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

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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-α*) 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-α* 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

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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.

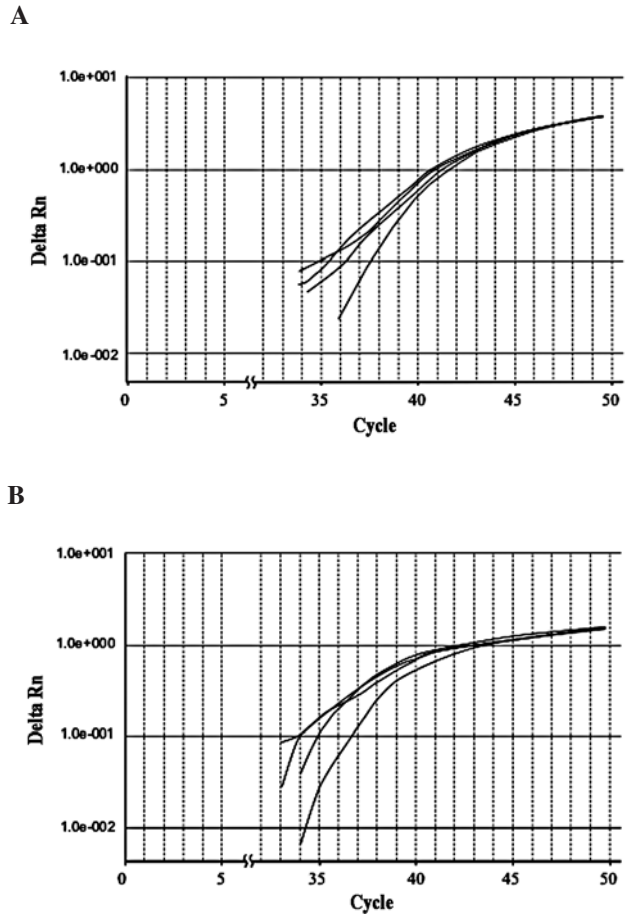

Figure 1. Amplification plots for the *p16* real-time PCR analysis of blood DNA samples (A). X-axis, the cycle number of quantitative PCR; Y-axis, Delta Rn, fluorescence intensity over the background. Amplification plots for the *ESR1* real-time PCR analysis of blood DNA samples (B). X-axis, the cycle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

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