Biochemical and Biophysical Research Communications 458 (2015) 381–386

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NPTX1 is a novel epigenetic regulation gene and associated with prognosis in lung cancer

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ARTICLE INFO

Article history:
Received 18 January 2015
Available online 31 January 2015

Keywords:
NPTX1
Promoter methylation
Prognosis
Lung cancer

ABSTRACT

Background: CpG island hypermethylation of gene promoters is a well-known mechanism of epigenetic regulation of tumor related-genes and is directly linked to lung carcinogenesis. Alterations in the pattern of methylation of the NPTX1 gene have not yet been studied in detail in human lung cancer.
Methods: Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) were used to analyze promoter methylation status, and real-time quantitative reverse transcription-PCR (qRT-PCR) examined mRNA levels. Subsequently, we compared the methylation profile of NPTX1 in samples of neoplastic and non-neoplastic lung tissue taken from the same patients by using quantitative methylation specific PCR (QMSP).
Results: CpG island hypermethylation in promoter of NPTX1 was confirmed in lung cancer cell lines. A significant increase in NPTX1 methylation was identified in lung cancer specimens compared to adjacent noncancerous tissues and that it was negatively correlated with its mRNA expression. The overall survival time among patients carrying methylated NPTX1 tumors was significantly shorter as compared to those with unmethylated NPTX1 tumors ($P = 0.011$). Moreover, methylation of NPTX1 gene was found to be an independent prognostic factor for poor overall survival based on multivariate analysis models ($p = 0.021$), as was age $\geq$60 years old ($p = 0.012$) and TNM stage ($p < 0.001$).
Conclusions: These results suggest that NPTX1 hypermethylation and consequent mRNA changes might be an important molecular mechanism in lung cancer. Epigenetic alterations in NPTX1 may serve as potential diagnostic and prognostic biomarkers in lung cancer.

1. Introduction

Lung cancer is one of the most common malignant tumors with highest incidence and mortality [1,2]. Although the overall prognosis for patients with lung cancer is poor with a five-year survival of <15%, patients diagnosed with early stage disease have a much more favorable prognosis [3,4]. Therefore, many efforts have been made in searching and validating novel and meaningful molecular diagnostic or prognostic biomarkers for lung cancer [5,6].

Methylation, one of the most common molecular alterations in human neoplasia refers to the addition of a methyl group to the cytosine ring of those cytosines that precede a guanosine (referred to as CpG dinucleotides) to form methyl cytosine (5-methylcytosine). A growing body of evidence indicates that aberrant methylation of cytosine-guanine dinucleotide (CpG) islands in the promoter regions of tumor-related genes downregulates these genes by suppressing transcription [7]. Promoter methylation in various tumor-related genes has been demonstrated to play a vital role in the development of lung cancer [8], and has thus been used as a molecular biomarker to predict the outcome of cancer [9].

Neuronal pentraxin 1 (NPTX1), mapping to chromosome 17q25.3, encodes a binding protein involved in the regulation of cell immortalization [10] and neural induction [11]. NPTX1 was first

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http://dx.doi.org/10.1016/j.bbrc.2015.01.124
0006-291X/

identified as an epigenetic target by the genome scanning technique which provides a global analysis of methylation events in human pancreatic cancers [12]. Since then, NPTX1 has been suggested as a methylation marker in cervical cancers [13,14] and colorectal cancer [15]. However, data concerning the relationship between promoter methylation of NPTX1 and lung cancer were not reported yet. Therefore, our present studies provide the first evidence for epigenetic regulation of NPTX1 in lung carcinogenesis.

In this study, we identify the methylation status of NPTX1 promoter in lung cancer cell lines and primary lung tumor tissues, and compare the methylation profile and mRNA expression level of NPTX1 in samples of malignant and non-malignant lung tissue obtained from the same participants.

2. Materials and methods

2.1. Cell culture

The human lung cancer cell lines A549, HCC827, H1975, and H460 were purchased from American Type Culture Collection ATCC (Manassas, VA, USA) and were cultured in 5% CO_2 at 37 °C in RPMI 1640 medium, supplementary with 10% fetal bovine serum (Life Technologies, Carlsbad, CA), 100 unit/ml penicillin, and 100 g/ml streptomycin.

2.2. Patients and tissue samples

A total of 188 (primary tumor samples, n = 94; corresponding nonmalignant lung tissues, n = 94) formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from patients with lung cancer who had been diagnosed between July 2009 and February 2011 with curative resectional surgery at the Department of Medicine, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University.

Patients who were enrolled for present study should fulfill the criteria as follows:(1) no radiotherapy or chemotherapy was given before surgery; (2) no concurrent primary cancer of any other organs and no prior cancer history. All adjacent noncancerous specimens were harvested freshly from morphologically normal appearing tissue located at least 3 cm from the tumor margin. A surgical pathologist performed histological evaluation of the tumor and non-tumor tissues. All cases of cancer were staged according to the guideline of the tumor–node– metastasis (TNM) classification of the Union for International Cancer Control (UICC). This study was approved by the ethics committee of the hospital and informed consent was obtained from all participants before surgery. Clinical details are listed in Supplementary Table 1.

2.3. DNA extraction and bisulphite conversion

DNA was isolated from FFPE tissue samples and lung cancer cell lines using DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany). Then, the extracted DNA was subjected to bisulfite treatment, which was performed using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions.

2.4. Analysis of methylation specific PCR (MSP) in lung cancer cell lines

The methylation status of NPTX1 promoter in cell lines and tissue samples was detected by conventional MSP by using specific primer pairs for both the methylated and unmethylated promoter sequences. The MSP reaction was performed using a Technet-512 (Technet, Staffordshire, UK), included an initial incubation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 56 °C for 20 s and 72 °C for 20 s, followed by one cycle of 72 °C for 10 min. Each reaction was performed in a total volume of 20 ml. In all, 2 μl of bisulphite-converted DNA was added into a 18 μl reaction mixture that contained 600 nmol/L of each primer, 1unit of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 200 μmol/L of each dATP, dCTP, dGTP, and dTTP (Invitrogen); 16.6 mmol/L of ammonium sulfate; 67 mmol/L of Trizma, 6.7 mmol/L of magnesium chloride; 10 mmol/L of mercaptoethanol; and 0.1% dimethyl sulfoxide. Each sample was amplified in two separate reactions, one with a set of primers specific for the methylated and one for the unmethylated NPTX1 promoter sequences. Primer sequences are shown in Supplementary Table 2. The products were visualized on 2% agarose gels.

2.5. Bisulphite sequencing

The fragment containing the transcription start site of human promoter of NPTX1 was analyzed using Methyl Primers Express software (Applied Biosystems, Foster City, CA) to identify CpG islands, and corresponding primers (Supplementary Table 2) were used for bisulfite sequencing. These primers were used to amplify bisulphite-converted DNA spanning −605 to −144 bp upstream of the NPTX1 transcription start site. The PCR product was TOPO-cloned into the pCR4 vector (Life Technologies) and 5 positive clones were selected for sequencing. The methylation result produced from bisulfite sequencing were analyzed using BiQ Analyzer software to generate a lollipop diagram. The lollipop diagram presented the percent of methylation for each CpG. Analyses of non-CpG cytosines indicated that the efficiency of bisulfite conversion was ~99%.

2.6. 5-Aza-2-deoxycytidine treatment

The demethylation treatment was performed with two different concentrations (2.5 and 5 μM) of 5-aza-2-deoxycytidine (5-AZA) (Sigma, St. Louis, MO). The medium containing 5-AZA was changed every 24 h for 3days and treated cells were harvest at 72 h.

2.7. RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from primary lung cancers, paired noncancerous tissues, and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The synthesized cDNA were subsequently used as a template to measure the mRNA expression of NPTX1 by qRT-PCR. The primer sequences used for amplification of NPTX1 and ACTB are listed in Supplementary Table 2. The SYBR green Premix Ex Taq TM kit (Takara Bio Inc., Shiga, Japan) was used for qRT-PCR assay which was performed using an ABI 7500 Sequence Detector (Applied Biosystems). NPTX1 mRNA levels were normalized to ACTB and the $2^{-\Delta\Delta Ct}$ method was used to analyze the relative levels of NPTX1 expression.

2.8. Quantitative methylation specific PCR (QMSP) assay of NPTX1 methylation status in lung cancer tissues

The bisulfite-modified DNA samples were used as a template for fluorescence-based QMSP. In brief, primers and probes (Supplementary Table 1) were designed to specifically amplify the bisulfite converted sequence of NPTX1. Amplifications were carried out in 384-well plates in a 7900 Sequence Detector System (Applied Biosystems). The relative level for NPTX1 in each sample was determined as a ratio of QMSP-amplified NPTX1 to ACTB and then multiplied by 1000 for easier tabulation (average value of triplicates of gene of interest divided by the [average value of triplicates of

ACTB] × 1000). The samples were categorized as unmethylated or methylated based on the sensitivity of the assay. QMSP reactions were carried out in a reaction volume of 20 μl consisting of 1.2 μmol/L of forward and reverse primers; 200 nm/L of the probe; 1U of Platinum Taq DNA polymerase (Invitrogen); 200 μmol/L of each dATP, dCTP, dGTP, and dTTP (Invitrogen); 16.6 mmol/L of ammonium sulfate; 67 mmol/L of Trizma; 8.7 mmol/L of magnesium chloride; 10 mmol/L of mercaptoethanol; and 0.1% dimethyl sulfoxide, and 20 ng of bisulphite-modified DNA. The condition of the QMSP was as follows: 95 °C for 10 min for denaturation, 50 cycles of amplification (95 °C, 30 s; 58 °C, 15s; 72 °C, 20 s) with a final elongation step of 10 min at 72 °C. Each plate contained tissue samples, water blanks, and positive controls. Leukocyte DNA from a healthy individual was methylated in vitro with excess SssI methyltransferase (New England Biolabs Inc., Beverly, MA) to generate completely methylated DNA, and serial dilutions (50–0.005 ng) of this DNA were used to construct a calibration curve. Each reaction was done in triplicate, the average of the triplicate was considered for analysis.

2.9. Statistical analysis

Statistical analysis was performed using the SPSS 17.0 (IBM, Armonk, NY) and Prism 5 (GraphPad Software, San Diego, CA) software. The methylation and expression of NPTX1 in paired lung tissues was analyzed using paired-samples t test. Associations between the methylation and clinicopathological characteristics were analyzed using a chi-square test or Fisher exact test. Overall survival (OS) was measured from the day of surgery until the date of death. Survival time estimates were done by using the Kaplan–Meier method, and the differences in OS were compared using the log-rank test. Cox's proportional hazard regression analysis was used to analyze the hazard ratios (HRs) and 95% confidence intervals (CIs) of independent factors for patients' survival, with adjustments for NPTX1 methylation status (negative vs positive), age (<60 vs $\geq$ 60 years), gender (female vs male), smoking condition (former, never-vs current-smoker), histological type (adenocarcinoma vs squamous cell carcinoma), lymph node metastasis (N_0 vs $N_{1\text{-}3}$), and TNM stage (I,II vs III, IV). A p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. NPTX1 promoter hypermethylation in lung cancer cell lines

To initially examine if the NPTX1 gene is an epigenetic target in lung cancer, we tested NPTX1 promoter methylation status in four lung cancer cell lines and two nonmalignant lung tissues. We observed that NPTX1 promoter was hypermethylated in H1975, HCC827, H460 and A549 cell lines, but only weakly methylated in nonmalignant lung tissues (Fig. 1A). In contrast, two nonmalignant tissue samples were strongly expressed unmethylated NPTX1 promoter fragment.

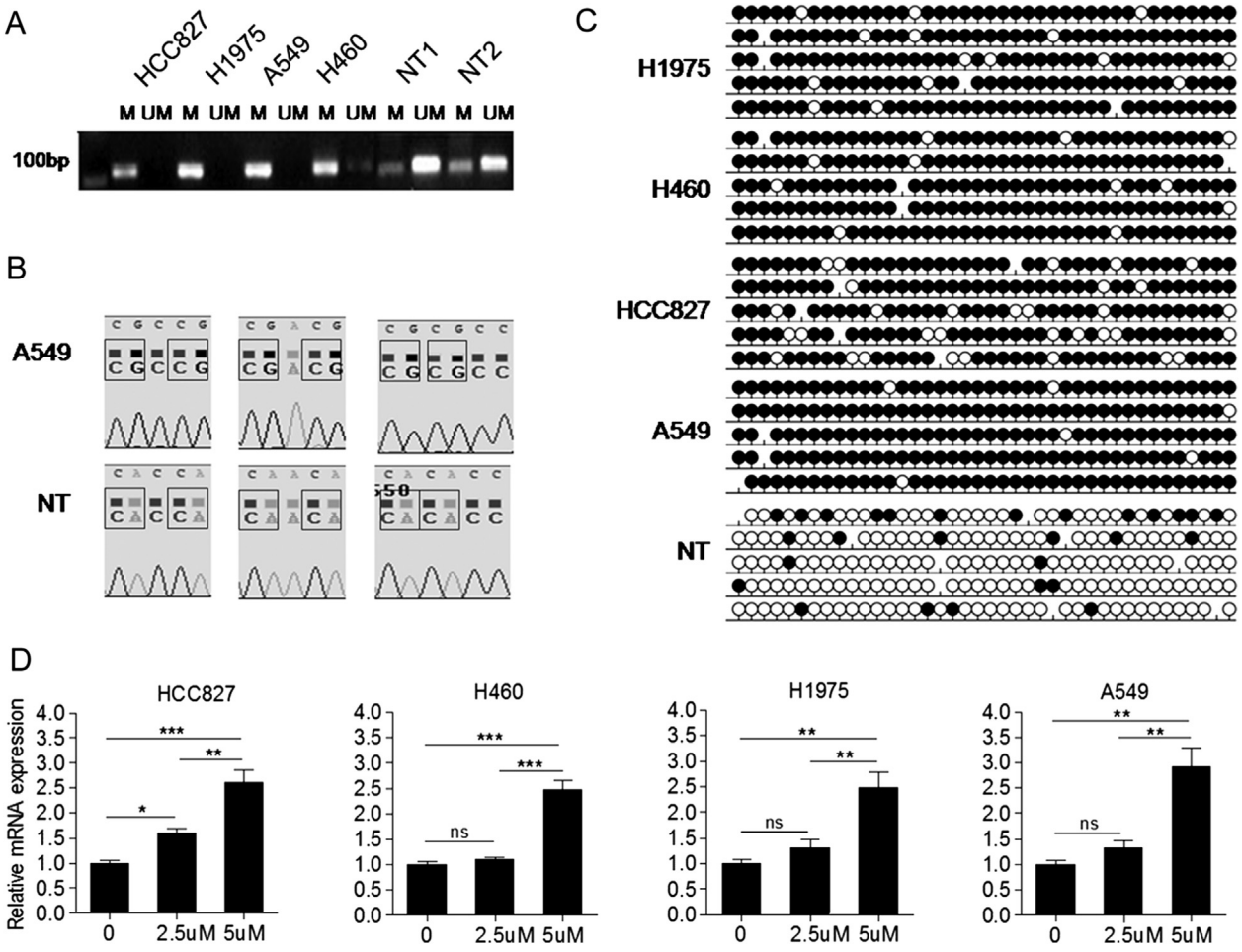

Fig. 1. Methylation analysis of NPTX1 by MSP, BSP, and 5-AZA treatment. (A) MSP of NPTX1 in lung cancer cell lines and nonmalignant normal tissues. (B) Representative sequences of BSP in A549 cell line and nonmalignant normal tissues. CpG dinucleotide loci in normal tissue were unmethylated while all CpG loci were methylated in A549 cells. (C) Methylation status of 56 CpG dinucleotides in the promoter region of NPTX1. Black dot, methylated CG; white dot, unmethylated CG; stub, not available. (D) qRT-PCR analysis of NPTX1 mRNA expression after 0, 2.5 or 5 μM 5-AZA treatment for 72 h *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance; independent t-test.

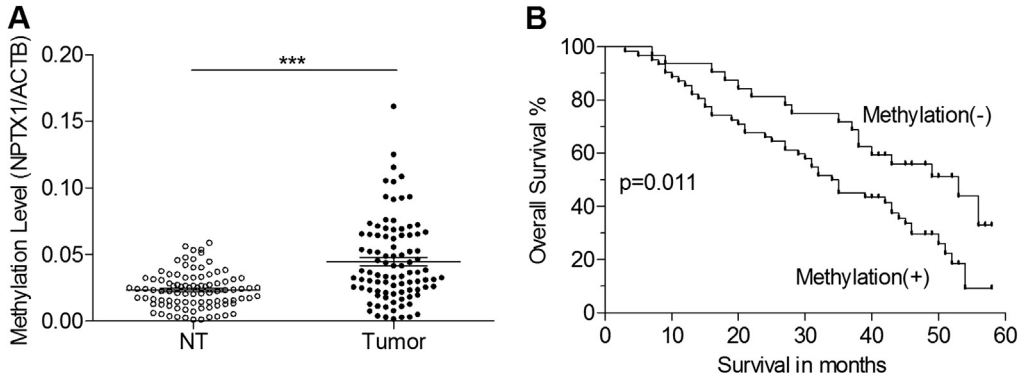

Fig. 2. Clinical significance of NPTX1 methylation. (A) Comparison of NPTX1 methylation between primary lung cancers and adjacent normal tissues by QMSP. NT, normal tissues; ***, P < 0.001; paired-samples t test. (B) The overall survival analysis of lung cancers with negative or positive NPTX1 methylation by Log–Rank test.

In order to define the specific CpG site methylation status, we performed bisulfite sequencing analysis of the CpG islands in the NPTX1 promoter region. The promoter region (−605 to −144) including 56 CpG sites was amplified by PCR and sequenced. The 56 CpG sites were identified to be hypermethylated in all four cell lines, whereas nonmalignant tissue showed partial methylation in this region (Fig. 1B and C). These results demonstrated NPTX1 promoter hypermethylation status in lung cancer cell lines.

To further analysis whether hypermethylation of the NPTX1 promoter regulates its mRNA expression, cancer cell lines were treated with the demethylating agent 5-AZA. After 5-AZA treatment, NPTX1 mRNA expression was up-regulated in all four cell lines (Fig. 1D). These results indicate that hypermethylation of the NPTX1 promoter directly regulates its transcriptional repression in lung cancer cell lines.

3.2. Aberrant methylation level of NPTX1 is observed in lung cancer

The methylation level of NPTX1 in primary lung tumors and corresponding nonmalignant tissues was measured by QMSP. We observed a significantly increased methylation level in primary tissues (0.043 ± 0.029) compared with nonmalignant tissues (0.023 ± 0.013) (p < 0.001, Fig. 2A). Primary tumor tissues exhibited higher methylation frequency of NPTX1 than adjacent noncancerous tissues (66% vs 29%, p < 0.001, Table 1). However, it was also found that the frequency of NPTX1 promoter methylation did not correlate with gender, age, histological type, or the clinical stage of patients. All results are shown in Table 1.

In order to confirm that NPTX1 is down-regulated in tumor tissues by promoter hypermethylation, we performed qRT-PCR to measure the mRNA expression levels in lung cancers samples (n = 72) and matched nonmalignant lung tissues (n = 72). The mRNA levels were significantly down-regulated in cancer tissues as compared to the normal tissues (70.553 ± 15.972 vs 77.839 ± 17.057, p = 0.006). More importantly, the mRNA level of NPTX1 in advanced lung cancers (66.390 ± 12.591) was significantly lower than that in early stage of lung cancers (75.206 ± 16.185) (p = 0.047, Table 2). The expression of NPTX1 did not associate with other clinicopathological features. Furthermore, we identified a negative correlation between NPTX1 methylation and expression (Pearson's correlation, r = −0.580, p = 0.000, Table 3).

3.3. Promoter methylation of NPTX1 in lung cancer correlates with poor survival

To study if there is an association between patient overall survival time and promoter methylation of NPTX1, the completely clinical follow-up data were analyzed. We found that promoter methylation of NPTX1 correlated with a significantly worse prognosis (Fig. 2B). Kaplan–Meier survival analysis revealed that lung cancer patients with methylated NPTX1 had a shorter survival time than the patients with unmethylated NPTX1 (means for survival time (month):32.048 ± 9.899 vs. 40.188 ± 0.707, P = 0.011).

The correlation of clinicopathological characteristics and promoter methylation statue of NPTX1 with clinical outcome was also analyzed in lung cancer patients. Cox proportional hazard regression analysis showed prognostic significance for the age above 60 years old, current smoker, higher TNM stage, and the presence of lymph node metastasis (p = 0.030, 0.011, 0.001 and 0.010, respectively, Table 4). The presence of methylation of NPTX1 was associated with a relative of death of 2.01 [95% confidence interval (95% CI): 1.104–3.660, p = 0.022]. We also performed multivariate analysis, these dates revealed that only hypermethylation of NPTX1 (HR, 1.974; 95% CI, 1.107–3.517), higher TNM stage (HR, 3.420; 95% CI, 1.951–5.956), and the age above 60 years old (HR, 2.091; 95% CI, 1.179–3.708) was independently associated with a significantly increased risk of death (Table 4). Gender, smoking condition,

Table 1
Correlation between NPTX1 methylation and clinicopathological characteristics.

Characteristics	No. of patients (%)	Methylation status	p value[b]
		Positive (%)	
Gender			0.481
Male	60(64)	38(63)	
Female	34(36)	24(71)	
Age (years)			0.353
< 60	35(37)	21(60)	
≥60	59(63)	41(69)	
Smoking condition			0.114
Former, never	40(43)	30(75)	
Current	54(57)	32(59)	
Histological type			0.441
Adenocarcinoma	47(50)	29(62)	
Squamous cell carcinoma	37(39)	26(70)	
Other	10(11)	7(70)	
TNM[a] stage			0.307
I, II	43(46)	26(60)	
III, IV	51(54)	36(71)	
Lymph node metastasis			0.524
N_0	34(36)	21(62)	
N_{1-3}	60(64)	41(68)	
Tissue			
Tumor tissue	94(100)	62(66)	< 0.001
Adjacent normal tissues	94(100)	27(29)	

[a] Union for International Cancer Control; Former, stop smoking over two year; Never, no smoking history; Current, current smoker.
[b] Calculated by Fisher exact test.

Table 2
The expression level of NPTX1 in seventy-two lung cancer patients.

Characteristics	No. of patients (%)	NPTX1 mRNA	p value
Gender			
Male	48(67)	71.448 ± 15.197	0.612[a]
Female	24(33)	68.763 ± 17.621	
Age(years)			
< 60	29(40)	68.356 ± 16.134	0.38[a]
≥60	43(60)	72.133 ± 15.221	
Smoking condition			
Former/never	28(39)	67.264 ± 14.791	0.35[a]
Current	44(61)	72.646 ± 16.501	
Histological type			
Adenocarcinoma	38(53)	70.471 ± 16.287	0.675[b]
Squamous cell carcinoma	30(42)	71.847 ± 15.806	
Other	4(5)	61.630 ± 15.087	
TNM stage			
I, II	34(47)	75.206 ± 16.185	0.047[a]
III, IV	38(53)	66.390 ± 12.591	

[a] Calculated by Mann–Whitney test.
[b] Calculated by Kruskal–Wallis test.

Table 3
Association of NPTX1 expression with the gene promoter methylation.

	Expression	Methylation
Tumor tissues	70.553 ± 15.972	0.043 ± 0.029
Adjacent normal tissues	77.839 ± 17.057	0.023 ± 0.013
Pearson's correlation, r = −0.580, p = 0.000		

histological type, and Lymph node metastasis were not independent significant risk factors.

4. Discussion

The prevalence and mortality of lung cancer have shown a rising trend and appear to have stabilized the leading cause of cancer-related death among men in China [16]. Although improvements in treatment of lung cancer patients, a substantial percentage of patients still have a poor prognosis with a 5-year survival only for % 15, mainly due to the lack of effective early diagnosis. Increasing evidence supporting that promoter hypermethylation of tumor-related gene can be used as a sensitive marker for lung cancer early diagnosis and prognosis prediction [8,9]. However, the molecular events responsible for lung cancer initiation and progression are still largely unclear. Thus, searching for novel epigenetic target may be help for understanding of pathogenesis in lung cancer. To our knowledge, this is the first study of NPTX1 methylation in lung cancer.

In the present study, hypermethylation of the NPTX1 promoter was found in 4/4 lung cancer cell lines, indicating a high prevalence of NPTX1 methylation in lung cancer. In addition, NPTX1 promoter methylation was observed in 3/7 pancreatic cancer cell lines [12]. The methylation status of NPTX1 was different in various cancer cell lines might be due to tissue-specificity and the primer set which we used was different from the others. Considering epigenetic silencing of cancer-related genes has been shown to be reversible [17,18]. Consequently, we analyzed NPTX1 expression in lung cancer cells cultured in the presence of the demethylation agent 5-AZA. As our supposed, the expression level of NPTX1 in lung cancer cell lines was significantly up-regulated after 5-AZA treatment. In general, these results reveal that the promoter methylation of NPTX1 has a critical role in its transcriptional downregulation, which may be involved in lung cancer development.

In order to investigate the clinical significance of NPTX1 promoter methylation, we determined NPTX1 methylation level in 94 pairs of primary lung cancers and mRNA expression in 72 pairs of primary lung cancers. As our shown, the methylation level of NPTX1 was markedly increased in primary lung tumors, indication hypermethylation of NPTX1 might be a potential biomarker for lung cancer diagnosis. Similarly, methylation of NPTX1 was detected with high frequency in colorectal cancer [15] and cervical carcinomas [14]. Methylation of NPTX1 has previously been reported to be more common in colorectal cancers compared to adenomas [15], but our data revealed no substantial differences in NPTX1 methylation frequency between squamous cell carcinomas and adenocarcinomas. Further analysis regarding on correlations between methylation of NPTX1 and histological typing of cancer may be provide more information. Furthermore, the expression of NPTX1 was inversely correlated with promoter methylation level, indicating that NPTX1 is an epigenetic regulation gene in primary lung tumors. Interestingly, we observed a significant down-regulation of mRNA expression and no significant difference of methylation frequency between advanced cancer and the early stage of cancer. Recently, it was found that NPTX1 gene is a target of as a hypoxia-inducible factor and it regulate NPTX1 expression by binding to hypoxia responsive elements (HREs) in its promoter region [19]. Thus, we supposed that HIF-1α or other mechanisms may be involved in the transcription regulation of NPTX1 in the development of lung cancer.

Epigenetic regulation of NPTX family has been reported in association with various types of tumors [20–22]. Hypermethylation of NPTX2 in the tissue samples or serum of pancreatic cancer could be a promising diagnostic marker [21,23]. Moreover, down-regulation of NPTX2 played an anti-tumor effect on pancreatic cancer cells [24], while overexpression induced apoptosis and inhibited proliferation in glioblastoma [25]. These findings suggest that the NPTX family plays an important role in tumorigenesis. Further study concerning on the biological function of NPTX1 would greatly promote our knowledge of the molecular mechanisms of lung cancer.

In conclusion, this is the first study to describe NPTX1 as a novel methylation gene in lung cancer, thereby providing a potential marker for early diagnosis and prognosis prediction. Moreover, our findings indicate that the promoter hypermethylation contributes

Table 4
Clinical characteristics of patients correlate with overall survival by Cox proportional hazard regression analysis.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	p value	HR	95% CI	p value
NPTX1 methylation status	2.010	1.104–3.660	0.022	1.974	1.107–3.517	0.021
Age	1.930	1.067–3.491	0.030	2.091	1.179–3.708	0.012
Gender	0.833	0.463–1.683	0.705			
Smoking condition	2.294	1.214–4.335	0.011			
Histological type	1.324	0.926–1.893	0.124			
TNM stage	2.842	1.566–5.155	0.001	3.420	1.951–5.956	< 0.001
Lymph node metastasis	2.134	1.208–3.769	0.010			

HR, hazard ratio; CI, confidence interval.

to lower NPTX1 expression in lung cancer, which may lead to cancer pathogenesis.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was funded by National Key Scientific & Technology Support Program: Collaborative innovation of Clinical Research for chronic obstructive pulmonary disease and lung cancer (NO. 2013BAI09B09).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.101.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.101.

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