Clinical Significance of the Detection of the Homozygous Deletion of P16 Gene in Malignant Pleural Effusion

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Abstract

Objective To assess the role of the p16 gene exon 2 homozygous deletion in malignant pleural effusions.

Methods The homozygous deletion of p16 gene was determined in 34 pleural effusions due to non-small cell lung cancer (NSCLC) and in 21 cases with tuberculous pleuritis by polymerase chain reaction (PCR), compared with the determination of exfoliated cytology in the same specimens.

Results The PCR analysis showed that the homozygous deletion of p16 exon 2 was identified in 15 of 34 malignant pleural effusions (44.11%), including 8 negative cytology and it was not found any tuberculous pleural effusions. The exfoliated cytology of pleural effusion was positive in 19 of 34 malignant cases (55.88%). By combining two methods, the diagnostic sensitivity was enhanced, from 55.88% (19/34) to 79.41% (27/34), whose positive rate was higher than only determination of p16 exon2 homozygous deletion or exfoliated cytology in malignant pleural effusions (p<0.001, p<0.05 respectively).

Conclusion Our data suggested that combining the examination of exfoliated cytology and homozygous deletion of p16 gene exon2 in pleural effusion can recruit and enhance the diagnostic value of pleural effusion cytology. The detection of the homozygous deletion of the p16 gene in pleural effusion may be a useful adjunct to the cytological and histological examinations of pleural effusion. In cases of undiagnosed exudative pleural effusion with a high clinical suspicion for malignancy, it is reasonable to examine the homozygous deletion of pleural fluid p16 gene. With p16 gene homozygous deletion in pleural effusion, it may be strongly highly likely to be malignant and have a higher metastatic potential.

Key words: malignant pleural effusion, p16, homozygous deletion, lung cancer, polymerase chain reaction, cytology

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Introduction

Pleural effusion is a common clinical problem in respiratory diseases and determining its etiology may be difficult because of the great variety of diseases that can elicit pleural effusion. Malignancy is a common cause as are benign diseases such as tuberculosis. Both benign and malignant effusions share the same lymphocytic exudative profile making it more difficult to determine a specific diagnosis. Furthermore, the differentiation between malignant and benign effusions is very important for further management plans.

Although cytological examination of the pleural fluid is a rapid, efficient and minimally invasive method of diagnosing cancer, the diagnostic yield is about 60% (1). Therefore, various tumor markers in pleural fluid have been studied in order to find a test with high sensitivity and acceptable specificity that can differentiate malignant from benign pleural fluids. Despite being less invasive, the efficiencies of tumor markers, such as CA-19.9, CA-125 and NSE, are not good enough to differentiate malignant and benign pleural effusions (2). Taking together these facts, the genetic analysis of pleural effusion may be useful for diagnosis and determining treatment strategies.

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Since the multiple tumor suppressor gene1 (MTS1/p16) was reported by Kamb et al (3), it has been a research focus in many fields such as molecular oncology, molecular genetics and clinical medicine and it had been shown that p16 gene is frequently deleted in a variety of human cancers including lung cancer (4). Some researchers have indicated that the homozygous deletion of the p16 gene is found in about 20~50% of lung cancer cell lines. Deletion and mutation of the p16 gene can be detected in the neoplasm samples of 12~30% non-small cell lung cancer (3, 5-9), however, examination of the homozygous deletion of pleural fluid p16 gene has not been reported to date in the diagnosis of malignant pleural effusion. Therefore, the aim of this study was to assess the role of the homozygous deletion of pleural fluid p16 gene in the diagnosis of malignant pleural effusion in attempt to determine its clinical significance.

### Methods

#### Subjects

From among patients who were hospitalized because of pleural effusions due to lung cancer as a substitute for malignant effusion or tuberculous pleuritis as a substitute for benign effusion, in the Department of Respiratory Medicine, the first affiliated hospital, Anhui Medical University, Hefei, Anhui, P.R.China, from January 1999 to December 2001, 55 patients were consecutively selected. The validity of pleural fluid p16 gene was determined in these 55 patients (27 males and 28 females) with exudative pleural effusion (34 malignant and 21 tuberculous). The diagnosis of malignant pleural effusion due to non-small cell lung cancer (NSCLC) was defined by cytological or histological results (20 adenocarcinoma, 5 squamous cell carcinoma, 9 unclassified) (Table 1).

Pleural fluid was submitted for cytological examination and the homozygous deletion of p16 gene in every case and closed pleural biopsy or medical thoracoscopy was performed in selected cases. Of all the 34 malignant pleural effusions, the diagnostic yield was 19 cases by pleural fluid cytology, 8 cases by closed pleural biopsy and 7 cases by medical thoracoscopy. In all these cases, metastatic diseases were defined by computed tomography (CT) or magnetic resonance imaging (MRI). There are 16 cases with metastases spread in the thoracic lymph nodes or distant extrathoracic metastases (6 mediastinal lymphatic node metastases, 4 bone metastases, 2 hepatic metastases, 4 adrenals metastases) and 18 cases without metastasis. Tuberculous pleuritis was diagnosed if at least one of the following criteria were established: (i) positive staining from pleural fluid; (ii) positive culture from pleural fluid or pleural tissue; (iii) pleural tissue histology consistent with tuberculosis.

#### Polymerase chain reaction (PCR) for exon 1 and exon2 of p16 gene

To investigate the status of p16 gene homozygous dele-
tion, a cell pellet was obtained from pleural effusion by centrifugation, white blood cells obtained from health volunteer as control, and DNA was extracted by standard proteinase K-phenol/chloroform method (10). The primers were designed according to the published sequence of p16 and the report of Kamb et al (3). The sequences of primers for PCR amplification were as follows: p16 exon 1: 3’ primer: 5’GCG CTAC CTG AAT CTC, 5’ primer: 5’GAA GAA AGA GGA GGG GCT G. p16 exon 2: 3’ primer: 5’AGC TTG AGC CAG CAG CAG, 5’ primer: 5’TGG CTC TGA CCA TTC TGT. CDK4 was used as an internal control. 3’ primer: 5’GGA GGT CGG TAC CAG AGT G ,5’ primer: 5’CAT GTC GAC CAG GAC AGG. The reaction solution, including 10 mmol/L Tris, 50 mmol/L KCl, 2 mmol/L MgCl2, 0.001% Gelatin, 200 mmol/L dNTPs, 5% DMSO and 0.5 mmol/L primers, was added to 50 ng of DNA template. On the PCR thermocycler, the reaction was performed under the following conditions: one initial cycle at 97°C for 5 minutes, then chilling on ice, addition of 3 U of Taq diluted with 1×PCR reaction buffer, and continuation of the PCR program. Denaturing was performed at 95°C for 1 minute, annealing at 60°C for 1 minute, and polymerase reactions at 72°C for 1 minute, 35 cycles in all. Eight μl of PCR products was separated on 1.5% agarose gel. If there was no specific band of p16<sup>exon1</sup> and p16<sup>exon2</sup> while a specific band of CDK4 appeared, it was regarded as homozygous deletion of p16 gene. The presence of a band or the absence of p16 gene homozygous deletion was confirmed by Southern blot. The sensitivity of PCR in the examination of the homozygous deletion of p16 gene was detected by examining the mixture of normal cells and cancer cells at different ratios (100:0, 99:1, 67:33, 50:50, 33:67, 1:99, 0:100).

**Southern blot**

A human p16 cDNA clone (donate by Dr. Beach, Laboratory of Cold Spring, NY, USA) was picked up and inoculated in LB/Amp media, then shaken at 37°C for overnight. The plasmid was extracted using a QIAGEN column, quantitated and cut with EcoRI/SalI. The p16 cDNA was purified by electrophoresis. The p16 cDNA was labeled with digoxin according to the manual (Boehringer, Mannheim, Germany). The PCR products of p16 gene were separated on 2% agarose gel. Subsequently, they were transferred onto the nitrocellulose membrane. Prehybridization, hybridization and development were performed.

**Statistical analysis**

Statistical analysis was performed using chi-square test. <p><0.05 was considered as statistically significant.

**Results**

**Analysis of the homozygous deletion of p16 gene**

The PCR was performed by using 50 ng DNA extracted from pleural effusion as a template and CDK4 as an internal control. The sensitivity of PCR for p16 exon2 homozygous deletion was detected at a ration of 36:67 of HepG2 containing normal p16 gene and A549 containing homozygous deletion p16 gene (Fig. 1). The result showed that a specific band of amplified p16<sup>exon1</sup> and p16<sup>exon2</sup> was observed in every tuberculous pleural fluid and white blood cell sample, and so was p16<sup>exon2</sup> in every malignant case, while the specific band of p16<sup>exon1</sup> was found in 19 of 34 malignant pleural fluid samples (Fig. 2). The homozygous deletion of p16<sup>exon2</sup> was present in 15 malignant pleural effusions, 8 cases of which were negative by pleural fluid cytology. The incidence of homozygous deletion of p16<sup>exon2</sup> in malignant pleural effusions was 44.11% (15/34) (Table 2).

**The comparison between examination of the homozygous p16 exon2 deletion and the cytological method**

Of all 34 malignant pleural effusions, the diagnostic yield was 19 cases by cytology, positive rate being 55.88% (19/34), and 15 cases by examination of the homozygous p16 exon2 deletion, positive rate 44.11% (15/34). The combined methods were diagnostic in 79.41% (27/34) of the malignant pleural effusions. The statistical analysis showed that there
Figure 2. The agarose gel electrophoresis picture of the PCR analysis of the homozygous deletion of p16 gene. A, PCR products of p16 gene exons2. B, PCR products of CDK4. C, the Southern Blot of PCR products p16 gene. DNA sample from volunteer white blood cells (1), tuberculosis pleural effusion (2), no DNA (3), malignant pleural effusion (4-8). M, 100 bp DNA ladder.

was no obvious difference between the results of the homozygous deletion of p16\textsuperscript{exon2} and the pleural fluid cytology. But the diagnostic sensitivity by combined methods was obviously higher than that of the homozygous deletion of p16\textsuperscript{exon2} or exfoliated cytological analysis (p<0.001, p<0.05, respectively). Meanwhile, we also noted that the homozygous deletion of p16\textsuperscript{exon2} was present in 13 of 16 cases with metastases spread in thoracic lymph nodes or distant extra-thoracic metastases (6 mediastinal lymph node metastases, 4 bone metastases, 2 hepatic metastases, 4 adrenals gland metastases), while only 2 cases of the homozygous deletion of p16\textsuperscript{exon2} in were found among 18 malignant pleural fluids without metastases (Table 2).

One of the 15 cases with homozygous deletion of p16 gene had been misdiagnosed as tuberculous pleurisy for over 8 months because of negative results over ten times by pleural fluid exfoliated cytology method, but the p16\textsuperscript{exon2} was deleted. And 9 months later, the tumefied lymphatic nodes were found and confirmed as metastatic adenocarcinoma by puncture of the node with a needle for cytology analysis. Another young patient, hospitalized for 3 months, due to his young age and the repeated negative results for cytology, was treated as a case of tuberculous pleuritis until the tumefied lymphatic node above the right clavicle was found and the pathological diagnosis of puncture showed as adenocarcinoma, but the homozygous deletion of p16\textsuperscript{exon2} was detected at the beginning.

Discussion

Nearly all neoplasms have been reported to involve the pleura. In most studies, however, lung carcinoma has been the most common neoplasm, accounting for approximately one-third of all malignant effusions (1). The definitive diagnosis of malignant effusion is largely based on positive cytological and histological results but the sensitivity is only about 60% (11, 12). Therefore, some studies have attempted to increase the sensitivity of the cytological examination by using the determination of various tumor markers in pleural fluid and differentiate malignant from benign pleural fluid (13, 14). Among them, CEA is the most commonly studied and used, with an accuracy in pleural fluid greater than that of other tumor markers (15). Nevertheless, in almost all series on CEA in pleural fluid, high false positive results have been reported (16, 17). Thus, it is necessary to find a test with higher sensitivity in the diagnosis of malignant pleural effusions. Recently, genetic studies of pleural effusion have been reported (18). But the detection of p16 gene homozygous deletion in pleural fluid has not been reported to date.

P16 gene, the well-known tumor suppressor gene, is located on chromosome 9p21, which is a frequent target of homozygous deletion in many tumor types. The proteins encoded by p16 gene inhibit progression through the G1 phase of the cell cycle and deletion or functional inactivation of p16 gene is expected to promote cell proliferation. Initial studies of p16 gene revealed homozygous deletions in many tumor lines (3, 19-21). Suppressed gene expression and inactivation of its function due to hypermethylation of the 5'-CpG island in the 5'-flanking region, and loss of heterozygosity or mutation were also reported (22-24). Previous studies indicated that homozygous deletion was the main mechanism for inactivation of p16 gene in human primary lung carcinoma, especially in non-small cell lung carcinoma (23). Thus, we analyzed the homozygous deletion of p16 gene in 34 malignant pleural effusions due to non-small lung carcinomas by the PCR method. To avoid false-positive results (no PCR product) due to PCR error, we used CDK4 as an internal control and enrolled only samples that yield a clear band in electrophoresis and then confirmed by southern blot analysis.

In patients with a pleural effusion that cannot be diagnosed with non-invasive methods, when physicians use a combination of clinical and laboratory characteristics suggesting a high risk of malignancy before proceeding to a thoracoscopy, it is reasonable to examine the homoygous deletion of pleural fluid p16 gene. The present study has shown a good diagnostic value of the detection of homozygous deletion of p16 gene in malignant pleural effusion. Among 34 malignant pleural fluids due to non-small cell

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>No</th>
<th>Deletion (%)</th>
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<tbody>
<tr>
<td>Benign</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Malignant Pleural Effusions</td>
<td>34</td>
<td>15 (44.12)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>20</td>
<td>10 (50.00)</td>
</tr>
<tr>
<td>Squamous Cell Carcinoma</td>
<td>5</td>
<td>3 (60.00)*</td>
</tr>
<tr>
<td>Unclassified</td>
<td>9</td>
<td>2 (22.00)*</td>
</tr>
<tr>
<td>With Metastases</td>
<td>16</td>
<td>13 (81.25)</td>
</tr>
<tr>
<td>Without Metastases</td>
<td>18</td>
<td>2 (11.11)**</td>
</tr>
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Compared with Adenocarcinoma * p<0.05, Compared with Metastases Cases ** p<0.001
p16 gene homozygous deletion occurred frequently in malignant pleural effusions with metastasis but not frequently in tumor without metastasis. This result is in keeping with a study by Okamoto et al. (24). These data strongly suggested that homozygous deletion of p16 gene is a late event in tumor progression, probably related to the acquisition of metastatic phenotype and could represent a useful marker of tumor aggressiveness in NSCLC.

In conclusion, our study has shown that the homozygous deletion of p16 gene exists in malignant pleural effusion and combining the detection of exfoliated cytology and homozygous deletion of p16 gene exon2 in pleural effusion can recruit and enhance the diagnostic value of pleural effusion cytology. The detection of the homozygous deletion of p16 gene in pleural effusion should be a useful adjunct to cytological and histological examination of pleural fluid. In cases of undiagnosed exudative pleural effusion with a high clinical suspicion for malignancy, it is reasonable to examine the homozygous deletion of pleural fluid p16 gene. The combination of usefulness of p16 gene homozygous deletion and clinical characteristics of patients can be used to predict the risk of malignant etiology of pleural effusions with negative cytology. With p16 gene homozygous deletion of pleural effusion, it may be strongly suggested to be malignant and have a higher metastatic potential.

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