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Original Article

A RASSF1A 133 single-nucleotide polymorphism is associated with increased susceptibility and unfavorable prognosis in non-small cell lung cancer

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Abstract: The Ras-association domain family 1 gene (RASSF1) is involved in various carcinogenetic pathways. The aim of the present study was to investigate the relationship between a single-nucleotide polymorphism (SNP) at codon 133 of the RASSF1 gene and non-small cell lung cancer (NSCLC) susceptibility, epidermal growth factor receptor (EGFR) mutation status and prognosis. From January 2011 to December 2012, 225 pathologically diagnosed NSCLC patients and 225 healthy controls from Shanghai Pulmonary Hospital were enrolled. Clinicopathological features, including sex, age, smoking status, pathologic type, lymphatic metastasis status, EGFR mutation status and TNM stage, were collected. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to explore polymorphisms in the RASSF1 gene. Compared to healthy controls, the risk of having the Ala/Ser and Ala/Ser+Ser/Ser genotypes was increased in NSCLC patients (adjusted P=0.028 and 0.013, respectively). The increased frequency of individuals with the Ala/Ser genotype was associated with being male (adjusted OR=3.84, 95% CI 1.407-10.482) and smoking status (adjusted OR=11.967, 95% CI 2.195-17.475); however, no significant associations with EGFR mutations were observed. According to the Kaplan-Meier survival curve (log-rank, χ^2=11.503, P<0.01) and multivariate Cox regression model, patients with Ala/Ser or Ser/Ser genotypes (HR=4.028, 95% CI 2.520-6.439, P<0.001) have an unfavorable prognosis. These results suggest that the RASSF1 Ala133Ser SNP contributes to non-small cell lung cancer susceptibility and poor survival. Significant associations of the RASSF1 Ala133Ser SNP with male and smoking status were observed, but no significant associations between the RASSF1 SNP and EGFR mutations were observed.

Keywords: RASSF1A SNP, prognosis, tumor-suppressor gene (TSG), NSCLC, EGFR mutation

Introduction

Lung cancer is considered to be the leading cause of cancer-related deaths worldwide. The frequency of lung cancer diagnosis is the highest among common cancers in males, and it ranks fourth in females [1]. The pathogenesis of lung cancer involves interactions between the environment and genetic factors, such as the allelic loss of the short arm of a chromosome, which occurs early in lung cancer. The RAS-association domain family, which includes 10 members (RASSF1-10), plays an important role in apoptosis, cell cycle regulation and microtubule stability [2]. RASSF1, which is located at 3p21.3, was first cloned by Dammann in 2000, and it is often found to be unstable in lung cancer [3]. RASSF1 has eight transcripts (A-H) [4]. RASSF1A, one of the most extensively studied transcripts, is predicted to encode a 39-kDa polypeptide. RASSF1A contains a Ras-association domain in the carboxy (C)-terminal region and a diacylglycerol (DAG)-binding domain in the amino (N)-terminal region [5]. The N- and C-terminal regions of RASSF1A are necessary for the microtubule localization of RASSF1A [6]. RASSF1A has been associated with cellular migration, apoptosis, microtubule stability and nuclear factor kappa B (NFκB)-related inflammation [4, 7-9]. RASSF1A is thought to have a key role in DNA damage control, as supported by evidence from ataxia telangiectasia mutated (ATM) phosphorylation. An ATM phosphorylation site on RASSF1A that

contains an N-terminal protein kinase C conserved region 1 (C1) domain has been shown to meditate a connection between RASSF1A and death receptor complexes [6, 7]. This ATM phosphorylation site can regulate a RASSF1-dependent DNA damage response [10]. RASSF1A is missing in all small cell lung cancer (SCLC) cell lines, and a missense mutation has also been found in some primary lung tumors [3]. Hypermethylation of the RASSF1A promoter is one of the most common epigenetic inactivations in human cancers, including lung cancer [11, 12]. RASSF1A is a known tumor suppressor gene (TSG) [3, 12].

The epigenetic activation of RASSF1A by the hypermethylation of CpG islands within its promoter and first exon is frequent [11]. A single-nucleotide polymorphism (SNP) has been identified that causes an alanine residue (Ala: GCT) to be substituted with a serine residue (Ser: TCT) [13]. Previous studies have demonstrated that polymorphisms in the RASSF1A gene increase lung cancer susceptibility [14]. In addition to lung cancer, the RASSF1A 133 polymorphism has also been reported in breast cancer, hepatocellular carcinoma, esophageal cancer and gastric cardia cancer [11, 15, 16]. The hypermethylation of RASSF1A predicts a poor recurrence-free survival (RFS) in node-negative stage I-II NSCLC patients [17], and the RASSF1A 133 Ala/Ser polymorphism is associated with poor survival in cancers such as hepatocellular cancer and soft tissue carcinoma [18, 19]. In the past decade, the epidermal growth factor receptor (EGFR), which principally activates the RAS/RAF/MAPK pathway, has been considered as the first mutation-based tumorigenic driver of lung cancer that is treatable. Treatment with EGFR tyrosine kinase inhibitors (TKIs) has become the standard care for lung cancer patients with EGFR-mutations [20]. Low expression of RASSF3 is significantly associated with wild-type EGFR status [21]. However, the association between RASSF1A polymorphisms and EGFR mutations has not yet been investigated. The aim of this study was to detect a possible relationship between RASSF1A polymorphisms and EGFR mutation status in lung cancer.

Materials and methods

Subjects

All participants in this study were recruited at Shanghai Pulmonary Hospital between January 2011 and December 2012. Two hundred twenty-five cases were pathologically confirmed lung cancer patients, and another 225 healthy controls with no recorded tumor complication who visited the hospital for routine health checkups were enrolled. All of the recruited cancer patients consented to surgery and were not treated with radiation, chemical or immune therapy before surgery. All the control subjects were age- (±5 years) and sex-matched to the cases. All participants gave written informed consent before this study. Approval for this research was obtained from the institutional ethics committee of Shanghai Pulmonary Hospital. The detailed clinicopathological features of patients were collected, including sex, age, smoking status, pathologic type, lymphatic metastasis status, EGFR mutation status and TNM stage. Nonsmokers were defined as those who smoked <1 cigarette/day for <1 year; otherwise, participants were recorded as smokers.

DNA extraction and detection of EGFR mutations

Cancer patient genomic DNA was isolated from formalin-fixed, paraffin-embedded histologic specimens according to the standard protocol for the Omega FFPE DNA kit (Omega, USA). Control genomic DNA was extracted from venous blood samples that were obtained from the control group (Tiangen, Dalian, China). The venous blood was stored at -80°C until DNA extraction. The concentration of genomic DNA was determined spectrophotometrically. The EGFR mutation status was evaluated by the Department of Pathology, Shanghai Pulmonary Hospital, and the mutation was detected using the human EGFR Gene Mutations Fluorescence Polymerase Chain Reaction Diagnostic Kit (AmoyDx, Xiamen, China). The Ct values used to determine whether a sample was positive or negative were based on extensive validation, as described previously [22].

Genetic analysis

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach was used to detect the RASSF1A gene. We used Primer 5.0 software (manufactured by McGraw-Hill) to design sequence-specific primers (forward: 5'-CAA AGG CCT GCA GTG CGC GCA-3' and reverse: 5'-GAT CAA CAG CAA CCT CTT CAT-3'). PCR was performed in a 20-μl

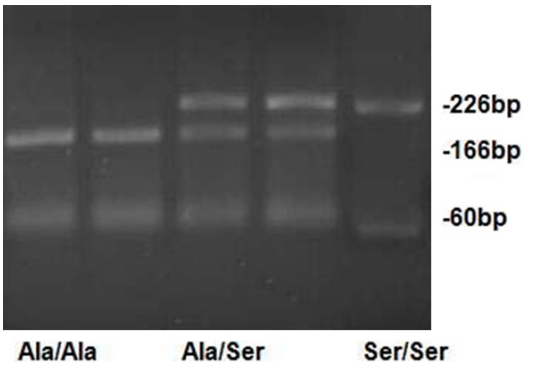

Figure 1. Analysis of PCR-RFLP for the RASSF1 Ala-133Ser SNP.

reaction volume using a Premix Taq PCR kit (Takara, Japan) with the following components: 20 ng of genomic DNA, 10 μl of premix buffer, 0.5 μl each of forward and reverse primer and pure water to bring the total volume to 20 μl. The conditions for amplification were as follows: initial incubation for 5 min at 94°C; 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min; and a final step at 72°C for 5 min. The PCR products were digested with Alu I (Takara, Japan) overnight at 37°C and then subjected to electrophoresis on a 2% agarose gel. The Ala/Ala genotype was determined by the appearance of two DNA bands of 166 and 60 bp. If a SNP was present in RASSF1A exon 3 Ala133Ser, the Ser/Ser genotype was represented by a primary band at 226 bp; the heterozygous genotype displayed three bands at 226, 166, and 60 bp (**Figure 1**). The genotype was analyzed using a Gel Image Analysis System (Bio-Rad, USA).

Statistical analysis

Hardy-Weinberg analysis between cancer patients and healthy controls was performed using the χ^2 test. A logistic regression model was used to calculate the odds ratio (OR) for association with the Ala/Ser and Ser/Ser genotypes compared to those with the Ala/Ala genotype in this case-control study. ORs were expressed with a 95% confidence interval (CI) and a *P*-value. Kaplan-Meier and multivariate Cox regression analyses were used to detect an association between the presence of a RASSF1A SNP and the NSCLC prognosis. All *P*-values were two-sided, and the statistical significance threshold was $P<0.05$. Age and sex were the adjusted factors for the multivariate logistic regression analysis. SPSS 19.0 (IBM, Armonk, NY, USA) was used for statistical analyses.

Results

RASSF1A polymorphism is a risk factor for lung cancer

The frequencies of the RASSF1A genotype distributions in NSCLC patients and controls are listed in **Table 1**. In both NSCLC patients and controls, the distribution of genotypes conformed to Hardy-Weinberg equilibrium. The percentages of Ala/Ala, Ala/Ser and Ser/Ser genotypes in NSCLC patients were 82.2%, 16%, and 1.8%, respectively, while they were 90.7%, 8.9%, and 0.4%, respectively, in healthy controls. On the basis of *P*-value and adjusted OR, the heterozygous Ala/Ser (adjusted OR=1.96; 95% CI 1.076-3.672, $P=0.028$) and Ala/Ser+Ser/Ser (adjusted OR=2.098; 95% CI 1.172-3.755, $P=0.013$) genotypes demonstrated an increased risk for NSCLC relative to the Ala/Ala genotype.

Association between heterozygous RASSF1A genotypes and clinicopathological features

The results of association analyses between lung cancer and clinical parameters such as age, sex, smoking status, pathologic type, lymphatic metastasis status, TNM stage and EGFR mutation status are exhibited in **Table 2**. The associations between lung cancer and the presence of the Ala/Ser and Ala/Ala genotypes were also analyzed. The increased frequency of individuals with the Ala/Ser genotype was associated with male sex (adjusted OR=3.84, 95% CI 1.407-10.482) and smoking status (adjusted OR=11.967, 95% CI 2.195-17.475). After taking age, sex and smoking status into consideration, the association between RASSF1A SNPs and lymphatic metastasis status disappeared (the *P*-value changed from 0.035 to 0.123).

RASSF1A SNP and EGFR mutation status are not significantly associated

We found no association between RASSF1A SNPs and EGFR mutations, even after taking age, sex, and smoking status into consideration. Detailed information concerning the RASSF1A SNP and EGFR mutation status is listed in **Table 3**.

Table 1. Distributions of RASSF1 Ala133Ser SNP genotypes in lung cancer patients and healthy controls

Genotypes	Controls	Lung cancer patients	*P* value	Crude OR (95% CI)	Adjusted *P* value	Adjusted OR (95% CI)[a]
Ala/Ala	204	185	-	1	-	1
Ala/Ser	20	36	0.021	1.985 (1.109, 3.551)	0.028	1.96 (1.076, 3.672)
Ser/Ser	1	4	0.186	4.411 (0.489, 8.821)	0.213	4.863 (0.325, 9.012)
Ala/Ser+Ser/Ser	21	40	0.01	2.1 (1.194, 3.693)	0.013	2.098 (1.172, 3.755)

[a]Adjusted for age, sex and smoking status.

Table 2. RASSF1 Ala133Ser SNP status and clinicopathological parameters of lung cancer patients

Groups	n			*P* value	Crude OR (95% CI)	Adjusted *P* value	Adjusted OR (95% CI)[a]
	Ala/Ala	Ala/Ser	Ser/Ser				
Age							
≥60	101	18	3				
<60	84	18	1	0.916	1.04 (0.507, 2.132)	0.58	0.809 (0.382, 1.715)[b]
Sex							
Male	118	31	3				
Female	67	5	1	0.013	3.52 (1.307, 9.484)	0.009	3.84 (1.407, 10.482)[c]
Smoking							
Yes	119	34	3				
No	66	2	1	0.003	9.429 (2.195, 15.21)	0.002	11.967 (2.195, 17.475)[d]
Pathological type							
ADC	158	30	4				
SCC	27	6	0	0.103	0.292 (0.067, 1.28)	0.13	0.308 (0.067, 1.413)
Lymphatic metastasis status							
Yes	138	33	4				
No	47	3	0	0.035	3.746 (1.098, 12.784)	0.123	2.723 (0.762, 9.727)
Stage							
III+IV	45	2	0				
I+II	140	34	4	0.142	3.032 (0.689, 13.341)	0.412	1.922 (0.404, 9.134)
EGFR							
Mutated	82	19	2				
Wild type	103	17	2	0.353	1.404 (0.686, 2.872)	0.303	1.486 (0.857, 2.888)

SCC: Squamous cell carcinoma; ADC: Adenocarcinoma; [a]Adjusted for age, sex and smoking status. [b]Adjusted for sex and smoking status. [c]Adjusted for age and smoking status. [d]Adjusted for age and sex.

RASSF1A 133 Ala/Ser polymorphism is associated with poor survival in NSCLC

According to the Kaplan-Meier survival curve (**Figure 2**, log-rank, χ^2=11.503, *P*<0.01) and a multivariate Cox regression model, patients with Ala/Ser or Ser/Ser genotypes (HR=4.028; 95% CI 2.520-6.439, *P*<0.001) have an unfavorable prognosis.

Discussion

In our study, we found that individuals with a heterozygous Ala/Ser genotype have an increased incidence of lung cancer, as indicated by comparing the RASSF1A genotype distributions of lung cancer patients and healthy controls (adjusted OR=1.96; 95% CI 1.076-3.672, *P*=0.028). Additionally, the significant associations between the heterozygous genotype and male sex (adjusted OR=3.84; 95% CI 1.407-10.482, *P*=0.009) and smoking status (adjusted OR=11.967; 95% CI 2.195-17.475, *P*=0.002) were found. The RASSF1A 133 Ala/Ser or Ser/Ser genotypes indicated a poor prognosis in NSCLC patients. In particular, we found that the relationship between the Ala/Ser genotype and lymphatic metastasis status was significant (with a crude *P*-value <0.05), but after con-

Table 3. Distributions of RASSF1 Ala133Ser SNPs in the presence of EGFR mutations

EGFR mutation	n	*P* value	Crude OR (95% CI)	Adjusted *P* value	Adjusted OR (95% CI)[a]
Wild type	122				
Exon19 deletion	39	0.29	1.668 (0.646, 4.305)	0.317	1.574 (0.647, 3.829)
Exon20 T790M	20	0.948	0.957 (0.255, 3.586)	0.898	0.915 (0.235, 3.556)
Exon21 L858R	44	0.46	1.394 (0.578, 3.364)	0.482	1.391 (0.555, 4.486)

[a]Adjusted for age, sex and smoking status.

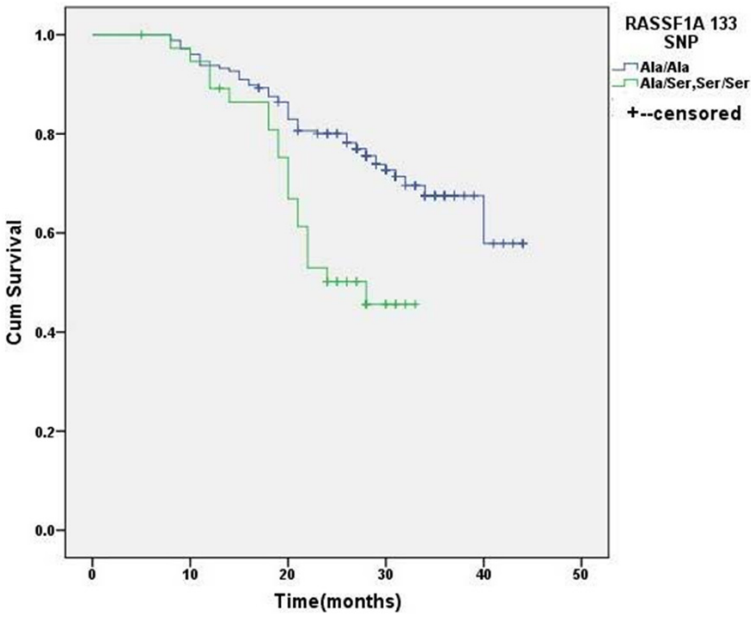

Figure 2. Overall survival in patients with the Ala/Ser and Ser/Ser genotypes was significantly lower than in patients with the Ala/Ala genotype.

trolling for age, sex and smoking status, the association was no longer significant ($P>0.05$). Thus, we conclude that there is no significant association between the Ala/Ser genotype and lymphatic metastasis status. We also analyzed the correlation between the RASSF1A SNP and the EGFR mutation status. To our knowledge, our present study is the first study to report the RASSF1A genotype distributions among NSCLC patients with EGFR mutations. Unfortunately, we did not find significant associations between RASSF1A SNPs and EGFR mutations. The RASSF1A Ala133Ser SNP lies in a bona fide phosphorylation site and adjacent to the N-terminal domain that is highly homologous to the cysteine-rich diacylglycerol/phorbol ester-binding domain [23, 24]. Kanzaki and his colleagues [14] first assumed that this SNP influences the risk of lung cancer incidence by preventing protein phosphorylation or dimerization, and the result of their pilot study showed an increased incidence of lung cancer in persons with the Ala133Ser SNP. The result of our study is consistent with the experimental results of Kanzaki.

Studies on loss of heterozygosity (LOH) have identified chromosome 3p.21; this locus includes the RASSF1 gene, which is considered a tumor suppressor gene [25]. RASSF1A is often silenced in lung cancer and plays an important role in regulating the stability of mitotic cyclins and the timing of mitotic progression through its interaction with Cdc20, which can result in the inhibition of APC/C (anaphase-promoting complex/cyclosome). APC/C is a large multisubunit complex that regulates ubiquitin conjugation [3, 26]. RASSF1A can induce cell cycle arrest involving Ras family-associated G_1/S cell cycle progression by preventing cyclin D1 accumulation [27]. However, the Ala133Ser-substituted protein cannot induce cell cycle arrest by preventing cyclin D1 accumulation [12]. The role of RASSF1A in microtubule stabilization through acetylation has been demonstrated. The RASSF1A Ala/Ser mutant may be the only mutant that can associate with β-tubulin to interfere with α/β tubulin dimers. There, it may probably affect the stabilization of tubulin and the tumor-suppressive function of RASSF1A [6]. Diseases involving the RASSF1A polymorphism likely affect the function of RASSF1A through microtubule stabilization [6, 28]. The DAG-binding domain, which is located in the N-terminal region, plays a key role in dimeriza-

tion [5]. The position of the RASSF1 Ala133Ser SNP is extremely close to the DAG-binding domain [14]. The most common polymorphism of RASSF1A, Ala/Ser, is located within the ATM DNA damage checkpoint site [28]. The above mechanisms may contribute to an increased incidence of lung cancer.

We also explored whether the RASSF1A 133 Ala/Ser polymorphism is associated with poor survival outcomes in NSCLC patients using Kaplan-Meier and multivariate Cox regression analyses. Compared to patients with Ala/Ala, patients with Ala/Ser and Ser/Ser showed an unfavorable prognosis. The EGFR signaling pathway, which modulates cell cycle progression, induces angiogenesis, inhibits apoptosis and promotes metastasis, is crucial for the development of cancer [29]. The EGFR/PI3K/AKT pathway can control cellular function [20]. The classical MAPK (RAS/RAF/MEK/ERK) and PI3K (PI3K-AKT) pathways and the interaction between them influence cell growth and survival [2]. Our study uncovered no associations between the RASSF1 Ala133Ser SNP and mutation of EGFR.

In conclusion, the RASSF1A Ala/Ser SNP is associated with increased susceptibility to and unfavorable prognosis in non-small cell lung cancer. Meanwhile, significant associations were found between the heterozygous genotype and male sex and smoking status. Equally important, we also found no association between the heterozygous RASSF1 Ala/Ser SNP and EGFR mutations. However, the sample size in this study was small; additional research is needed to further explore the associations between RASSF1 SNPs and NSCLC.

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Disclosure of conflict of interest

None.

Abbreviations

RASSF, Ras-association domain family; NSCLC, non-small cell lung cancer; TSG, tumor suppressor gene; SNP, single-nucleotide polymorphism; EGFR, epidermal growth factor receptor; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; ADx-ARMS, ADx amplification refractory mutation system; HR, hazard ratio; OR, odds ratio; CI, confidence interval; NFκB, nuclear factor kappa B; DAG, diacylglycerol; ATM, ataxia telangiectasia mutated; SCLC, small cell lung cancer; Ala, alanine; Ser, serine; RFS, recurrence-free survival; TKIs, tyrosine kinase inhibitors; LOH, loss of heterozygosity; APC/C, anaphase-promoting complex/cyclosome.

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