Usefulness of detection of *p16* promoter methylation of tumor suppressor genes in blood DNA from non–small cell lung cancer patients using real–time PCR

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Abstract

**Introduction**

The application of molecular markers specific for lung cancer offers new possibilities for early detection. Recent evidence suggests that tumor cells may release DNA into the circulation, and is enriched in the serum and blood. We examined peripheral blood in this study. *p16* tumor suppressor gene can be inactivated by promoter lesion methylation in many tumor types, including lung cancer. We examined whether *p16* aberrant methylation might also be found in the blood of patients with non–small cell lung cancer (NSCLC) using real–time PCR.

**Materials and Methods**

The specimens obtained from healthy volunteers and benign disease patients and patients with lung cancer. Peripheral blood samples were obtained from 95 patients of NSCLC, including 66 with adenocarcinoma, 23 with squamous cell carcinoma, 6 with large cell carcinoma and 30 controls consisting of normal subjects or benign disease patients.

**Results**

*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. The difference in the ratios between the NSCLC patients and controls was statistically significant (*p*<0.01). 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%. In cases with Stage I lung cancer, there was a statistically significant difference in the methylation positive rate *p16* and the CEA positive rate (*p*<0.05). We examined *p16* methylation ratios in 26 lung cancer patients before and 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 less than the median preoperative methylation level.

**Conclusion**

Detection of aberrant *p16* promoter methylation in blood samples using real–time PCR appears to be useful in the diagnosis of lung cancer, especially early lung cancer detection and follow up after curative surgery 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 countries. 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.

Recent evidence suggests that tumor cells may release DNA into the circulation, causing the serum to become enriched with the DNA. In lung cancer, promoter hypermethylation has been detected in blood, bronchial lavage, sputum and pleural fluid of lung cancer patients. Thus, DNA methylation in blood might represent one of field defect of change. We tested p16 promoter hypermethylation in blood by real-time PCR, which is more sensitive than methylation-specific PCR. p16 is a well-known D-type cyclin-dependent kinase (cdk) inhibitor gene that interferes with the interaction of cdk4 with cyclin D1, 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 in the blood of lung cancer patients and patients with benign diseases.

Materials and Methods

1. Collection samples

We examined whether aberrant p16 methylation might be found in the blood of non-small cell lung cancer (NSCLC) patients by real-time PCR in 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 NSCLC, 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 were obtained from 16 normal subjects 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 patients of Stage I cases, 8 Stage II cancer, 23 Stage III cancer, and 12 Stage IV cancer. Patients with multiple primary cancers were excluded. The diagnosis in all the patients was made by the pathologists at our hospital.

2. DNA extraction, bisulfite treatment and methylation-specific real-time PCR

Peripheral blood samples were collected to investigate the methylation status of blood DNA. Theuffy coat was isolated after centrifugation at 3,000 rpm for 10 minutes and blood DNA was extracted using phenol/chloroform extraction from blood (EDTA • 2Na). Bisulfite conversion of DNA samples was carried out and was based on the principle. The bisulfited DNA, p16 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 TaqMan analysis were used to represent the relative level of methylated p16 DNA in a given sample. The sequences of the primers and probes used to amplify and detect methylated p16 and MYOD were as follows, and have been described previously. p16: 5'-CGCAACCGCC-GAAGC-3' (forward primer), 6'FAM-5'-CGCGATCCGGCCCACCTT-TAMRA-3' (probe) and 5'-TTTTTCGGTTTAGATCGGAGGAGA-3' (reverse primer). MYOD: 5'-TGATTTAGATTGG-GTTTAGAGAAGGA-3' (forward primer), 6'FAM-5'-TCCCTTCTATCCCTAAATCCAAC-CTAAATACCTCC-TAMRA (probe) and 5'-CCAACCTCAATCCCCCTCTCTAT-3' (reverse primer).

Real-time PCR is based on continuous optical monitoring of a progressive fluorogenic PCR (Fig. 1). We used Prism 7000 (Applied Biosystems Inc.) for this study. The methylation ratio was defined as the ratios of the fluorescence emission intensity values for the p16 PCR products to those of the MYOD PCR products obtained by TaqMan analysis, multiplied by 100,000.

3. Statistical analysis

The correlation between the methylation ratios p16 was evaluated by χ² test and Welch’s t-test using the SPSS. The relationship between p16 promoter methylation in blood and the clinicopathological characteristics of the patients was assessed by χ² test and Welch’s t-test using the same software. Statistical significance was assumed to be indicated by p<0.05.

Results

1. p16 methylation in blood of controls, benign disease patients 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 MYOD. 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. 2). The difference in the ratios between the NSCLC patients and controls was statistically significant (p<0.01). We determined the cutoff value
Fig. 1 Amplification plots for the p16 real-time PCR analysis of blood DNA samples. X axis, the cycle number of quantitative PCR; Y axis, Delta Rn, fluorescence intensity over the background.

Table 1  

| p16 methylation positive rates in controls and NSCLC patients |
|-----------------|-----------------|-----------------|
| Sensitivity     | 26.3% (25/95)   |
| Specificity     | 96.7% (29/30)   |
| Accuracy        | 43.2% (54/125)  |
| Positive predictive value | 96.2% (25/26)   |
| p               | p < 0.01        |

P: controls vs. NSCLC patients: t test

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positive predictive value was 96.2% (Table 1).

2. \textbf{Associations with clinicopathological variables of the p16 methylation positive rates}

We analyzed the correlations between the DNA methylation positive rates of DNA in blood and various clinicopathological variables. In each group, controls and NSCLC patients, there were no statistically significant correlations between the p16 methylation rates and gender, age, smoking history, histological type or clinical stage (Table 2).
3. Comparison between the p16 methylation positive rates and positive rates of serum protein tumor markers

In this study, the p16 methylation positive rates were compared with the positive rates of the serum protein tumor markers. The commonly evaluated tumor markers in the clinical situation are CEA, CA19-9, SLX, SCC and CYFRA. Since CEA is the most commonly evaluated serum protein tumor marker in patients of NSCLC, we examined the methylation positive rates and the CEA positive rate. In cases with Stage I lung cancer, there was a statistically significant difference in the p16 methylation positive rate and the CEA positive rate (p<0.05) (Table 3).

4. The change of methylation ratio before and after curative operation

We examined p16 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, 18 cases were negative both before and after the operation. In 4 cases (Group A), methylation ratios increased after the operation and in 3 cases (Group B), it decreased. The methylation ratio of the cases of chronic obstructive pulmonary disease (COPD) in Group C got worse, increased after curative surgery. Two of the four cases in which it increased, including one with p1Stage IA, were found to have recurrence. The other p1Stage 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 less than the median preoperative methylation level (Fig. 3).
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. These aberrant hypermethylation 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. 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.

We examined peripheral blood 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. 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 changes.

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 methylation ratios between NSCLC patients and controls (p<0.01), suggesting that the test may be potentially useful for mass screening of lung cancer and follow-up of lung cancer patients. 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. However our results could not demonstrate statistically significant differences between p16 promoter methylation positive rates and any clinicopathological variables.

DNA methylation can be 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 p16 were higher than the positive rates of CEA or any other tumor marker. 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 found to have tuberculosis, was a 78-year-old man who was a heavy smoker (58 pack-years). Old age and smoking have been reported as strong risk factors for lung cancer and to bear strong correlations with the DNA methylation change. 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. 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.

In conclusion, detection of aberrant p16 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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References

Real-time PCR を用いた非小細胞肺癌症例の血中 DNA における
p16 プロモーターメチレーション検出の有用性

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目的：肺癌の死亡者数は年々増加傾向にあり、2002 年には年間 5.6 万人に達し、すべての悪性疾患の中で第 1 位となってい
る。このような中、肺癌の早期発見につながるに実用的な分子生物学的マーカーが求められている。最近のエビデンス
では血漿や血液に release DNA が多く含まれていることが示唆されている。肺癌を含む多くの腫瘍におけるプロモーター
領域のメチル化は、p16 病抑制遺伝子を不活化させる。我々は、real-time PCR を用いて非小細胞肺癌症例の血液中に p16
変異メチレーションが認められるか否かを実験した。試料は健常者、良性疾患症例および非小細胞肺癌症例より集められ
た。95 例の非細胞肺癌症例のうち 66 例は肺癌、23 例は扁平上皮癌、6 例は大細胞癌であり、30 例のコントロール群は 16
例の健常者と 14 症例の良性疾患であった。

結果：p16 メチレーション値の中央値がコントロール群では 1.3±0.9 (mean±SE) で、非小細胞肺癌症例群では 18.6±5.1
であり、統計学的に有意差を認めた (p<0.01)。またこの p16 メチレーション陽性率を用いた肺癌診断の感度は 26.3%、特
異度は 96.7%、正確度は 43.2%、陽性的中率は 96.2% であった。Stage I の肺癌症例に関して、p16 のメチレーション陽性率
は CEA よりも高く、統計学的に有意差を認めた (p<0.05)。また、26 症例について術前・後のメチレーション値を測定
した。術後再発を認めた症例および重複症例症例のメチレーション値の平均は術前後で 17.6 倍上昇しており、術後再発の
ない症例群では 3 分の 1 に低下していた。結論：Real-time PCR を用いた血液中の変異 p16 メチレーションの検出は肺癌
の診断、早期発見および術後の Follow up に有用であると考えられた。

キーワード 非小細胞肺癌、p16、メチレーション、real-time PCR