RESEARCH ARTICLE

Combined Effects Methylation of FHIT, RASSF1A and RARβ Genes on Non-Small Cell Lung Cancer in the Chinese Population

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Abstract

Epigenetic modifications of tumour suppressor genes are involved in all kinds of human cancer. Aberrant promoter methylation is also considered to play an essential role in development of lung cancer, but the pathogenesis remains unclear.We collected the data of 112 subjects, including 56 diagnosed patients with lung cancer and 56 controls without cancer. Methylation of the FHIT, RASSF1A and RAR-β genes in DNA from all samples and the corresponding gene methylation status were assessed using the methylation-specific polymerase chain reaction (PCR, MSP). The results showed that the total frequency of separate gene methylation was significantly higher in lung cancer compared with controls (33.9-85.7 *vs* 0 %) ($p<0.01$).Similar outcomes were obtained from the aberrant methylation of combinations of any two or three genes ($p<0.01$). There was a tendency that the frequency of combinations of any two or three genes was higher in stageI+II than that in stage III+IV with lung cancer. However, no significant difference was found across various clinical stages and clinic pathological gradings of lung cancer ($p>0.05$).These observations suggest that there is a significant association of promoter methylation of individual genes with lung cancer risk, and that aberrant methylation of combination of any two or three genes may be associated with clinical stage in lung cancer patients and involved in the initiation of lung cancer tumorigenesis. Methylation of FHIT, RASSF1A and RARβ genes may be related to progression of lung oncogenesis.

Keywords: Methylation - non-small cell lung cancer - combined effect - FHIT - RASSF1A gene - RARβ gene

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Introduction

Lung cancer is the most common malignant tumor originating and the most common cause of death from cancer (Missaoui et al., 2010), and increasing numbers of individuals suffer from the disease every year. Lung cancer was found to be the first highest malignant disease in Chinese population in 2007 (Jemal et al., 2009; Missaoui et al., 2010; Bodoor et al., 2014). The prognosis of non-small cell lung cancer (NSCLC) patients remains poor because that no curative treatments are available, in despite of multimodal therapies including surgery, radiotherapy, and chemotherapy (Toyooka et al., 2004; Jemal et al., 2009; Bodoor et al., 2014). Therefore, the optimal molecular analyses and treatment modality are essential for each NSCLC patient with developing technology.

DNA methylation is located 5' to a guanosine in a CpG dinucleotide. And DNA methylation of CpG sites in the promoter regions or the first exon of genes is a frequent epigenetic event in cancers of the breast, liver, lung and others (Botana-Rial et al., 2012; Fujiwara et al., 2012). Gene silencing or inactivation associated with aberrant methylation of tumor-suppressor genes (TSGs) is a common mechanism of tumorigenesis in NSCLC and DNA methylation also plays an important role in process of tumorigenesis (Fujiwara et al., 2005; Deng et al., 2012). Thus, the mechanisms of multiple DNA hypermethylation of TSGs should be elucidated in order to understand and manage NSCLC.

The Fragile histidine triad (FHIT) gene located on chromosome 3 at band p14.2 (3p14.2) (Ki et al., 2008; Jeong et al., 2013; Liu et al., 2013). The Ras association domain family 1 A (RASSF1A) gene located on chromosome 3 at band p21.3 (3p21.3) (Neyaz et al., 2008; Liu et al., 2013; Vo et al., 2013), and retinoic acid receptor beta (RARβ) is to the p24 band of chromosome 3 (Virmani et al., 2000; Li et al., 2012). Aberrant methylation of FHIT, RASSF1A and RARβ were reported separately in lung cancer. These findings suggest that FHIT, RASSF1A and RARβ are a putative tumor suppressor gene and are likely to be involved in the genesis of lung cancer, which plays an important role in the pathogenesis and progression of lung cancer (Zochbauer-Muller et al., 2004; Deng et al., 2012;

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Dumitrescu et al., 2012). As epigenetic modification in the short arm of chromosome 3p loci genes including FHIT, RASSF1A and RARβ together have been implicated in the progression of lung tumorigenesis in one study on a Chinese population (Fujiwara et al., 2005; Li et al., 2012).

In the present study, FHIT, RASSF1A and RARβ genes are known to be TSGs in various tumors, their role in tumorigenesis of NSCLC needs to be clarified. we used the methylation-specific polymerase chain reaction (MSP) method to examine the methylation status these genes in South-Central Chinese population Han.17 We correlated our results with clinicopathological features of the NSCLCs.

Materials and Methods

Patients

Between August 2010 and October 2012, blood samples were collected from 112 subjects, including 56 diagnosed patients with lung cancer and 56 controls without cancer.

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: 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 International Contrele Cancer (UICC) in 2003. The mean age of the patients was 55.1±15.7 years and the mean age of the controls was 51.2±15.2 years.

The clinic pathological features of samples are shown in Table 1. Fifteen of the tumors were stage I, 7 were stageII, 20 were stage III, and 14 were stage IV histologically; 29 of the 56 tumors were squamous cell carcinomas, 17 were adenocarcinomas, and 10 were other carcinomas.

All study subjects provided written consents and were ethnic South-Central Chinese population Han, and the research protocol was approved by the Institutional Review Board of the hospital.

DNA extraction and Bisulfite Treatment

Genomic DNA was extracted from peripheral blood lymphocytes using a standard kit-based method (Gentra Systems, Minneapolis, MN). and Genomic DNA was treated with sodium bisulfite using the EZ DNA methylation-Gold kit (Zymo Research, USA) to modify unmethylated cytosine 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. Water blank was used as a negative control. Genomic DNA was treated with sodium bisulfite.

Methylation-specific PCR

Three sets of primers were derived from several reports, using to amplify methylated and unmethylated alleles (Virmani et al., 2000; Ki et al., 2008; Li et al., 2012; Jeong et al., 2013). The PCR conditions of FHIT gene were 35 cycles of 94˚C for 45sec, 70˚C for 30sec and 72˚C for60sec, followed by 72˚C for 8min; The PCR conditions of RASSF1A gene were 35 cycles of 94˚C for 45sec, 62˚C for 30sec and 72˚C for60sec, followed by 72˚C for 5min; The PCR conditions of methylated RARβ gene was 35 cycles of 94˚C for 45sec, 64˚C for 30sec and 72˚C for 60sec, followed by 72˚C for 5 min; The PCR conditions of unmethylated RARβ gene was 35 cycles of 94˚C for 45sec, 59˚C for 30sec and 72˚C for 60sec, followed by 72˚C for 10min.All PCR products were analyzed on 2% agarose gel.

Statistical analysis

Statistical analyses were performed using Statistical software SPSS13.0. The frequency of methylation status in FHIT, RASSF1A and RARβ genes among cases and clinical outcome was statistically analyzed using x2-test. The association between the methylation of those genes and lung cancer was determined using the logistic regression method to assess odds ratio (ORs) and 95% confidence intervals (95%CI). P-values less than 0.05 were considered statistically significant.

Results

FHIT, RASSF1A and RARβ genes promoter hypermethylation profile

We analyzed the methylation pattern of FHIT, RASSF1A and RARβ promoter region in lung cancer cases and controls and the results were listed in Table 1. The aberrant promoter methylation of the RASSF1A gene was detected to 85.71% (48/56) of cases, the RARβ gene was detected to 80.36% (45/56) of cases, and the FHIT gene was detected to33.93 % (19/56) of cases. Moreover, The frequency of methylation was 30.36% (17/56) between FHIT and RARB genes , and The frequency of methylation was 75.87% (44/56) in between RARB and RASSF1A genes. whereas it was 30.36 % (17/56) in FHIT and RASSF1A, and the frequency of combined three genes was 26.79% (15/56). In contrast, no aberrant promoter methylation of FHIT, RASSF1A and RARβ was found in either of these genes with the 56 normal samples (Table 1). There was a significant statistical association of the promoter methylation of separate genes between with the lung cancer compared with controls (p<0.01) .and the combined effect of the presence of methylation in all genes demonstrated significantly association with the lung cancer compared with controls (p<0.01).

Clinicopathological correlation

The relationship between methyaltion of these genes and clinicopathlogical of lung cancer was analyzed and the results were listed in Table 2. The RASSF1A methylation was 86.7% (13/15) in stageI and 100% (7/7) in stageII, whereas it was 85% (17/20) in stage III and 78.6% (11/14) in stage IV of lung cancer cases (Table2). The frequency

of methylation was 89.7 % (26/29) in Squamouscell carcinoma, 82.4 % (14/17) in adenocarcinoma and 80.0 % (8/10) in other carcinomas including the small cell, large cell, and mixed cell carcinomas or undifferentiated carcinomas. No difference was found in the methylation rate of the RASSF1A gene between clinical stage and organization type in lung cancer.

The RARβ methylation was 66.7% (10/15) in stageI and 85.7% (6/7) in stageII, whereas it was 80% (16/20) in stage III and 90.9% (13/14) in stage IV of lung cancer cases (Table2). The frequency of methylation was 79.3 % (23/29) in Squamouscell carcinoma, 88.2 % (15/17) in adenocarcinoma and70.0% (7/10) in other carcinomas. No difference was found in the methylation rate of the RARβ gene between clinical stage and organization type in lung cancer.

Likewise, for the FHIT gene, the incidence of methylation was 13.3% (2/15) in stageI and 28.6% (2/7) in stageII, whereas it was 50% (10/20) in stage III and 35.7% (5/14) in stage IV of lung cancer cases (Table2). The frequency of methylation was 31.0 % (9/29) in squamouscell carcinoma, 35.3 % (6/17) in adenocarcinoma and 40.0 % (4/10) in other carcinomas. No difference was found in the methylation rate of the FHIT gene between clinical stage and organization type in lung cancer.

Table 1. Methylation Status of FHIT RASSF1A and RARβ Ggenes in Lung Cancer and Controls

Gene Methylation status	Case N (%)	Controls N (%)	OR (95%CI)	*p* value
FHIT				
Methylated	19 (33.93)	0	2.514	$p<0.01^a$
Unmethylated	37 (66.07)	56 (100)	(1957-3.228)[a]	
RASSF1A				
Methylated	48 (85.7)	0	8	$p<0.01^a$
Unmethylated	8 (14.3)	56 (100)	(4.184-15.297)[a]	
RARβ				
Methylated	45 (80.4)	0	6.091	$p<0.01^a$
Unmethylated	11 (19.6)	56 (100)	(3.549-10.455)[a]	
FHIT+ RARβ				
Methylated	17 (30.36)	0	2.436	$p<0.01^b$
Unmethylated	39 (69.64)	56 (100.0)	(1.914-3.100)[b]	
RARβ+RASSF1A				
Methylated	44 (78.57)	0	5.667	$p<0.01^b$
Unmethylated	12 (21.43)	56 (100.0)	(3.391-9.469)[b]	
FHIT+RASSF1A				
Methylated	17 (30.36)	0	2.436	$p<0.01^b$
Unmethylated	39 (69.64)	56 (100.0)	(1.914-3.100)[b]	
FHIT+RASSF1A+				
Methylated	15 (26.79)	0	2.366	$p<0.01^b$
RARβ				
Unmethylated	41 (73.21)	56 (100.0)	(1.875-2.985)[b]	

OR, odds ration; CI, confidence interval; *p*-Vale,probability from the Fisher's exact test comparing the methylation status for cases and control;acomparing the frequency of methylated versus unmethylated gene among cases and controls; bcomparing the frequency of methylated versus toal (methylated and unmethylated) gene among cases and control

Table 2. Methylation Status of FHIT RASSF1A and RARβ Genes according to Clinical Staging and Histological Grading in Lung Cancer

	No.of cases	RASSF1A methylation (%)	RARβ methylation (%)	FHIT methylation (%)
Histological grad				
Squamouscell carcinoma	29	26 (89.7)	23 (79.3)	9 (31.0)
adenocarcinoma	17	14 (82.4)	15 (88.2)	6 (35.3)
other carcinomasc	10	8 (80.0)	7 (70.0)	4 (40.0)
Clinical stage				
I	15	13 (86.7)	10 (66.7)	2 (13.3)
II	7	7 (100.0)	6 (85.7)	2 (28.6)
III	20	17 (85.0)	16 (80.0)	10 (50)
IV	14	11 (78.6)	13 (90.9)	5 (35.7)

Combined effects methylation of FHIT, RASSF1A and RARβ genes

FHIT and RARB methylation occurred in stageI (6.7%%), stageII (28.57%), stage III (50%), and stage IV (28.57%), FHIT and RASSF1A methylation was found in stageI (13.3%), stageII (28.57%), stage III (45%), and stage IV (28.57%), RARβ and RASSF1A methylation was found in stageI (66.67%), stageII (85.7%), stage III (75%), and stage IV (78.6%), and the aberrant methylation of combined three genes was 6.7% (1/15) in stageIand 28.57% (2/7) in stageII, whereas it was 45% (9/20) in stage III and 21.43% (3/14) in stage IV.and the results were shown in Table 3.No relationship was found between genes methylation and pathologic staging ($p>0.05$).

The frequency of methylation with FHIT and RARB genes was 27.59% (8/29) in Squamouscell carcinoma, 29.41 % (5/17) in adenocarcinoma and 40.0 % (4/10) in other carcinomas. Similar results were obtained for the methylation between FHIT and RASSF1A genes. The frequency of methylation with RASSF1A and RARB genes was 72.4% (21/29) in Squamouscell carcinoma, 82.40 % (14/17) in adenocarcinoma and 70.0 % (7/10) in other carcinomas. and the frequency of methylation with combined three genes was 24.14% (7/29) in Squamouscell carcinoma, 23.53 % (4/17) in adenocarcinoma and 40.0 % (4/10) in other carcinomas. No relationship was found between genes methylation and histological grad ($p>0.05$).

Table 3. Methylation Status of Combination of any two or three Gene According to Clinical Staging and Histological Grading in Lung Cancer

		No.of cases	FHIT+ RARβ methylation (%)	FHIT+RASSF1A methylation (%)	RASSF1A+RARβ methylation (%)	FHIT+RASSF1A+ RARβ methylation (%)
Clinical stage	I	15	1 (6.7)	2 (13.33)	10 (66.67)	1 (6.67)
	II	7	2 (28.57)	2 (28.57)	6 (85.7)	2 (28.57)
	III	20	10 (50.0)	9 (45.0)	15 (75.0)	9 (45.0)
	IV	14	4 (28.57)	4 (28.57)	11 (78.6)	3 (21.43)
Histological grad	Squamouscell carcinoma	29	8 (27.59)	8 (27.59)	21 (72.4)	7 (24.14)
	adenocarcinoma	17	5 (29.41)	5 (29.41)	14 (82.4)	4 (23.53)
	other carcinomas	10	4 (40.0)	4 (40.0)	7 (70.0)	4 (40.0)

Discussion

Pathogenesis of lung cancer is a complicated biological process including multiple genetic and epigenetic changes (Virmani et al., 2000; Fujiwara et al., 2005; Dumitrescu et al., 2012; Liu et al., 2013). The role of the genetic mechanisms has become an increasing concern to global investigators at present. Methylation is major epigenetic modification in mammal, and changes in methylation patterns play a key role in tumor genesis with humans (Virmani et al., 2000; Dumitrescu et al., 2012; Fiolka et al., 2013). In particular, the promoter CpG island hypermethylation is closely related between inactivation and silencing, resulting in tumor suppressor loss of gene expression and X-chromosome inactivationmay, and affect development of carcinogenesis (Ki et al., 2008; Fiolka et al., 2013; Al-Temaimi et al., 2013). The aberrant promoter region methylation of tumor suppressor genes is associated with mechanism for carcinogenesis (Grote et al., 2006; Neyaz et al., 2008; Dumitrescu et al., 2012). Therefore, the aberrant promoter methylation is considered to be a novel and promising marker of previous carcinogen exposure and cancer risk.

The FHIT, RARβ and RASSF1A genes were scientific researched correlating the development in human lung, gastrointestinal, liver and cervical cancer, as well as other malignant tumors as antioncogenes (Virmani et al., 2000; Neyaz et al., 2008; Ki et al., 2008; Deng et al., 2012; Li et al., 2012; Jeong et al., 2013; Liu et al., 2013; Vo et al., 2013). Although the aberrant promoter region methylation was a common epigenetic event with lung cancer, combined detection of these genes may vastly improve the positive detection rate of tumors or prognostic judgment (Deng et al., 2012; Dumitrescu et al., 2012; Vo et al., 2013; Al-Temaimi et al., 2013; Zuo et al., 2013). Several studies showed separately that methylation of CpG islands of FHIT, RASSF1A and RARβ genes has a significant role in the process of lung cancer (range, 20-90%, depending on tumor type), this figure identified the these genes as the most commonly modified gene in human cancers (Ki et al., 2008; Neyaz et al., 2008; Deng et al., 2012; Jeong et al., 2013; Liu et al., 2013; Al-Temaimi et al., 2013; Zuo et al., 2013). however, there are only a few studies reporting hypermethylation of combined FHIT, RASSF1A and RARβ genes together in cancers, especially in non-small cell lung cancer (Li et al., 2012; Zuo et al., 2013; Mengxi et al., 2013; Ko et al., 2013). Thus, In this study, we showed the association between combined effects methylation of FHIT, RASSF1A and RARβ genes and several clinical characteristics in Chinese population Han.

The aberrant promoter methylation of FHIT gene was significantly associated with an increased risk of lung cancer ($p<0.01$), the frequency of the methylation was far higher in lung cancer as compared with control. Similar results were obtained for the methylation of RARβ and RASSF1A genes ($p<0.01$). However, we also found no correlation between the promoter methylation of FHIT gene and clinicopathological features at high risk for lung cancer such as tumor histological type and histological grade.

The aberrant promoter region methylation of FHIT, RARβ and RASSF1A genes are a common epigenetic event in chromosome 3 with lung cancer. The combined effect of the presence of methylation in all genes was first reported in Chinese population. In the present study, the frequency of methylation was 78.57 (44/56) in RARβ + RASSF1A genes together, 30.36 (17/56) in FHIT+ RARβ genes together, 30.36 (17/56) in FHIT+ RASSF1A genes together, and 26.79 (15/56) in FHIT+ RASSF1A+ RARβ genes together. The combined effect of the presence of methylation in all genes demonstrated a better association with the lung cancer compared with controls. And the combination of any two also being significantly associated ($p<0.01$).

Furthermore, there is a tendency that the frequency of the combination of any two or three genes was higher in stageI+II than that in stage III+IV with lung cancer. The frequency of combined FHIT and RARβ was 13.63% (3/22) in stageI+II, and 41.18% (14/34) in stage III+IV, The frequency of combined FHIT and RASSF1A was 18.18% (4/22) in stageI+II, and 38.23% (13/34) in stage III+IV, The frequency of combined RASSF1A and RARβ was 77.27% (17/22) in stageI+II, and 79.41% (27/34) in stage III+IV, The frequency of combined all genes was 13.63% (322) in stageI+II, and 35.29% (12/34) in stage III+IV. However, we also found no correlation between the promoter methylation gene and different clinical stage / histological grade of lung cancer. These observations suggest that there were a significant statistical association of the promoter methylation of the individual gene with lung cancer risk, the aberrant methylation of combination of any two or all together genes may be associated with clinical stage in lung cancer patients and involved in the initiation of lung cancer tumorigenesis (Grote et al., 2006; Hesson et al., 2007; Missaoui et al., 2010; Li et al., 2012; Ko et al., 2013; Moison et al., 2013; Liu et al., 2013; Vo et al., 2013; Chen et al., 2013; Bodoor et al., 2014). The methylation of FHIT, RASSF1A and RARβ genes may be a molecular marker for early diagnosis of lung cancer. Accordingly, it needs further work to understand the function of those genes at the molecular level.

In conclusion, we believe that FHIT, RASSF1A and RARβ genes methylation is closely related to the development process of NSCLC. In future studies, we will carry out further research with additional studies analyzing a larger number of individuals regarding the relationships between gene methylation and clinicopathological characteristics.

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