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Original Article

Down-regulation of *PAX6* by promoter methylation is associated with poor prognosis in non small cell lung cancer

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Abstract: Background: Promoter methylation is an alternative mechanism of gene silencing in human tumorigenesis. Although a number of methylated genes have been found in non small cell lung cancer (NSCLC), useful methylation markers for early prognostic evaluation of NSCLC remain largely unknown. Methods: Using methylation-specific PCR (MSP), we examined promoter methylation status of *PAX6* gene, and explored their association with clinical features in NSCLC via chi-square test. NSCLC patient survival was assessed by Kaplan-Meier analyses and a Cox proportional hazard model was employed for multivariate analyses. Results: The methylation level of *PAX6* gene was higher in tumor tissues than that in normal tissues. In addition, *PAX6* promoter methylation showed a very significant correlation with differentiation ($P = 0.002$), distant metastasis ($P = 0.024$), and TNM stage ($P = 0.002$). *PAX6* gene promoter hyper-methylation was found to be significantly associated with poor overall survival ($P = 0.018$) and to serve as an independent marker for prognosis using multivariate Cox regression analysis (HR: 2.254, 95% CI: 1.088-4.667, $P = 0.029$). Conclusion: We found that *PAX6* gene was specifically methylated in NSCLC, and demonstrated the effect of promoter methylation of *PAX6* gene on clinical outcome in NSCLC, indicating the methylated *PAX6* may be useful biomarkers for prognostic evaluation in NSCLC.

Keywords: Non-small cell lung cancer, prognosis, *PAX6*, methylation

Introduction

In recent years, primary lung cancer occupies the leading cause of cancer death in the world [1]. Among primary lung cancer cases, approximately 80% of the cases are non-small-cell lung cancer (NSCLC), which is the most common form of lung cancer [2, 3]. Despite of advances in surgical techniques and treatment strategies, the survival of patients with NSCLC has been improved. However, long-term survival after surgical resection remains poor owing to the high rate of recurrence and metastasis [4, 5]. Therefore, it is considered important to clarify the mechanism of tumor biology to improve the clinical outcome of patients with NSCLC.

Epigenetic alterations are important for carcinogenesis, tumor maintenance, and development of therapeutic resistance, including DNA methylation [6-9]. In lung cancer, it has been reported that the clinical outcome is relevance to methylation at several loci [10-12]. Because the survival rates of patients with lung cancer are influenced enormously by epigenetic alterations [13], methylated genes may be used as molecular markers for disease outcome [14].

Paired-box 6 (*PAX6*), located on chromosome 11p13, encodes a transcription factor that is involved in various developmental processes of the eye and central nervous system [15]. *PAX6* is an important transcription factor during embryogenesis and a stem cell factor [16], and *PAX6* expression was recently found in tumors, therefore, *PAX6* may play an important role in tumorigenesis. To the best of our knowledge, correlations between *PAX6* gene promoter methylation and its relation to NSCLC clinicopathological parameters have so far not been addressed.

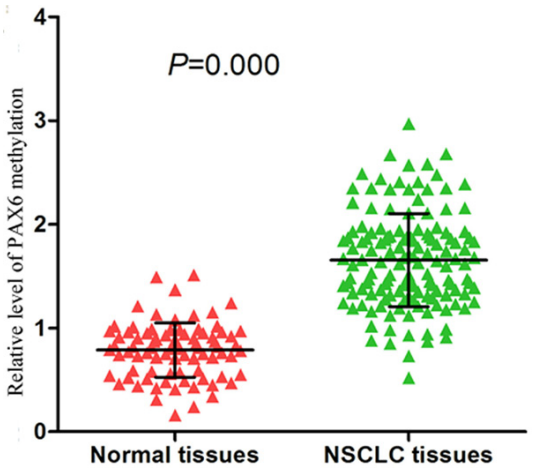

Figure 1. *PAX6* promoter methylation was detected in normal tissues and NSCLC tissues. The t-test analysis showed methylation of *PAX6* in NSCLC tissues was significantly higher than normal tissues (*P* = 0.000).

In the present study, we assessed the level of *PAX6* promoter methylation in NSCLC tissues and normal tissues. 143 NSCLC tissues were used to assess *PAX6* gene promoter methylation and its clinicopathological significance. In addition, it has been reported that *PAX6* expression may serve as an independent prognostic biomarker for improved survival in NSCLC patients [17]. Together, these observations prompted us to assess *PAX6* methylation as a possible prognostic biomarker of NSCLC patients.

Methods and materials

Patients and tissue samples

NSCLC tissue and their adjacent tissue samples were collected from 143 NSCLC patients who underwent pneumonectomy at the First Clinical Hospital Affiliated to Harbin Medical University (Harbin, China). None of the patients had undergone any medical treatment before surgery. As a measure of prognosis we analyzed overall survival (OS) rates, defined as the time from surgery to death by NSCLC, or to last contact. All recruited patients were subjected to periodic followed-up until due date. This study was approved by the institutional review board of China Medical University and each patient signed a consent form to participate in this study. Specimens were collected immediately after surgical excision, frozen in liquid nitrogen and stored at -80°C until DNA/RNA extraction. All NSCLC cases were pathologically confirmed. The mean age of the patients was 63.5 years (range: 25-79 years), and 61 of them were female and 82 were male. Non-malignant lung tissues were collected as control tissues, and were retrieved at least 5 cm away from the original tumor sites.

DNA extraction

The tissue samples were deparaffinized in xylene followed by ethanol incubation. Genomic DNA was isolated using a GENE ALL™ Tissue SV Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's recommendation. Briefly, the tissue samples were digested with proteinase K, and the DNA samples were bound to columns, washed and eluted. All paraffin-fixed tissues were centrifuged with 1,200 µL xylene and washed with ethanol. After being mixed with 20 µL proteinase K solution, the deparaffinized tissues were incubated at 56°C for 2 h. Finally, SV column and buffer were added in the tubes and centrifuged with the tissue samples. Supernatants were used for sodium-bisulfite modification.

Sodium-bisulfite modification

Extracted DNA was modified with sodium bisulfite using the EZ DNA Methylation™ Kit (Zymo Research, Orange, USA) following the kit protocols. Purified DNA was denatured with a dilution buffer and incubated with the CT conversion reagent (Zymo research) at 50°C for 12 to 16 h. The modified DNA was applied to columns (Zymo-Spin™ IC Column; Zymo Research) and centrifuged with 100 µL washing buffer. After the washing phase, 200 µL desulphonation buffer was added to the column, and the DNA was incubated at room temperature (20-30°C) for 20 min. Finally, the substrates were centrifuged for 30 s with an elution buffer. In this modification, the unmethylated cytosines were converted to uracils, whereas the methylated cytosines were unaffected in the reaction and remained as cytosines.

Methylation specific polymerase chain reaction (MSP)

The sodium bisulfite-converted DNA was amplified with Blend Taq®Plus (Toyobo, Osaka, Japan), using specific primers. The following *PAX6* primers were used to detect the methylated (M) or unmethylated (U) alleles of the

Table 1. Association of the level of *PAX6* methylation with clinicopathologic characteristics in NSCLC

Characteristics	No.	*PAX6* methylation status		X^2	*P* values
		Unmethylation	Methylation		
Age (years)					
≤ 70	56	26	30	3.295	0.069
> 70	87	28	61		
Gender					
Male	82	30	52	0.004	0.949
Female	61	22	39		
Tumor size					
≤ 3 cm	75	32	43	2.708	0.100
> 3 cm	68	20	48		
Histology					
Adenocarcinoma	79	25	54	1.698	0.193
Other	64	27	37		
Differentiation					
Well or moderate	86	40	46	9.602	0.002
Poor	57	12	45		
Lymphatic duct infiltration					
Yes	35	15	20	0.844	0.358
No	108	37	71		
Vessel infiltration					
Yes	45	20	25	1.853	0.173
No	98	32	66		
Distant metastasis					
Yes	50	12	38	5.079	0.024
No	93	40	53		
Smoking history					
Smoker	86	32	54	0.067	0.796
Never smoked	57	20	37		
TNM stage					
I	102	45	57	9.243	0.002
II-III	41	7	34		

PAX6 promoter: for methylated alleles, *PAX6*-MF 5'-TTTTTGGGGATCGATTTTAC-3', *PAX6*-MR 5'-G ACTAATTTTCCGACCGAAC-3'; for unmethylated alleles, *PAX6*-UF 5'-GTTTT TTGGGGATTGATTTTAT-3', *PAX6*-UR 5'-CCAACTAATTTTCCAACCAAAC-3'. The DNA samples were predenatured at 94°C for 2 minutes. Subsequently, the polymerase chain reaction (PCR) amplification was accomplished using a 35 cycles of denaturation at 94°C for 30 s, 54°C annealing for 30 s and extension at 72°C for 1 min, followed by 72°C for 10 min, according to the manufacturer's indication. The PCR products were analyzed on 2.5% agarose gels, stained with ethidium bromide, and then visualized by UV illumination. Results were confirmed by repeating the bisulfite reaction and MSP for all samples.

Statistical analyses

All the statistical analyses were performed by 18.0 SPSS statistical software (SPSS Inc, IL, USA). Chi-square test was used to compare the *PAX6* methylation rate in tissue samples with different clinicpathologic parameters. Survival curves were performed by the Kaplan-Meier model, and differences between different clinicpathologic parameters were determined by the log-rank test. The independent prognostic factors were identified by multivariate analysis based on the Cox proportional hazard model. A *P* value < 0.05 was considered to be statistically significant.

Results

Frequency of methylation status of PAX6 gene in NSCLC tissues and normal tissues

We examined promoter methylation of *PAX6* by using MSP approach in a total of 143 NSCLC tissues and 143 adjacent normal tissues. As shown in **Figure 1**, the overall methylation level of *PAX6* was higher in tumor tissues than those in normal tissues. The MSP product, the length of bp, was directly loaded onto a 2.5% agarose gel under the UV illumination.

Correlation of methylation status and clinicopathologic features

The association of *PAX6* methylation with clinicopathological characteristics was analyzed in NSCLC patients via chi-square test. As shown in **Table 1**, the methylation level of *PAX6* gene

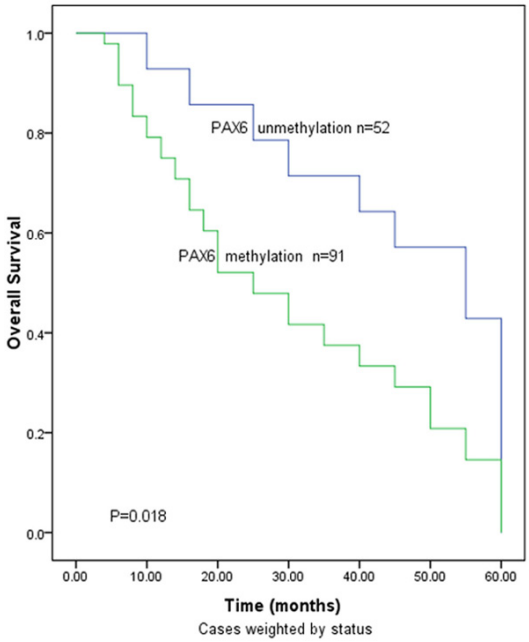

Figure 2. Kaplan-Meier survival curves according to *PAX6* methylation level. Patients with *PAX6* methylation expression (n = 91) had a significantly poorer prognosis than those with *PAX6* unmethylation expression (n = 52, *P* = 0.018). *P* value was calculated by Log-rank test.

Table 2. Multivariate analysis for factors influencing the overall survival rate of NSCLC patients

Characteristics	HR	95% CI	*P* values
PAX6 methylation	2.254	1.088-4.667	0.029
Differentiation	1.079	0.502-2.320	0.846
Distant metastasis	1.098	0.598-2.019	0.762
TNM stage	1.028	0.544-1.945	0.932

was found to be significantly associated with differentiation (*P* = 0.002), distant metastasis (*P* = 0.024), and TNM stage (*P* = 0.002). However, there was no relationship with other clinical parameters, such as age, gender, tumor size, histology, lymphatic duct infiltration, vessel infiltration, Smoking history (all *P* > 0.05).

Methylation status and clinical outcomes

Kaplan-Meier analysis confirmed that NSCLC patients with *PAX6* methylated tumors had a low overall survival rate than the patients with unmethylated tumors (log rank test, *P* = 0.018, **Figure 2**). Cox regression analysis revealed that methylation status of *PAX6* was an independent predictor in the prognosis of NSCLC patients (HR: 2.254, 95% CI: 1.088-4.667, *P* = 0.029, **Table 2**).

Discussion

DNA methylation is one of the most widely researched epigenetic events [18, 19] and represents one of the most common epigenetic changes in human cancers. DNA hyper-methylation could, among others, lead to the down-regulation or silencing of tumor suppressor genes (TSGs). It has been reported that DNA methylation of the 5'-CpG island is a major mechanism of tumor suppressor gene inactivation in NSCLC [8, 20]. Kim et al. have suggested that loss of the *Wnt7a* gene induced by promoter methylation might be another prognostic factor for NSCLC [21]. Considered the fact that epigenetic alterations such as DNA methylation usually occur before the generation of genetic alterations, aberrant methylation patterns at CpG islands of TSG promoters may serve as biomarkers for the prognosis of NSCLC.

PAX transcription factors regulate developmental processes, which belong to the paired box gene family [22-24]. As a transcription factor, *PAX6* has been shown to regulate the expression of a broad range of molecules, hormones and structural proteins [15], and act as a critical regulator in several kinds of cancers [23, 25-28]. Additionally, *PAX6* function as either oncogene or tumor suppressor seems to be tissue specific and is discussed controversially [22, 23, 29]. However, methylation promoter of *PAX6* in NSCLC has never been illustrated. In this study, we detected *PAX6* promoter methylation status in patients with NSCLC by MSP. The results revealed that methylation *PAX6* level was higher in NSCLC tissues than those in normal tissues. Pesek *et al.* have found that *PAX6* exhibited methylation status frequently in NSCLC patients [30]. Tibor *et al.* have showed that there were 12 CpG islands of *PAX6* gene confirmed to be methylated in 85% to 100% of the squamous cell carcinomas [31]. Our findings were consistent with above research. However, the viewpoint was controversial. Zhao *et al.* have reported that *PAX6* mRNA levels were significantly higher in primary lung cancer tissues compared to their matched adjacent tissues, which accelerated cell cycle progression by activating MAPK signal pathway [29].

This is the first study to analyze the relationship between *PAX6* methylation status and clinicopathological factors in NSCLC through chi-square test. The results showed that *PAX6* methylation status was significantly association with differentiation, distant metastasis and TNM stage. However, there was no relationship with other clinical characteristics, including age, gender, tumor size, adenocarcinoma, lymphatic duct infiltration, vessel infiltration and smoking history.

We also analyzed the prognostic effects of *PAX6* methylation on overall survival using Kaplan-Meier method and log-rank test. The results revealed that the patients with *PAX6* methylation have shorter overall survival time than that with *PAX6* unmethylation. Yang *et al.* reported that *PAX6* hypermethylation predict worse survival in gastric cancer [32]. We thought *PAX6* methylation might be related to the prognosis of NSCLC, so we investigated the prognostic values of *PAX6* methylation status in NSCLC in multivariate analysis using Cox regression model. Interestingly, we observed that the patients with *PAX6* methylation levels in cancerous tissues trended toward unfavorable outcomes.

In conclusion, this study demonstrated that the methylation level of *PAX6* was higher in NSCLC tissues than that in normal tissue. In addition, the methylation status of *PAX6* was associated with a poor overall survival in cancer tissues. We suggest that the methylation status of the *PAX6* gene may be a promising biomarker for assessing the prognosis of NSCLC patient.

Disclosure of conflict of interest

None.

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