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Original Article

Prognostic significance of BRCA1 and RASSF1A promoter hypermethylation in non-small cell lung cancer patients

Weiwei Gao, Zheng Zhou, Ying Liu, Daijian Liu, Qingqing Feng, Yalai Xu

Department of Respiratory Medicine, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450014, Henan, China

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Abstract: Background: Promoter methylation has been found in several genes in cancer development and progression. Promoter methylation mediated silencing of different tumor suppressor genes found to be associated with lung cancer progression. The aim of this study was to explore the role of promoter methylation in tumor suppressor genes (BRCA1 and RASSF1A) and their association with clinicopathological features as well as survival of non-small cell lung cancer (NSCLC) patients. Methods: Promoter methylation status of BRCA1 and RASSF1A was observed in peripheral blood samples of 50 histopathologically confirmed newly diagnosed cases of NSCLC as well as 50 matched healthy controls by using Methylation Specific-Polymerase Chain Reaction (MS-PCR). The association of promoter methylation and NSCLC mortality was evaluated by Cox-proportional hazards models. Kaplan-Meier survival analysis was performed for overall survival of NSCLC patients. Results: Our data showed that there was a significant association of BRCA1 and RASSF1A with methylation levels of 54% ($P<0.001$), 62% ($P<0.001$), respectively. In addition, we found a strongly correlation between tumor suppressor genes (BRCA1 and RASSF1A) hypermethylation with histological grade and lymph nodes metastasis of NSCLC patients. Furthermore, our results indicated that NSCLC specific mortality was significant correlated with promoter methylation of BRCA1 [HR and 95% CI: 2.053 (0.973-6.748)] and RASSF1A [HR and 95% CI: 3.291 (1.287-8.316)]. Conclusion: Our data demonstrated that promoter methylation of BRCA1 and RASSF1A genes was associated with advanced clinical features and poor prognosis of NSCLC patients, indicating that promoter methylation of BRCA1 and RASSF1A may play important roles in NSCLC progression.

Keywords: Promoter methylation, BRCA1, RASSF1A, non-small cell lung cancer, MS-PCR

Introduction

Lung cancer is one of the leading causes of cancer related death worldwide, with high possibilities of recurrence and metastasis [1]. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancers, and the majority (60-80%) of patients are diagnosed at an advanced stage, for which the prognosis of NSCLC patients remains very poor with a 5-year survival of 15% [2, 3].

DNA methylation has attracted intensive investigation in recent years and it is seen to play an important role in the regulation of genes related to cancer [4]. In particular, aberrant promoter methylation occurs in numerous genes in cancer progression [5]. Among these genes, BRCA1 and RASSF1A are frequently methylated in NSCLC [6, 7]. The mechanism of gene deactivation by methylation and its significance to cancer pathogenesis is well described, with hypermethylation of tumor suppressor genes, affecting transcriptional activity of the genes, considered to be one of the most important drivers of carcinogenesis [8].

Recently, more and more attention is paid to the phenomenon of hypermethylation of disease related genes in peripheral blood (PB) DNA and its involvement in the pathology of cancer [9, 10]. Those studies indicated that detection of tumor DNA in the blood may serve as an early and more accessible marker of

diagnosis and prognosis of cancer [11]. However, the frequency of aberrant methylation in peripheral blood (PB) has not been extensively investigated. BRCA1 status may potentially be used as a prognostic marker as studies have shown that higher levels of BRCA1 expression were significantly associated with shorter disease-free survival in stage I NSCLC patients [12]. BRCA1 promoter methylation was found be positively associated with recurrence-free survival in patients with curatively resected stage I NSCLC [6]. DNA methylation markers have been applied as an alternative approach to molecular profiling of RASSF1A promoter methylation provides important prognostic information in NSCLC cancer patients [13]. These studies suggested that DNA methylation profiling correlated with clinical status in NSCLC and could play important roles in NSCLC progression.

In the present study, we examined the aberrant methylation status of BRCA1 and RASSF1A in peripheral blood with clinicopathological features and NSCLC specific mortality in NSCLC patients.

Materials and methods

Study population

The present study was approved by The Second Affiliated Hospital of Zhengzhou University Ethics Committee. Prior informed consent was obtained from each patients and healthy volunteers in accordance with the guidelines of The Second Affiliated Hospital of Zhengzhou University, China. All specimens were handled and made anonymous according to the ethical and legal standards.

Current study was performed on 50 histopathologically confirmed NSCLC patients and 50 healthy volunteers. Samples were collected from The Second Affiliated Hospital of Zhengzhou University during January 2011 to December 2012. 5 ml of peripheral blood sample was collected from each patients and healthy volunteers and stored at -80°C until use. None of the patients had received chemotherapy or radiotherapy before surgery.

Patient data collection and follow-up

All patients' survival information of 60 month post operative follow-up was received by telephone and mail. Patients' characteristics were obtained from the medical records. Patients with a history of any other malignancy or metastasized cancer from any other organs were excluded. The median follow-up period was 33.7 months (range: 12-60 months).

DNA extraction and bisulfite modification

DNA extraction was performed using Blood DNA extraction kit (Geneaid) on peripheral blood samples collected in EDTA vials from each patient and healthy volunteer by using manufacturer's protocol. DNA concentrations were measured and 1 μg of DNA was used for bisulfite modification. DNA bisulfite modification was performed using Bisul flash DNA modification kit (Epigenetek) according to the manufacturer's instructions. Bisulfite treated DNA was immediately stored at -20°C.

Methylation specific-polymerase chain reaction (MS-PCR) analysis

After bisulfite conversion, Qualitative methylation analysis of different genes were analyzed by Methylation Specific Polymerase Chain Reaction (MS-PCR). Primers for MS-PCR were as shown in previous studies [6, 7]. PCRs were run in a volume of 25 μl, containing 2 ul bisulfite-modified DNA, 12 μl of 2x Hot Start PCR Master mix (Fermentas), 0.25 μl sense primer, 0.25 μl antisense primer, and 12.5 μl H_2O. The PCR profile was 95°C for 10 minutes, 40 cycles at 95°C for 45 seconds, primer annealing at 56°C to 60°C for 45 seconds, 72°C for 45 seconds, and a final extension step at 72°C 10 minutes. The amplified PCR products were further electrophoresed on 2% agarose gels and evaluated under ultraviolet light.

Statistical analysis

The software of SPSS version 18.0 for Windows was used for statistical analysis. The association between the promoter methylation status and the clinicopathological features was analysed using chi-squared test. The effect of methylation on patient survival was estimated by the Kaplan-Meier method and the differences between two groups were compared using the log-rank test. The Cox proportional hazard regression model was used to estimate the

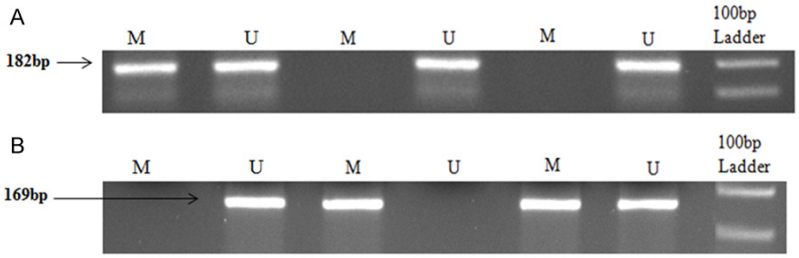

Figure 1. Representative results of MS-PCR analysis for (A) BRCA1 and (B) RASSF1A in NSCLC patients. M (methylated) and U (unmethylated).

Table 1. Association between promoter methylation of Tumor suppressor genes and clinicopathological features

	BRCA1 POSITVE (%)	P	RASSF1A POSITVE (%)	P
Cases (50)	27 (54)	<0.001	31 (62)	<0.001
Controls (50)	0 (0)		0 (0)	
Age (years)				
Age ≤60 (22)	11 (50)	0.615	13 (59.09)	0.707
Age >60 (28)	16 (57.14)		18 (64.29)	
Gender				
Male (31)	17 (54.84)	0.879	19 (61.29)	0.895
Female (19)	10 (52.63)		12 (63.15)	
Tumor size (cm)				
Size ≤3 (21)	10 (47.62)	0.441	11 (52.38)	0.233
Size >3 (29)	17 (58.62)		20 (68.96)	
Histology				
Adeno (16)	6 (37.5)	0.108	9 (56.25)	0.566
Squamous (34)	21 (61.76)		22 (64.71)	
Histological grade				
I (18)	5 (27.78)	0.005	6 (33.33)	0.002
II-III (32)	22 (68.75)		25 (78.13)	
Lymph nodes metastasis				
Positive (14)	12 (85.71)	0.005	12 (78.57)	0.031
Negative (36)	15 (41.67)		19 (55.56)	

hazard ratio (HR) of factors influencing patient's survival. Differences were considered statistically significant when P was less than 0.05.

Results

Methylation status of the CpG islands at the promoter regions of BRCA1 and RASSF1A genes was analysed by MS-PCR in peripheral blood samples of NSCLC patients (**Figure 1**). Hypermethylation of the BRCA1 and RASSF1A promoters was found in 27/50 (54%) (P< 0.001), 31/50 (62%) (P<0.001), respectively (**Table 1**).

We found significant correlation between tumor suppressor gene (BRCA1 and RASSF1A) hypermethylation with histological grade and lymph nodes metastasis of NSCLC patients (P<0.05) (**Table 1**). No significant association were observed between tumor suppressor genes (BRCA1 and RASSF1A), gender, age, tumor size, and histology (P>0.05).

We then analyzed the effect of BRCA1 and RASSF1A-promoter methylation on the patient survival. The group of patients with tumors containing BRCA1 promoter methylation had significantly poorer overall survival rates then the patients with tumors that did not contain BRCA1 promoter methylation (P<0.05, **Figure 2A**). Moreover, the group of patients with tumors containing RASSF1A promoter methylation also had significantly poorer overall survival rates then the patients with tumors that did not contain RASSF1A promoter methylation (P<0.05, **Figure 2B**). The results were confirmed using Cox proportional hazard regression model (HR 2.053 for BRCA1 and HR 3.291 for RASSF1A) (**Table 2**).

Only part of the variation in the risk of mortality among lung cancer can be explained by known pathologic and clinical parameters [14]. Thus, to effectively reduce the disease burden of lung cancer, it is important to identify etiologic factors of the disease as well as factors that predict survival [15]. Promoter DNA methylation plays an important role in tumor development by regulating the expression of specific genes [16].

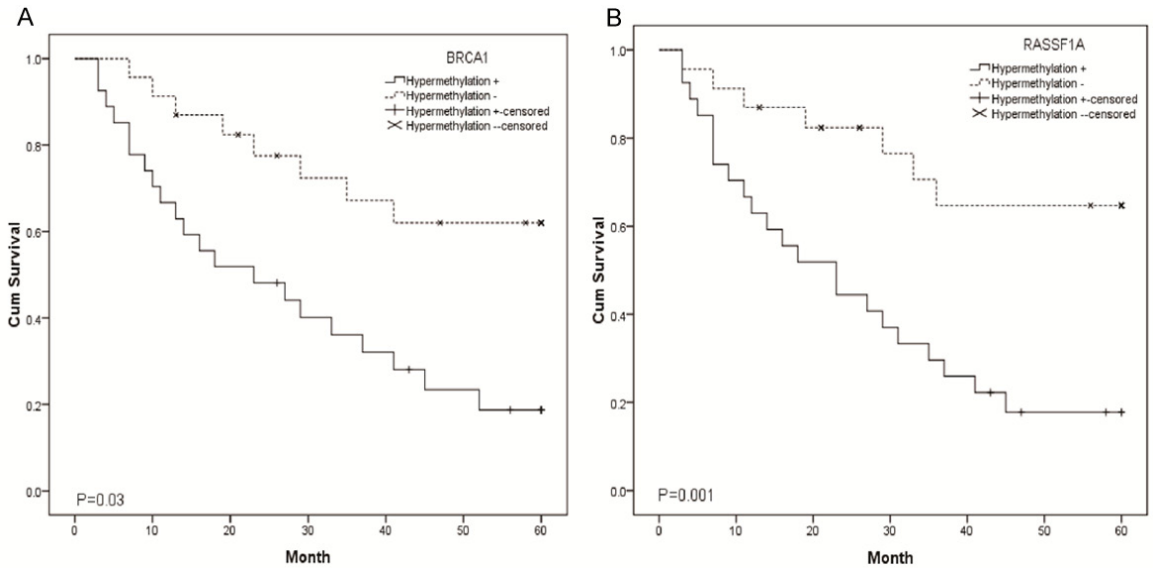

Figure 2. Kaplan-Meier Survival plot for NSCLC patients by (A) BRCA1 and (B) RASSF1A promoter methylation status in peripheral blood samples.

Table 2. Hazard ratios (HRs) and 95% confidence intervals (CIs) for the associations of gene promoter methylation status and mortality among NSCLC patients

Genes	No. of Cases	No. of Deaths	Hazard ratio (95% CI)
BRCA1			
Unmethylated	23	8	1.00 (Ref)
Methylated	27	16	2.053 (0.973-6.748)
RASSF1			
Unmethylated	19	7	1.00 (Ref)
Methylated	31	20	3.291 (1.287-8.316)

In tumor progression, methylation has been observed in tumor suppressor genes BRCA1 and RASSF1A. For example, Xu et al found that BRCA1 promoter methylation was associated with increased mortality among women with breast cancers [17]. Du et al reported that methylation of RASSF1A gene promoter and the correlation with DNMT1 expression that may contribute to esophageal squamous cell carcinoma [18]. Klacz et al showed that RASSF1A was hypermethylation in clear cell renal cell carcinoma tissues and associated with worse prognosis of clear cell renal cell carcinoma patients [19]. Wu et al indicated that there was a strong association between RASSF1A methylation and nasopharyngeal and highlighted a promising potential for RASSF1A methylation in nasopharyngeal risk prediction of Chinese [20]. However, the role of BRCA1 and RASSF1A methylation in NSCLC remains unclear.

In the present study, we explored the relationship between the promoter methylation of BRCA1 and RASSF1A tumor suppressor genes, and clinicopathological parameters and prognosis of NSCLC patients. We found significant difference between promoter methylation of cases than controls for BRCA1 and RASSF1A. Frequencies for the methylation of BRCA1 and RASSF1A were 54%, 62% respectively. Furthermore, we found significant correlation between promoter methylation of the two genes and histological grade and lymph nodes metastasis of NSCLC patients. Apart from histological grade and lymph nodes metastasis, we are not able to find any correlation between promoter methylation of these tumor suppressor genes and clinicopathological features of NSCLC patients. In addition, we also studied the prognostic significance of BRCA1 and RASSF1A in surgically treated NSCLC patients. We found that the patients with tumors showing the BRCA1 and RASSF1A promoter methylation had significantly poorer overall survival rates than the patients with tumors that did not show the BRCA1 and RASSF1A promoter methylation.

Limitations of the this study are: Firstly, we have a small samples, which is the biggest limitation. Secondly, we have not analyzed gene expression of these tumor suppressor genes, so we could not predict the exact effect of these promoter methylation in functional mechanism of gene silencing.

In conclusion, our data indicated that there have a significant association of BRCA1, and RASSF1A genes promoter methylation with NSCLC patients and they play important roles in lung cancer pathogenesis. Moreover, we also showed that promoter methylation of BRCA1 and RASSF1A was associated with increased advanced clinical stages and poor survival of NSCLC patients. Our results suggested that promoter methylation of BRCA1 and RASSF1A genes might provide additional values in NSCLC progression.

Disclosure of conflict of interest

None.

Address correspondence to: Zheng Zhou, Department of Respiratory Medicine, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450014, Henan, China. E-mail: zhouzheng8327@163.com

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancerstatistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Ettinger DS, Akerley W, Bepler G, Blum MG, Chang A, Cheney RT, Chirieac LR, D'Amico TA, Demmy TL, Ganti AK, Govindan R, Grannis FW Jr, Jahan T, Jahanzeb M, Johnson DH, Kessinger A, Komaki R, Kong FM, Kris MG, Krug LM, Le QT, Lennes IT, Martins R, O'Malley J, Osarogiagbon RU, Otterson GA, Patel JD, Pisters KM, Reckamp K, Riely GJ, Rohren E, Simon GR, Swanson SJ, Wood DE, Yang SC; NCCN Non-Small Cell Lung Cancer Panel Members. Non-small cell lung cancer. J Natl Compr Canc Netw 2010; 8: 740-801.

[3] Ettinger DS, Akerley W, Borghaei H, Chang AC, Cheney RT, Chirieac LR, D'Amico TA, Demmy TL, Ganti AK, Govindan R, Grannis FW Jr, Horn L, JahanTM, Jahanzeb M, Kessinger A, Komaki R, Kong FM, Kris MG, Krug LM, LennesIT, Loo BW Jr, Martins R, O'Malley J, Osarogiagbon RU, Otterson GA, Patel JD, Pinder-Schenck MC, Pisters KM, Reckamp K, Riely GJ, Rohren E, SwansonSJ, Wood DE, Yang SC, Hughes M and Gregory KM; NCCN (National Comprehensive Cancer Network). Non-small cell lungcancer. J Natl Compr Canc Netw 2012; 10: 1236-1271.

[4] Smith ZD and Meissner A. DNA methylation: roles in mammalian development. Nat Rev Genet 2013; 14: 204-220.

[5] De Carvalho DD, Sharma S, You JS, Su SF, Taberlay PC, Kelly TK, Yang X, Liang G and Jones PA. DNA methylation screening identifies driver epigenetic events of cancer cell survival. Cancer Cell 2012; 21: 655-667.

[6] Harada H, Miyamoto K, Yamashita Y, Nakano K, Taniyama K, Miyata Y, OhdanH and Okada M. Methylation of breast cancer susceptibility gene 1 (BRCA1) predicts recurrence in patients with curatively resected stage I non-small celllung cancer. Cancer 2013; 119: 792-798.

[7] Mengxi D, Qian W, Nan W, Xiaoguang X and Shijun L. Effect of DNA methylation inhibitor on RASSF1A genes expression in non-small cell lung cancer cell line A549 and A549DDP. Cancer Cell Int 2013; 13: 91.

[8] Portela A, Liz J, Nogales V, Setien F, Villanueva A and Esteller M. DNA methylation determines nucleosome occupancy in the 5'-CpG islands of tumor suppressor genes. Oncogene 2013; 32: 5421-5428.

[9] Wojdacz TK, Thestrup BB, Overgaard J and Hansen LL. Methylation of cancer related genes in tumor and peripheral blood DNA from the same breast cancer patient as two independent events. Diagn Pathol 2011; 6: 1596-1596.

[10] Brennan K, Garcia-Closas M, Orr N, Fletcher O, Jones M, Ashworth A, Swerdlow A, Thorne H, Riboli E and Vineis P. Intragenic ATM methylation inperipheral blood DNA as a biomarker of breast cancer risk. Cancer Res 2012; 72: 2304-2313.

[11] Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, Dunning MJ, Gale D, Forshew T, Mahler-Araujo B, Rajan S, Humphray S, Becq J, Halsall D, Wallis M, Bentley D, Caldas C and Rosenfeld N. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013; 368: 1199-1209.

[12] Lafuente-Sanchis A, Zuniga A, Galbis JM, Cremades A, Estors M, Martinez-Hernandez NJ and Carretero J. Prognostic value of ERCC1, RRM1, BRCA1 and SETDB1 in early stage of non-small cell lung cancer. Clin Transl Oncol 2016; 18: 798-804.

[13] Lee JU, Sul HJ and Son JW. Promoter methylation of CDKN2A, RARβ, and RASSF1A in non-small cell lung carcinoma: quantitative evaluation using pyrosequencing. Tuberc Respir Dis 2012; 73: 11-21.

[14] Tomasetti C and Vogelstein B. Variation in cancer risk among tissues can be explained by the

number of stem cell divisions. Science 2015; 347: 78-81.
[15] Dela Cruz CS, Tanoue LT, Matthay RA. Lung cancer: epidemiology, etiology, and prevention. Clin Chest Med 2011; 32: 605-44.
[16] Hackett JA, Reddington JP, Nestor CE, Dunican DS, Branco MR, Reichmann J, Reik W, Surani MA, Adams IR and Meehan RR. Promoter DNA methylation couples genome-defence mechanisms to epigenetic reprogramming in the mouse germline. Development 2012; 139: 3623-3632.
[17] Xu X, Gammon MD, Zhang Y, Bestor TH, Zeisel SH, Wetmur JG, Wallenstein S, Bradshaw PT, Garbowski G and Teitelbaum SL. BRCA1 promoter methylation isassociated with increased mortality among women with breast cancer. Breast Cancer Res Treat 2009; 115: 397-404.
[18] Du Z, Ma K, Sun X, Li A, Wang H, Zhang L, Lin F, Feng X and Song J. Methylation of RASSF1A gene promoter and the correlation with DNMT1expression that may contribute to esophageal squamous cell carcinoma. World J Surg Oncol 2015; 13: 141.
[19] Klacz J, Wierzbicki PM, Wronska A, Rybarczyk A, Stanislawowski M, SlebiodaT, Olejniczak A, Matuszewski M and Kmiec Z. Decreased expression of RASSF1A tumor suppressor gene is associated with worse prognosis in clear cell renal cell carcinoma. Int J Oncol 2016; 48: 55-66.
[20] Wu K, Xu X, Chen Y, Pu X, Wang B and Tang X. RASSF1A Gene Methylationis Associated with Nasopharyngeal Carcinoma Risk in Chinese. Asian Pac J Cancer Prev 2014; 16: 2283-2287.