RESEARCH COMMUNICATION

Aberrant Promoter Methylation Profile in Pleural Fluid DNA and Clinicopathological Factors in Patients with Non–small Cell Lung Cancer

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Abstract

The aim of this study was to investigate the prognostic value of hypermethylation of tumor suppressor genes in patients with non-small cell lung cancer (NSCLC). In samples from 34 lung patients with malignant pleural effusions, we used a methylation-specific polymerase chain reaction to detect aberrant hypermethylation of the promoters of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT), p16INK4a, ras association domain family 1A (RASSF1A), apoptosis-related genes, death-associated protein kinase (DAPK), and retinoic acid receptor β (RARβ). There is no association between methylation status of five tumor suppressor genes including MGMT, p16INK4a, RASSF1A, DAPK and RARβ in pleural fluid DNA and clinicopathological parameters including clinical outcome. Aberrant promoter methylation of tumor suppressor genes in pleural fluid DNA could not be a valuable prognostic marker of NSCLC patients with malignant pleural effusion.

Key words: MGMT - p16INK4a - RASSF1A - DAPK - RARβ - malignant pleural effusion - survival

Introduction

Lung cancer is the leading cause of cancer death in men and women in Japan, with a 5-year survival rate of only 15%, a statistic that has changed very little over the past two decade. This dismal survival could be improved through earlier detection or through identification of prognostic markers, which could identify subsets of patients with worse prognosis who might benefit from a more aggressive treatment strategy. Epigenetic changes such as hypermethylation increasingly appear to play a role in carcinogenesis. DNA methylation is one form of epigenetic variability in mammalian cells (Baylin et al., 1998; Baylin et al., 2001; Merlo et al., 1995). As aberrant hypermethylation in CpG-rich promoter regions of many tumor suppressor genes interferes with gene transcription, hypermethylation can contribute to development and progression of various cancers by abolishing tumor suppressor gene function (Baylin et al., 1998; Baylin et al., 2001; Merlo et al., 1995).

A number of studies have demonstrated frequent methylation in lung cancer cells of several tumor suppressor genes including the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) (Zochbauer-Muller et al., 2001; Esteller et al., 2001), p16INK4a (Kashiwabara et al., 1998; Kersting et al., 2000), ras association domain family 1A (RASSF1A) (Burbee et al., 2001; Dammann et al., 2001), apoptosis-associated genes such as death-associated protein kinase (DAPK) (Tang et al., 2000), and retinoic acid receptor β (RARβ) (Virmani et al., 2000); in contrast, methylation of these genes was rare in nonmalignant lung tissue (Esteller et al., 2001). We previously reported that identification of promoter methylation of these five tumor suppressor genes in pleural fluid DNA could be useful for differential diagnosis of malignant pleural effusion (Katayama et al., 2007).

In the present study, we attempted to analyze the correlation between these methylation status in pleural fluid DNA and clinicopathological parameters including clinical outcome.

Patients and Methods

Study population

Subjects were 34 NSCLC patients with pleural effusions who were admitted to the Sanyo National Hospital between August 2000 and August 2004. Patient characteristics are summarized in Table 1. The study group included 24 men and 10 women, and had a median age of 70 years. 32 had adenocarcinoma and 1 had squamous cell carcinoma. One had unclassified NSCLC.

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Clinical signs and symptoms, laboratory data, and radiologic results were recorded. Malignant pleural effusions were diagnosed by either pleural fluid cytologic findings or malignant cells identified in a pleural biopsy specimen. Patients with potentially precancerous cytologic finding such as dysplasia or metaplasia were excluded from methylation analysis.

Sample collection and DNA extraction
Each sample of pleural fluid was collected in a syringe during thoracentesis performed after written informed consent. Supernatant was isolated from the pleural fluid by centrifugation at 2000 rpm for 10 min, and then stored at −80°C until use. DNA from 1 ml of pleural effusion was extracted using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Methylation-specific polymerase chain reaction
Sample DNA was treated with sodium bisulfite using a CpGenome DNA Modification Kit (Intergen, NY) according to the manufacturer’s instructions. This reagent converts unmethylated cytosine but not 5-methylcytosine to uracil. All bisulfite-modified DNA was resuspended in TE buffer (10 mM Tris-HCl / 0.1 mM EDTA, pH 7.5) and either analyzed immediately or stored at −20°C until subsequent methylation-specific polymerase chain reaction (MSP). Target sequences were amplified by polymerase chain reaction (PCR) using specific primers to yield fragments in which uracil and thymine residues are amplified as thymine and 5-methylcytosines are amplified as cytosine. Primers specific for either methylated or unmethylated sequences in the MGMT, p16INK4a, RASSF1A, DAPK, and RARß promoter regions were reported previously. The PCR mixture contained 10 µl of PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2], deoxyribonucleotide triphosphates (each at 2.5 mM), 0.5-µM concentrations of each primer, 0.5 units of Taq DNA Polymerase Hot Start Version (Takara Bio, Shiga, Japan), and 1.5 µl of bisulfite-modified DNA in a final volume of 20 µl. Initial denaturation at 95°C for 1 min was followed by 50 cycles of a denaturation step at 95°C for 30 sec, a 30-sec annealing step at an annealing temperature appropriate for the individual sequence, and an extension step at 72°C for 30 sec: a final extension step at 95°C for 10 min then was added. After amplification, each PCR product was electrophoresed through a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. All tests were duplicated to confirm the results.

Statistical analysis
The methylation status of five genes (MGMT, p16INK4a, RASSF1A, DAPK and RARß) in each patient was scored as the total number of methylated genes. Chi-squared or Fisher’s exact tests were applied to examine distributions for categorical variables. Overall survival in relation to methylation status was evaluated by Kaplan-Meier survival curves and log-rank tests. Statistical significance was defined by a p value less than 0.05.

Results
The relationship between methylation status and clinicopathological factors is examined. As shown in Table 2, no statistically significant correlation was observed between methylation status each five genes and clinicopathological factors including gender, age, clinical stage, LDH, CEA, or location in NSCLC patients with pleural effusions.

Next we examined the relationship between methylation status and survival in the patients with malignant effusion. As shown in Figure 1A, survival of the patients with at least one methylation is similar to that of the patients without methylation (Median survival time [MST] of 5.0 month vs. 4.7 month; p = 0.93). In addition, there are no impact of MGMT RASSF1A, DAPK and RARß methylation status on survival of NSCLC patients.
Survival of the patients with methylated p16INK4a tends to be shorter survival than that of patients without it, but it was not statistically significant (MST of 5.6 months vs. 3.7 months; p = 0.28).

**Discussion**

In the present study we found that there is no association between methylation status of five tumor suppressor genes including MGMT, p16INK4a, RASSF1A, DAPK and RARβ in pleural fluid DNA and clinicopathological parameters including clinical outcome. Using newer molecular biology technologies, many studies for genetic abnormality of lung cancers including p53 or K-ras has been extensively investigated in cancer pathogenesis and for their effects on clinical outcome (Fukuyama et al., 1997; Mitsudomi et al., 2000). It is now well established that epigenetic changes play an important role in cancer development and the distinct profile and varying levels of tumor suppressor genes methylations among different cancers have shown promise as a tool for the detection and diagnosis of cancer as we previously reported (Katayama et al., 2007; Fujiwara et al., 2005). However, the clinical significance, especially impact on survival, of the methylation of tumor suppressor genes has been undetermined. Gu et al. have carefully reviewed the literature on the association between tumor suppressor genes methylation and survival in patients with NSCLC (Gu et al., 2006). Most studies used MSP and investigated a single or a handful of genes. All studies reported either negative results or a single significant association between a specific tumor suppressor genes methylation and survival. With the exception of p16INK4a and RASSF1A, the other significant results have not been reproduced in a second independent study.

In our analysis, only p16INK4a methylation of the five genes tended to be correlated with poor prognosis in patients with NSCLC, but it was not statistically significant. p16INK4a promoter methylation is a widespread epigenetic alteration and plays a significant role inactivating p16INK4a in many tumor types. In particular, p16INK4a methylation is an early event in lung carcinogenesis, occurring frequently in all stages of NSCLC, even in cancer-free individuals exposed to tobacco carcinogens. In addition, our results are consistent with other reports that loss of p16INK4a expression was correlated with poor prognosis (Kratzke et al., 1996; Kawabuchi et al., 1999; Niklinski et al., 2001; Kim et al., 2001; Toyooka et al., 2004).

In conclusion, we found that no association between aberrant promoter methylation of these tumor suppressor genes in pleural fluid DNA may not be a valuable marker to aid in the prediction of consequent clinical outcome. Further studies including a large number of patients are warranted to confirm these results. Careful assessment of the effect of each methylated gene on survival is needed.

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**Figure 1. Relation between Methylation Status and Overall Survival in NSCLC Patients.** (Kaplan-Meier Analysis). (A) Survival curves by all methylation status, (B) Survival curves by methylation status of p16INK4a, (C) Survival curves by methylation status of RASSF1A, (D) Survival curves by methylation status of RARβ, (E) Survival curves by methylation status of DAPK. The resulting curves were compared using log-rank test.
References


