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Identification of epigenetic aberrant promoter methylation of *RASSF1A* in serum DNA and its clinicopathological significance in lung cancer

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Summary *RASSF1A* is a novel putative tumor suppressor gene located in 3p21.3 region. The most common inactivation mechanism of *RASSF1A* is promoter hypermethylation, which is observed in multiple solid tumors including lung cancer. In the present study, we identified the methylation status of *RASSF1A* in lung cancer sera using methylation-specific PCR and analyzed its clinicopathological significance. Hypermethylation of *RASSF1A* was detected in 27 of 80 (33.8%) cancer patients but no benign pulmonary disease patients or healthy donors ($P < 0.001$). *RASSF1A* hypermethylation was preferentially observed in small cell lung cancer ($P = 0.042$), while no statistical difference was found among methylation frequencies of different subtypes of non-small cell lung cancer. *RASSF1A* methylation status was associated with differentiation ($P = 0.009$) and stage ($P = 0.013$), but not with gender, age or treatment. These findings suggest that serum *RASSF1A* hypermethylation is a promising molecular biomarker for lung cancer diagnosis.

1. Introduction

Lung cancer is the most frequent cause of cancer-related death in the world, and causes over 1 million deaths worldwide each year [1]. Despite new diagnostic techniques,

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most lung cancers are detected late, with an estimated 5-year survival rate remaining below 18 and 7% in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), respectively [2]. Thus, the development of efficient diagnostic methods to enable the early detection is clearly imperative. The application of molecular biomarkers represents a new approach to improve lung cancer diagnostics [3]. Recent studies have suggested the use of aberrant CpG island methylation in the promoter region of tumor suppressor genes (TSGs) as a molecular marker system for early detection of lung cancer.

Ras association domain family 1 A (*RASSF1A*) gene is a newly identified candidate tumor suppressor gene located in 3p21.3 region [4]. The most common inactivation mechanism of *RASSF1A* is promoter hypermethylation [4–6]. Recent studies on resected tumors, cell lines, sputum and bronchial aspirates have reported hypermethylation of the *RASSF1A* promoter in up to 60% of NSCLC and 100% of SCLC [4,5,7,8]. These findings give rise to the hypothesis that *RASSF1A* hypermethylation may be a promising molecular biomarker for lung cancer diagnosis.

It has been early demonstrated that cancer patients have increased levels of free DNA in their sera, which is released from cancer cells [9,10]. In the present study, we attempted to identify hypermethylated *RASSF1A* gene in serum DNA of lung cancer patients by methylation-specific polymerase chain reaction (methylation-specific PCR, MSP), and to analyze the correlation between aberrant *RASSF1A* methylation and clinicopathological parameters.

Table 1 Characteristics of the adopted 80 patients with primary lung cancer

Characteristics	No.
Gender	
Male	49
Female	31
Age (years)	
<60	35
⩾60	45
Histological type	
Small cell lung cancer	5
Non-small cell lung cancer	75
Adenocarcinoma	40
Squamous cell carcinoma	26
Adenosquamous carcinoma	9
Differentiation	
Well	17
Moderately	34
Poorly/undifferentiated	29
Stage	
I	9
II	18
III	29
IV	24

2. Materials and methods

2.1. Study population

Eighty patients who were diagnosed as primary lung cancer in Departments of Cardiothoracic Surgery, Respiratory Diseases and Medical Oncology of Jinling Hospital from June 2005 to June 2006 were included in this study. All diagnoses were based on pathological and/or cytological evidence. The age of the patients ranges from 36 to 79, with a median 62 and the average 62.3. Histological classification was conducted according to ''Histological typing of lung and pleural tumors: third edition'' of the World Health Organization (WHO) in 1999, and the tumor stage was determined according to the TNM staging guideline suggested by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC) in 2003. Detailed information of the patients was summarized in Table 1. Thirty-five patients with benign pulmonary diseases (pneumonia, tuberculosis, etc., data not shown) and 15 healthy donors were adopted as control. Ethical approval was obtained from the hospital and fully informed consent from all patients prior to sample collection.

2.2. Sample collection

Five milliliter peripheral blood was collected from each patient described above on the next day after admission. At this time spot, a pretreatment (surgery, chemotherapy or radiotherapy), if existed, was within 4 weeks, and the new treatment had not been conducted yet. The blood samples were let stand in 4 °C refrigerator with the presence of clot activator for 2 h, and sera were isolated subsequently after centrifugation at 3000 rpm for 10 min. Fifteen serum samples of healthy donors were obtained from Blood Center of Jinling Hospital as normal control. All serum samples were stored at −80 °C until use.

2.3. DNA extraction and bisulfite treatment

Serum DNA was extracted using QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's instructions, and stored at −80 °C until use. Around 300 ng DNA was extracted from 2.5 mL serum in cancer patients. Extracted DNA was modified as previously described [11] with slight change, converting all unmethylated cytosines to uracils. Briefly, extracted sample DNA, together with 1 μg of salmon sperm DNA (Sigma, St. Louis, MO, USA) added as carrier, in a volume of 50 μL was denatured by NaOH (final concentration, 0.3 mol/L) for 15 min at 40 °C. Thirty microliter of 10 mmol/L hydroquinone (Sigma) and 520 μL of 3 mol/L sodium bisulfite (Sigma) at pH 5.0, both freshly prepared, were added and mixed, and samples were incubated under mineral oil at 55 °C for 14 h. Modified DNA was purified using Wizard DNA Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's protocol. Modification was completed by NaOH (final concentration, 0.3 mol/L) treatment for 10 min at room temperature, followed by ethanol precipitation. DNA was resuspended in 30 μL sterile deionized water and used immediately or stored at −80 °C.