The value of chest CT scan and tumor markers detection in sputum for early diagnosis of peripheral lung cancer

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Abstract Objective To investigate the diagnostic value of chest CT scan combined with telomerase activity and p16 gene methylation from exfoliated cells of sputum in 55 cases of solitary pulmonary nodules suspected early peripheral lung cancer. Methods The sputum specimens from 34 cases of cancer nodules and 21 cases of benign lesion were detected for telomerase activity by TRAP-PCR-ELISA and p16 gene methylation by PCR-based methylation analysis. Results The qualitative diagnostic accuracy of CT scan was 61.8% [34/55] for SPN provided by pathology. Cytology analysis of sputum was positive in 13 cases [8.2%]. Telomerase activity was positive in 29 cases [sensitivity was 79.4% [specificity was 90.5% accuracy was 83.6%] p16 gene methylation was found in 11 cases [sensitivity was 32.4% [specificity was 100.0% accuracy was 58.2%]. The sensitivity was increased to 86.1% by combination of telomerase activity and p16 gene methylation. Compared with nodules without malignant CT signs expression of telomerase activity and p16 methylation of SPN with malignant CT sign lobulation or spiculate protuberance or spicule sign had a significant difference [P < 0.01]. Conclusion The results suggest that chest CT scan combined with telomerase activity and p16 gene methylation detection in sputum for patients with peripheral lung cancer may enhance the diagnostic value of radiology and conventional cytology.

Key words Lung neoplasms Sputum Telomerase activity p16 gene Methylation Tomography X-ray computed

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1 Introduction

The most important means to reveal and diagnose peripheral lung neoplasm are X-ray photography and other imaging studies. It’s well known that it is easy to find a nodule or a mass but difficult to make the diagnosis by radiography. Even computer tomography CT and magnetic resonance image MRI technology could hardly make precise judgement of a solitary pulmonary nodule SPN. Investigators have come to realize that the alterations of oncogenes and anti-oncogenes are important factors in the oncogenesis and progression of neoplasm so people try to make earlier diagnosis of malignant neoplasm by using gene detection combined with qualitative and quantitative imaging analysis. Recent years several investigators carried out some prospective studies on the diagnosis of early peripheral lung cancer by using both imaging and molecular biology methods [1-5]. In the present study telomerase activity and p16 gene methylation from 55 sputum specimens collected 24 h after lung biopsy from patients with SPN revealed by CT scan which were suspected as early peripheral lung cancers T1N0M0 were detected by PCR-TRAP-ELISA silver staining PCR-TRAP and methylation related PCR.

2 Materials and Methods
2.1 Clinical and CT data

Fifty-five patients enrolled in this study at the Second Affiliated Hospital of Xuzhou Medical College between January 2000 and December 2001 were found to have SPN by chest CT scan and were suspected as early peripheral lung cancer male 37; female 18; 23--75 year old. A high resolution computerized tomography (HRCT) and enhancing chest CT scan slice thickness and spacing are 2 mm continuous scan 120 KV 130 mA 1.8S 340 × 340 matrix were taken in all patients for the SPN and were analyzed for their forms density and relationship with bronchi carefully. Then 22 patients accepted CT guided percutaneous needle cutting lung biopsy 33 patients accepted bronchofibroscope biopsy. Of all the 55 patients 40 patients accepted curative lobectomy. Finally 34 SPN were diagnosed as peripheral lung cancer by histopathology including 17 squamous cell carcinoma 13 adenocarcinoma 2 small cell carcinoma 1 adenosquamous carcinoma and 1 carcinoid WHO Histologic Typing of Lung Tumors. The pathologic TNM stage was determined according to the newly revised classification of the American Joint Committee on Cancer and the Union Internationale Centre of Cancer in 1997. Of all the 32 non-small-cell lung cancer NSCLC 19 were stage I disease 10 stage II 1 stage IIIb 2 stage IV. The other 2 small cell lung cancer SCLC were limited disease. The 21 SPNs were confirmed as pulmonary benign lesions 16 pneumonia 3 pulmonary tuberculosis 1 lung abscess 1 silicosis by more than 1 year’s follow-up survey and histopathology of biopsy 15 and operation 6 specimens.

2.2 Specimens collection and management

Sputum specimens were collected in a poly-trisathylene beaker within 24 hours after biopsy incubated 30 minutes on ice after adding 20–30 ml solution containing trypsin then the mixture was diluted with 50 ml PBS filtrated with double-deck gauzes. The filtrate was divided into 3 aliquot 2500 r/min centrifuged for 5 minutes then the supernatant was discarded the precipitants were washed with PBS for 2 times then one of the precipitants was used to make 3 smears for cytology diagnosis the other two were kept in a –80 °C refrigerator for telomerase activity and p16 methylation detection.

2.3 Telomerase activity detection

Telomerase activity was semi-quantitatively assessed by PCR-TRAP-ELISA described by the manufacturer Huamei Inc. P. R. China. Silver staining PCR-TRAP was applied to the qualitative detection of telomerase activity according to the method of Gu et al.[1][2].

2.4 Aberrant methylation detection of exon-1 of p16 gene

Genome DNA was extracted from the exfoliated cells with the genomic DNA purification kit provided by Sangon Inc. P. R. China. The quantity of the DNA was evaluated by spectrophotometry. All oligonucleotide primers used for PCR were synthesized by Sangon Inc. Exon-1 of p16 specific primers sequences were as follows 5'–GAA GAA AGA GGA GGG GCT G-3' 5'-GCG GTA CCT GAT TCC AAT TC-3'. The PCR product migrated as a 340 bp fragment in 1.8% agarose gel. A PCR assay with specific for the gene β-actin was carried out in each case served as an inner control its primers sequences were 5’-CAC TGT GTT GGC GTA CAG GT-3’ 5’-TCA TCA CCA T TG GCA ATG AG-3’ the PCR product migrated as a 154 bp fragment in 1.8% agarose gel.

2.5 Enzyme cutting of genomic DNA

Two-stepped enzyme cutting methods using restriction endonuclease Smal I /Sac II Promega Inc were carried out for the genomic DNA with a volume of 25 μl reaction mixture containing 16 μl ddH2O 0.4 μl 10 × buffer 1 μl restriction endonuclease 10 U/μl 10 μl genomic DNA 0.1 g/L covered with 30 μl liquid paraffin. 25°C for Smal I 37°C for Sac II incubated for 16 hr. The product was precipitated by pre-cooled anhydrous ethanol then the DNA was re-purified for next procedure.

2.6 PCR-based methylation analysis

The PCR was performed in a 30 μl reaction mixture containing 200 ng of DNA template 3 μl of 10 × buffer 1 μmol/L of each primers 2 U of Taq polymerase 2.5
mmol/L MgCl₂ and 200 μmol/L dNTP. PCR were performed in a thermocycler BIO-RAD Gene Cycler™. The amplification conditions were set up as follows: 1 cycle of denaturing at 95°C for 5 min followed by 32 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min before a final extension at 72°C for 5 min. A volume of 6 μl PCR product was transferred to 1.8% agarose gel for electrophoresis and analyzed on the UVP system.

2.7 Statistical analysis

χ² test or Fisher’s exact probability test and t test were used for the data analysis. P < 0.05 was considered to have statistical significance.

3 Results

3.1 Qualitative diagnosis of SPN by CT scan

All 55 SPN were revealed by CT scan the qualitative diagnostic accuracy was only 61.8% 34/55 Tab 1. Compared with the post surgical TNM staging the quantitative diagnostic TNM accuracy of CT scan was only 59.4% 19/32 two cases of SCLC could not be accurately staged. Tab 1

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▲ P < 0.01 ▲ P < 0.05.

3.2 Telomerase activity analysis of sputum

Twenty-nine sputum samples were positive for telomerase activity by PCR-TRAP-ELISA semi-qualitative analysis. Significant difference was found between the telomerase activity rate of lung cancer group 27 out of 34 79.4% and pulmonary benign disease group 2 out of 21 9.5%. The telomerase activity level absorb value were 0.305 ± 0.0203 in lung cancer and 0.105 ± 0.104 in benign pulmonary lesions respectively t = 4.18 P = 0.000 1. When detected by PCR-TRAP silver staining method the product migrated as a succession brown-yellow fragment intermitted with 6 bp strap in an 1.8% agarose gel Fig 1. The diagnostic sensitivity for peripheral lung cancer by detection of telomerase activity was 79.4% specificity was 90.5% and accuracy was 83.6%.

3.3 Aberrant methylation detection of exon-1 of p16 in sputum

The aberrant methylation rate of exon-1 of p16 in sputum obtained from patients with lung cancer was 32.4% 11/34 in which 5 samples were positive for Sma I site abnormal methylation 7 positive for Sac I site abnormal methylation and 1 positive for both sites. There was no positive for abnormal methylation in 21 sputum samples obtained from patients with pulmonary benign disease Fig 2 and Fig 3. The diagnostic sensitivity specificity and accuracy of aberrant methylation analysis in sputum for patients with peripheral lung cancer were 32.4% 100.0% and 58.2% respectively.

3.4 Cytology analysis of sputum

Of the 34 sputum samples from patients with lung cancer 13 38.2% were positive for malignant cells and 21 61.8% were negative. Compared the detection results of telomerase activity or abnormal methylation of p16 gene there was significant difference between the two groups Tab 2. None of the 21 sputums obtained from patients with pulmonary benign diseases was positive for malignant cells.
metylation of p16 analysis sputum samples

In 55 sputum samples one achieved positive results the others were negative. Compared with the results of histopathology examination the genuine positive cases of the combined test were 29 pseudo-positive cases were 2 genuine negative cases were 19 and false-negative cases were 5. These results indicated that the sensitivity specificity and accuracy of the combined test method were 86.1% 90.5% and 87.3% respectively. Compared with single chest CT scan or sputum cytology the combined test method increased the diagnostic accuracy of lung cancer significantly Tab 1.

4 Discussion

X-ray photography and CT scan are the most important methods to reveal peripheral lung cancer. However the qualitative diagnosis must be made by sputum cytology CT guided lung biopsy and surgery. Cytology is the most important mean for the early diagnosis of lung cancer. Due to the sample collection treatment and other causes tumor cells in sputum are rare and often deformed. Therefore the positive rate of cytology examination was low 38.2% in this study. Imaging technics could reveal SPN whose diameter is more than 2 mm but it is difficult to make the qualitative diagnosis. In this study the diagnostic positive rate of imaging method is only 61.8% [34/55]. New possibility for the early diagnosis of lung cancer is coming to reality in virtue of the development of molecular biology. For example the detection of tumor markers telomerase activity methylation status of p16 etc. could make up the deficiency of cytology.

Telomerase a ribonucleoprotein enzyme that functions in the maintenance of telomeres has been reported to be one of the most broad-spectrum diagnostic markers for malignant diseases. Kumaki et al. had analyzed the telomerase activity in 115 lung cancer tissues and their corresponding normal lung tissues 93% cancer tissues were positive for telo-merase activity but no positive activity was observed in their corresponding normal lung tissues. In addition the positive rate of telomerase activity in poor differentiated squamous cell carcinoma and adeno-

![Fig 2](image1.png)

**Fig 2** Aberrant methylation analysis in exfoliated cells from sputum Sac II enzyme cutting.

M DNA Marker 2 sputum sample from patients with adenocarcinoma positive for methylation of exon-1 of p16 5 sputum sample from patients with squamous cell carcinoma positive for methylation of exon-1 of p16 1 3 4 6 sputum samples from patients with lung cancer negative for methylation of p16.

![Fig 3](image2.png)

**Fig 3** Aberrant methylation analysis in exfoliated cells from sputum Sma I enzyme cutting.

M DNA Marker 2 sputum samples from patients with squamous cell carcinoma positive for methylation of exon-1 of p16 5 sputum sample from patients with adenocarcinoma positive for methylation of exon-1 of p16 1 3 4 6 sputum samples from patients with adenocarcinoma negative for methylation of p16

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<th>Tab 2</th>
<th>Results of telomerase activity assay methylation of p16 detection and cytologic assay of sputum from patients with lung cancer</th>
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▲P < 0.01.

3.5 Detection of Telomerase activity combined with...
carcinoma tissues was significantly higher than that in the well differentiated group it the total survival rate in the high expression group was lower than that in the low expression group. Sen et al.\(^{7}\) reported that diagnostic sensitivity of telomerase activity detection of lung cancer was 81.6% in sputum\(^{8}\) 68.4% in bronchovesicular lavage fluid\(^{9}\) BALF\(^{10}\) and 86.8% in bronchofiberscope biopsied samples respectively. Most investigators suggest that telomerase activity analysis is a non-invasive method\(^{11}\) with potential value for early diagnosis of lung cancer\(^{12,13}\). Therefore\(^{14}\) in patients who were clinically diagnosed as peripheral lung cancer by CT scan\(^{15}\) but cytology assay of sputum were negative\(^{16}\) telomerase activity detection may make up the deficiency of cytology so as to shorten the times to final diagnosis\(^{17}\) and improve the positive diagnostic rate\(^{18}\) and may apply to the early diagnosis of peripheral lung cancer.

p16 is the first gene which may suppress the genesis of neoplasm directly\(^{19}\) it is closely associated with the genesis and progression of many neoplasms\(^{20}\) so it is called as multiple tumor suppressor gene\(^{21}\) MTS1\(^{22}\) and it is a regulator and repressor of cell proliferation\(^{23}\) Belinsky et al.\(^{24}\) reported that aberrant methylation of the p16 promoter region could be detected in DNA from exfoliated cells in sputum of patients with lung cancer. In their study\(^{25}\) 43%\(^{26}\) 3/7 sputum samples from patients with lung cancer were positive for the p16 promoter region methylation detection\(^{27}\) and 19%\(^{28}\) 5/26 sputum samples from cancer-free\(^{29}\) high-risk subjects were detected the same abnormal alteration. Application the sensitive method of methylation-specific PCR\(^{30}\) MSP\(^{31}\) Palmisano et al.\(^{32}\) had analyzed p16 and MGMT\(^{33}\) O6-methylguanine-DNA methyltransferase\(^{34}\) gene\(^{35}\) their results demonstrated that aberrant methylation of the p16 and/or MGMT could be detected in DNA from sputum in 100% of patients with squamous cell lung carcinoma\(^{36}\) not only at the time of diagnosis\(^{37}\) but also in all sputum samples taken from patients 5--35 months before clinical tumor detection. Therefore\(^{38}\) they thought aberrant methylation of p16 was an early and frequent alteration of lung cancer and suggested that detection of aberrant methylation constitute a promising approach to the early detection and population-based screening of lung cancers and other common human cancers. In this present investigation the abnormal p16 methylation rate in sputum obtained from patients with lung cancer after lung biopsy was 32.4%\(^{39}\) though lower than the previous protocols\(^{40}\) but compared with the positive rate of pulmonary benign disease\(^{41}\) 0 out of 21 it\(^{42}\) 0% the difference was significant\(^{43}\) which supports the viewpoint that the aberrant methylation of p16 is a specific marker for lung cancer. Besides the two sites\(^{44}\) Sma I/Sac II\(^{45}\) analyzed in this study The CpG methylation also has other sites Hha I\(^{46}\) Hha II\(^{47}\) Eag I\(^{48}\) Acc II\(^{49}\) Cfo I\(^{50}\) ethanol\(^{51}\) so the rate of aberrant methylation of p16 may be higher than our result. Provided combined with detection of other tumor markers methylation assay of p16 is a satisfactory marker for diagnosis of lung cancer.

Attribute to the difficult to process sputum up to now\(^{52}\) few report is with regard to telomerase activity-detection in exfoliated cells of sputum. Considering that telomerase activity was detectable in urine\(^{53}\) bronchial washings\(^{54}\) and oral cavity washings\(^{55}\) it is possible to analyze telomerase activity in sputum the committed step is the procession of sputum. Our method is as follows: collect sputum within 24 hours after lung biopsy and process the sample on ice within 30 minutes all these measures greatly prevent telomerase from degradation by RNA Lyase. In choice of samples for p16 methylated and telomerase detection the collection of sputum is convenient non-invasive and acceptable to patients and there are more cells in samples collected after lung biopsy. Aberrant methylation of p16 is highly specific marker and telomerase is a greatly sensitive one for peripheral lung cancer the combined use of the two markers may increase the diagnostic efficiency of peripheral lung cancer. Our results indicate that using sputum as detection sample telomerase activity assay is highly sensitive than CT scan and cytology. Though pseudo-positive\(^{56}\) 9.5%\(^{57}\) has presented in our assay of telomerase activity and the positive rate of p16 methylation\(^{58}\) 32.4%\(^{59}\) is low the combined use of the two assay increases the diagnostic efficiency. The combined assay’s diagnostic sensitivity specificity and accuracy for peripheral lung cancer were 86.1%\(^{60}\) 90.5% and 87.3% respectively. Yahata et
reported telomerase activity in exfoliated cells from BALF had no correlation with the location or size of the lung tumor. Therefore we think that detection of aberrant methylation of p16 and telomerase activity assay are more valuable for diagnosis of peripheral lung cancer.

It should be noted that though CT examination is hardly to make a qualitative diagnosis of peripheral lung cancer its function of location and quantitative estimate for the tumor is un-replaceable. To make accurate staging of TNM and appropriate plan for treatment it is important to perform the enhancing CT scan and carry out a complete examination of liver kidney and other organs for patients with lung cancer. Our results demonstrate that to assay telomerase activity and aberrant methylation of p16 in sputum could provide a new and complementary method to the differential diagnosis of SPN revealed by CT scan.

References