tasectomy; and follow-up. Between March 1984 and May 2006, 57 patients underwent resection of pulmonary metastases from primary esophageal cancer. These cases were registered in the Metastatic Lung Tumor Study Group of Japan database and were retrospectively reviewed from the registry. Preoperative examination, surgical indication, and operative procedure were at the discretion of each institution.

After excluding eight cases because of missing information such as number of resected metastases, age, or DFI, we examined the remaining 49 cases (46 males and 3 females) in our study. Surgery alone for the primary tumor was performed in 26 cases (53%), surgery and chemoradiotherapy were performed in 7 cases (14%), surgery and radiotherapy were performed in 6 cases (12%), surgery and chemotherapy were performed in 3 cases (6%), radiotherapy alone was performed in 2 cases (4%), and treatment data were not available for 5 cases (10%). We examined the following variables (Table 1): age (≥70 or <70), number of resected metastases (solitary or multiple), resected side (unilateral or bilateral), tumor size (≥3 or <3cm), DFI (≥12 or <12 months), surgical procedure (partial resection, segmentectomy, or lobectomy), and curability (complete or incomplete).

The present study was analyzed using anonymized data that were collected in each institution. Therefore, informed consent was not specifically obtained and institutional review board approval was not necessary.

Statistical Analysis

Overall survival was analyzed by the Kaplan-Meier method, and differences in variables were calculated by the log-rank test. The date of pulmonary resection was defined as the starting point. Cox's proportional hazards model was used for multivariate analysis. The data were calculated using version 5.0 of the StatView software package (SAS Institute Inc, Cary, NC). A p value of less than 0.05 was defined as indicative of statistical significance.

TABLE 1. Survival of 49 Patients According to Clinical Factors of Pulmonary Metastases

Variables	n (%)	5-yr Survival (%)	p
Age (yr)			
≥70	13 (27)	32.9	0.928
<70	36 (73)	27.8	
Number			
Solitary	39 (80)	27.4	0.797
Multiple	10 (20)	42.9	
Resected side			
Unilateral	44 (90)	29.3	0.621
Bilateral	5 (10)	30.0	
Tumor size[a]			
≥3 cm	10 (21)	40.0	0.640
<3 cm	38 (79)	26.7	
DFI			
≥12 mo	28 (57)	39.2	0.048
<12 mo	21 (43)	15.7	
Surgical procedure			
Partial and segment	31 (63)	36.4	0.338
Lobectomy	18 (37)	22.9	
Curability			
Complete	45 (92)	31.4	0.990
Incomplete	4 (8)	25.0	

[a] No cases were available.
DFI, disease-free interval.

RESULTS

The median interval between treatment of esophageal cancer and diagnosis of pulmonary metastasis (disease-free interval) was 14 months (range: 0–124 months). There were no perioperative deaths. The median age of patients at the time of pulmonary metastasectomy was 65 years (range: 35–82). The median number of resected metastatic lesions per patient was one (range: 1–5). The metastases ranged in size from 0.4 to 5.5 cm, and the median size was 2.0 cm. The metastases were squamous cell carcinoma in 48 cases and adenocarcinoma in one case. The surgical procedure was wedge resection in 23 cases (47%), lobectomy in 16 cases (33%), segmentectomy in 8 cases (16%), and bilobectomy in 2 cases (4%). The median follow-up period after the first pulmonary resection was 18 months (range: 0–206 months). Recurrence developed in 16 (33%) of the 49 patients. Recurrences were as follows: lung, nine; lymph node, three; neck, one; distant metastasis, one; stomach, one; and unknown, two. The overall 5-year survival after pulmonary metastasectomy was 29.6% (Figure 1). Median survival time was 27 months. We investigated the relationships between prognostic factors and survival (Table 1). Patients with a DFI less than 12 months had a significantly worse prognosis, as assessed by survival rates, than patients with a DFI greater than 12 months (Figure 2). Multivariate analysis of these variables was performed using Cox's proportional hazards model for disease-specific survival. A DFI less than 12 months was shown to be an independent prognostic factor (p = 0.04) (Table 2). At the time of submission, 28 patients examined in our study have died. Although 23 patients died of esophageal cancer, 7 patients were not available for recurrent sites. Five patients have died of other diseases (two cases

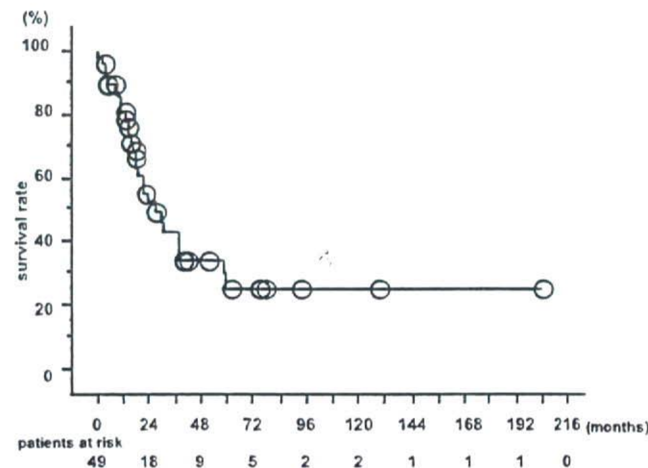

FIGURE 1. Overall survival of the 49 patients after pulmonary metastasectomy. The 5-year survival was 29.6%.

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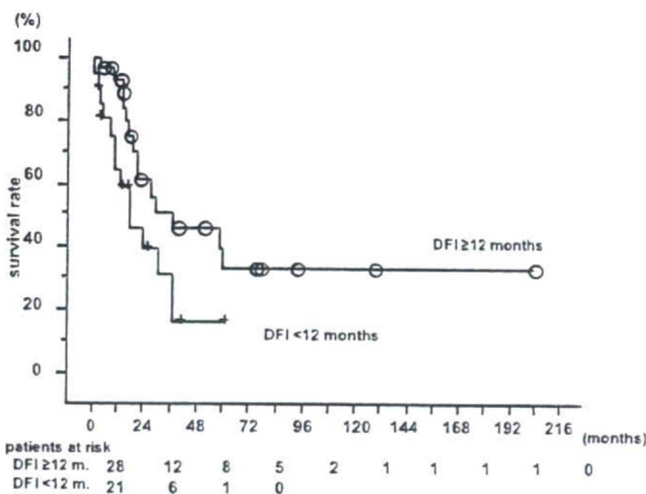

FIGURE 2. Overall survival after pulmonary metastasectomy according to DFI. Survival curves of patients with DFI <12 months and ≥12 months. DFI, disease-free interval.

TABLE 2. Relationships of Individual Variables to Survival (Cox's Proportional Hazards Model)

Variable	Risk Ratio	95% CI	p
≥70 yr	1.01	0.41–2.50	0.983
Multiple metastasis	1.67	0.30–9.19	0.557
Bilateral metastasis	1.19	0.19–7.53	0.853
Tumor size ≥3 cm	0.76	0.25–2.35	0.635
DFI <12 mo	2.30	1.04–5.09	0.040
Partial and segment	0.60	0.22–1.65	0.180
Incomplete resection	1.00	0.22–4.56	0.881

CI, confidence interval; DFI, disease-free interval.

were pneumonia, two cases were cerebral infarction, and one case was myocardial infarction).

DISCUSSION

Patients who are candidates for pulmonary metastasectomy for metastases from esophageal cancer are a minority. Analysis of the outcomes of surgery for pulmonary metastases from esophageal cancer has not been published. Quint et al.[4] showed that 29 of 147 (20%) patients with newly diagnosed metastasized esophageal cancer had lung metastasis. Although autopsy studies showed that the frequency of esophageal lung metastasis was 50%,[5] there was not a high percentage of esophageal cancer relapse after esophagectomy. Kyriazanos et al.[6] revealed that 12 of 151 (8%) patients who underwent a curative esophageal resection had lung metastases. Within our study the number of adenocarcinoma of the esophagus was very small. Because the frequency of adenocarcinoma of the esophagus is low in Japan, we do not speculate about the scarce incidence of lung metastasis from adenocarcinoma of the esophagus.

Matsubara et al. showed that 38 of 230 patients (17%) who underwent surgery for esophageal cancer with extended lymph node dissection had distant metastases and 14 (6%) patients had lung metastases. In their article, the outcomes after recurrence were dismal, and no patients were alive 5 years after detection of recurrence. Nevertheless, they showed that the 1-year survival of the patients who had recurrent lesions and were treated with resection and adjuvant therapy was 83%. They concluded that when recurrent lesions were localized macroscopically, surgical removal of the recurrent lesions was an effective treatment.[7] Through our analysis, we found a 5-year survival of 29.6% after pulmonary metastasectomy, which indicates that pulmonary metastasectomy is a promising treatment for metastases from esophageal cancer. Nevertheless, as it is not easy to differentiate esophageal metastases from primary lung squamous cell carcinomas, it is possible that our data might include primary lung squamous cell carcinoma. Survival after metastasectomy might be lower than what our data indicate. Virgo et al mentioned that genetic markers are needed to confidently distinguish between metastases and primary solitary nodules.[8] Further investigation is needed to clarify this matter.

An article from the international registry of lung metastases states that the 5-year survival was 37% after pulmonary metastasectomy. In addition, the article showed that among cases of complete resection, the 5-year survival was 33% for patients with a DFI of 0 to 11 months and 45% for those with a DFI of more than 36 months. Furthermore, the 5-year survival was 43% for single lesions and 27% for 4 or more lesions.[1] DFI and number of pulmonary metastases are significant prognostic factors. Because our present data show that the median DFI is 14 months, we categorized DFI as ≥12 or <12 months. Regarding the DFI, our study suggests that patients with a DFI less than 12 months have a poor prognosis. Osugi et al. showed that 83% of recurrences presented within 24 months after esophagectomy and that the chance of survival of patients whose disease recurred within 24 months after esophagectomy was better than that of patients who suffered recurrence within 24 months. Regarding follow-up studies after esophagectomy, meticulous care should be taken to detect hematogenous recurrence.[9]

In general, incomplete resection is a dismal prognostic factor in lung metastasectomy. We could not demonstrate whether surgical curability is a prognostic factor. McDonald et al. reported that incomplete resection appeared to have no influence on overall survival in metastatic breast cancer. They suggested that this could be due to the systemic nature of the disease at the time of thoracotomy with unsuspected occult metastasis in other areas.[10] Nevertheless, in our study, only four patients underwent incomplete resection. Because the report from The International Registry of Lung Metastases stated that cases with incomplete resection clearly had worse prognoses,[1] we speculate that patients with lung metastases from esophageal cancer have the same tendency.

Although our present study was multi-institutional, we could not analyze in detail all of the records for each patient. From this point of view, because our findings were based on a limited number of cases, pulmonary metastasectomy for lung metastases from esophageal cancer is still highly controversial. Nevertheless, we identified that patients with a DFI less than 12 months had a worse prognosis, as assessed by

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survival rates, than patients with a DFI greater than 12 months.

Consequently, although metastases from esophageal cancer are a minority, we think that pulmonary metastasectomy for esophageal cancer should be considered for selected patients with a DFI ≥12 months. As this study is small, further clinical studies will be needed.

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Quantitative *p16* and *ESR1* methylation in the peripheral blood of patients with non-small cell lung cancer

YASUHIRO SUGA, KUNIHARU MIYAJIMA, TAKEFUMI OIKAWA, JUNICHI MAEDA, JITSUO USUDA, NAOHIRO KAJIWARA, TATSUO OHIRA, OSAMU UCHIDA, MASAHIRO TSUBOI, TAKASHI HIRANO, HARUBUMI KATO and NORIHIKO IKEDA

Department of Thoracic Surgery, Tokyo Medical University Hospital, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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Abstract. Inactivation of the *p16* and *ESR1* tumor suppressor genes by promoter lesion methylation has been reported in many tumor types, including lung cancer. We examined the blood of 95 non-small cell lung cancer patients (66 cases of adenocarcinoma, 23 of squamous cell carcinoma and 6 of large cell carcinoma) and 30 controls consisting of normal subjects and benign disease patients to determine the methylation ratios of *p16* and *ESR1* using real-time PCR. For both genes, there was a statistically significant difference in the methylation ratio between non-small cell lung cancer patients and controls (*p16*; $p<0.01$, *ESR1*; $p<0.001$). In addition, there was a strong correlation between the methylation ratio of each gene and old age (*p16*; $p<0.01$, *ESR1*; $p<0.001$ and *p16* or *ESR1*; $p<0.001$), and between *p16* or *ESR1* methylation rate and smoking history ($p<0.01$). Moreover in Stage I cases, the methylation positive rate of each gene (*p16*, *ESR1* and *p16* or *ESR1*) was higher than the CEA positive rate ($p<0.05$, $p<0.001$, $p<0.001$). Evaluation of *p16* and *ESR1* promoter methylation in blood using real-time PCR appears to be very useful for lung cancer diagnosis and there is some possibility that these methylated genes might come to represent useful biomarkers for the early detection of lung cancer. Our study results also suggested that comparative evaluation of the methylation ratio before and after surgery might be a powerful tool to predict the prognosis of lung cancer patients.

Introduction

Lung cancer is the leading cause of cancer-related deaths in Japan. Lung cancer screening by chest X-ray and sputum cytology has not resulted in any improvement of the mortality rate of this cancer, either in Japan or in any other country. Therefore, it is very important to identify and develop reliable diagnostic and prognostic markers of early-stage lung cancer. We would like to suggest some new possibilities for early detection.

Previous evidence suggests that tumor cells may release DNA into the circulation, causing the serum to become enriched with the DNA (1). In lung cancer, promoter hypermethylation has been detected in blood, bronchial lavage, sputum and pleural fluid of lung cancer patients (2-5). Thus, DNA methylation in blood might represent a field defect of change. We tested *p16* and *ESR1* (*ESR1-a*) promoter hypermethylation in the blood by real-time PCR, which is more sensitive than methylation-specific PCR (6-8). *p16* is a well-known D-type cyclin-dependent kinase (cdk) inhibitor gene that interferes with the interaction of cdk4 with cyclin D_1, stimulating the progression of eukaryotic cells through the G1 phase of the cell cycle. The relation between *p16* methylation and lung cancer has been confirmed in tumor, tissue and serum samples (9).

ESR1 has been mapped to chromosome 6p25 and belongs to the superfamily of transcription activators (10,11). *ESR1* has also been shown to have growth-suppressive functions. *ESR1* promoter hypermethylation has been shown to be associated with irreversible inhibition of gene transcription in many cancers, including colon cancer, breast cancer, cervical cancer, and hematopoietic neoplasms (12-14). A previous study indicated that *ESR1-a* may play a more important role than *ESR1-β* in lung cancer. Recently, hypermethylation of *ESR1* was reported in a lung cancer cell line as well as in lung cancer specimens (11,15). Some studies showed that loss of *ESR1* expression was associated with aberrant 5'CpG island hypermethylation in breast cancer, colon cancer and lung cancer (11,12,16,17). Therefore, we tested *p16* and *ESR1* methylation in blood of lung cancer patients and patients with benign diseases.

Materials and methods

Collection samples. We examined whether aberrant *p16* and *ESR1* methylation might be found in the blood of non-small cell lung cancer (NSCLC) patients by real-time PCR in

Correspondence to: Dr Kuniharu Miyajima, Department of Thoracic Surgery, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
E-mail: miyajima@wb3.so-net.ne.jp

Key words: p16, ESR1, methylation, real-time PCR, non-small cell lung cancer

Table I. *p16*, *ESR1*, and *p16* or *ESR1* methylation positive rates (%) in normal control and benign disease patients and NSCLC patients.

	p16	*ESR1*	*p16* or *ESR1*
Sensitivity	26.3 (25/95)	52.6 (50/95)	62.1 (59/95)
Specificity	96.7 (29/30)	90.0 (27/30)	86.7 (26/30)
Accuracy	43.2 (54/125)	61.6 (77/125)	68.0 (85/125)
Positive predictive value	96.2 (25/26)	94.3 (50/53)	93.7 (59/63)
P-value	p<0.01[a]	p<0.001[a]	p<0.001[b]

[a]t-test, [b]χ^2 test. P-value, benign and normal control cases vs. NSCLC patients.

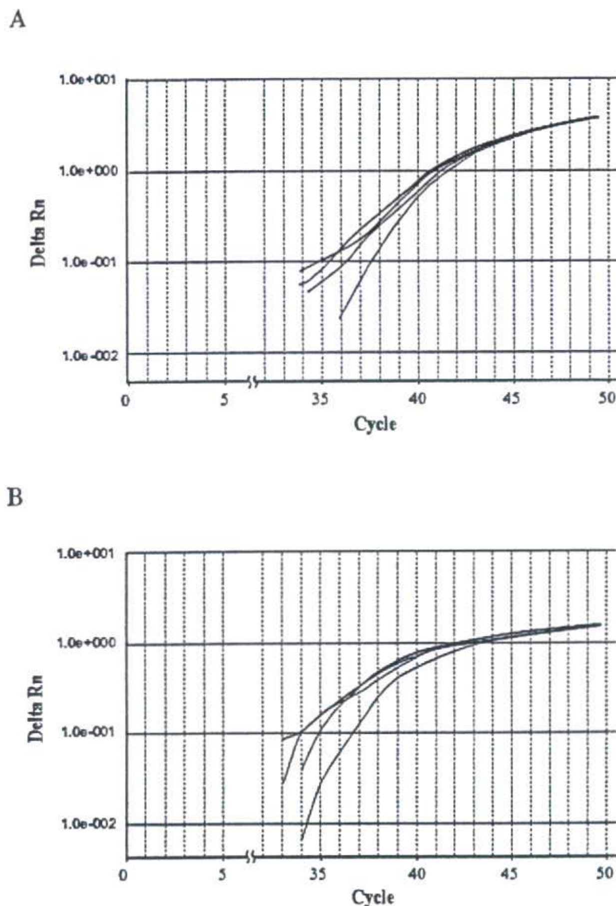

Figure 1. Amplification plots for the *p16* real-time PCR analysis of blood DNA samples (A). X-axis, the cycle number of quantitative PCR; Y-axis, Delta Rn, fluorescence intensity over the background. Amplification plots for the *ESR1* real-time PCR analysis of blood DNA samples (B). X-axis, the cycle number of quantitative PCR; Y-axis, Delta Rn, fluorescence intensity over the background.

specimens obtained from healthy volunteers and benign disease patients and patients with lung cancer. With written informed consent and in accordance with the Declaration of Helsinki, peripheral blood samples were obtained from 95 patients with NCSLC, including 66 with adenocarcinoma, 23 with squamous cell carcinoma, 6 with large cell carcinoma and 30 controls consisting of normal subjects and benign disease patients. This last group of 30 samples was obtained from 16 normal controls and 14 benign disease patients, including 5 with tuberculosis, 2 with mediastinal tumors, 2 with pneumonia, and 5 with other diseases. Among lung cancer patients, there were 52 Stage I cases, 8 Stage II, 23 Stage III, and 12 Stage IV. Patients with multiple primary cancers were excluded. The diagnosis in all the patients was made by the pathologists at our hospital.

DNA extraction, bisulfite treatment and methylation-specific real-time PCR. Peripheral blood samples were collected to investigate the methylation status of blood DNA. The buffy coat was isolated after centrifugation at 3,000 rpm for 10 min and blood DNA was extracted using phenol/chloroform extraction from blood (EDTA·2Na). Bisulfite conversion of DNA samples was carried out as previously described (18). The bisulfited DNA, *p16* and *ESR1*, as well as the internal reference gene *MYOD1*, were used as the templates for real-time PCR. The ratios between the values for the gene of interest versus the internal reference gene obtained by the TaqMan analysis were used to represent the relative level of methylated *p16* and *ESR1* DNA in a given sample. The sequences of the primers and probes used to amplify and detect methylated *p16*, *ESR1* and *MYOD* were as follows, and have been described previously (19,20). *p16*: 5'-CGCAA CCGCCGAACG-3' (forward primer), 6FAM-5'-CGCGAT CCGGCCCACCCT-TAMRA-3' (probe) and 5'-TTTTTT CGTTAGTATCGGAGGAAGA-3' (reverse primer). *ESR1*: 5'-GGCGTTCGTTTTGGGATTG-3' (forward primer), 6FAM-5'-CGATAAAACCGAACGACCCGACGA-TAMRA-3' (probe) and 5'-GCCGACACGCGAACTCTAA-3' (reverse primer). MYOD: 5'-TGATTAATTTAGATTGGGT TTAGAGAAGGA-3' (forward primer), 6FAM-5'-TCC CTTCCTATTCCTAAATCCAACCTAAATACCTCC-3'-TAMRA (probe) and 5'-CCAACTCCAAATCCCCTCTC TAT-3' (reverse primer).

Real-time PCR is based on continuous optical monitoring of a progressive fluorogenic PCR. We used Prism 7000 (Applied Biosystems) for this study. The methylation ratio was defined as the ratios of the fluorescence emission intensity values for the *p16* and *ESR1* PCR products to those of the *MYOD1* PCR products obtained by TaqMan analysis, multiplied by 100,000 and 10,000.

Statistical analysis. The correlation between the methylation ratios *p16* and *ESR1* was evaluated by χ^2 test and Welch's

Table II. *p16*, *ESR1*, and *p16* or *ESR1* methylation positive rates (%) classified according to the gender, age, smoking history, histological type and clinical stage.

	p16	*ESR1*	*p16* or *ESR1*
Gender			
Male	23.2 (19/82)	40.2 (33/82)	51.2 (42/82)
Female	16.3 (7/43)	46.5 (20/43)	46.5 (21/43)
Age (years)			
<63	9.1 (5/55)[a]	31.0 (17/55)[c]	34.5 (19/55)[c]
≥63	30.0 (21/70)	51.4 (36/70)	62.9 (44/70)
Smoking history			
Non-smoker	16.2 (12/74)	36.5 (27/74)	40.5 (30/74)[b]
Smoker	27.5 (14/51)	51.0 (26/51)	65.0 (33/51)
Histological type			
ADC	21.2 (14/66)	53.0 (35/66)	60.6 (40/66)
SCC	39.1 (9/23)	47.8 (11/23)	65.2 (15/23)
LCC	33.3 (2/ 6)	50.0 (3/ 6)	66.7 (4/ 6)
Clinical stage			
I	21.2 (11/52)	55.8 (29/52)	61.5 (32/52)
II	37.5 (3/ 8)	25.0 (2/8)	50.0 (4/8)
III	26.1 (6/23)	43.5 (10/23)	56.5 (13/23)
IV	41.6 (5/12)	66.6 (8/12)	83.3 (10/12)

[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, χ^2 test. ADC; adenocarcinoma, SCC; squamous cell carcinoma, LCC; large cell carcinoma

t-test using the SPSS. The relationship between *p16* and *ESR1* promoter methylation in blood and the clinicopathological characteristics of the patients was assessed by χ^2 test and Welch's t-test using the same software. Statistical significance was assumed to be indicated by $p<0.05$.

Results

p16 methylation in blood of controls and NSCLC patients. *p16* methylation ratios in controls and NSCLC patients are shown. The ratios were corrected by the value for the internal reference gene MYOD1. *p16* median methylation level in the controls was 1.3±0.9 (mean ± SE), while that in the NSCLC patients was 18.6±5.1 (Fig. 1A). The difference in the ratios between the NSCLC patients and controls consisting of normal subjects and benign disease patients was statistically significant ($p<0.01$). We determined the cutoff value as 3.0, and based on this cutoff value, the sensitivity of the determination for the diagnosis of lung cancer was 26.3%, specificity was 96.7%, accuracy was 43.2% and positive predictive value was 96.2% (Table I).

ESR1 methylation in blood of controls and NSCLC patients. The *ESR1* median methylation level in controls was 1.2±0.6 (mean ± SE), while that in the NSCLC patients was 9.0±2.0 (Fig. 1B). The difference between the values in the NSCLC controls was statistically significant ($p<0.001$). We determined the cutoff line as 2.5, and based on this cutoff value, the sensitivity of the determination for the diagnosis of lung cancer was 52.6%, specificity was 90.0%, accuracy was 61.6% and positive predictive value was 94.3% (Table I).

Associations with clinicopathological variables of the methylation positive rates of p16 and ESR1. We analyzed the correlations between the DNA methylation positive rates of DNA in blood and various clinicopathological variables. There was a strong statistically significant difference between *p16* and *ESR1* methylation positive rates in patients <63 years old and those ≥63 years old ($p<0.01$, $p<0.001$). However, there were no statistically significant correlations between the methylation rates of *p16* and *ESR1* and gender, smoking history, histological type or clinical stage (Table II).

p16 or ESR1 methylation positive rates in the blood of controls and NSCLC patients. There were statistically significant differences in the methylation positive rates of *p16* or *ESR1* between the NSCLC patients and controls ($p<0.001$). The sensitivity of *p16* or *ESR1* methylation positive rates for the diagnosis of lung cancer was 62.1%, specificity was 86.7%, accuracy was 68.0%, and positive predictive value was 93.7% (Table I). In addition, we found significant correlation between *p16* or *ESR1* methylation rates and old age ($p<0.001$), smoking history ($p<0.01$) (Table II).

Comparison between the methylation positive rates and tumor marker positive rates. In this study, the methylation positive

Table III. Comparison of *p16*, *ESR1*, and *p16* or *ESR1* methylation positive rates (%) and positive rates (%) of tumor markers in each clinical stage.

Stage	I	II	III	IV
p16	21.2[a]	37.5	26.1	41.6
ESR1	55.8[b]	25.0	43.5	66.6
p16 or *ESR1*	61.5[b]	50.0	56.5[c]	83.3
CEA	5.8	25.0	17.4	41.6
Tumor markers	19.2	50.0	47.8	50.0

Tumor markers, at least one serum protein marker (CEA, CA19-9, SLX, SCC and CYFRA). [a]vs. CEA: $p<0.05$, [b]vs. CEA or tumor makers: $p<0.001$, [c]vs. CEA: $p<0.03$.

rates of *p16*, *ESR1*, and *p16* or *ESR1* were compared with the positive rates of the serum protein tumor makers. The most commonly evaluated tumor markers in the clinical situation are CEA (carcinoembryonic antigen), CA19-9 (carbohydrate antigen 19-9), SLX (Sialyl Lewisx-i antigen), SCC (squamous cell carcinoma antigen) and CYFRA (cytokeratin 19 fragment). We investigated the correlations between the clinical stage and the methylation positive rates of the two genes (*p16*, *ESR1*, and *p16* or *ESR1*) and of the serum protein tumor markers.

Since CEA is the most commonly evaluated serum protein tumor marker in patients of NSCLC, we examined the methylation positive rates of each of the genes in blood and of the CEA positive rate. In cases with Stage I lung cancer, there was a statistically significant difference in the methylation positive rate of each gene (*p16*, *ESR1*, and *p16* or *ESR1*) and the CEA positive rate ($p<0.05$, $p<0.001$, $p<0.001$). In cases with Stage III also, a statistically significant difference was detected between the CEA positive rate and *p16* or *ESR1* methylation positive rate ($p<0.03$) Furthermore, in Stage I cases, there were statistically significant differences between at least one of the serum protein tumor markers and *ESR1* and *p16* or *ESR1* positive rates ($p<0.001$, $p<0.001$) (Table III).

Discussion

Previous studies have shown that aberrant promoter hypermethylation can be detected in specimens obtained from patients with no evidence of histopathological malignancy. In lung cancer patients, promoter hypermethylation has been detected in blood, bronchial lavage, sputum and pleural fluid of lung cancer patients (2-5). These aberrant hypermethylations have also been reported to be present in the primary tumor and thus may represent a field defect of changes that occur early in tumorigenesis, just like the presence of microsatellite instability in the majority of colorectal carcinomas (21,22).

Malignant associated changes have been reported as subtle morphological changes in the nuclei of normal cells found in the vicinity of the malignant growth. We regard this as one type of field cancerization (23). We used peripheral blood as the specimens in this study. In a previous study in which peripheral blood buffy coat specimens were used as the specimens to evaluate *p16* methylation status in cases of hepatocellular carcinoma, the median *p16* methylation ratio in the postoperative buffy coat specimens was significantly lower than that in the preoperative samples. The tendency of the methylation ratio to decline was shown to be greater in the buffy coat than in plasma specimens (8). It has been suggested that the degree of apoptosis and necrosis might affect the amount of tumor DNA released into the bloodstream. Thus, we believe that circulation DNA might be present in peripheral blood of cancer patients and represent a field defect of preneoplastic change.

This study showed the possibility of methylated DNA evaluated in peripheral blood samples becoming a useful biomarker for detection of lung cancer. There was a statistically significant difference in *p16* and *ESR1* methylation ratios between NSCLC patients and controls ($p<0.01$, $p<0.001$). We found that the sensitivity and specificity improved with evaluation of a combination of the two genes (Table I), and that the test may be potentially useful for mass screening of lung cancer and follow-up of lung cancer patients (24). The main factors influencing the ratio of DNA methylation in lung cancer patients have been shown to be the presence of malignant disease, including that of double cancer, old age and smoking history. Our results demonstrated the strong statistically significant differences of *p16*, *ESR1*, and *p16* or *ESR1* promoter methylation positive rates between patients aged <63 years and those aged ≥63 years ($p<0.01$, $p<0.001$, $p<0.001$). The finding of a significant difference in *p16* or *ESR1* methylation positive rate between smokers and non-smokers is also of interest ($p<0.01$). Previous studies have shown old age and smoking as being strong risk factors for lung cancer and also as bearing strong correlations with the DNA methylation change. (12,25,26) DNA methylation was detected in lung cancer patients of all stages in this study.

Comparison of the DNA methylation positive rates with the CEA positive rate in Stage I cases showed that the DNA methylation positive rates of the genes (*p16*, *ESR1*, *p16* or *ESR1*) were higher than the positive rates of CEA or any other tumor markers. Therefore, there is some possibility that evaluation of DNA methylation might become a more powerful tool in the early detection of lung cancer and lung cancer screening than the evaluation of tumor makers. There was only one false-positive case of *p16* methylation in our study; this case, who was diagnosed to have tuberculosis, was a 78-year-old man who was a heavy smoker (58 pack-years: p.y.). On the other hand, there were 3 false-positive cases of *ESR1* methylation. One of the cases had pneumonia, and the patient was an extraordinarily heavy smoker (92.5 p.y.). In the second case, the serum level of the tumor maker CYFRA was increased in the absence of any cancer. The third false-positive case was diagnosed to have emphysema and was an 80-year-old man who was a heavy smoker (60 p.y.). Old age and smoking have been reported as strong risk factors for lung cancer and to bear strong correlations with the DNA methylation change (25,26). Based on our results, we believe that old age is an especially strong risk factor for *p16* methylation, and the association warrants a careful investigation to determine if cases showing *p16* methylation are likely to be found to have malignancy in the near future.

We examined *p16* and *ESR1* methylation ratios in 26 lung cancer patients before and after curative surgery. The mean period from post operation to obtaining blood samples was 11.9±9.0 months. In relation to *p16* methylation, 19 cases were negative both before and after the operation. In 4 cases, methylation ratios increased after the operation and in 3 cases, it decreased. Two of the four cases in which it increased, including one with p-Stage IA, were diagnosed to have recurrence. The other p-Stage IA case had prostate cancer after curative surgery. The median postoperative methylation level in cases with recurrence or double cancer was shown to be 17.6-fold higher than the overall median preoperative methylation level. On the other hand, in cases without recurrence, the median postoperative methylation level was about one-third lower than the median preoperative methylation level. In relation to *ESR1* the methylation ratios were increased in 7 cases and decreased in 9 cases after curative surgery. Of the 7 cases in which it was increased, including 2 cases with p-Stage I, 4 were diagnosed to have recurrence, whereas no evidence of recurrence was found in the remaining 3 cases. We propose to carefully follow up these latter 3 cases to detect possible recurrence. Thus, quantitative analysis of the methylation ratio may allow follow-up of longitudinal changes of the methylation ratios in lung cancer patients (27).

In clinical situations, adjuvant chemotherapy has been established by consensus as efficient standard therapy. Evaluation of the methylation ratio before and after surgery may be useful to determine whether or not adjuvant chemotherapy must be administered. We consider that methylation-positive patients after curative surgery should receive adjuvant chemotherapy (28,29).

In conclusion, detection of aberrant *p16* and *ESR1* promoter methylation in blood samples using real-time PCR appears to be useful in the diagnosis of lung cancer, early lung cancer detection and also clinical follow-up of lung cancer patients.

Acknowledgements

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Can ischemic preconditioning enhance to protection of ischemia-reperfusion injury of the lung ?

Takafumi KONO, Masahiro TAIRA, Masakazu KIMURA, Akihiko SUZUKI, Hiroki HAYASHI, Kuniharu MIYAJIMA, Osamu UCHIDA, Naohiro KAJIWARA, Norihiko IKEDA, Harubumi KATO

Department of Surgery, Tokyo Medical University

Abstract

Background : Ischemic preconditioning (IP) has been focused on as a novel strategy to overcome ischemia-reperfusion injury. Steroid administration has also been routinely employed to reduce ischemia-reperfusion injury in lung transplantation. Therefore, we investigated whether ischemic preconditioning could enhance the effects of steroid on ischemia-reperfusion injury. **Methods** : Twenty-four Japanese white rabbits were randomly divided into four groups ($n=6$ in each group). Group I was the warm ischemia group (after 3 hours of warm ischemia, the left lung. was reperfused for 2 hours). Group II was the steroid group (10 mg/kg of methyl predonisolone was administered just before reperfusion). Group III was the IP group (3 cycles of IP were performed prior to left lung warm ischemia). Group IV was the combination group (3 cycles of IP and steroid were given before warm ischemia). The hemodynamics, airway pressure and blood gas measurement were assessed during reperfusion period. In addition, histological and immunohistochemical analyses were performed. **Results** : In group I, warm ischemia led to rapid and severe deterioration PaO_2 after reperfusion. Group IV had significantly higher PaO_2 than the other groups ($p<0.05$). $PaCO_2$ was significantly lower in Group IV than the other groups ($p<0.05$). Histologically, the left lung in group I showed diffuse alveolar damage and interstitial edema. However, groups II, III, and IV showed almost normal structures. **Conclusion** : This study demonstrates ischemic preconditioning can enhance the effects of steroid on ischemia-reperfusion injury.

Background

Lung transplantation has been accepted as treatment for end-stage pulmonary disease[1)2)]. However, early allograft dysfunction caused by ischemic and reperfusion injury remains an important and unpredictable issue to be solved. Although a number of strategies have evolved to limit lung ischemic injury during storage[3-9)], most clinical lung transplantation programs do not accept ischemic times in excess of 8 hours for organ storage. Even with short ischemic times, graft dysfunction occasionally occurs[1)8)]. Inadequate preservation of the donor lung may result in acute ischemia-reperfusion injury, characterized by increased pulmonary capillary, pulmonary edema, impaired gas exchange, and sometimes right heart dysfunction following an acute rise in pulmonary vascular resistance. Ischemic preconditioning (IP) describes the phenomenon by which a brief period of ischemia is used to induce tissue protection against subsequent ischemia and reperfusion injury. This has been intensively investigated with regard to myocardial ischemia[11)12)]. Recently it has been applied

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Corresponding author : Takafumi KONO M.D, First Department of Surgery Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo, 160-0023 Japan
Tel : 048-474-7211 Fax : 048-472-7581

to other organs such as liver, kidney, and lung[13-18]. It has also been reported effective in preventing apoptosis[19)20]. Since corticosteroids are routinely employed to reduce acute lung injury in lung transplantation, we investigated whether ischemic preconditioning could enhance the effects of steroid on ischemia-reperfusion injury, using a rabbit lung reperfusion model.

Materials and method

Operative procedure

All animals received human care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. (NIH publication No. 86-23, revised 1985). Twenty-eight female Japanese white rabbits (3.80±0.40 kg) were premedicated with subcutaneous atropine sulfate (0.25 mg/kg), ketamine hydrochloride (35 mg/kg). Isovolemia was maintained by intravenous infusion of modified Ringer's lactate, in which 4 ml of 7% sodium hydrocarbonate was added to 500 ml normal Ringer's lactate to maintain the pH of the fluid at 7.4. The infusion was given at a rate of 15 ml/kg/h via a peripheral ear vein. They were anesthetized with intravenous sodium pentobarbital (25 mg/kg) and 0.5 mg/kg of pancronium bromide. After cervical tracheostomy and placement of an endotracheal tube (interior diameter 4 mm), animals were ventilated with 100% O_2, tidal volume was maintained to ensure good inflation (15-25 ml/kg), With a respiratory rate of 30/min and positive end-expiratory pressure 1.0 cm H_2O, using a volume-cycled ventilator (Shinano SN-480-5 Tokyo). Core temperature was maintained at 38～40°C with warming blankets. Intravenous anesthesia was maintained by sodium pentobarbital and pancronium bromide. After median sternotomy and thymectomy, a 20 gauge catheter was placed in the right carotid artery to monitor systemic blood pressure. A 6 F introducer inserted through a 5-0 nylon pursestring suture in the right ventricule to place a 6 F Swan-Ganz catheter in the trunk of the pulmonary artery. After pleurectomy, the pulmonary hila were carefully dissected bilaterally and atraumatically encircled with rubber vascular tapes. Tourniquets were applied loosely over these tapes.

Experimental groups

The rabbits were divided into 4 groups of 6 each. In the warm ischemia group, 500 IU/kg of sodium heparin was given via a fluid line before the tourniquet was firmly tightened around the right pulmonary hilum. The ventilator was readjusted for unilateral ventilation. To keep minute ventilation roughly unchanged, tidal volume was reduced the 12-20 ml/kg, and the rate increased to 40/minute. After 5 minutes of stabilization, the baseline measurement was obtained, and the

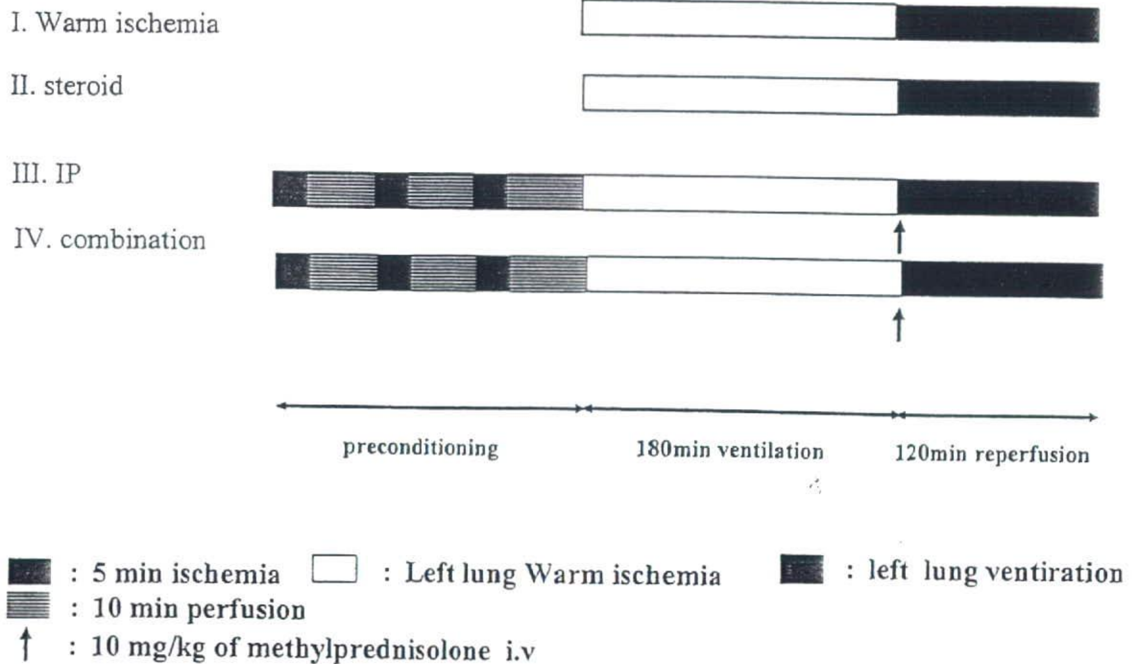

Fig. 1 Time course of the experimental groups. The warm ischemic group received 2 hours of single left lung perfusion after ischemia for 3 hours. The IP group 3 received cycles of 5 minutes IP followed by 10 minutes intervals before the experiment. In steroid group 10 mg/kg methylprednisolone sodium succinate was given i.v. just before reperfujsion. In the combination group IP and steroid injection was performed in the same manner as in the to the manner of IP and steroid groups.

tourniquet around the right hilum released. The left pulmonary hilum was then occluded for 180 minutes at end inspiration to keep the lung at fully inflated tidal volume. Three hours later the tourniquet was released and reperfusion performed for 2 hours, while during this reperfusion time the contralateral side was occluded in the same way. The IP group was preconditioned with 3 cycles of short periods of ischemia, (5 minutes each) by clamping the left hilum with reperfusion four 10 minutes after the cycles before performing ischemia and then reperfusion as in warm ischemia group. In the steroid group, after 3 hours of left lung ischemia 10 mg/kg of methylprednisolone sodium succinate was injected, followed by reperfusion for 2 hours. The combination group was subjected that of the IP group followed by the same treatment as the steroid group (Fig. 1).

Measurement of lung function

Assessments, blood gas analysis, mean systemic blood pressure (AoP), mean pulmonary arterial pressure, heart rate and peak inspiratory pressure were performed before left hilar clamping and after lung ischemia. The first post-ischemia assessment was made after 5 min of reperfusion. Subsequent assessments were made at 20-min intervals during the 120 min of reperfusion. During the 2 hours of reperfusion, the level of anesthesia was maintained with pancronium bromide and sodium pentobarbital intravenously. Blood pH was normalized as necessary by administration of sodium bicarbonate.

Histological studies

For light microscopic studies, the middle portion of the lower lobe was removed from the left lung. One sample was fixed in 10% neutral buffered formalin for H-E staining and another sample was fixed in 4.0°C of 99% acetone for TUNEL staining of immunohistochemically detect apoptosis. These sections were embedded in paraffin.

***In situ* detection of cell damage**

After deparaffinization, the 5-μm sections were placed in 1×phophate-buffered saline for 10 minutes at room temperature and digested by proteinase K for 15 minutes at 37°C. Intrinsic peroxidase activity was quenched by the addition of 3% hydrogen peroxide in methanol for 5 minutes. Staining procedures followed the instructions of the apoTACS™-DAB In Situ Apoptosis Detection Kit (Trevigen®).

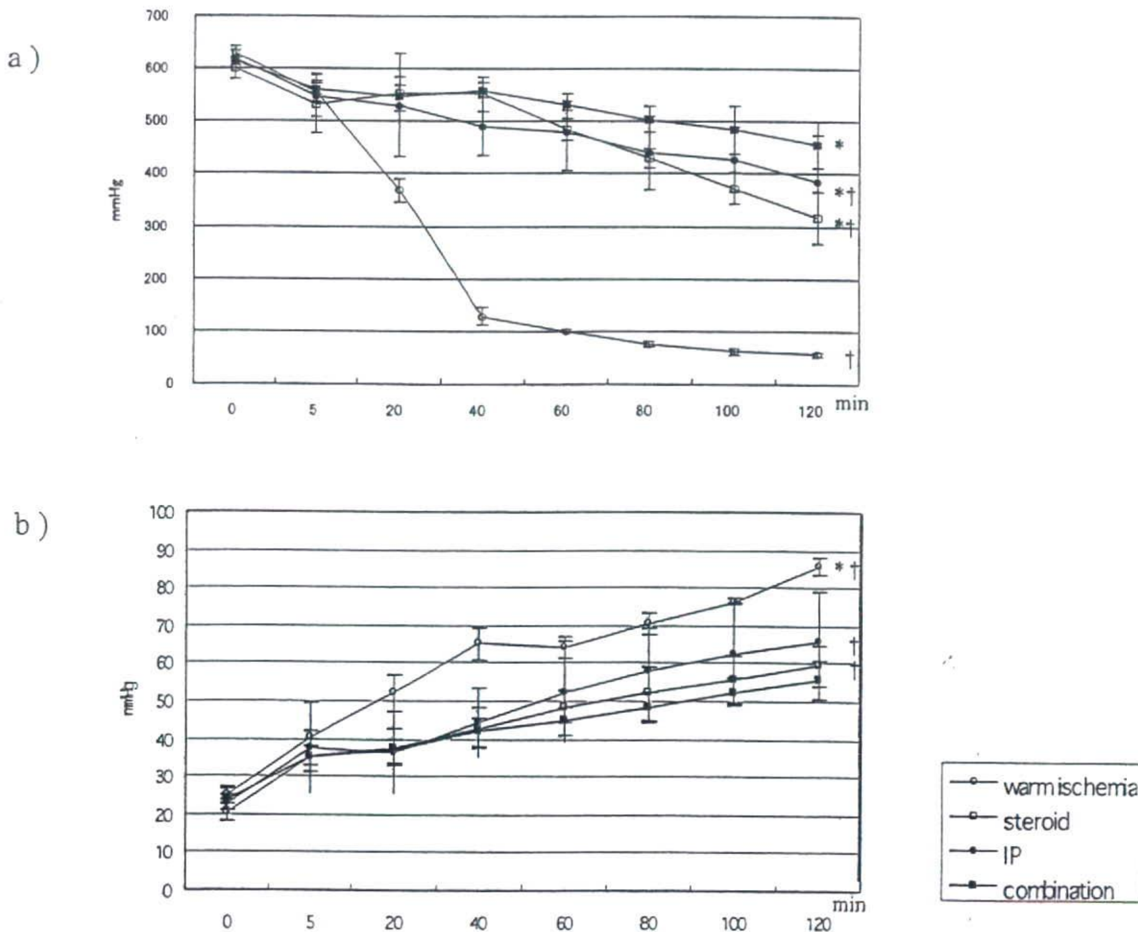

Fig. 2 Changes in a) PaO_2 and b) $PaCO_2$ in all experimental group, Each point is mean±SEM. *$p<0.01$ vs. warm ischemia group, by repeatedly measured ANOVA. †$p<0.05$ vs. combination group, by repeatedly measured ANOVA.

Statistical analysis

All statistical analysis was performed using StatView® (Abacus Concepts Inc.) A p value of less than 0.05 was considered to indicate statistically significant difference. All data ware presented as means±SEM. Continuously recorded data were compared among groups over time using repeated measurement analysis of variance (ANOVA) with a polynomial transformation applied to time.

Results

Hemodynamic, Airway Pressure, and Blood Gas Measurements

In each animal, following reperfusion there was a brief period of hypotension, which in most cases recovered spontaneously within minutes. However, two rabbits died within 40 minutes of reperfusion, one in the IP group due to hilar bleeding, another rabbit in the control group died of cardiac failure of unknown cause. Two rabbits in the control group and one rabbit in the steroid group died in the warm ischemic period due to cardiac failure. Data from these animals were excluded from the analysis.

Arterial blood gas analysis

Among the four groups, there were significant overall differences in the trends of partial arterial oxygen pressure (PaO_2). In the warm ischemia group, 3 hours of warm ischemia led to rapid and severe decrease in gas exchange after reperfusion. In the 3 other groups, PaO_2 at the end of 2 hours reperfusion was significantly higher ($p<0.01$) than in the warm ischemia group. And there was significant difference in PaO_2 at the end of 2 hours of reperfusion in the combination group and other two treatment group ($p<0.05$). A significant difference in the arterial carbon dioxide pressure ($PaCO_2$) level was seen in the combination group and other three groups at the end of 2 hours of reperfusion (Fig. 2).

Hemodynamics and Airway Pressure

There were no significant differences in pulmonary artery pressure in each groups after 2 hours of reperfusion, but only the warm ischemia group showed a significantly higher level after 2 hours of reperfusion

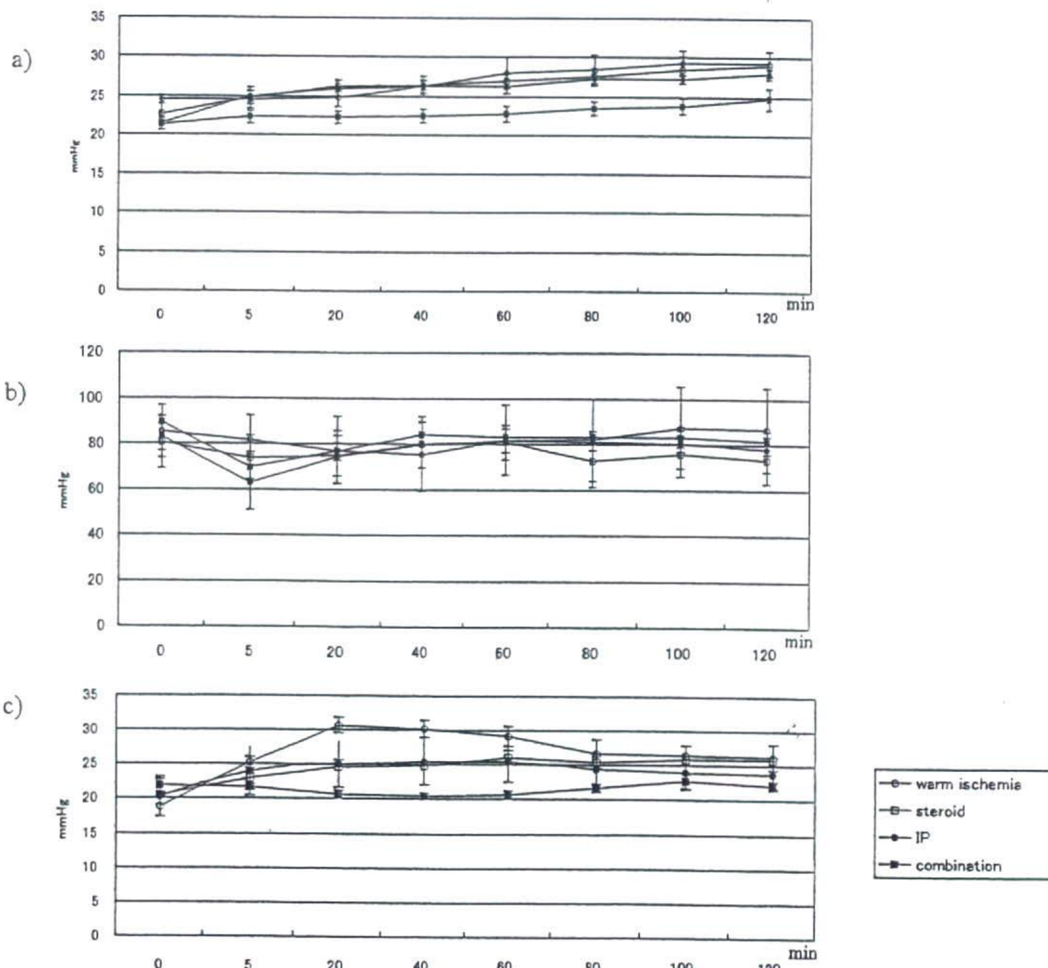

Fig. 3 Changes in a) airway pressure, b) systemic blood pressure and c) pulmonary arterial pressure of all experimental group, Each point is mean±SEM. *$p<0.01$ before vs. 2 hours after warm ischemia, by repeatedly measured ANOVA. †$p<0.05$ vs. combination group, by repeatedly measured ANOVA.

compared to before ischemia. No significant difference in systemic pressure or airway pressure was seen in any groups, after 2 hours of reperfusion (Fig. 3).

Wet to Dry Lung Weight Ratio

The wet to dry lung weight ratios of the reperfusion lung were 6.00±1.00 in the combination group, which was significantly lower than in the other groups ($p<0.05$). The ratio in the steroid group and IP group were significantly lower than in the warm ischemia group ($p<0.05$) (Fig. 4).

Histological Findings

After 2-hour assessment, the left lung in the warm ischemia group showed severe interstitial edema and thickening of the alveolar septum, and markedly increased intraalveolar red cells and granulocytes. The warm ischemia group showed diffuse alveolar damage and interstitial edema. The steroid group and the IP group showed mild interstitial edema, while the combination group showed normal structures and no sign of pulmonary edema (Fig. 5).

TUNEL staining

The warm ischemia group showed severe cell damage but the other groups had less alveolar cell damage, especially in the combination group (Fig. 6).

Discussion

We attempted to determine whether ischemic preconditioning is useful for lung transplantation. Our in situ warm ischemic lung model for this study was based on the report by Qayumi et al. They reported that ischemia-reperfusion injury after warm ischemia resulted in very similar conditions biochemically, functionally and morphologically to those recognized after lung transplantation following cold ischemia[21]. Our model is suitable to investigate the effects of ischemic preconditioning. In lung transplantation, early allograft dysfunction caused by ischemic and reperfusion injury remains an important and unpredictable issue. In IP, solid organs are subjected to repeated periods of short ischemia and reperfusion to protect against ischemia-reperfusion injury following prolonged otherwise lethal ischemia. This phenomenon was first reported in myocardium by Murry and colleagues in 1986[11], has the mechanism of the effects of IP is not clear. In his summary of cardiac preconditioning, Meldrum stated that stress hormones like adenosine, nor epinephrine and high-level of intracellular calcium were propagated as signaling mechanisms in acute preconditioning, leading to activation of protein kinase C, ecto-5'-nucleotidase, and K_{ATP} channels[12]. The results suggest that it is good strategy for ischemia-reperfusion injury not only in cardiomyocytes but also for other solid organs[13-18]. These reports suggested the possible usefulness of IP for organ transplantation. However, clinically, steroid what may inhibit the production of proinflammatory cytokines and the sequestration of PMNs[22], is given intraoperatively just prior to graft perfusion in lung transplantation. We investigated whether ischemic preconditioning enhances the protective power of steroids against ischemic reperfusion injury of the lung. Histologic findings 2 hours after reperfusion in the warm ischemic group revealed severe alveolar damage. In the steroid group and IP group, the damage was mild and the combination group showed almost no damage. Blood gas analysis data showed similar results with regard to PaO_2 2 hours after reperfusion and there were significant differences between the combination group and the steroid group. The combination group showed the best results in comparison to all other groups. Though we found a difference in each group in blood gas analysis and histological assessment by this study, no difference was found in the hemodynamic and airway

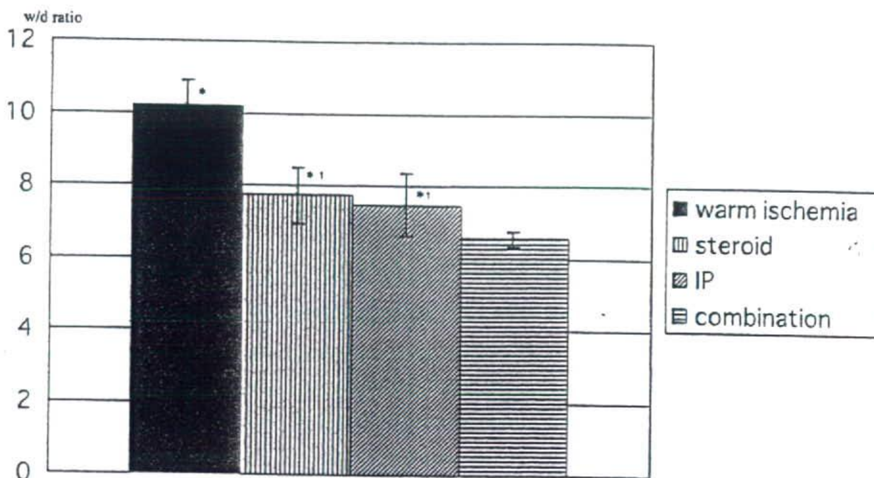

Fig. 4 Wet to dry weight ratio after 2 hr single left lung perfusion, After assessment, the wet to dry ratio of the reperfusion rabbit left lung in the combination group was greater than in the other group. *$p<0.05$ vs. combination group, by repeatedly measured ANOVA. †$p<0.05$ vs. warm ischemia group, by repeatedly measured ANOVA.

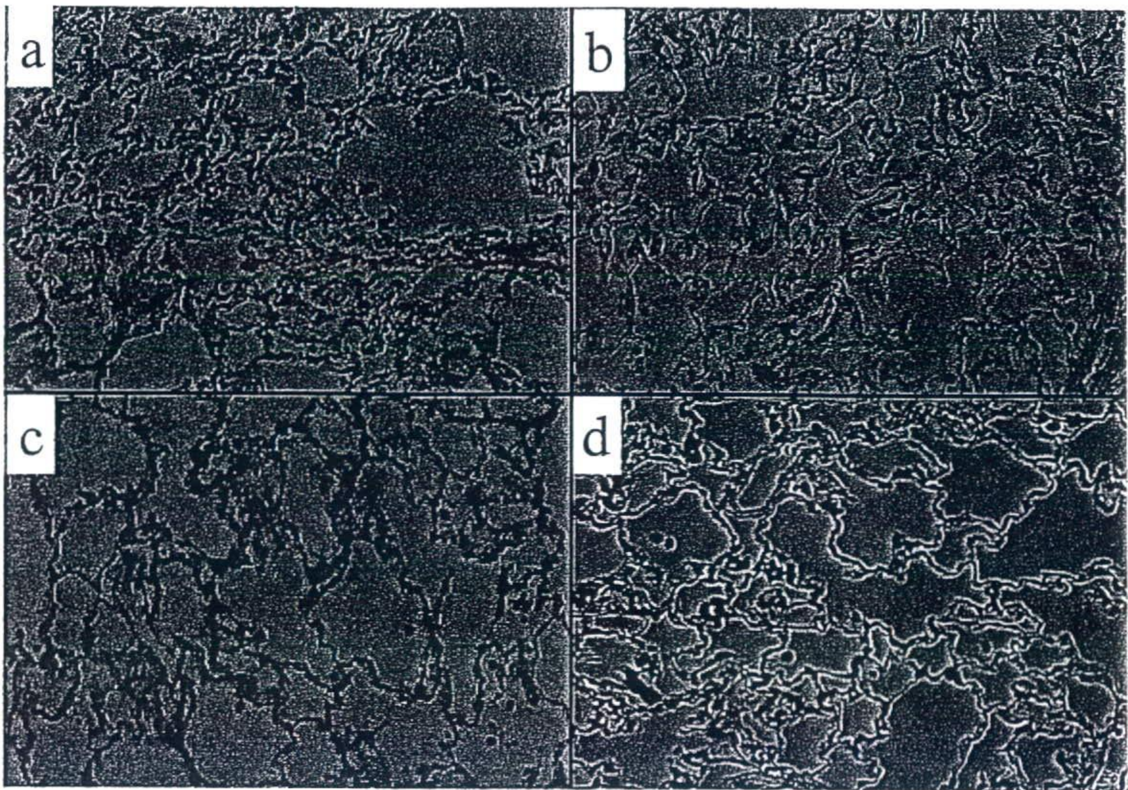

Fig. 5 Histrogical finding in a left lung after 2-hour reperfusion, (a) The warm ischemic group showed severe interstitial edema and thickening of the alveolar septum. (b) The IP group and (c) The steroid group showed almost no abnormal structure and slight thickening of the alveolar septum. (d) The combination group showed almost no abnormal structure and no sign of pulmonary edema. (HE×400)

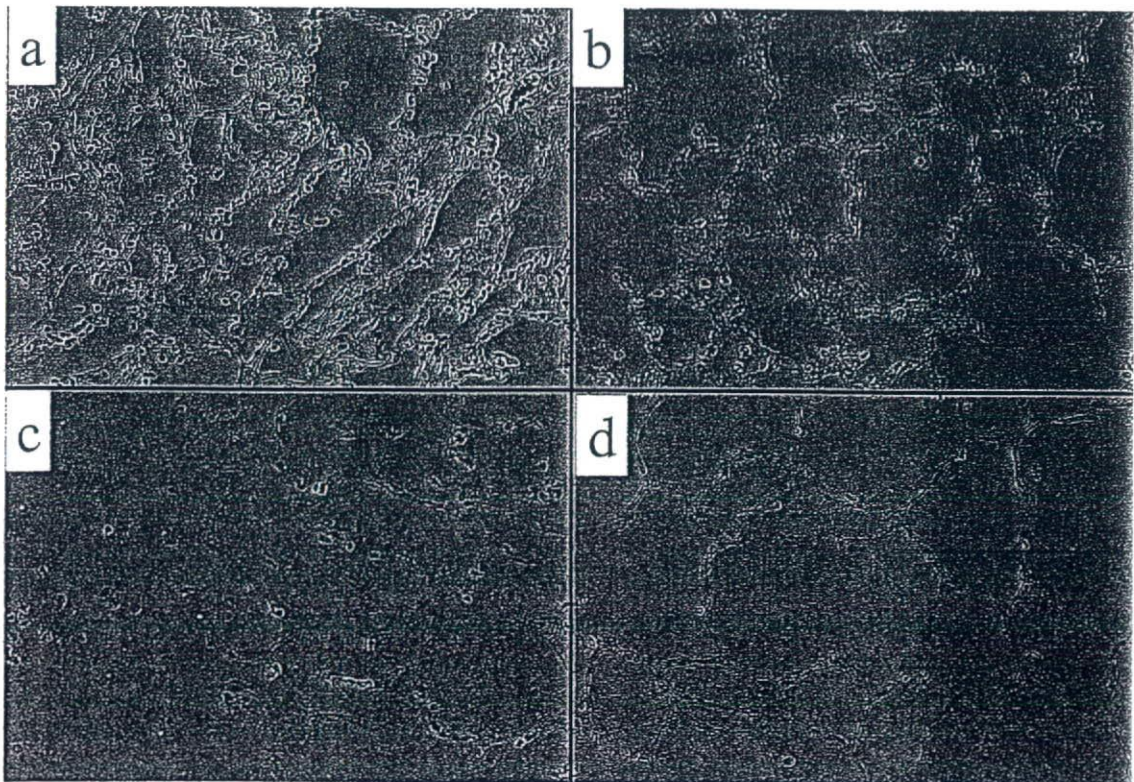

Fig. 6 In TUNEL staining, the warm ischemic group showed severe cell damage (a). (b) The IP group and (c) the steroid group showed mild cell damage. (d) The combination group was almost normal. (×400)

pressure. It is thought that may be these results reflect an injury in peripheral tissue, and the influence did not yet extend at this stage to the general state.

Ischemic preconditioning in lung transplantation with reperfusion injury in rabbit lung after a three-hour warm ischemic period was less pronounced when the lung was previously subjected to repetitive periods of short ischemia and reperfusion and IP can also enhance steroid effectiveness. This study demonstrates that IP can enhance the protective effect of steroids against ischemia-reperfusion injury in the rabbit lung.

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虚血前短時間虚血による肺虚血再潅流障害抑制の増強効果に関する検討

河野貴文　平良真博　木村雅一
鈴木明彦　林　博樹　宮島邦治
内田　修　梶原直央　池田徳彦
加藤治文

東京医科大学外科学第一講座

【要旨】 肺移植後の肺虚血再潅流障害の対策として、ステロイド投与が、第一に行われているが、虚血前短時間虚血を付加することにより、より効果的に再潅流障害を抑制するとの仮定に基づき以下の実験を行った。【方法】日本白兎を用い、左肺における虚血再潅流障害を以下の4群にて比較した。1（温阻血）群：開胸後左肺門3時間遮断、温阻血の後右肺門を遮断し、同時に左肺門を解放2時間再潅流せしめた。2（ステロイド投与）群：温阻血群に準じ開胸後左肺門3時間遮断し、左肺解放直前にステロイドを経静脈的に投与した。3（虚血前短時間虚血）群：開胸し左肺門の5分遮断10分解放（虚血前短時間虚血）を3回繰り返した後、温阻血群に準じて左肺門の遮断及び解放を行った。4（併用群）：虚血前短時間虚血群に準じ虚血前短時間虚血を行った後にステロイド投与群に準じステロイドを経静脈的に投与した。それぞれの群に対して再潅流後経時的に血行動態、気道内圧、血液ガス測定を施行した。また湿乾燥重量比、病理組織学的検討を行った。【成績】再潅流2時間後の血液ガス測定では、虚血前短時間虚血群、ステロイド投与群、併用群ともに温阻血群に対し良好な成績を得た。併用群は虚血前短時間虚血群、ステロイド投与群に対しても良好な成績を得た。湿乾燥重量比においても同様の傾向を認めた。気道内圧は併用群において、良好な成績を得た。病理組織像においても温阻血群に対し虚血前短時間虚血群、ステロイド投与群、併用群では良好な結果が得られた。【結語】虚血前短時間虚血はそれ自体に虚血-再潅流障害軽減効果を持つが、ステロイドによる同様の効果を更に増強させる傾向が示され、臨床的付加価値がある可能性が示唆された。

〈キーワード〉 虚血-再潅流障害、ischemic preconditioning、lung transplantation

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免疫染色法による *EGFR* 遺伝子変異 (L858R) の検出についての検討

坂田義詞[1)]　大平達夫[1)]　名和公敏[1)]
稲田秀洋[1)]　前田純一[1)]　西條天基[1)]
本多英俊[1)]　垣花昌俊[1)]　中嶋英治[1)]
野村将春[1)]　坪井正博[1)]　池田徳彦[2)]
平野　隆[1)]　加藤治文[1)]

[1)]東京医科大学外科学第一講座
[2)]国際医療福祉大学三田病院呼吸器センター

【要旨】 目的：ゲフィチニブは EGFR のチロシンキナーゼ阻害剤 (Tyrosine Kinase Inhibitor; TKI) であり、非小細胞肺癌 (non-small cell lung cancer; NSCLC) においてその有効性が示された分子標的治療薬である。特定の *EGFR* 遺伝子変異を有する症例に、より高い感受性を示すことが分かっている。現在、変異型 *EGFR* については 20 種類以上報告されているが、Exon 18、19、21 に集中しており、その中でも主な変異は Exon 19 の deletion と Exon 21 の point mutation で、全体の 80～90% を占めるといわれている。今回我々は、従来の direct sequence 法に代わり、変異検出法としてより簡便な免疫染色を用いて Exon 21 の point mutation の検出を試みた。

対象と方法：術後再発症例に対してゲフィチニブを投与した非小細胞肺癌患者 42 例を対象とした。切除病理標本に対し、Exon 21 の point mutation (L858R) に特異的な抗体を用いて免疫染色を行い、現在広く用いられている direct sequence 法による結果との比較を行った。

結果：direct sequence 法では、42 例中 Exon 21 の突然変異は 6 例 (14.3%) に認め、一方 EGFR L858R 抗体を用いた免疫染色では、7 例 (16.6%) が陽性となった。 direct sequence 法で変異を検出した 6 例は、いずれも免疫染色法にて陽性となった。

結論：免疫染色法は簡便かつ安価であり、今回の実験で従来の direct sequence 法との高い相関性も得られたため、今後有用な変異検出法となり得ると考えられる。

はじめに

我が国における肺癌死亡率は年々増加し、1998 年には胃癌死亡数を上回り悪性新生物の中で第 1 位となった。非小細胞癌は肺癌の 80～85% を占め、その治療の中心は手術であるが、たとえ早期であっても術後の再発例は少なくなく、化学療法の必要性は高い。近年、化学療法の進歩は目覚ましいが、依然十分な効果が得られていないのが実情である。こうした臨床的背景から、症例毎に腫瘍の性格や薬剤感受性を把握し最適な治療を選択する個別化学療法の実現が急務となっている。現在、新たな薬剤として分子標的治療薬

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Tel：03-3342-6111 (5070)　Fax：03-3349-0326

が注目を集めている。分子標的治療薬は作用点が明確なため、ターゲットとなる機構の解明により、個別化学療法を目指すうえでは非常に有用な薬剤と考えられる。

上皮成長因子受容体（Epidermal Growth Factor Receptor；EGFR）はチロシンキナーゼ型受容体で、ErbB familyに属する分子量170 kDaの膜貫通型糖タンパクである[1-3]。細胞外領域はリガンド結合部位をもち、上皮成長因子（Epidermal Growth Factor；EGF）や、TGF-α、Amphiregulin、ヘパリン結合EGF様増殖因子（Heparin-binding EGF-like Growth Factor；HB-EGF）、Betacellulin、Epiregulinなどと結合することで活性化し、細胞膜上を移動してEGFR同士、あるいは他のErbBファミリー受容体と二量体を形成する[4]。二量体を形成すると、細胞内ドメインのチロシンキナーゼの自己リン酸化により活性化される。そしてその活性化シグナルは、PI3K（Phosphoinositide-3 Kinase）/Akt、Grb2/SOS/Ras/Raf/MEK/MAPK（Mitogen-Activated Protein Kinase）、Jak/STATなどの経路により伝達され、癌細胞の増殖、血管新生、転移形成等を促進する[5]。Grb2/SOS/Ras/Raf/MEK/MAPK経路は、主に細胞増殖と生存に関与し、PI3K/Akt経路は主に細胞成長や抗アポトーシス、浸潤、遊走などに関与する[6]。

EGFRの発現は上皮系、間葉系、神経系起源の多様な細胞でみられ、正常組織においては、細胞の分化、発達、増殖、維持の調節に重要な役割を演じている[7]。このEGFRに遺伝子増幅や遺伝子変異、構造変化が起きると、発癌、および癌の増殖、浸潤、転移などに関与するようになる[8][9]。EGFRはさまざまな悪性腫瘍で過剰発現がみられ、非小細胞肺癌では40〜80%で過剰発現がみられる[10]。EGFR過剰発現は癌の予後不良因子であり、これまでに、低分化な組織型、進行した臨床病期、抗癌剤耐性などとの相関が報告されている[11-14]。また、リン酸化型EGFRの発現が非小細胞肺癌の予後と逆相関することが示されており[15]、これらの結果より、EGFRが非小細胞肺癌の治療における格好の標的分子であると考えられている。

ゲフィチニブはEGFRのATP競合的チロシンキナーゼ活性阻害剤（Tyrosine Kinase Inhibitor；TKI）であり、EGFRのシグナル伝達をブロックする[16][17]。非小細胞肺癌においてその有効性が示されており、現在臨床的に用いられている分子標的治療薬である。

近年、非小細胞肺癌患者のEGFRチロシンキナーゼドメインにある、Exon 18〜23の遺伝子解析により、同部位の遺伝子変異が同定された。これらの遺伝子変異が、非小細胞肺癌患者のうち腺癌や女性、非喫煙者、そしてゲフィチニブ著効例等に多く認められたことにより、ゲフィチニブの有用な感受性予測因子と考えられている[18-21]。

現在、変異型 *EGFR* については20種類以上報告されているが、Exon 18、19、21に集中しており、その中でも主な変異は、Exon 19のcodon 746-750を中心とする部位のdeletion（E746-A750del）と、Exon 21のcodon 858におけるロイシンからアルギニンへのpoint mutation（L858R）で、全体の80〜90%を占めるといわれている。

EGFR 遺伝子変異を有する進行肺癌において、ゲフィチニブによる初回治療で70%以上の奏功率が報告されている[22][23]。一方で変異を持たない患者では奏功率が低く、また重喫煙者などでは他の抗癌剤と比べ、急性肺障害の発生率が高い。これらより、ゲフィチニブは効果が期待でき、重篤な合併症のリスクの低い特定の患者に対して、投与が推奨されると考えられる。

今回我々は、ゲフィチニブが有効な症例群を同定するために、従来のdirect sequence法に代わり、変異検出法としてより簡便な免疫染色を用いてExon 21のpoint mutationの検出を試みた。

材料と方法

本研究は、ヒトゲノム・遺伝子解析研究に関する指針（文部科学省・厚生労働省・経済産業省の三省共同指針、および関連学会の指針）に基づき、全症例とも当院の倫理委員会で認められた承諾書を得たうえで、倫理的配慮を図って行った。

患者

東京医科大学病院で1992年から2004年に切除された非小細胞肺癌42症例の切除標本（腺癌35例、扁平上皮癌4例、大細胞癌2例、多形癌1例）を使用した。これらの切除標本は全て術後再発に対してゲフィチニブが使用され治療効果が評価された。患者の年齢は35から91歳（平均61.8歳）。男性は26人で女性は16人であった。非喫煙者（喫煙歴のない症例）は18例（42.9%）で、男性は26人中6人（23.1%）、女性は16人中12人（75.0%）が非喫煙者であった。術後の病理病期はそれぞれ、IA期7人（16.7%）、IB期10人（23.8%）、IIA期0人（0%）、IIB期4人（9.5%）、IIIA期

Table 1　The characteristics of NSCLC patients treated with gefitinib

42 non-small-cell lung cancer (NSCLC) treated with gefitinib		
PATIENTS' CHARACTERISTICS	$N=42$ cases	%
Age at beginning of IRESSA therapy (Median ; Range)	61.8	35-91
Gender : M/F	26/16	61.9/38.1
Histology : ADC/SCC/La/Pleomorphic	35/4/2/1	83.3/9.5/4.8/2.4
Pathological Stage : IA/IB/IIA/IIB/IIIA/IIIB/IV	7/10/0/4/11/6/4	16.7/23.8/0/9.5/26.2/14.3/9.5
Smoke : Never/Former+Current	18/24	42.9/57.1

11人（26.2%）、IIIB期6人（14.3%）、IV期4人（9.5%）であった（Table 1）。

治療効果判定

イレッサの治療効果判定はRECIST (Response Evaluation Criteria In Solid Tumors) の基準にしたがって、complete response (CR)、partial response (PR)、stable disease (SD)、progressive disease (PD)、not evaluable (NE) に分類した。

免疫組織化学染色法

免疫組織化学染色法は特別な言及がない限り室温内で一連の手順が行われた。パラフィン固定標本ブロックから4-μmに薄切した検体組織をスライドガラスに貼り付け、60°Cで30分間乾燥させた。100%キシレンで脱パラフィン後エタノールにて脱水し、その後0.3%過酸化水素水に30分間浸し内因子ペルオキシターゼをブロッキングした。抗原賦活化処理のため、クエン酸緩衝液（10 mM、pH 6.0）に浸しオートクレーブにかけ（120°C 10分間）、冷却後にPBSにて洗浄した。50倍希釈した一次抗体（EGFR L858R Rabbit mAb : Cell Signaling Technology, Danvers, MA, USA）100 μlで検体組織を覆い、4°Cで一晩反応させた。

スライドをPBSで洗浄後、200倍希釈した二次抗体（Biotinylated Anti-Rabbit IgG : Vector Laboratories, Burlingame, CA, USA）200 μlで覆い、60分間反応させた。さらにPBSで洗浄し、2%NSS (Normal Swine Serum : Vector Laboratories, Burlingame, CA, USA) にて200倍希釈したアビジン-ビオチン標識酵素複合体（VECTASTAIN ABC kit : Vector Laboratories, Burlingame, CA, USA）200 μlで覆い、30分間反応させた。

最後に切片をPBSで洗い、0.02% 3, 3'-ジアミノベンジジン、Trizma Base、Trizma Hydrochloride、および0.006%過酸化水素水の混合液に入れ、15分間反応させ、その後ヘマトキシリンで染色し脱水して封入した。

免疫染色の判定は東京医科大学外科学第一講座が症例の標本を盲目判定にて評価した。

EGFR 突然変異の分析

ゲノムDNAは切除標本の腫瘍細胞から採取された。我々は以下のプライマーリストにしたがって、EGFRのチロシンキナーゼ領域のexon 18-21のシークエンスを行った。4.5 μlの反応促進物と0.125 μlのTaKaRa Ex Taq HS (Takara Bio, Otsu-shi, Shiga, Japan)、100 ngのDNA検体と0.5 μlのそれぞれのプライマー（SRL, Tachikawa-shi, Tokyo, Japan）を使用し、72°C、30サイクルにて反応させた。PCR反応物は、the 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) にて解析した。

プライマーは以下のものを使用した。

Exon 18 (Fw) 5'-CCTTGTCTCTGTGTTCTTGT-3'
Exon 18 (Re) 5'-CTGCGGCCCAGCCCAGAGGC-3'
Exon 19 (Fw) 5'-CATGTGGCACCATCTCACA-3'
Exon 19 (Re) 5'-CCACACAGCAAAGCAGAAAC-3'
Exon 21 (Fw) 5'-CAGGGTCTTCTCTGTTTCAG-3'
Exon 21 (Re) 5'-TAAAGCCACCTCCTTACTTT-3'

結　果

direct sequence法による*EGFR*遺伝子変異は、非小細胞肺癌42例中15例に認められた。15例の遺伝子変異は、Exon 18のpoint mutation (G719S) が1例、Exon 19のdeletion (E746-A750del) が8例、Exon 21のpoint mutation (L858R) が6例であった。

EGFR L858R抗体を用いた免疫染色にて、42例中7例（16.6%）が染色された（Fig. 1、Fig. 2）。陽性症例7例の中で、3例が男性、4例が女性であった。また、2例が喫煙者で5例が非喫煙者であった。組織型は全