot analysis and immunodetection using anti-RCN mAbs (mAb clone 6A1 recognized the N-terminal of RCN and mAb clone M23A2 recognized the C-terminal of RCN) on H69 cells were carried out after 2-DE.

Evaluation of the expression levels of RCN and GST-π in cultured cells

H69 cells (parent strain), H69 CDDP-resistant cells and H69 CDDP-resistant strain transfected with RCN-c DNA were subjected to 1D-SDS-PAGE, and proteins were transferred and immunodetected using the same method described above. We used anti-RCN mAb (6A1) and anti-GST-π mAb for the immunodetection of each molecule.

Surgical specimens with non-small cell lung cancer and immunohistochemical staining

A total of 126 surgical specimens were obtained from patients with NSCLC resected at Tokyo Medical University Hospital between 1994–1997. Sixty-seven patients (Stage IA: 2 patients, Stage IB: 7 patients, Stage IIA: 7 patients, Stage IIB: 10 patients, Stage IIIA: 29 patients, Stage IIIB: 12 patients) were treated using platinum-based adjuvant chemotherapy (either cisplatin [CDDP] or carboplatin [CBDCA] combined with vindesine sulfate [VDS]) after complete resection. The clinicopathological background of the 126 patients with NSCLC is summarized in Table I. Four micrometer-thick tissue sections of those surgical specimens were prepared from acetone-fixed, paraffin-embedded specimens (AMeX specimens) and collected on glass slides. After deparaffinization, the specimens were stained immunohistochemically by the ABC method using mAb against RCN (clone 6A1) and GST-π (clone 353-10, DakoCytomation, Glostrup, Denmark). Meyer's hematoxylin was used for counterstaining.[11] All AMeX specimens, which had been prepared during this period, were available for the immunohistochemical investigation, and we evaluated all AMeX specimens except in the Stage IV cases (5 patients).

Statistical analysis

Statistical analysis was carried out using the StatView software system (StatView 5.0.1, SAS Institute Inc., Cary, NC). Disease-free survival curves were calculated from the day of operation by the Kaplan-Meier method and the significance of the difference in the survival rates between the patient groups was calculated by log-rank test. A p-value of <0.05 was taken to indicate a statistically significant difference.

Results

Identification of differential proteins by 2-DE

Two-dimensional polyacrylamide gel electrophoresis findings of H69 cultured cells are shown in Figure 1. The intensity of 44 kDa protein (Spot a) decreased remarkably as the CDDP resist-

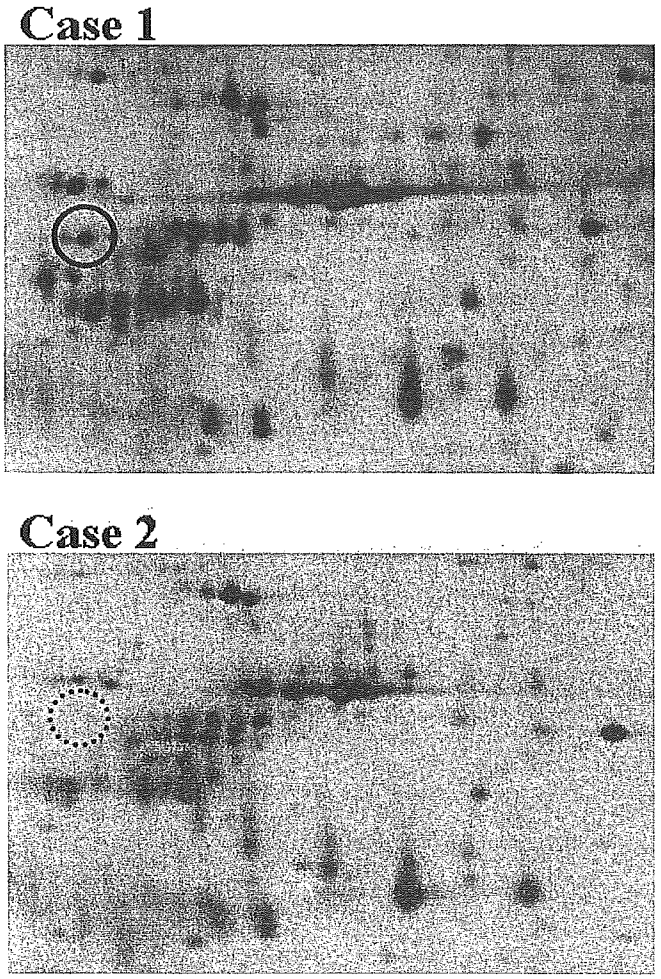


FIGURE 2 – Spot a was clearly recognized in Case 1. This case received CDDP-based adjuvant chemotherapy, and was disease-free at 5 years after complete resection. In Case 2 Spot a could not be recognized. Although Case 2 was also diagnosed as Stage IIIA as in Case 1, recurrence was recognized at 20 months after surgery.

ance was obtained. The elevation of the intensity of 23 kDa protein (Spot b) was observed. The same findings were recognized on 2-DE gels of PC14 cells. We recognized the same relationship between the intensity of these spots and responsiveness to platinum-based chemotherapy in clinical materials. Spot a was clearly detected in the cases with NSCLC that showed good prognosis with postoperatively adjuvant chemotherapy using platinum-based drugs (Fig. 2).

N-terminal amino acid sequence of Spot a and b

A 22 amino-acid N-terminal sequence of spot a was determined except for the sixth amino acid. The sequence of Spot a was KPTVR( )ERVVRPDSELGGRPPE. Also, a 20-amino acid N-terminal sequence of Spot b was determined except for amino acids 13 and 14. The sequence of Spot b was PPTYVVYFPVRG( ) ( )AALRML. Protein database examination suggested that Spot a and b was homologous with reticulocalbin (RCN) and glutathione-*S*-transferase-π (GST-π), respectively.

Microcytotoxicity assay of H69 CDDP-resistant strain transfected with RCN compared to H69 parent strain and H69 CDDP-resistant strain

We evaluated the drug sensitivity of H69 CDDP-resistant strain transfected with RCN cDNA using MTT assay comparing to parent strain and CDDP-resistant strain. RCN transfectant showed the intermediate of cell proliferation between parent strain and CDDP-resistant strain (Fig. 3). When the viability at 50 μM of CDDP was compared in our assay, there was a statistically significant difference between H69 cells (parent strain) and H69 CDDP-resistant strain transfected with vector only (Vector/H69/CDDP) ($p < 0.005$). We could not, however, detect a statistically significant difference in cellular viability between H69 cells and H69 CDDP-resistant strain transfected with RCN c DNA ($p = 0.500$). RCN transfection enhances the sensitivity of H69 CDDP-resistant strain to CDDP.

Western blot analysis using mouse mAb against RCN after 2-DE

We produced 2 kinds of mouse MAbs against N-terminal and C-terminal of RCN and designed them in mAb-6A1 and M23A2, respectively. Using each mAb against RCN, Western blot analysis of sample from H69 parent strain was carried out after 2-DE, and we confirmed that mAbs recognized Spot a (Fig. 4).

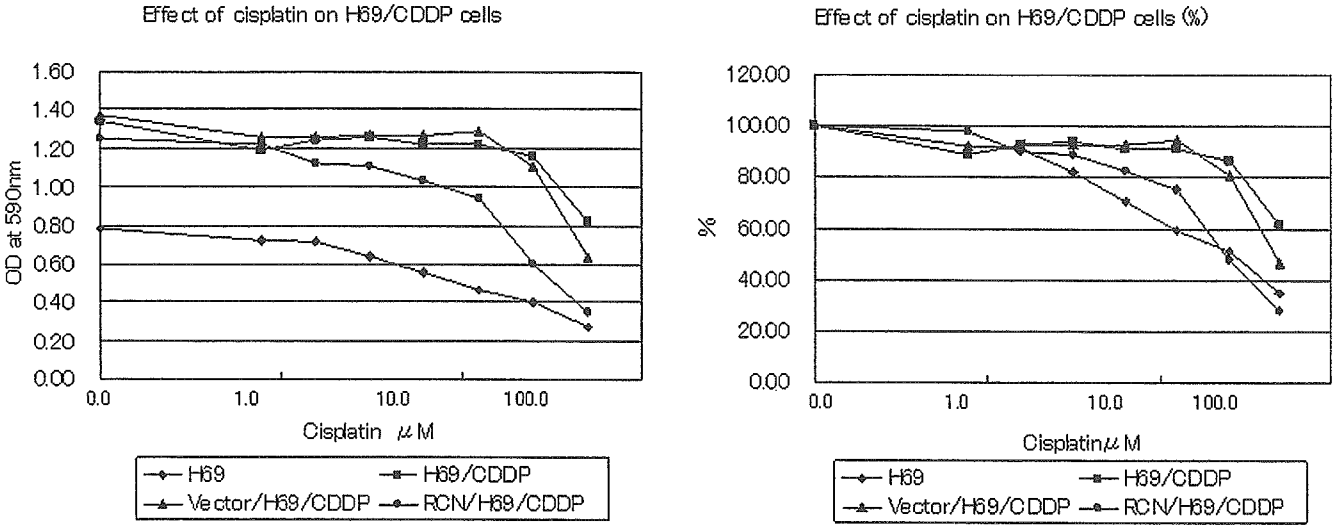


FIGURE 3 – Effects of cisplatin on H69 parent strain (H69), H69 CDDP-resistant strain (H69/CDDP) and H69 CDDP-resistant strain transfected with RCN cDNA (RCN/H69/CDDP). Exponentially growing cell suspension of H69, H69/CDDP and RCN/H69/CDDP or H69/CDDP transfected only with vector (Vector/H69/CDDP) were seeded in 96-well microtiter plates with CDDP at various concentration and incubated for 4 days. After incubation, living cells were measured by MTT assay (Material and Methods.) OD at 590 nm of each cells (*left*) and percentages (%) of each OD 590 against OD 590 without CDDP (*right*) were shown. □, H69 cell; ■, H69/CDDP cell; ▲, Vector/H69/CDDP; ●, RCN/H69/CDDP.

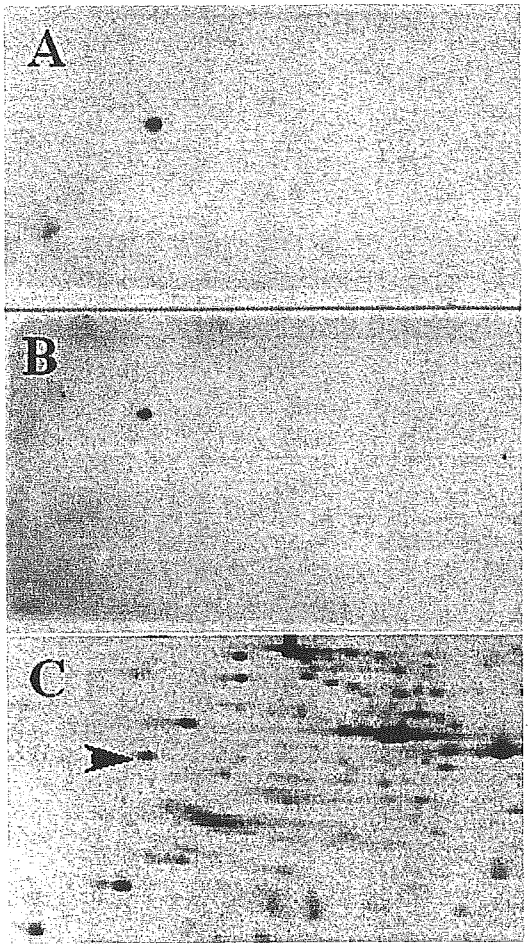


FIGURE 4 – Western blot analysis of H69 parent strain using MAbs against RCN. (*a*) mAb clone 6A1 recognized the N-terminal of RCN. (*b*) mAb clone M23A2 recognized the C-terminal of RCN. (*c*) After Western blotting, the residual proteins on the gel were visualized by silver staining. We confirmed that the spots visualized by Western blot corresponded with the RCN-spot (showing with arrowhead) on 2-DE gel.

Evaluation of the expression levels of RCN and GST-π in H69 CDDP-resistant strain transfected with RCN-c DNA using Western blot analysis

H69 CDDP-resistant strain transfected with RCN-c DNA showed high expression levels of RCN as well as GST-π (Fig. 5). In this strain GST-π expression level is similar to that of the H69 CDDP-resistant strain. The transfection of RCN-cDNA did not affect the expression levels of GST-π.

Immunohistochemical analysis of RCN and GST-π

Representative staining of RCN and GST-π is shown in Figure 6. Cytoplasmic staining was observed in RCN and GST-π. The average labeling index was 15.4% and 42.7% in reticulocalbin and GST-π, respectively. We evaluated the cases with <6% immunohistochemical reactivity of RCN as having reduced expression of RCN (negative). We also evaluated the cases with >43% immunohistochemical reactivity of GST-π as overexpression of GST-π (positive). According to these criteria, 87 of 126 cases (69.0 %) showed reduced expression (negative) of RCN and 62 cases (49.2 %) showed overexpression (positive) of GST-π. There were no relationship between pathological stage and the expression rate of either RCN or GST-π (Table II).

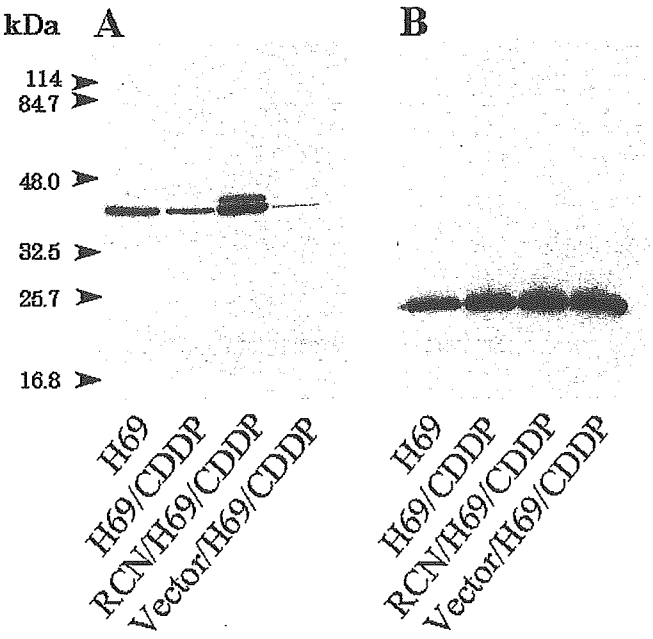


FIGURE 5 – Comparison of the expression levels of RCN and GST-π in H69 parent strain, H69/CDDP, RCN/H69/CDDP and Vector/H69/CDDP. (*a*) Western blot analysis using mAb against RCN (clone 6A1). (*b*) Western blot analysis using mAb against GST-π. H69, H69 parent strain; H69/CDDP, H69 CDDP resistant strain; RCN/H69/CDDP, H69 CDDP transfected RCN; Vector/H69/CDDP, H69/CDDP transfected with vector. RCN/H69/CDDP cells exhibited higher expression of RCN than H69 CDDP. We could not recognize a remarkable change of expression level of GST-π compared to H69/CDDP.

Disease-free survival and the expression of RCN or GST-π

RCN. Non-relapse mortality curves of cases without adjuvant chemotherapy are shown in Figure 7*b*. We did not detect statistically significant differences between cases with and without the expression of RCN. In cases with platinum-based adjuvant chemotherapy, however, there was a statistically significant difference between cases with and without RCN expression ($p = 0.007$) (Fig. 7*a*).

GST-π. The non-relapse mortality curves of cases with/without adjuvant chemotherapy are shown in Figure 8. There was a statistically significant difference between cases with and without expression of GST-π only in cases with platinum-based adjuvant chemotherapy ($p = 0.0219$).

Disease-free survival and co-evaluation of RCN and GST-π

When we evaluated pathological Stage IIA–IIIB cases with postoperative adjuvant chemotherapy, the non-relapse mortality rate in the cases with positive RCN expression and negative expression of GST-π was 66.0%. It was <20% in the cases with the other combinations of RCN and GST-π expressions. There were statistically significant differences recognized between cases with positive expression of RCN and negative expression of GST-π and cases with other combination of these proteins ($p = 0.0150$) (Fig. 9).

Discussion

Distant metastases occur frequently in patients with advanced stage-NSCLC who undergo only surgery, perhaps because micrometastasis exists at the time of surgical treatment. We believe that the concept of postoperatively adjuvant chemotherapy for control of micro-metastasis does not conflict with the improvement of the prognosis in NCLC. The efficacy of postoperative adjuvant chemotherapy in completely resected NCLC, however, used to be controversial.[12,13] According to a previous meta-analysis, postoperative adjuvant chemotherapy using platinum-based

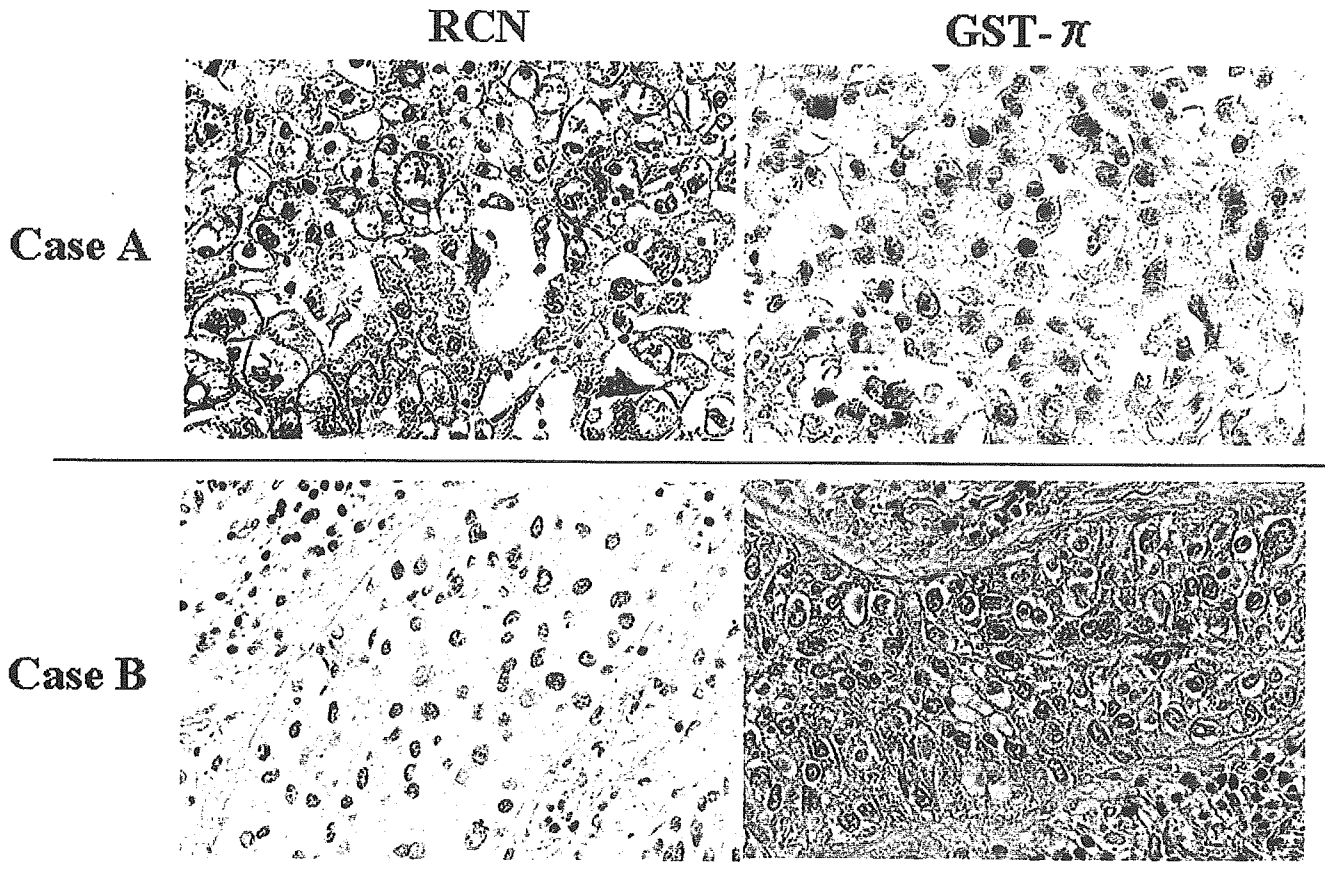


FIGURE 6 – Immunohistochemical reactivity of representative cases using anti-RCN mAb (clone 6A1) and anti-GST-π mAb (clone 353-10, DakoCytomation). Case A showed positive cytoplasmic staining of RCN and negative immunohistochemical reactivity of GST-π. Case B showed negative immunohistochemical reactivity of RCN and positive cytoplasmic staining of GST-π.

agents is generally not effective for survival improvement of patients with NSCLC. Several adjuvant chemotherapy trials in the late 1990s after an individual data-based meta-analysis suggested a 5% increase in survival at 5 years.[1] The results of 2 randomized trials to assess the benefit of adjuvant chemotherapy in patients with early-stage NSCLC were reported at the 40th Annual Meeting of the American Society of Clinical Oncology.[3,4] Furthermore, a recent large scale randomized clinical trial launched just after the publication of the meta-analysis concluded that patients assigned to CDDP-based postoperative adjuvant chemotherapy had a statistically significantly higher survival rate than those assigned to postoperative observation (44.5% *vs.* 40.4% at 5 years; $p < 0.03$).[2] Even though a statistically significant difference was found in this study, 5-year-survival rate benefit was approximately 5–15%. It may seem that leading lung cancer experts have reached a consensus concerning postoperatively adjuvant chemotherapy.

We propose that rather than focusing only on improving chemotherapy regimens, we should also make efforts to identify patients who will show good response for already established chemotherapy regimens. We believe that this proposal is justified based only on the results of evidence-based medicine. We attempted to detect novel biomarkers for selection of well-responders using proteomic analysis.

Two-dimensional polyacrylamide gel electrophoresis enables simultaneous evaluation of a large number of polypeptides.[14] Most cellular functions are controlled by protein–protein interaction and we believe that proteomic analysis is the best strategy for understanding various kinds of clinical phenomenon. Our study simultaneously detected 2 kinds polypeptides related to drug resistance by comparing 2-DE patterns of parent strains of lung cancer cultured cells and their CDDP-resistant strains. When strains obtained CDDP-drug resistance, one polypeptide showed reduced expression, whereas the other one showed overexpression. N-terminal of amino-acid sequence analysis showed that the former was RCN and that the latter was GST-π.

TABLE II – RELATIONSHIP BETWEEN PATHOLOGICAL STAGE AND IMMUNOHISTOCHEMICAL REACTIVITY OF RCN AND GST-π

Stage	Reduced expression of RCN	Overexpression of GST-π
I A	19/25 (76.0%)	10/25 (40.0%)
I B	15/24 (62.5%)	12/24 (50.0%)
II A	6/10 (60.0%)	5/10 (50.0%)
II B	12/16 (75.0%)	5/16 (31.3%)
III A	28/37 (75.7%)	22/37 (59.5%)
III B	7/14 (50.0%)	8/14 (57.1%)
Total	87/126 (69.0%)	62/126 (49.2%)

GST-π is an enzyme involved with cellular detoxification of many xenobiotic substances through conjugation to glutathione and degrading oxygen free radicals.[15–17] It is already well-known that GST-π overexpression is associated with increased resistance to platinum-based chemotherapy and poor outcome in NSCLC.[18,19]

RCN is an endoplasmic reticulum-resident Ca^{2+}-binding protein with 6 repeats of a domain containing the EF-hand motif. RCN is reported to be a luminal protein with molecular weight 44 kDa.[20–23] The RCN molecule is probably necessary for normal behavior of cells because homozygous deletion in mice of RCN could contribute to the lethality.[24] Although the detailed physiological functions of RCN are still unknown, a few investigators reported that RCN expressed in malignant neoplasms. Yu et al.[25] reported overexpression of RCN in hep-

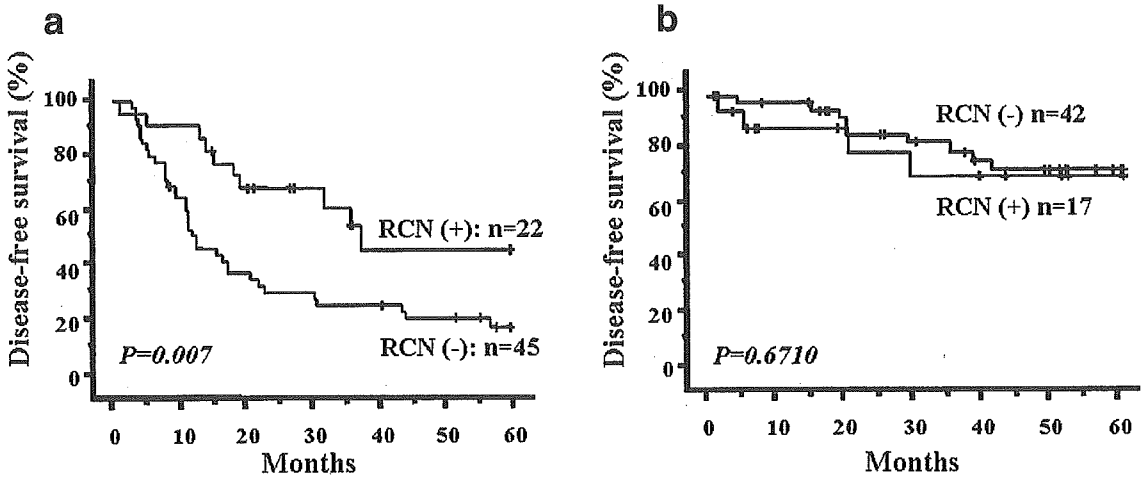


FIGURE 7 – Kaplan-Meier curves for disease-free survival from complete resection in pathological Stage IA–IIIB NSCLC cases with postoperative adjuvant chemotherapy using platinum (*a*) or without any adjuvant chemotherapy (*b*). When we evaluated cases with platinum-based adjuvant chemotherapy, RCN-positive cases showed statistically significant higher survival than RCN-negative cases ($p = 0.007$). In cases without adjuvant chemotherapy, there was no statistically significant difference in disease-free survival between 2 groups with or without RCN expression.

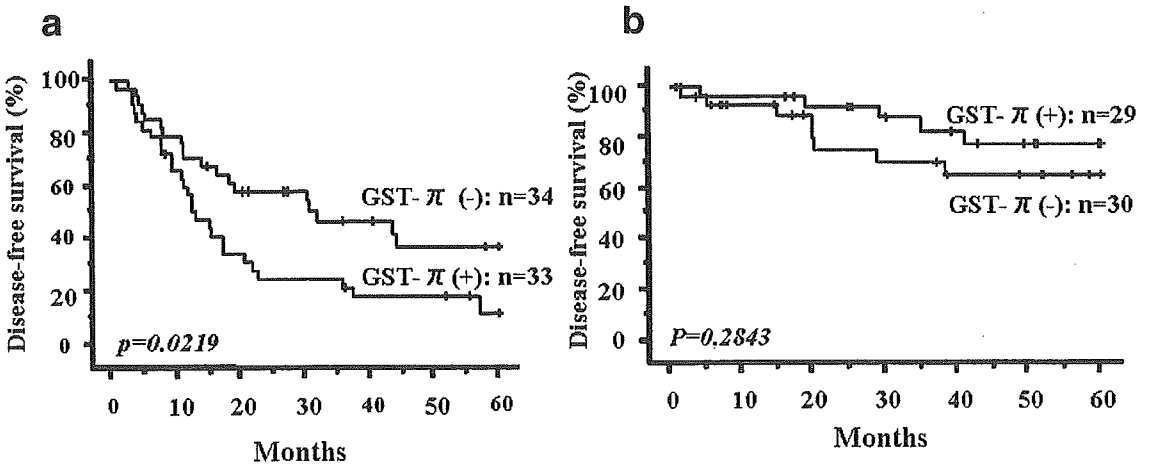


FIGURE 8 – Kaplan-Meier curves for disease-free survival from complete resection in pathological Stage IA–IIIB NSCLC cases with postoperative adjuvant chemotherapy using platinum (*a*) or without any postoperative adjuvant chemotherapy (*b*). When we evaluated cases with platinum-based adjuvant chemotherapy, GST-π-negative cases showed statistically significant higher survival than GST-π-positive cases ($p = 0.0219$). In cases without adjuvant chemotherapy, there was no statistically significant difference in disease-free survival between 2 groups with or without GST-π expression.

atoma compared to normal liver cells. It was also reported that RCN was expressed in highly invasive breast cancer cell lines but not in poorly invasive lines in matrigel invasion assays.[26] Liu *et al.*[26] suggested the high expression of RCN correlated with a low expression of the Ca^{2+}-dependent cell adhesion molecule cadherin in these cell lines. There are no reports, however, concerning the relationship between the expression of RCN and drug-resistance in malignant neoplasms.

The 2-DE findings of lung cancer cultured cells suggested that a decreased expression level of RCN was deeply related with drug-resistance to CDDP. Furthermore, as CDDP-resistant strain transfected RCN c-DNA, the transfectant showed an intermediate sensitivity to CDDP between the sensitivity of the parent strain and that of the CDDP-resistant strain, indicating that RCN c-DNA transfection enhanced the sensitivity to CDDP. We evaluated the outcome of the patients that received postoperative adjuvant chemotherapy using platinum based agents. We recognized a statistically significant association between positive RCN expression and good outcome. No such association was observed in cases without postoperative adjuvant chemotherapy. Furthermore, we showed no relationship between pathological stages and the expression rate of RCN in Table II. Therefore, we concluded that RCN is an important molecule related to CDDP-drug resistance.

We attempted to evaluate the relationship between the co-expressions of RCN and GST-π and disease-free survival of more advanced NSCLC cases (pathological Stage IIA–IIIB) because remarkable changes of these 2 kinds of polypeptides were observed simultaneously on 2-DE gels. These changes were also commonly shown and reproducibly observed in 2 kinds of lung cancer cell lines. Disease-free survival probability showed that only the group with positive RCN expression and negative expression of GST-π was associated significantly with a good outcome. The 5-year disease free survival rate of the group was 66.0%. This favorable survival rate showing positive RCN and negative GST-π corresponds to that of Stage I-NSCLC. Positive RCN and GST-π-negative expression cases should be considered as good indications of platinum-based postoperative adjuvant chemotherapy.

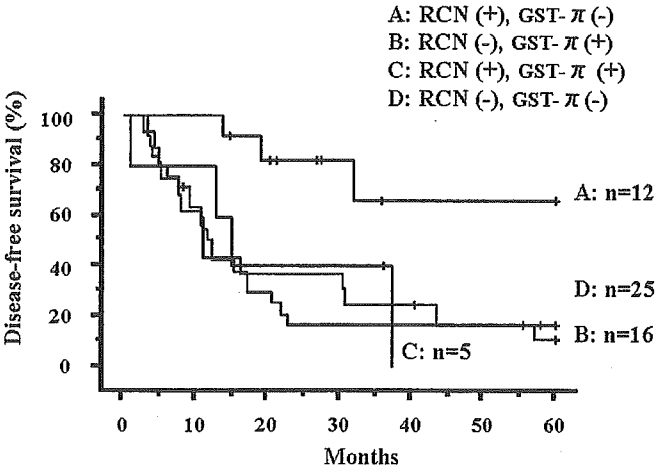


FIGURE 9 – Kaplan-Meier curves for disease-free survival from complete resection in pathological Stage IIA–IIIB NSCLC cases with postoperative adjuvant chemotherapy using platinum. (*a*) Cases with positive RCN-expression and negative GST-π expression (non-relapse mortality rate at 5-years = 66.0%). (*b*) Cases with negative RCN-expression and positive GST-π expression (non-relapse mortality rate at 5-years = 11.3%). (*c*) Cases with positive expression of both RCN and GST-π (non-relapse mortality rate at 5-years = 0 %). (*d*) Cases with negative expression of both RCN and GST-π (non-relapse mortality rate at 5-years = 16.7 %). Group A showed statistically significant higher survival than the other groups (A–B, $p = 0.0007$; A–C, $p = 0.0388$; A–D, $p = 0.0065$).

Therefore, we concluded that this result provided an important information concerning pre-selection of patients who would benefit from postoperatively adjuvant chemotherapy using platinum.

Until the results of 2 randomized trials to assess the benefit of adjuvant chemotherapy in patients with early-stage NSCLC were reported at the 40th Annual Meeting of the American Society of Clinical Oncology,[3,4]) we had no evidence concerning the efficacy of adjuvant chemotherapy. We believed that severe adjuvant chemotherapy led to poor prognosis. In our study, we have enough Stage I NSCLC patients to evaluate the relationship between efficacy and the expression of these molecules. We could not avoid excluding Stage I NSCLC cases in the evaluation of the relationship between the co-expressions of RCN and GST-π and disease-free survival.

Individual therapy for lung cancer patients is attracting attention. Therapeutic strategies should be different for each patient. We do not know, however, that individual therapy for lung cancer is realized routinely in clinical. For more than 20 years it has been believed that platinum based-chemotherapy is one of the most important therapeutic strategies for lung cancer patients who lack indications of surgical treatment, and complete response has been shown in some patients untreated previously. At the same time, we believe that platinum-based chemotherapy is one of the most useful strategies to control systemic micro-metastasis after complete resection. Co-evaluation of RCN and GST-π might provide valuable information concerning the selection of postoperatively platinum-based adjuvant chemotherapy and we believe that this kind of proteomic evaluation would be realizing postoperative individual therapy based upon medical evidences.

We emphasize that downregulation of RCN was independent of GST-π expression and our study is the first article concerning the relationship between the efficacy of platinum-based adjuvant chemotherapy and co-expression of RCN and GST-π.

Acknowledgements

We would like to thank Professor J.P. Barron of the International Medical Communications Center of Tokyo Medical University for reviewing this manuscript. We are also grateful to Dr. Saijo, National Cancer Center, for the gift of CDDP-resistant strains of lung cancer cultured cells.

References

1. Non-Small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomized clinical trials. Br Med J 1995; 311:899–909.
2. Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J. (International Adjuvant Lung Cancer Trial Collaborative Group) Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. N Engl J Med 2004; 350:351–60.
3. Winton TL, Livingston R, Johnson D, Rigas J, Cormier Y, Butts C, Ding K, Seymour L, Magoski N, Shepherd F. A prospective randomized trial of adjuvant vinorelbine (VIN) and cisplatin (CIS) in completely resected stage IB and II non small cell lung cancer (NSCLC) Intergroup JBR. 10. Proc Am Soc Clin Oncol 2004;23:621.
4. Strauss GM, Herndon J, Maddaus MA, Johnstone DW, Johnson EA, Watson DM, Sugarbaker DJ, Schilsky RL, Green MR. Randomized clinical trial of adjuvant chemotherapy with paclitaxel and carboplatin following resection in stage IB non-small cell lung cancer (NSCLC): report of Cancer and Leukemia Group B (CALGB) Protocol 9633. Proc Am Soc Clin Oncol 2004;23:621.
5. Kato H, Ichinose Y, Ohta M, Hata E, Tsubota N, Tada H, Watanabe Y, Wada H, Tsuboi M, Hamajima N, Ohta M, for the Japan Lung Cancer Research Group on Postsurgical Adjuvant Chemotherapy. A randomized trial of adjuvant chemotherapy with Uracil-Tegafur for adenocarcinoma of the lung. N Engl J Med 2004;350:1713–21.
6. Hirano T, Franzen B, Uryu K, Okuzawa K, Alaiya AA, Vanky F, Rodrigues L, Ebihara Y, Kato H, Auer G. Detection of polypeptides associated with the histopathological differentiation of primary lung carcinoma. Br J Cancer 1995;72:840–8.
7. Franzen B, Auer G, Alaiya AA, Eriksson E, Uryu K, Hirano T, Okuzawa K, Kato H, Linder S. Assessment of homogeneity in polypeptide expression in breast carcinomas shows widely variable expression in highly malignant tumors. Int J Cancer 1996;69:408–14.
8. Hirano T, Auer G, Maeda M, Hagiwara Y, Okada S, Ohira T, Okuzawa K, Fujioka K, Franzen B, Hibi N, Seito T, Ebihara Y, Kato H. Human tissue distribution of TA02, which is homologous with a new type of aspartic Proteinase, napsin A. Jpn J Cancer Res 2000;91: 1015–21.
9. Franzen B, Linder S, Okuzawa K, Kato H, Auer G. Non-enzymatic extraction of cells from clinical tumor material for analysis of gene expression by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 1993;14:1045–53.
10. Banno S, Yoshikawa K, Nakamura S, Yamamoto K, Seito T, Nitta M, Takahashi T, Ueda R, Sero M. Monoclonal antibody against PRAD1/cyclin D1 stains nuclei of tumor cells with translocation or amplification at BCL-1 locus. Jpn J Cancer Res 1994;85:918–26.
11. Shou Y, Hirano T, Gong Y, Kato Y, Yoshida K, Ohira T, Ikeda N, Konaka C, Ebihara Y, Zhao F, Kato H. Influence of angiogenetic factors and matrix metalloproteinases upon tumour progression in non-small-cell lung cancer. Br J Cancer 2001;85:1706–12.
12. Pisters KM. Stage I/II non-small cell lung cancer: is there a role for combined-modality therapy? American Society of Clinical Oncology (2002 educational book), ASCO Publications, Alexandria, 2002:465–70.
13. Scagliotti GV, Fossati R, Torri V, Giaccone G, Silvano G, Martelli M, Clerici M, Cognetti F, Tonato M. Randomized study of adjuvant chemotherapy for completely resected stage I, II, or IIIA non-small-cell lung cancer. J Natl Cancer Inst 2003;95:1453–61.
14. Okuzawa K, Franzen B, Lindholm J, Hirano T, Bergman T, Ebihara Y, Kato H, Auer G. Characterization of gene expression in clinical lung cancer material by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 1994;15:382–90.
15. Mannervik B. The enzymes of glutathione metabolism: an overview. Biochem Soc Trans 1987;15:717–8.
16. Tew KD. Glutathione-associated enzymes in anticancer drug resistance. Cancer Res 1994;54:4313–20.
17. Goto S, Kamada K, Soh Y, Ihara Y, Kondo T. Significance of nuclear glutathione S-transferase pi in resistance to anti-cancer drugs. Jpn J Cancer Res 2002;93:1047–56.
18. Arai T, Yasuda Y, Takaya T, Hayakawa K, Toshima S, Shibuya C, Kashiki Y, Yoshimi N, Shibayama M. Immunohistochemical expression

of glutathione transferase-pi in untreated primary non-small cell lung cancer. Cancer Detect Prev 2000;24:252–7.
19. Bai F, Nakanishi Y, Kawasaki M, Takayama K, Yatsunami J, Pei XH, Tsuruta N, Wakamatsu K, Hara N. Immunohistochemical expression of glutathione S-transferase-pi can predict chemotherapy response in patients with non small cell lung carcinoma. Cancer 1996;78:416–21.
20. Ozawa M, Muramatsu T. Reticulocalbin, a novel endoplasmic reticulum resident Ca2+-binding protein with multiple EF-hand motifs and a carboxyl-terminal HDEL sequence. J Biol Chem 1993;268:699–705.
21. Ozawa M. Cloning of a human homologue of mouse reticulocalbin reveals conservation of structural domains in the novel endoplasmic reticulum resident Ca2+-binding protein with multiple EF-hand motifs. J Biochem 1995;117:1113–9.
22. Scherer PE, Lederkremer GZ, Williams S, Fogliano M, Baldini G, Lodish HF. Cab45, a novel Ca2+-binding protein localized to the Golgi lumen. J Cell Biol 1996;133:257–68.
23. Tachikui H, Navet AF, Ozawa M. Identification of the Ca2+-binding domains in reticulocalbin, an endoplasmic reticulum resident Ca2+-binding protein with multiple EF-hand motifs. J Biochem 1997;121:145–9.
24. Kent J, Lee M, Schedl A, Boyle S, Fantes J, Powell M, Rushmere N, Abbott C, van Heyningen V, Bickmore WA. The reticulocalbin gene maps to the WAGR region in human and to the Small eye Harwell deletion in mouse. Genomics 1997;42:260–7.
25. Yu LR, Zeng R, Shao XX, Wang N, Xu YH, Xia QC. Identification of differentially expressed proteins between human hepatoma and normal liver cell lines by two-dimensional electrophoresis and liquid chromatography-ion trap mass spectrometry. Electrophoresis 2000;21:3058–68.
26. Liu Z, Brattain MG, Appert H. Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. Biochem Biophys Res Commun 1997;231:283–9.

Review

Present Strategy of Lung Cancer Screening and Surgical Management

Norihiko Ikeda, MD,[1] Aeru Hayashi, MD,[1] Yoshifumi Miura, MD,[1] Tetsuya Okunaka, MD,[2] Ken Okuzawa, MD,[2] Masahiro Tsuboi, MD,[3] Yasufumi Kato, MD,[3] Yasuhiro Suga, MD,[3] and Harubumi Kato, MD[3]

Previous lung cancer screening trials in the United States (US) employing chest X ray and sputum cytology did not demonstrate reductions in lung cancer mortality. However, recent case control studies in Japan demonstrated a decrease in lung cancer mortality in the computed tomography (CT) screened group. Lung cancer screening using chest CT detected more cancers at an earlier stage than chest X ray. Before CT screening is widely performed, lung cancer mortality reduction should be proved in a scientific manner. The problem of a much higher false positive rate of this method should be solved. The subtypes of adenocarcinoma; bronchioloalveolar carcinoma (BAC) tends to show specific CT findings called ground glass opacity (GGO) and a favorable prognosis can be expected. BAC is usually invisible by chest X ray and detected only by CT. Recent studies have shown the proportion of GGO is strongly related to biological malignancy of small adenocarcinoma. Based on this fact, thoracic surgeons wish to identify the possibility of limited resection for minimally invasive cancers. Lung cancer researchers are interested in evaluating the nature of small adenocarcinoma as well as the carcinogenic process. A comprehensive understanding of screening-detected cancers including the CT images, pathology and genetic analysis is necessary for optimum management of such nodules. (Ann Thorac Cardiovasc Surg 2005; 11: 363–6)

Key words: lung cancer screening, computed tomography screening, ground glass opacity, limited surgery

Lung Cancer Screening: Past and Present

Lung cancer is the primary cancer killer worldwide due to delayed diagnosis. The five year survival rate is less than 15% for those with lung cancer. However, ~80% of stage IA cases survive more than five years. Early detection may be the best way to improve prognosis. Mass screening is a strategy for early detection. However, the effect in decreasing lung cancer mortality has not been demonstrated. Three randomized controlled trials (RCTs) in the United States (US) (Mayo, Johns Hopkins, Memorial-Sloan Kettering) screened high risk individuals using chest X ray and sputum cytology in the 1980's and failed to show reduction of lung cancer mortality.[1-5]

Since these pessimistic results were reported, public lung cancer screening has not been recommended. Recently, fair quality case-control studies in Japan showed lung cancer mortality benefit using chest X ray and sputum cytology[6-11] (Table 1). These results might be due to good quality control as well as improvement of diagnosis and treatment of detected cancer compared to those of 20-30 years ago. However, as those studies were retrospective, a recommendation for screening could not be made.

From [1]Department of Thoracic Oncology and Surgery, International University of Health and Welfare, Mita Hospital, [2]Respiratory Disease Center, Sanno Hospital, International University of Health and Welfare, and [3]Department of Surgery, Tokyo Medical University, Tokyo, Japan

Received August 18, 2005; accepted for publication September 9, 2005.

Address reprint requests to Norihiko Ikeda, MD: Department of Thoracic Oncology and Surgery, International University of Health and Welfare, Mita Hospital, 1-4-3 Mita, Minato-ku, Tokyo 108-8329, Japan.

Table 1. Case control study of lung cancer screening in Japan

Group (ref.)	Method	No. of cases	No. of controls	% of screened cases	% of screened controls	odds ratio (95% CI)
Miyagi[11]	x–p+sp	328	1,886	73.5	82.6	0.54 (0.41-0.73)
Okayama[8]	x–p+sp	412	3,490	32.3	43.9	0.59 (0.46-0.74)
Niigata[9]	x–p+sp	174	801	35.0	56.2	0.40 (0.27-0.59)
Gunma[10]	x–p	121	536	57.9	67.2	0.68 (0.44-1.05)

Case: died of lung cancer between the age of 40 and 79.

Table 2. Lung cancer screening using low-dose spiral CT

Author (ref.)	No. of cases	Age	% of cancer	% of stage I
Sobue[13]	1,611	40-79	0.87	78
Sone[14]	5,483	40-74	0.4	100
Nawa[16]	7,956	50-69	0.44	89
Henschke[12]	1,000	60-	2.5	83
Swensen[23]	1,520	50-85	2.7	85

It is reported that the sensitivity of chest X ray is low in detecting early stage lung cancer. The application of chest computed tomography (CT) to screening was firstly performed by the Anti-Lung Cancer Association (ALCA) in Tokyo. Much higher sensitivity in detection of lung cancer, especially in localized cases, was reported. Recently, a number of studies suggest that CT screening may increase the lung cancer detection rate by 3-10 times compared with chest X ray and that 70-80% of detected cancers are stage I[12-16] (Table 2). The prevalence of lung cancer in each study might change according to the risk (smoking status, age, etc.) of subjects. However, the mean tumor size is less than 2 cm in each study and it is obvious most of such cases survive longer than those detected by chest X ray.

The question has been evoked whether CT screening decreases lung cancer mortality.

The preclinical stage of lung cancers detectable by CT screening should be much earlier than that of cancers detected by chest X ray. Evaluation based on survival will be biased by lead-time bias and length-bias. To evaluate the efficacy of CT screening, prospective randomized control trials have started in the US and Europe. The endpoints of these trials are mortality reduction. The National Lung Screening Trial (NLST) sponsored by National Cancer Institute (NCI) has been scheduled to accrue 50,000 current and former smokers with age range of 55-74. These are randomized to chest X ray group versus spiral CT group and will be followed up until 2009. Reduction of lung cancer mortality in the CT group will be analyzed. In Netherlands, a total of 20,000 high risk individuals will be randomized to undergo chest CT or standard care (NELSON trial). Such a randomized trial is not being performed in Japan. However, a large scale prospective cohort study of CT screening has already started and mortality rates due to lung cancer will be analyzed based on the data of 50,000 subjects.

Small Cancers Detected by CT Screening

Most small cancers detected by CT screening are adenocarcinomas. Based on high resolution CT findings, small adenocarcinoma is usually classified into three groups; Solid type, Mixed type and ground glass opacity (GGO) type. Bronchioloalveolar carcinoma (BAC) seldom shows abnormalities on chest X ray because it grows without destroying alveolar structure. Lung cancers with a large GGO component tend to be BAC or minimally invasive adenocarcinomas which have good prognoses.[17] The Noguchi's classification is routinely used to classify the subtypes of small adenocarcinomas (Table 3).[18] Type A and B are considered to be non-invasive cancer and Type D, E, F, to be invasive cancer. Cases with enormous proportion of GGO tend to be type A or B and have a favorable prognosis. Hence, the solid dominant cancers tend to be types D, E, F and have a poor prognosis. There are several reports indicating that the ratio of the size of GGO and that of consolidation on the high resolution computed

Table 3. Noguchi's classification and lymph node involvement

	Nodal meta. (%)
A: Localized BAC (LBAC)	0
B: LBAC with foci of collapse of alveolar structure	0
C: LBAC with foci of active fibroblast proliferation	28.4
D: Poorly differentiated adenoca.	47.7
E: Tubular adenoca.	55.6
F: Papillary adenoca. with destructive growth	25
	Cancer 1995

tomography (HRCT) is strongly related to the stage and prognosis.[19-22]

Intervention on the Non-calcified Nodule

The biggest potential difficulty in CT screening has been reported to be a higher false positive rate compared to that of chest X ray. Non-calcified nodules were detected in 15-50% of all screens.[12-16,23] Most of these were followed up and had invasive diagnostic procedures as the protocol required, which often showed negative for malignancy. The follow-up and/or invasive procedure causes physical and emotional discomfort. The definitive protocol for diagnostic work-up should be established. The sophisticated algorism of intervention of International Early Lung Cancer Action Program (IELCAP) is shown in their website.[24]

Surgery for Screening Detected Cancer

Lobectomy and locoregional lymph node dissection have been recommended as standard lung cancer operation procedures. This is based on the fact that nearly 20% of adenocarcinomas less than 2 cm in diameter were reported to be node positive and 5% of cases less than 1 cm were also N1 or N2 disease.[25-28] Also, the Lung Cancer Study Group failed to demonstrate positive results of limited resection for clinical T1 lung cancers. The limited surgery group showed a local recurrence rate 5-6 times higher than the lobectomy group.[29] However, many thoracic surgeons postulated that GGO dominant cases might be candidates for limited resection. The Noguchi's classification is useful in evaluating the aggressive potential in individual cases, but these criteria are based on postoperative pathologic findings and could not have a strong impact on the choice of treatment. Therefore we need criteria which are available preoperatively to define early minimally invasive cancers. Lesser resection mainly for GGO dominant tumors showed favorable results in some registry studies.[30,31] Also, the Japan Clinical Oncology Group (JCOG) recruited resected T1 cases to analyze the relationship between CT findings and pathological results including lymph node metastasis as well as nodal and vascular involvement. The results of these studies may contribute to reveal characteristics of minimally invasive tumors which can be cured by limited resection. Small cancers with a high GGO ratio might be candidates for limited resection, however, prospective randomized studies comparing lobectomy versus limited resection will be necessary to confirm this.

Comprehensive research including pathology, CT images and molecular analysis are needed to define non-invasive adenocarcinoma and will alter conventional method of management of tiny cancers. We are in the midst of a historic evolution of the study of lung adenocarcinoma, which was triggered by CT screening.

References

1. Melamed MR, Flehinger BJ. Detection of lung cancer: highlights of the Memorial Sloan-Kettering Study in New York City. *Schweiz Med Wochenschr* 1987; **117**: 1457–63.
2. Melamed MR, Flehinger BJ, Zaman MB, et al. Screening for early lung cancer. Results of the Memorial Sloan-Kettering study in New York. *Chest* 1984; **86**: 44–53.
3. Tockman MS. Survival and mortality from lung cancer in a screened population. The Johns Hopkins Study. *Chest* 1986; **89**: 324–5.
4. Fontana RS, Sanderson DR, Woolner LB, et al. Lung cancer screening: the Mayo program. *J Occup Med* 1986; **28**: 746–50.
5. Fontana RS, Sanderson DR, Woolner LB, et al. Screenig for lung cancer. A critique of the Mayo Lung Project. *Cancer* 1991; **67**: 1155–64.

6. Sobue T, Suzuki T, Naruke T. A case-control study for evaluating lung-cancer screening in Japan. Japanese Lung-Cancer-Screening Research Group. *Int J Cancer* 1992; **50**: 230–7.
7. Okamoto N, Suzuki T, Hasegawa H, et al. Evaluation of a clinic-based screening program for lung cancer with a case-control design in Kanagawa, Japan. *Lung Cancer* 1999; **25**: 77–85.
8. Nishii K, Ueoka H, Kiura K, et al. A case-control study of lung cancer screening in Okayama Prefecture, Japan. *Lung Cancer* 2001; **34**: 325–32.
9. Tsukada H, Kurita Y, Yokoyama A, et al. An evaluation of screening for lung cancer in Niigata Prefecture, Japan: A population-based case-control study. *Br J Cancer* 2001; **85**: 1326–31.
10. Nakayama T, Baba T, Suzuki T, et al. An evaluation of chest X-ray screening for lung cancer in Gunma Prefecture, Japan: a population-based case-control study. *Eur J Cancer* 2002; **38**: 1380–7.
11. Sagawa M, Tsubono Y, Saito Y, et al. A case-control study for evaluating the efficacy of mass screening program for lung cancer in Miyagi Prefecture, Japan. *Cancer* 2001; **92**: 588–94.
12. Henschke CI, McCauley DI, Yankelevitz DF, et al. Early Lung Cancer Action Project: overall design and findings from baseline screening. *Lancet* 1999; **354**: 99–105.
13. Sobue T, Moriyama N, Kaneko M, et al. Screening for lung cancer with low-dose helical computed tomography: anti-lung cancer association project. *J Clin Oncol* 2002; **20**: 911–20.
14. Sone S, Takashima S, Li F, et al. Mass screening for lung cancer with mobile spiral computed tomography scanner. *Lancet* 1998; **351**: 1242–5.
15. Kaneko M, Eguchi K, Ohmatsu H, et al. Peripheral lung cancer: screening and detection with low-dose spiral CT versus radiography. *Radiology* 1996; **201**: 798–802.
16. Nawa T, Nakagawa T, Kusano S, et al. Lung cancer screening using low-dose spiral CT: results of baseline and 1-year follow-up studies. *Chest* 2002; **122**: 15–20.
17. Travis WD, Garg K, Franklin WA, et al. Evolving concepts in the pathology and computed tomography imaging of lung adenocarcinoma and bronchioloalveolar carcinoma. *J Clin Oncol* 2005; **23**: 3279–87.
18. Noguchi M, Morikawa A, Kawasaki M, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995; **75**: 2844–52.
19. Suzuki K, Asamura H, Kusumoto M, et al. "Early" peripheral lung cancer: prognostic significance of ground glass opacity on thin-section computed tomographic scan. *Ann Thorac Surg* 2002; **74**: 1635–9.
20. Aoki T, Tomoda Y, Watanabe H, et al. Peripheral lung adenocarcinoma: correlation of thin-section CT findings with histologic prognostic factors and survival. *Radiology* 2001; **220**: 803–9.
21. Matsuguma H, Yokoi K, Anraku M, et al. Proportion of ground-glass opacity on high-resolution computed tomography in clinical T1 N0 M0 adenocarcinoma of the lung: A predictor of lymph node metastasis. *J Thorac Cardiovasc Surg* 2002; **124**: 278–84.
22. Ikeda N, Maeda J, Yashima K, et al. A clinicopathological study of resected adenocarcinoma 2 cm or less in diameter. *Ann Thorac Surg* 2004; **78**: 1011–6.
23. Swensen SJ, Jett JR, Hartman TE, et al. Lung cancer screening with CT: Mayo Clinic experience. *Radiology* 2003; **226**: 756–61.
24. *http://www.IELCAP.org/*
25. Asamura H, Nakayama H, Kondo H, et al. Lymph node involvement, recurrence, and prognosis in resected small, peripheral, non-small-cell lung carcinomas: are these carcinomas candidates for video-assisted lobectomy? *J Thorac Cardiovasc Surg* 1996; **111**: 1125–34.
26. Konaka C, Ikeda N, Hiyoshi T, et al. Peripheral non-small cell lung cancers 2.0 cm or less in diameter: proposed criteria for limited pulmonary resection based upon clinicopathological presentation. *Lung Cancer* 1998; **21**: 185–91.
27. Yoshida J, Nagai K, Yokose T, et al. Primary peripheral lung carcinoma smaller than 1 cm in diameter. *Chest* 1998; **114**: 710–2.
28. Kondo D, Yamada K, Kitayama Y, et al. Peripheral lung adenocarcinomas: 10 mm or less in diameter. *Ann Thorac Surg* 2003; **76**: 350–5.
29. Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. Lung Cancer Study Group. *Ann Thorac Surg* 1995; **60**: 615–23.
30. Nakata M, Sawada S, Saeki H, et al. Prospective study of thoracoscopic limited resection for ground-glass opacity selected by computed tomography. *Ann Thorac Surg* 2003; **75**: 1601–6.
31. Yoshida J, Nagai K, Yokose T, et al. Limited resection trial for pulmonary ground-glass opacity nodules: fifty-case experience. *J Thorac Cardiovasc Surg* 2005; **129**: 991–6.

□疫学

□発見

■診断

□治療

画像無所見肺癌（喀痰細胞診陽性）の局在診断

はじめに

喀痰細胞診陽性，胸部X線陰性の肺癌は従来より“オカルト肺癌（occult lung cancer）”と呼ばれ，喫煙と高い関係があり，中枢気管支に発生し多発傾向がある，肺がんの中でも特殊なものと認識されている[1]．近年では特に内視鏡的レーザー治療により完治する症例も多く，発見することが治ることに直結することが多い[2~5]．しかし喀痰細胞診が陽性の場合，中枢気管支以外にも気管支鏡可視範囲外の細い気管支や末梢肺，耳鼻科領域に病変が存在することもあり局在同定に難渋することもしばしばある[6]．本稿では中心型早期肺癌の特性とともに喀痰細胞診陽性で画像陰性症例の検査上の注意点に関し詳述する．

❶…中心型早期肺癌と喀痰細胞診

中心型早期肺癌は胸部X線やCTでは異常所見が得られず，喀痰細胞診が唯一の発見動機となる[2,7]．喀痰細胞診は日常臨床において肺がんの危険群に対するスクリーニングや肺がんを疑った場合の診断補助に行われることが多い．肺がん検診では40歳以上の男女には胸部X線検査を施行し，50歳以上で喫煙係数（1日の平均喫煙数×喫煙年数）が600以上の人や40歳以上で6カ月以内に血痰を認めた人に対して喀痰細胞診を施行する[8]．加えて呼吸機能検査で閉塞性障害を認めた場合には胸部X線で異常がなくとも喀痰細胞診を奨励する報告もある[9]．感度は20～70％と報告されているが，肺癌の発生部位や進行度によって異なる[9~14]．宮城県の肺がん検診では，発見された扁平上皮癌のうち39％が喀痰のみで発見されており，高危険群に喀痰細胞診を行うと早期の肺癌の発見頻度が高いことが報告されている[15]．

❷…中心型早期肺癌と気管支鏡

喀痰細胞診で中心型早期肺癌の存在を疑う場合，気管支鏡による局在診断が必要となる．元来，中心型早期肺癌とは区域気管支より中枢に位置し，がんの浸潤が組織学的に気管支壁を越えないで，なおかつリンパ節転移，遠隔転移がないものと定義されている[16]．しかし，現在は後述する内視鏡基準を定義として用いることが一般的である[8]．本腫瘍は従来より喫煙などの発がん危険因子の長期暴露との関連が指摘されており，組織型はほとんどが扁平上皮癌である．早期がん，特に上皮内癌は肉眼的に気管支粘膜の微細な変化を呈するのみの場合が多く，内視鏡的にも局在同定が困難な症例が少なくない．また20～30％の症例は同時，異時的に多発する傾向がある[17]．

喀痰細胞診陽性，胸部X線，CTで無所見の場合，中心型早期肺癌の存在が強く疑われる．したがって，喀痰細胞診で肺がんを疑う細胞が得られた場合（検診ではD, E判定），気管支鏡検査を行うことが必須である．

日本肺癌学会肺癌取扱い規約では中心型早期癌の内視鏡基準を

1：気管から亜区域支までに限局する．

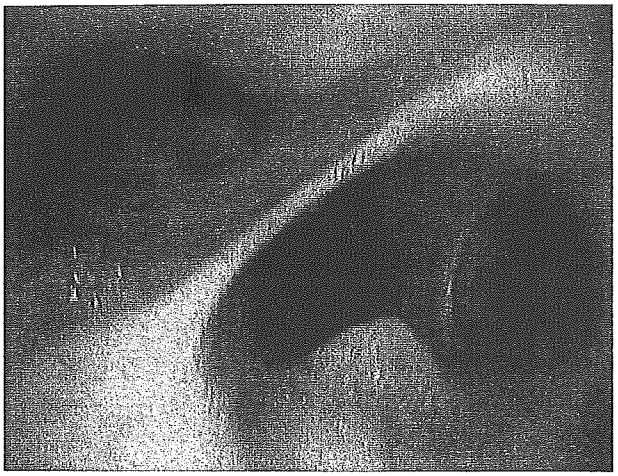


図 1　結節型早期肺癌（右下葉入口部）

2：病巣の末梢辺縁が，内視鏡的に可視できること．
3：病巣の長径が 2 cm 以下であること．
4：組織学的に扁平上皮癌であること．
とし，その所見を
1：無所見型
2：肥厚型
3：結節型（**図 1**）
4：ポリープ型と分類している[8]．

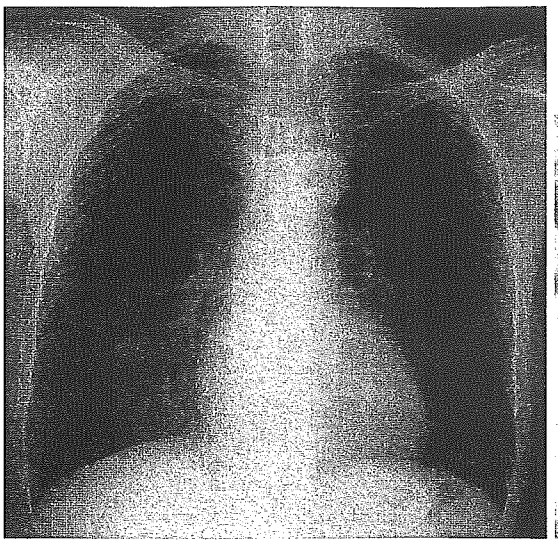
肉眼で局在が捉えられない無所見型では熟練の専門医でも診断困難で，複数回の気管支鏡検査を必要とすることもある．

中心型早期肺癌では浸潤範囲が気管支粘膜面で 1 cm 以下であれば上皮内癌の可能性が 90%程度とされ，粘膜面の隆起の程度や腫瘍径が増すに従い，気管支壁への深達進展が進むとされている[18]．

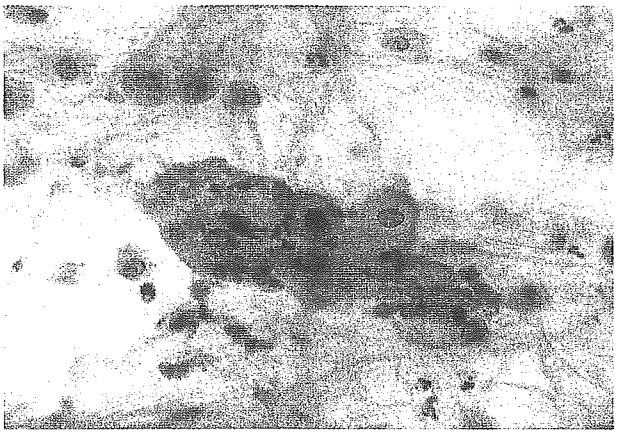
中枢気道を綿密に検査しても局在同定不可能な場合は無所見型を念頭に置くとともに，末梢型の肺癌の可能性も考慮する[6]（**図 2**）．この場合の局在診断にはすべての亜区域以降の気管支擦過細胞診や洗浄細胞診を順次行い，陽性気管支の範囲を決定する[19]．当初胸部 CT で所見が捉えられなくとも，経過観察中に末梢肺の異常影が発見可能な大きさになることも経験される．同時に声帯や副鼻腔などの耳鼻科領域に病変が存在することもあるため（**図 3**）副鼻腔の CT 検査や専門医による診察も行う[6]．診断の手順を**図 4** に示す．

❸…蛍光内視鏡

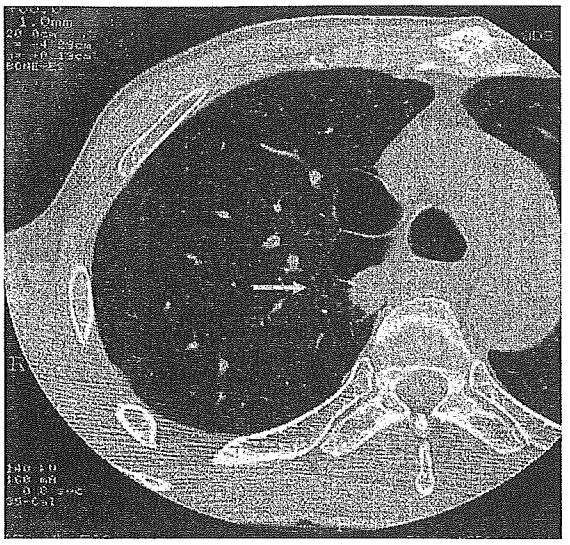
気管支の微細な病変を発見する方法として蛍光診断法が開発された．この原理は生体組織に含まれる各種成分は特定の波長の光を照射すると（光励起），それぞれその物質に特異的な波長を有する微弱な光（自家蛍光）を発する．特に青色波長の励起光を気管支の正常部に照射すると緑色波長領域の自家蛍光が発生するが，癌病巣ではこの波長領域の自家蛍光の強度は極端に低下している[20]．自家蛍光を高感度カメラで捕捉し image intensifier で増幅することにより，自家蛍光を発する部位＝正常部と自家蛍光が欠損している部位＝病変部のコントラストが鮮明になり微小な病変も発見しうる（**図 5, 6**）．蛍光診断は主として肺がんの治療前精査，喀痰細胞診異常症例，肺がん術後の経過観察，頭頸部領域の悪性腫瘍の治療後などが対象になる．通常の気管支鏡検査を行い，ひき続いて蛍光内視鏡検査を行って白色光単独の場合の診断率と蛍光を追加した場合の診断率を比較している．扁平上皮化生とがんの診断率は白色光単独より白色光＋蛍光の方が



a



b



c

図2　胸部X線陰性，喀痰細胞診陽性（末梢型扁平上皮癌）

胸部X線正常(**a**)．喀痰細胞診で扁平上皮癌(**b**)．内視鏡，耳鼻科診察で異常は発見されず，胸部CTにて右上葉に腫瘍性病変を発見(**c**)．手術にて扁平上皮癌．

1.5から6倍程度，優れていたとする報告が多い[20~27]．また，喀痰細胞診陽性症例の局在診断目的に本診断法を行った場合に限っても早期癌においては白色光では診断困難な微細な変化が蛍光診断を併用することにより鮮明に観察されたり，がんの進展範囲が客観的に評価されるなど，本検査の併用による利点が認められる[23]．がんの浸潤範囲を把握しうるため，切除線の術前決定やレーザーの照射範囲を正確に同定しうる利点がある[23]．また，喀痰細胞診異常症例に対する局在診断率は，蛍光診断導入以前の診断率と比較した場合，大幅に向上することが報告されている[23,26,27]．しかし，気管支粘膜が肥厚していたり，血管が増生している部位では蛍光画像では“異常”として認識されることがあり慢性炎症が偽陽性の原因となる[20~27]．

まとめ

喀痰細胞診陽性で画像無所見の症例に遭遇した場合，大きく3つの可能性がある．すなわち，中心型早期肺癌，末梢型肺癌，口腔—耳鼻科領域の癌である．このようながんは比較的進行が緩徐なこともあり，1

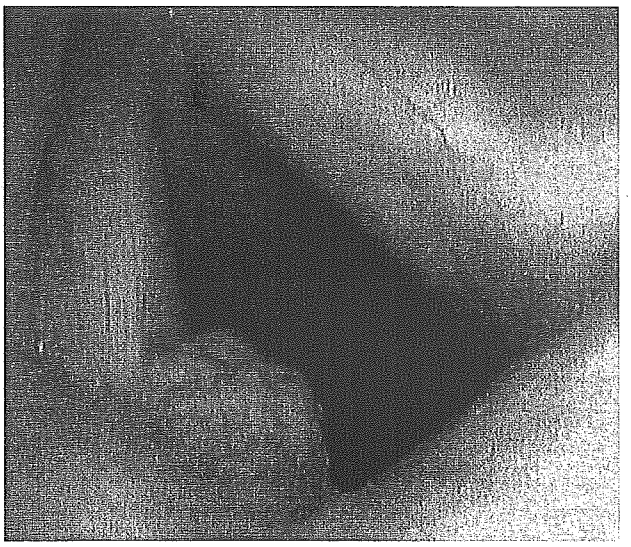


図3 喀痰細胞診陽性で発見された声帯癌

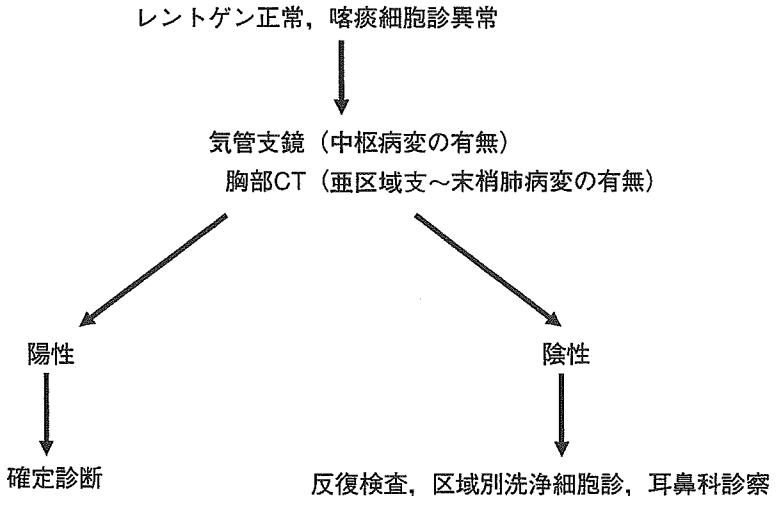


図4 レントゲンが正常で喀痰細胞診が異常な場合の精検

～2年後にやっと画像で感知できる大きさになることもある．したがって，一連の検査後に病変が発見できなくとも，定期的に検査を継続することが必要である．また特に，中心型早期肺癌で内視鏡的に腫瘍の全範囲が確認しうる長径1 cm以下の症例の場合，内視鏡的レーザー治療（光線力学的治療）の成績は完全寛解率95～98%ときわめて良好である[3~5]．早期診断の重要性を重ねて強調したい[28,29]．

文 献

1) Cortese DA, Pairolero PC, Bergstralh EJ, et al: Roentgenographically occult lung cancer: A ten-year experience. *J Thorac Cardiovasc Surg* **86**: 373–380, 1983
2) Kato H, Horai T: A color atlas of endoscopic diagnosis in early stage lung cancer. Wolfe, London, 1992
3) Kato H: Photodynamic Therapy for Lung Cancer: A review of 19 years experience. *J Photochem Photobiol* **42**: 96–99, 1998
4) Kato H, Okunaka T, Shimatani H: Photodynamic therapy for early-stage bronchogenic carcinoma. *J Clin Laser Med and Surg* **14**: 235–238, 1996
5) Furuse K, Fukuoka M, Kato H, et al: A prospective Phase II study on photodynamic therapy with Photofrin II for centrally located early stage lung cancer. *J Clin Oncol* **11**: 1852–1857, 1993