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Methylation of the RASSF1A and RARβ genes as a candidate biomarker for lung cancer

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Abstract. Promoter methylation of the RASSF1A and RARβ genes has been associated with susceptibility to different types of cancer. In addition, RASSF1A and RARβ methylation plays an important role in the pathogenesis of lung cancer. We investigated the aberrant promoter methylation of RASSF1A and RARβ in lung cancer patients using methylation-specific polymerase chain reaction (MSP). Aberrant promoter methylation of the RASSF1A gene was detected in 45 of 56 (80.36%) cancer patients and aberrant promoter methylation of the RARβ gene was found in 48 of 56 (85.71%) cases; promoter methylation of both genes was found in 42 of 56 (75%) lung cancer cases. None of the 52 samples from controls exhibited DNA methylation in these two target genes. Methylation was significantly associated with the lung cancer cases compared to controls for the RASSF1A gene (adjusted OR=7.50; 95% CI, 3.935-14.296; p<0.001); similar results were obtained for methylation of the RARβ gene (adjusted OR=5.727; 95% CI, 3.348-9.797; p<0.001). In addition, the association remained significant in these two target genes (adjusted OR=8.429; 95% CI, 4.205-16.896; p<0.001). Our results indicated that the high percentage of promoter methylation in the RARβ and RASSF1A genes indicate their important role in the development of lung cancer in the population studied, and that risk of lung cancer for carriers positive for both genes is higher than in single-gene positive carriers, which may serve as a useful marker for prognosis and a target for the treatment of lung cancer.

Introduction

Carcinoma of the lung is the most common malignancy worldwide; in 2007, it was found to have the highest incidence among malignant diseases in the Chinese population (1-3). Moreover, lung cancer causes over 1 million deaths worldwide each year (4). Despite the advent of new diagnostic techniques, most lung cancers are detected at a late stage, and the 5-year survival rate of lung cancer is less than 15% in the US (5). Once tumor cells have spread, the long-term prognosis is poor since no curative treatments are available. Thus, the development of biomarkers for effective early diagnosis of lung cancer is clearly necessary. The molecular biomarker is a new diagnostic technique for tumors (6). Aberrant CpG island methylation in the promoter region of tumor-suppressor genes is suspected of participating in the pathogenesis and progression of lung cancer, and its use as a biomarker offers a new approach to ensure the early diagnosis of lung cancer (7-10).

The Ras association domain family 1 A (RASSF1A) gene, located on chromosome 3 at band p21.3 (3p21.3), is a frequent target for aberrant methylation in lung cancer, and hypermethylation of the RASSF1A promoter was reported in up to 60% of non-small cell lung cancer and 100% of small-cell lung cancer cases (11-13). These findings suggest that RASSF1A is a putative tumor-suppressor gene and is likely to be involved in the genesis of lung cancer, and plays an important role in the progression of tumorigenesis. Epigenetic modification in the short arm of chromosome 3p loci genes, including RASSF1A and RARβ, together have been implicated in the progression of lung tumorigenesis in one study in a Chinese population (14).

In the present study, we used the methylation-specific polymerase chain reaction (MSP) method to examine the methylation status of RASSF1A and RARβ in a South-Central Chinese Han population. We analyzed the relationship of gene methylation patterns with clinical features and lung cancer risk.

Materials and methods

Patients. A total of 56 patients diagnosed with lung cancer and 52 controls without cancer were included in the present study. All patients were recruited from the Hunan Provincial Tumor Hospital in Changsha, China. Histological classification was conducted according to 'Histological Typing of Lung and Pleural Tumors, 3rd edition' of the World Health Organization (WHO), 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

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Table I. Sequences of primers used in MSP.

Gene	Primer sequence (5'-3')	Annealing temperature (˚C)	Product size (bp)
RASSF1A			
M F	GGGTTTTGCGAGAGCGCG	64	169
M R	GCTAACAAACGCGAACCG		
U F	GGTTTTGTGAGAGTGTGTTTAG	59	169
U R	CACTAACAAACACAAACCAAAC		
RARβ			
M F	TCGAGAACGCGAGCGATTCG	62	146
M R	GACCAATCCAACCGAAACGA		
U F	TTGAGAATGTGAGTGATTTGA	62	146
U R	AACCAATCCAACCAAAACAA		

M, methylated; U, unmethylated; F, forward; R, reverse.

Cancer (UICC) in 2003. Fifteen of the tumors were stage I, 7 were stage II, 20 were stage III and 14 were stage IV histologically; 29 of the 56 tumors were squamous cell carcinomas, 17 were adenocarcinomas and 10 included other carcinoma types. The mean age of the controls was 52.6±16.2 years.

All study subjects were of South-Central Chinese population Han ethnicity and provided written consents for participation; the research protocol was approved by the Institutional Review Board of the Hunan Provincial Tumor Hospital, Changsha, China.

DNA extraction and bisulfite treatment. Genomic DNAs were extracted from peripheral blood lymphocytes using a standard kit-based method (Gentra Systems, Minneapolis, MN, USA). Genomic DNA was treated with sodium bisulfite using the EZ DNA methylation-Gold kit (Zymo Research, USA) to modify unmethylated cytosines to uracil. The bisulfite-modified DNA was used immediately for PCR or stored at -70˚C.

Positive control for methylation. Lung cancer patient DNAs were treated *in vitro* with excess SssI methyltransferase (New England Biolabs, Beverly, MA, USA) to generate completely methylated DNA at all CpGs and was used as positive control for methylated alleles of each gene. DNA from a healthy control sample was used as the control for unmethylated alleles. Genomic DNA was treated with sodium bisulfite.

Methylation-specific PCR. Two sets of primers were used to amplify methylated and unmethylated alleles, as shown in Table I. The PCR condition for MSP assays were derived from several reports (15,16). Lymphocyte DNA, original or methylation treated *in vitro* with excess SssI methyltransferase (New England Biolabs), was used as the unmethylation- and methylation-positive controls, respectively. Water blank was used as a negative control.

Statistical analysis. Statistical analyses were performed using the SPSS13.0 statistical software. The association between the methylation status of the two genes and clinicopathological parameters was analyzed using the Fisher's or Chi-square exact test. The association between the methylation of the two genes and lung cancer was determined using the logistic regression method to assess odds ratio (ORs) and 95% confidence intervals (95% CI). $p<0.05$ was considered to indicate statistical significance.

Results

RASSF1A and RARβ gene promoter hypermethylation profile. We analyzed the methylation patterns of RASSF1A and RARβ promoter regions in lung cancer cases and controls. Fig. 1 shows a typical example of the MSP products analyzed on agarose gel for the RASSF1A and RARβ genes. We found that the aberrant promoter methylation of the RASSF1A gene was detected in 85.71% (48/56) of cases and the RARβ gene was detected in 80.36% (45/56) of cases. The promoter methylation of both genes was found in 75% (42/56) of lung cancers (Tables II and III). By contrast, none of the 52 controls was detected to exhibit methylation in either of the two genes (Table III). There was a significant statistical association of the promoter methylation of the RASSF1A gene with lung cancer risk (adjusted OR=7.50; 95% CI, 3.935-14.296; $p<0.001$). Similar results were obtained for methylation of the RARβ gene (adjusted OR=5.727; 95% CI, 3.348-9.797; $p<0.001$). Moreover, methylation of both genes was significantly associated with cancer risk in the cases when compared with the controls (adjusted OR=8.429; 95% CI, 4.205-16.896; $p<0.001$)

Clinicopathological correlation. The relationship between methylation of these two genes and clinicopathlogical characteristics of the lung cancer cases was analyzed and the results are documented in Table II. There was no relationship between RASSF1A methylation status and clinicopathological features. Similar results were obtained for methylation of the RARβ gene. Moreover, there was no relationship between the status of both genes being methylated and clinicopathological features.

Discussion

It is known that methylation is a major epigenetic modification in mammals, and changes in methylation patterns play a key role in tumorigenesis in humans. In particular, promoter

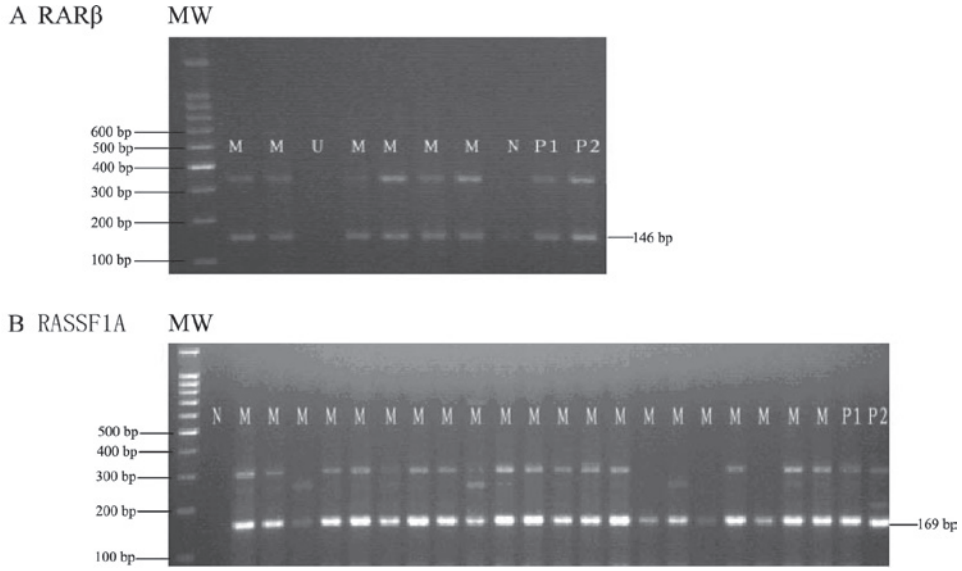

Figure 1. Methylation-specific PCR (MSP) of the promoter regions of RARβ and RASSF1A genes in lung cancer cases. (A) MSP of the RARβ promoter region. (B) MSP of the RASSF1A promoter region. MW, molecular weight DNA marker (100-bp DNA ladder). Lane M indicates the presence of the methylated gene promoter; lane U indicates the presence of the unmethylated gene promoter; lane N indicates the presence of the negative control. Lanes P1 and P2 indicate the presence of the positive control.

Table II. Methylation status of RASSF1A and RARβ genes according to clinical staging and histological grading in the lung cancer cases.

	No. of cases	RASSF1A methylation (%)	RARβ methylation (%)	Both RASSF1A and RARβ methylation (%)
Clinical stage				
I	15	13 (86.70)	10 (66.70)	10 (66.67)
II	7	7 (100.0)	6 (85.70)	6 (85.70)
III	20	17 (85.00)	16 (80.00)	15 (75.00)
IV	14	11 (78.60)	13 (90.90)	11 (78.60)
Total	56	48 (85.71)	45 (80.36)	42 (75.00)
Histological grade				
Squamous cell carcinoma	29	26 (89.70)	23 (79.30)	21 (72.40)
Adenocarcinoma	17	14 (82.40)	15 (88.20)	14 (82.40)
Other carcinoma types[a]	10	8 (80.00)	7 (70.00)	7 (70.00)
Total	56	48 (85.71)	45 (80.36)	42 (75.00)

[a]Including small-cell, large-cell and mixed-cell carcinomas or undifferentiated carcinomas.

Table III. Methylation status of the RASSF1A and RARβ genes in the lung cancer cases and controls.

Gene	Methylation status	Cases (%)	Controls (%)	p-value	OR (95% CI)
RASSF1A	Methylated	48 (85.71)	0	<0.01[a]	7.500 (3.935-14.296)[a]
	Unmethylated	8 (14.29)	52 (100)	<0.01[b]	1.929 (1.608-2.313)[b]
RARβ	Methylated	45 (80.36)	0	<0.01[a]	5.727 (3.348-9.797)[a]
	Unmethylated	11 (19.64)	52 (100)	<0.01[b]	1.929 (1.608-2.313)[b]
RASSF1A + RARβ (both genes)	Methylated	42 (75.00)	0	<0.01[a]	8.429 (4.205-16.896)[a]
	Unmethylated	7 (12.25)	52 (100)	<0.01[b]	2.061 (1.686-2.520)[b]

OR, odds ratio; CI, confidence interval; p-value, probability from the Fisher's exact test comparing the methylation status for cases and controls. [a]Frequency of methylated vs. unmethylated genes among cases and controls; [b]frequency of methylated vs. total (methylated and unmethylated) genes among cases and controls.

CpG island hypermethylation is closely related to inactivation and silencing, resulting in tumor suppressor loss of gene expression and X-chromosome inactivation, and affects the development of carcinogenesis (17,18). Aberrant promoter region methylation of tumor-suppressor genes is association with the mechanism for carcinogenesis. Aberrant methylation of RASSF1A within the promoter region has been reported in various tumor types, including lung cancers, similar to the RARβ gene (9,11-13). There are few studies reporting hypermethylation of both the RASSF1A and RARβ genes together in cancers, particularly in lung cancer (19,20,21). Thus, in the present study we aimed to determine whether aberrant promoter methylation of the RASSF1A and RARβ genes is of potential use as a molecular biomarker for lung cancer in a South-Central Chinese Han population using MSP.

Several studies have shown separately that methylation of CpG islands of the RASSF1A and RARβ genes has a significant role in the development of lung cancer (20-22), but no report in a South-Central Chinese Han population has examined the two genes simultaneously. In the present study, we found that the RASSF1A gene was hypermethylated in 48 out of 56 lung cancer samples. The frequency was consistent with previous studies (9,11-13), but higher than that found in the research of Wang *et al* in primary lung cancer in a Chinese population (14). The frequency of methylation was over 70% in all clinical stages. In agreement with this study, several reports have shown that there is no relationship with clinical stage and histological grade (11,14,19). The data suggest that aberrant promoter methylation of the RASSF1A gene is highly significantly associated with lung cancer when compared with normal samples ($p<0.01$). More importantly, the results showed that RASSF1A gene-positive carriers had a 7.5-fold increased risk of lung cancer (adjusted OR=7.50; 95% CI, 3.935-14.296; $p<0.001$) in a South-Central Chinese Han population. Thus, our data strongly support the theory that methylation of the RASSF1A gene in lung cancer cases in a South-Central Chinese Han population is a late event which may be associated with carcinogenesis. In addition, promoter methylation of the RASSF1A gene presented a significant relationship (adjusted OR=2.00; 95% CI, 1.662-2.407; $p<0.001$) with lung cancer when compared to the unmethylated and vs. the total of both (methylated + unmethylated) genes, thereby strengthening the relationship between lung cancer and methylation.

Several studies have suggested that epigenetic event of the RARβ gene may play a role in the development of lung cancer (23-25). Our study revealed that methylation of the RARβ gene was found in 80.36% of cases, higher than that found in the research of Virmani *et al* (26), but similar to that in small-cell lung cancers in a study by Zöchbauer-Müller *et al* (27). Likewise, there was no relationship between aberrant promoter region methylation of the RARβ gene and clinical stage/histological grade as well as the RASSF1A gene. No methylation was detected in the controls, and the aberrant promoter methylation of the RARβ gene had a highly significant correlation between the cases and controls ($p<0.01$). Moreover, the results showed that RARβ gene-positive carriers had a 5.7-fold increased risk of lung cancer (adjusted OR=5.727; 95% CI, 3.348-9.797; $p<0.001$) in our South-Central Chinese Han population. Thus, our data demonstrated that RARβ methylation was also important in the pathogenesis of lung cancer in our population. Similar to the RASSF1A gene, promoter methylation of the RARβ gene had a significant (adjusted OR=2.00; 95% CI, 1.662-2.407; $p<0.001$) association with lung cancer when ompared to the unmethylated and vs. the total of both (methylated + unmethylated) genes.

Aberrant methylation of the promoter region of RARβ and RASSF1A genes is a common epigenetic event in chromosome 3 in lung cancer. In the present study, the aberrant promoter methylation of both genes was found in 75% (42/56) of lung cancer cases. By researching the methylation profile of the RARβ and RASSF1A genes for different clinical stages/histological grades of lung cancer, no significant difference in methylation frequency was found. The association remained significant between cases and controls ($p<0.01$). The results demonstrated that positive carriers of both genes had an 8.4-fold increased risk of lung cancer (adjusted OR=8.429; 95% CI, 4.205-16.896; $p<0.001$) in our South-Central Chinese Han population. The risk of lung cancer for positive carriers with both genes was higher than for positive carriers of a single gene in the South-Central Chinese Han population. In addition, promoter methylation of both genes had a significant (adjusted OR=2.061; 95% CI, 1.686-2.520; $p<0.001$) relationship with lung cancer compared to the unmethylated and vs. the total of both (methylated + unmethylated) genes.

In conclusion, to our knowledge this is the first study to indicate an association between the results of the MSP analysis of RARβ and RASSF1A genes with lung cancer in a South-Central Chinese Han population. The high percentage of promoter methylation in the RARβ and RASSF1A genes indicates their important role in the development of lung cancer in the studied population. The risk of lung cancer for positive carriers of both genes was higher than for positive carriers of a single gene. This offers a potential marker for the prognosis as well as a target for the treatment of lung cancer.

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