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Methylation of *P16* in exhaled breath condensate for diagnosis of non-small cell lung cancer

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ABSTRACT

Background: Non-small cell lung cancer is the most frequently cause of cancer-related death in the world. To explore the technical feasibility, we detected aberrant promoter methylation of *P16* in exhaled breath condensate which was a new, non-invasive tool for diagnosis and screening program of NSCLC.
Methods: We analyzed aberrant promoter methylation of *P16* in 180 samples from 60 individuals, including 30 NSCLC patients (cancer tissues, adjacent normal lung tissues, blood plasma, and EBC), and 30 healthy controls (blood plasma and EBC) by fluorescent quantitative methylation-specific polymerase chain reaction (F-MSP).
Results: The positive rate of aberrant promoter methylation of *P16* was 26 of 30 (86.66%) in tumor tissues, 15 of 30 (50%) in blood plasma, and 12 of 30 (40%) in EBC, we have not observed the positive methylation of *P16* in the adjacent normal lung tissues, or in EBC or blood plasma from the healthy control group.
Conclusion: We found that detected promoter methylation of *P16* in EBC was feasibility, it should be an useful biomarker for diagnosis of NSCLC, it have potential prospect that detected the gene molecular in EBC because of noninvasive, specificity, convenient and repeatable.

1. Introduction

Non-small cell lung cancer (NSCLC) is the number one killer among cancer types in the United States [1,2]. Early diagnosis method of NSCLC which is more common and less aggressive has the highest potential for saving lives, this is a key factor in the high mortality rate of this disease. As yet, no routine screening method that enable early detection strategies exists. We need a new, noninvasive risk assessment, convenient and repeatable tool for diagnosis of NSCLC. In this study, we aimed to detect the aberrant promoter methylation of *P16* in exhaled breath condensate (EBC) from NSCLC, and we found that the technique was feasibility.

2. Materials and methods

2.1. Study population

The study design was approved by the Committee on Human Research of the Second Affiliated Hospital of Nantong University. Study participants gave informed consent prior to participation in the study. A total of 60 participants (30 NSCLC cases and 30 healthy controls) were included. The NSCLC patients included 19 males and 11 females who ranged in age from 47 to 78 years (average, 63.63 ± 7.38 years), of whom 20 had a history of smoking and 10 were nonsmokers. There were 9 cases of squamous carcinoma, and 21 cases of adenocarcinoma. The control group consisted of 9 males and 21 females who ranged in age from 35 to 79 years (average, 59.70 ± 13.06 years). The NSCLC patients had undergone no chemotherapy or radiotherapy prior to surgical resection, and the presence of cancer was histologically confirmed after surgery by histopathologic criteria. Staging was based on the TNM (tumor, node, metastasis) classification system of the International Union Against Cancer 2009(UICC). Information

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regarding age, gender, smoke history, etc., were obtained from an interviewer-administered questionnaire.

2.2. Tissue collection

Tumor specimens and adjacent normal lung tissues (≥3 cm) were collected by a pathologist from 30 patients undergoing surgical resection at the Second Affiliated Hospital of Nantong University, China. Tissue samples were immediately frozen in liquid nitrogen and stored at −70 °C until analysis.

2.3. Blood plasma collection

Blood samples were collected from patients before curative pulmonary resection. Plasma was collected after centrifugation at 3500 rpm for 3 min the day before surgery, and stored at −70 °C until analysis. The same procedure was followed for the healthy control participants.

2.4. Exhaled breath condensate (EBC) collection

Exhaled breath condensate (EBC) collection was performed using standard methods by the EcoScreen condenser (company of Eric Jaeger, German) before operation, after 15–20 min of quiet tidal volume breathing, with the exception that participants were asked to expectorate all saliva. Approximately 2–4 ml of EBC was collected from each participant, and stored at −70 °C until analysis. The details of collected procession been shown in Fig. 1.

2.5. DNA extraction

DNA was extracted from tissue, plasma, and EBC samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). For each sample, 20 mg of tissue and 1.6 ml each of plasma and EBC was used for DNA preparation. DNA was eluted in 200 μl buffer AE (Qiagen) for tissue, and in 50 μl buffer AE for plasma and EBC.

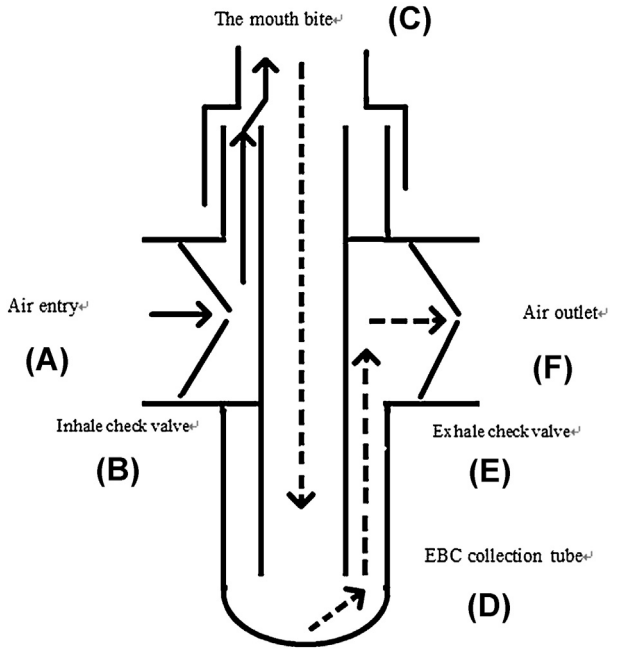

Fig. 1. Diagram of EBC collection procession, exhaled breathe air passed through the device in the sequence: (A) Air entry, (B) Inhale check valve, (C) The mouth bite, (D) EBC collection tub and then we got the water vapor after exhaled air passed the cooled tube (−20 °C), condensing apparatus, finally, the air passed the (E) exhale check valve and (F) air outlet. The whole process only need the volunteer keep quiet breath, we can obtain EBC 2–4 ml from each participant, without pollution of saliva and sputum.

2.6. Bisulfite treatment

Of the DNA extracts, 15 μl of the tissue extract, and 50 μl each of the plasma and EBC extracts, was used for bisulfite treatment. Bisulfite treatment was performed using the CpGenome™ DNA Modification Kit (Millipore, Billerica, MA, USA), with the reaction condition optimized to 50 °C for 14 h. Finally, DNA was eluted in 30 μl of TE.

2.7. Standards for absolute quantification

We established methylation and unmethylation standard curves using Raji cells and HeLa cells, which have methylation indices of 100% and 0%, respectively.

2.8. Fluorescence quantitative methylation-specific polymerase chain reaction (F-MSP)

F-MSP was carried out using the following primers: P16U forward, 5′-TTATTAGAGGGTGG-GGTGGATTGT-3′; P16U reverse: 5′-CAACCCCAAACCACAACCATAA-3′; P16UTaqman-probe (FAM): 5′-AGGTAGTGGGTGGTGG-GGAGTAGTATGGAGTTG-3′; P16M forward, 5′-TTATTAGAGGGTGGGGCGGATCGC-3′; P16M reverse, 5′-GACCCCGAACC GCGACC-GTAA3′; P16M Taqman-probe (FAM), 5′-AGTAGTATGGAG-TCGGCGGCGGG-3′; 0.5 M each), with an additional 40-cycle amplification (2 min at 95 °C, 10 s at 95 °C, and 30 s at 60 °C (data collection). The presence of genomic DNA was confirmed by PCR using 1.0 μl (for tissue), or 5.0 μl (for plasma and EBC) of sample, the experience of quantitative methylation-specific polymerase chain reaction based on s MJ opticon monitor analyzed system (Bio-Rad).

2.9. Data analysis

The association between gene methylation and clinical pathological variables was statistically tested. We acquired the equation $y = -3.4299x + 39$ for methylation standard curve, and equation $y = -3.3912x + 39.564$ for unmethylation standard curve, based on the C_T value, we calculated the target gene copy mean number after three times F-MSP, the methylation of P16 gene copy number more than 1 considered positive. Then the quantify data were analyzed by SPSS 17.0 software, with a 95% confidence interval (95% CI; $\alpha = 0.05$). The positive rate was completed by a X^2 Fisher's exact test and tested by the Chi-square test.

3. Results

3.1. Standard curves

We constructed the methylation standard curve using Raji cells, which have a methylation index of 100%, and derived the equation $y = -3.4299x + 39.103$, $r^2 = 0.9994$. We then used this equation to calculate copy concentration in our samples.

We constructed the standard curve for non-methylation using HeLa cells, which have a methylation index of 0%, and derived the equation $y = -3.3912x + 39.564$, $r^2 = 0.9994$. The value of r^2 indicated a strong linear relationship between the C_T values and the given concentrations.

We detected aberrant promoter methylation of the tumor suppressor gene *P16* in tumor tissue (26/30), serum (15/30), and EBC (12/30) from NSCLC by F-MSP; the positive rate was 86.66%, 50%, and 40%, respectively, for the 3 sample types. Promoter methylation of *P16* was not detected in adjacent normal tissues from