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Analysis of p16 gene mutations and their expression using exhaled breath condensate in non-small-cell lung cancer

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Abstract. The aim of the present study was to investigate the mutational status of exons 1 and 2 of the p16 gene in the exhaled breath condensate (EBC) of patients with non-small-cell lung cancer (NSCLC) and determine the feasibility and clinical significance of applying EBC in the diagnosis of NSCLC. Polymerase chain reaction and DNA sequencing were applied to detect exon 1 and 2 alterations of the p16 gene in EBC by comparing 58 samples from NSCLC patients and 30 from healthy controls. Of the 58 EBC samples from NSCLC patients, 54 were successfully tested and 8 cases of mutations were identified, of which 3 were in exon 1 and 5 in exon 2. The mutation rate was 14.81% (8/54). There were no p16 gene mutations in the 30 samples obtained from healthy controls. EBC p16 gene mutations exhibited no statistically significant differences according to gender, smoking history, pathological type, degree of differentiation and presence or absence of lymph node metastasis. The p16 gene mutation rate was proportional to the tumor stage ($P<0.05$). Therefore, the detection of the p16 gene mutation in EBC may be used as a novel molecular marker to assist in the diagnosis of NSCLC.

Introduction

Lung cancer is one of the most common malignancies, with a 5-year survival rate of patients with non-small-cell lung cancer (NSCLC) of only 15%. Early diagnosis and timely, appropriate treatment are crucial for improving the survival rates of patients with lung cancer (1-3). Therefore, the identification of lung cancer molecular markers to improve early diagnosis is a research field that has attracted significant attention over the last few years. As the exhaled breath condensate (EBC) is directly obtained from the respiratory tract and lungs, EBC component testing may reflect the pathological and physiological function of the lungs and signify airway inflammation, as well as other respiratory diseases, including lung cancer (4,5). The aim of the present study was to investigate the suppressor p16 gene mutation rate in the EBC of patients with NSCLC, determine its association with the occurrence and development of lung cancer and assess its clinical value in NSCLC diagnosis.

Patients and methods

Subjects. A total of 58 patients with NSCLC, who underwent surgery and pathological diagnosis at the Department of Thoracic Surgery of the Second Affiliated Hospital of Nantong University (Nantong, China) between March, 2010 and April, 2012, were included in this study. None of the patients had received radiotherapy and chemotherapy preoperatively. Of the 58 patients, 36 were male and 22 female, with a mean age of 63.6±7.4 years (range, 48-78 years). A total of 32 patients were smokers and the remaining 26 were non-smokers. Of the 58 cases, 32 were squamous cell carcinomas and the remaining 26 were adenocarcinomas. A total of 20 patients had poorly differentiated, 26 moderately differentiated and 12 well-differentiated tumors and 20 patients presented with lymph node metastases. According to the classification standards of the Union for International Cancer Control in 2009, the cases were classified as follows: Stage I, 18 patients; stage II, 24 patients; and stage III/IV, 16 patients. For the control group, 30 healthy subjects were selected, including 9 men and 21 women, with a mean age of 59.7±13.1 years (range, 35-79 years).

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Nantong University and all the participants signed the informed consent form.

EBC collection. EBC was collected from healthy controls and NSCLC patients prior to surgery with the EBC collector (HAAK EK20 EcoScreen; Eric Jaeger, Friedberg, Germany). The collector was pre-cooled for 20 min and the subjects were instructed to clean their mouths, wear a nose clip and breath quietly while biting the mouthpart. After 20 min of quiet breathing, the expiratory air was transformed into a snow-like

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Abbreviations: EBC, exhaled breath condensate; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction

Key words: non-small-cell lung cancer, exhaled breath condensate, p16 gene, mutation, polymerase chain reaction

Table I. p16 gene exon amplification primer sequences and parameters.

Primer name	Primer sequence (5'-3')	Fragment length (bp)	Annealing temperature (˚C)
Exon 1			
Forward	CAGCATGGAGCCTTCGGCTGA	216	55
Reverse	GCGCTACCTGATTCCAATTC		
Exon 2			
Forward	AGCTTCCTTTCCGTCATGC	264	55
Reverse	GCCAGGTCCACGGGCAGA		
GAPDH			
Forward	GTGAAGGTCGGAGTCAAC	356	56
Reverse	GAGATGATGACCCTTTTGGC		

Table II. p16 gene mutations in the exhaled breath condensate of patients with non-small-cell lung cancer.

Number	Exon	Mutation	Codon	Mutation type
1	1	GAA→GAC	94	Base substitution
2	1	TGG→AGG	15	Base substitution
3	1	AGC→AGG	78	Base substitution
4	2	GAG→GTG	88	Base substitution
5	2	CCC→CCG	81	Base substitution
6	2	GTG→CTG	59	Base substitution
7	2	GCC→GCG	86	Base substitution
8	2	GAG→GTG	69	Base substitution

substance by condensation. The collection tube was then removed and 1-3 ml of EBC was collected following melting of the specimen and transferred to a centrifuge tube to be stored at -80˚C.

Genetic testing. Genomic DNA was extracted from EBC using a DNA extraction kit (QIAamp Circulating Nucleic Acid Kit; Qiagen Co. Ltd, Hilden, Germany). GAPDH amplification was used as a positive control during polymerase chain reaction (PCR) amplification of the p16 gene exons 1 and 2 in EBC samples. The mutations were then contrasted using DNASTAR software (DNASTAR, Inc., Madison, WI, USA).

Primer synthesis. The PCR amplification kit was provided by Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). The p16 gene sequence data were retrieved from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA). With the assistance of the applied primer design software Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA), specific primers were designed, which were synthesized by Shanghai ShengGong Biological Engineering Technology Service Co., Ltd. (Shanghai, China). The p16 gene exon primer sequences and parameters are presented in Table I.

Statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The NSCLC patients were grouped according to gender, smoking history, pathological type, degree of differentiation, lymph node metastasis and tumor stage. The χ^2 test or Fisher's exact probability method were used to compare the differences in mutation rates among the EBC samples. $P<0.05$ was considered to indicate statistically significant differences.

Results

Electrophoresis of PCR amplification products of EBC DNA. DNA extracted from the EBC of NSCLC patients was used as the template. The internal gene GAPDH was amplified by PCR and agarose gel electrophoresis yielded clear GAPDH amplification product bands. The PCR amplification of the p16 gene exons 1 and 2 and agarose gel electrophoresis also yielded clear bands.

EBC p16 gene sequencing indicates mutations in NSCLC patients. The direct sequencing method was used to detect p16 gene mutations in the EBC of patients with NSCLC. Of the 58 EBC specimens from patients with NSCLC, 54 were successfully tested (the results of 2 cases could not be interpreted and 2 cases did not conform to the requirements of the test due to a low DNA concentration). Of the 54 EBC samples, 8 harbored mutations (3 in exon 1 and 5 in exon 2). Therefore, the mutation rate was 14.81% (8/54). p16 gene mutations in EBC were not detected in the 30 healthy controls. The p16 gene mutations in the EBC of patients with NSCLC are presented in Table II. Sequence diagrams are depicted in Figs. 1 and 2.

Table III. Association between p16 gene mutations in the exhaled breath condensate of patients with non-small-cell lung cancer and clinicopathological characteristics.

Clinicopathological characteristics	p16 gene mutation cases, n/total (%)	χ^2 value	P-value
Gender		1.487	>0.05
Male	3/34 (8.82)		
Female	5/20 (25.00)		
Smoking history		0.494	>0.05
Yes	6/31 (19.35)		
No	2/23 (8.70)		
Pathological type		0.936	>0.05
Squamous cell carcinoma	3/32 (9.40)		
Adenocarcinoma	5/22 (22.73)		
Degree of differentiation		3.076	>0.05
Well-differentiated	1/11 (9.09)		
Moderately differentiated	2/24 (8.33)		
Poorly differentiated	5/19 (26.32)		
Lymph node metastasis		0.181	>0.05
Yes	4/20 (20.00)		
No	4/34 (11.76)		
Tumor stage		7.122	<0.05
I	0/23 (0.00)		
II	4/17 (23.53)		
III/IV	4/14 (28.57)		

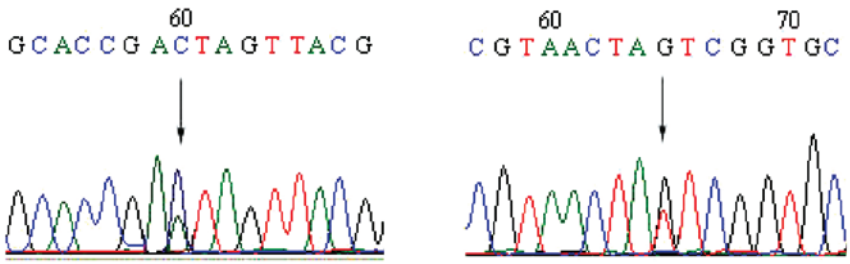

Figure 1. p16 gene exon 1 codon 94 in the exhaled breath condensate of a patient with non-small-cell lung cancer. GAA>GAC (arrows indicate mutation).

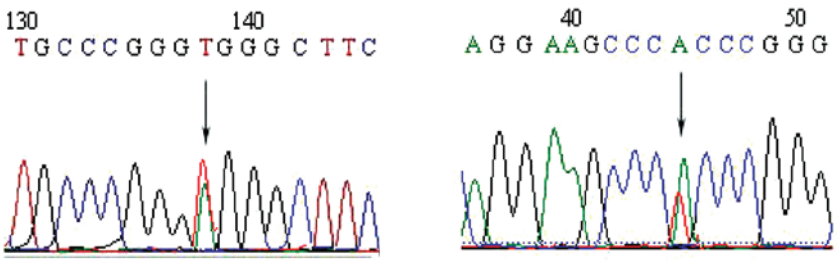

Figure 2. p16 gene exon 2 codon 88 in the exhaled breath condensate of a patient with non-small-cell lung cancer. GAG>GTG (arrows indicate mutation).

Association between p16 gene mutations and clinicopathological data of NSCLC patients. EBC p16 gene mutations exhibited no statistically significant differences according to gender, smoking history, pathological type, degree of differentiation and presence or absence of lymph node metastasis. However, EBC p16 gene mutations exhibited statistically significant differences among different tumor stages (P<0.05). The p16 gene mutation rate was proportional to the tumor

stage (Table III). The p16 gene mutation rate in stage I, II and in III/IV disease was 0.00, 23.53 and 28.57%, respectively.

Discussion

The prognosis of patients with lung cancer is closely associated with clinical stage at diagnosis. The 5-year survival rate postoperatively in patients with stage IA lung cancer is 60%, while the overall 5-year survival rate of patients with stage IV cancer is <5%. Early diagnosis and timely, appropriate treatment are crucial for improving the survival rates of patients with lung cancer (6). The identification of alterations in the gene or protein levels associated with certain types of tumor in the tissues or body fluids of patients during early tumor formation may significantly improve early diagnosis of lung cancer.

p16 is a tumor suppressor gene, located in human chromosome 9, composed of 2 introns and 3 exons. The p16 protein, encoded by the p16 gene, competes with the cyclin D1 protein for binding to the cyclin-dependent kinase 4 (CDK4), inhibiting cyclin D1/CDK4 compound activity and DNA synthesis and ultimately inhibiting proliferation. Low or no p16 protein expression results in uncontrollable cell proliferation and tumor growth. Deactivation of the p16 gene in patients with lung cancer is observed in 25-70% of the cases (7), suggesting that alterations of the p16 gene are closely associated with tumor occurrence.

EBC from the respiratory tract and lung may directly reflect the pathological and physiological function of the airway. Data reported by previous studies on lung cancer were mainly acquired by techniques including detection during surgery, fiberoptic bronchoscopy biopsy tissue, bronchoalveolar lavage fluid, bronchoscope brush-off substance, pleural effusion, plasma and sputum. Compared with the aforementioned methods, EBC detection technology has the following advantages: i) The EBC collection process is non-invasive and does not affect airway function or cause inflammation; ii) it may be repeated within a short period of time; iii) it may be used as a special detection method for lung disease specificity; iv) it is a simple, cost-effective and quick diagnostic method, readily accepted by the patients.

A previous study demonstrated that DNA may be detected in EBC (8) and Gessner *et al* (9) detected four cases of p53 gene mutations among 11 NSCLC patients through EBC collection. Our previous study also demonstrated that the detection of promoter methylation of p16 in EBC was feasible (10). These previous studies provide a theoretical basis for the feasibility of using EBC to detect genetic changes in patients with lung cancer. The present study detected p16 gene mutations in the EBC of NSCLC patients and applied PCR and DNA sequencing to analyze these mutations. According to the results, the p16 gene mutation rate was 14.81% (8/54) and p16 gene mutations were not detected in the EBC of healthy controls, indicating the high specificity of the detection of gene mutations for the diagnosis of NSCLC. Our results demonstrated that genetic testing in EBC samples is feasible. The p16 gene was not detected in certain EBC samples due to the low concentration of DNA, which did not conform to the concentration requirements of the sequencing protocol.

When comparing EBC p16 gene mutations according to gender, smoking history, pathological type, degree of differentiation and presence or absence of lymph node metastasis, no statistically significant differences were identified (P>0.05). However, the p16 gene mutation rate was found to be proportional to the tumor stage, exhibiting a clear tendency to increase with advancing stage. This proves that genetic testing is feasible in EBC samples and that p16 gene mutation detection in EBC may be used in the evaluation of the condition of the patient.

With the continuous advances in molecular biology, the application of the knowledge and technology of molecular biology in the diagnosis of lung cancer and the identification of novel methods for improving early cancer detection are research subjects that are attracting increasing attention. EBC appears to be promising as a novel non-invasive detection method and it may be used in the diagnosis of lung disease, patient condition assessment, evaluation of curative effect and prediction of prognosis. The identification of molecular markers associated with NSCLC in EBC should be the focus of future studies.

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References

1. Anglim PP, Alonzo TA and Laird-Offringa IA: DNA methylation-based biomarkers for early detection of non-small cell lung cancer: an update. Mol Cancer 7: 81, 2008.
2. Pillai RN and Ramalingam SS: Advances in the diagnosis and treatment of non-small cell lung cancer. Mol Cancer Ther 13: 557-564, 2014.
3. Koutsokera A, Loukides S, Gourgoulianis KI and Kostikas K: Biomarkers in the exhaled breath condensate of healthy adults: mapping the path towards reference values. Curr Med Chem 15: 620-630, 2008.
4. Chan HP, Lewis C and Thomas PS: Exhaled breath analysis: novel approach for early detection of lung cancer. Lung Cancer 63: 164-168, 2009.
5. Mozzoni P, Banda I, Goldoni M, *et al*: Plasma and EBC microRNAs as early biomarkers of non-small-cell lung cancer: Biomarkers 18: 679-686, 2013.
6. Padda SK, Burt BM, Trakul N and Wakelee HA: Early-stage non-small cell lung cancer: surgery, stereotactic radiosurgery, and individualized adjuvant therapy. Semin Oncol 41: 40-56, 2014.
7. Romagosa C, Simonetti S, López-Vicente L, *et al*: p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene 30: 2087-2097, 2011.
8. Carpagnano GE, Palladino GP, Gramiccioni C, *et al*: New biomolecular methodologies in diagnosis of lung cancer. Recenti Prog Med 99: 417-421, 2008 (In Italian).
9. Gessner C, Kuhn H, Toepfer K, *et al*: Detection of p53 gene mutations in exhaled breath condensate of non-small cell lung cancer patients. Lung Cancer 43: 215-222, 2004.
10. Xiao P, Chen JR, Zhou F, *et al*: Methylation of P16 in exhaled breath condensate for diagnosis of non-small cell lung cancer. Lung Cancer 83: 56-60, 2014.