The inactivation of tumor suppressor genes by promoter methylation plays an important role in the development of cancers; it can also be used as a marker to distinguish cancerous cells from non-cancerous cells. In this study, we investigated the aberrant methylation profile of the tumor suppressor genes P15, P16, APC and E-cadherin in the cells of body fluid. A methylation-specific polymerase chain reaction was performed in 31 cases of malignant effusion and 39 cases of non-malignant effusion. Aberrant promoter methylation of P15, P16, APC and E-cadherin genes was seen in 0%, 25.8%, 35.5% and 6.5% of malignant effusion cases, respectively, whereas the frequencies were 0%, 2.6%, 2.6% and 0%, respectively, for negative control effusion. There were statistically significant differences in the aberrant methylation of P16 ($p=0.008$) and APC ($p=0.018$) genes between cases of malignant effusion and controls. Methylation of one of three genes (P16, E-cadherin, APC) was found in 14 out of 31 (45.2%) cases of malignant effusion, and in two out of 39 (5.1%) cases of non-malignant effusion ($p=0.000004$). Concurrent methylation was found in nine out of 31 (29%) cases of malignant effusion, but in no non-malignant effusion sample. From these results, we suggest that methylation-specific polymerase chain reaction to analyze the promoters of tumor suppressor genes can distinguish between malignant effusion and benign effusion, and may help cytologists to make more accurate diagnoses.

**Key Words:** body fluid, cytologist, malignant cells, methylation-specific PCR, tumor suppressor gene


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coli (APC), Axin, and glycogen synthase 3β (GSK-3β). Unphosphorylated β-catenin then interacts with a transcriptional coactivator and T-cell factor/lymphoid-enhancer factor (Tcf/Lef) and activates the transcription of target genes [18–21]. The genes encoding APC, Axin and GSK-3β are thought to be tumor suppressor genes owing to their main function as regulators of β-catenin. E-cadherin has also been suggested to be a tumor suppressor gene. Mutation-induced suppression of E-cadherin expression has been associated with breast cancer and familial gastric cancer [14,19]. Under physiologic conditions, most β-catenin is bound to E-cadherin, suggesting a crucial regulatory role of E-cadherin in the Wnt signaling pathway [20]. The P15 and P16 genes are important tumor suppressors through cell cycle regulation, and previous reports have demonstrated hypermethylation of P16 and P15 in many cancers [21,22].

Cytologic examination of fluids obtained from the serous cavities is among the most common tasks performed in the practice of cytopathology. The diagnostic utility of effusion cytology is now well established, making it routine when trying to find the cause of fluid accumulation in a serous cavity. The most common reason for performing cytologic examination of fluids is to determine whether or not it contains malignant cells. In patients with clinically and/or pathologically proven pleural malignancy, cytologic examination of pleural fluid is positive in 71% of cases. The sensitivity of cytology for the diagnosis of malignancy is 58%. Due to the low specificity and sensitivity of routine cytologic examination of body fluids, especially to distinguish between benign and malignant mesothelial cells, it greatly affects clinical management and outcome.

Recent studies have shown that alterations in the tumor suppressor genes P15, P16 and APC-3, and in the adhesion molecule E-cadherin, were common in adenocarcinomas; however, no study has performed a concurrent analysis of the promoter methylation status of P15, P16, APC and E-cadherin genes in body fluids. Therefore, we used a panel of genes to aid in the identification of an epithelial population of cells in effusion.

**MATERIALS AND METHODS**

**Specimens**

Ascites fluid and pleural effusion were collected from 31 patients with metastatic adenocarcinomas and 39 patients with nonmalignant etiologies such as congestive heart failure, liver cirrhosis and infection. Samples were divided into two, one half for conventional cytology examination and the other for methylation study. This study was approved by the institutional review board of Changhua Christian Hospital and China Medical University Hospital.

**DNA extraction, methylation PCR and sequencing**

DNA extraction was performed as previously described [23,24]. Sodium bisulfite modification of DNA and methylation-specific polymerase chain reaction (PCR) were performed as previously described [23–25], with some modifications. The primer pairs used for the detection of methylated and unmethylated sequences on the promoters of various tumor-related genes are as previously described [23,24]. Generally, approximately 4 µg of genomic DNA in 40 µL H2O was denatured by incubation with 10 µL of 1 M NaOH at 37°C for 10 minutes, and then modified by 30 µL of 10 mM hydroquinone and 520 µL of 1.5 M sodium bisulfite (pH 5.0) at 50°C for 16 hours. DNA samples were eluted with 100 µL of pre-warmed H2O (65–70°C) using the wizard DNA purification kit (Promega, Madison, WI, USA). Then, 50 µL of 1 M NaOH was added to the eluant and the mixture was incubated at 65°C for 10 minutes. Finally, the sample was purified using the Wizard PCR Prep DNA purification kit (Promega, Madison, WI, USA).

**Table 1. Results of methylation-specific polymerase chain reaction analysis of P15, P16, APC-3 and E-cadherin genes**

<table>
<thead>
<tr>
<th></th>
<th>Malignant effusion (n=31)</th>
<th>Nonmalignant effusion (n=39)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylated</td>
<td>Unmethylated</td>
<td>Methylated</td>
</tr>
<tr>
<td>P16</td>
<td>8</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>2</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>APC-3</td>
<td>7</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>One of these genes</td>
<td>17</td>
<td>14</td>
<td>2</td>
</tr>
</tbody>
</table>
room temperature for 5 minutes. After precipitation with 150 µL of 100% isopropanol, the precipitate was washed with 70% ethanol. The DNA pellet was resuspended in 45 µL of H2O. Modified DNA pellets were amplified in a total volume of 20 µL containing 1X PCR buffer, 1 mM MgCl2, 100 ng of each primer, 0.2 mM dNTPs and 2.5 units of Taq polymerase. PCR was performed in a thermal cycler for 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at a specific temperature, as shown in Table 1, for 1 minute and extension at 72°C for 1 minute, followed by a final 5-minute extension at 72°C. PCR products were then loaded onto 3.5% agarose gels and electrophoresed, stained with ethidium bromide and visualized under UV illumination. CpG methylase (Sss I)-treated genomic DNA was used as a positive control for the methylation-specific primers because Sss I methylates all cytosines within the double-stranded dinucleotide CG. DNA samples from healthy individuals that produced no PCR products with methylation-specific primer sets, but did produce PCR products with unmethylation-specific primer sets, were used as positive controls for the unmethylation-specific primers. To ensure the specificity of methylation-or unmethylation-specific primer sets for bisulfite-modified DNA, amplification using unmodified genomic DNA samples from healthy individuals was also performed. For some representative cases, the PCR products were further analyzed by direct sequencing to confirm the results. Direct sequencing was performed on an ABI Prism 310 Genetic Analyzer using BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA).

**RESULTS**

The results of the methylation-specific PCR analysis of P15, P16, APC and E-cadherin are shown in the Figure. In total, 31 cases of malignant effusion and 39 cases of negative control effusion were analyzed for the methylation status of P15, P16, E-cadherin and APC (Table 1). The frequency of aberrant methylation was 0% for P15, 25.8% (8/31) for P16 (p = 0.008), 6.5% (2/31) for E-cadherin (p = 0.193) and 35.5% (11/31) for APC (p = 0.018). In nonmalignant effusion samples, less frequent methylation was detected in P15 (0%), P16 (2.6%), E-cadherin (0%) and APC (2.6%). There was a statistically significant higher frequency of aberrant methylation of P16 and APC genes in malignant compared to nonmalignant effusions. Methylation of one of the four genes analyzed (P15, P16, E-cadherin, APC) was found in 14 out of 31 (45.2%) cases of malignant effusion and two of 39 (5.1%) cases of negative control effusion (p = 0.000004). It is suggested that any one of these genes showing methylation in body fluid is a positive marker for malignancy. The results of concurrent methylation of P16, E-cadherin and APC are shown in Table 2. Of the 31 malignant effusion samples, one showed methylation of three genes (P16, E-cadherin and APC), two showed methylation of both P16 and E-cadherin, two showed methylation of both E-cadherin and APC, and four showed methylation of both APC and P16. Nine out of the 31 (29%) malignant effusion samples were positive for methylation changes in one of P16,

**Table 2. Number of cases showing concurrent methyla-
tion among P15, P16, E-cadherin and APC-3**

<table>
<thead>
<tr>
<th>Methylation gene</th>
<th>Tumor</th>
<th>Non-tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>All 4 genes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P16 + E-cadherin + APC-3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P16 + E-cadherin</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>P16 + APC-3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>E-cadherin + APC-3</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
E-cadherin or APC genes. None of the 39 negative control effusion samples showed concurrent methylation changes. Moreover, the frequency of concurrent gene methylation may also provide a more sensitive method for cancer detection. No methylation change in P15 was found in either malignant effusion samples or negative control effusions.

**DISCUSSION**

In the development of cancer, a number of tumor suppressor genes are inactivated by mutations and chromosomal deletions [26]. Aberrant methylation of CpG islands has been shown to serve as an alternative way of inactivating these genes in cancer. Recently, concurrent CpG-island hypermethylation has been reported in colorectal, pancreatic, gastric and oral carcinomas [12–19,27–30]. We have analyzed the methylation patterns of P15, P16, E-cadherin and APC in malignant pleural effusions and ascites, and negative control pleural effusion and ascites. Our results showed that aberrant methylation of P15 (0%), P16 (25.8%), E-cadherin (6.5%) and APC (35.5%) occurred in cases of malignant effusion. Nine cases of malignant effusion (29%) displayed concurrent hypermethylation.

Surprisingly, we found no aberrant methylation of P15 in either malignant or nonmalignant disorders. The P15 and P16 genes are important tumor suppressors through cell cycle regulation [21,22]. In our results, the aberrant hypermethylation rates of P16 and P15 were 25.8% and 0%, respectively. Aberrant methylation of P16 occurred frequently, and was significantly higher in malignant effusions than in nonmalignant effusions \( (p=0.008) \). No aberrant methylation of P15 was noted in either sample; therefore, this result suggested that methylation silencing of P15 might not be useful for the detection of cancer in body fluids.

The Wnt signaling pathway is commonly associated with the process of colon cancer tumorigenesis. Somatic mutations in the APC gene are present in 80% of sporadic colorectal cancers; however, 18% of sporadic colon cancers also demonstrated promoter methylation in 95% of the wild-type APC gene, suggesting an alternative mechanism of APC inactivation in colon tumorigenesis [31–33]. In our results, aberrant promoter methylation of the APC gene (35.5%) was much higher than seen previously. E-cadherin is a transmembrane glycoprotein that forms a complex with catenins, thereby linking E-cadherin to the actin cytoskeleton. There is compelling evidence from *in vitro* and *in vivo* experiments to suggest that the E-cadherin/catenin complex acts as an invasion suppressor [34]. It has been reported that loss of expression of E-cadherin by methylation of the promoter region was observed in 54.5% of poorly differentiated adenocarcinomas and mucinous carcinomas of the colon and rectum [35]. In our study, only 6.5% of malignant effusion cases showed aberrant promoter methylation. This discrepancy may be due to the fact that most (91%) of our patients had well to moderately differentiated adenocarcinomas. These findings suggest that aberrant promoter methylation of E-cadherin is not common in malignant effusions.

In conclusion, our study demonstrated the methylation profile of body fluid through a candidate gene approach. Our data indicated that aberrant promoter methylation is common in some tumor suppressor genes. The epigenetic silencing of multiple tumor suppressor genes might be a useful biomarker for diagnosing metastatic carcinomas in body fluids.

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**REFERENCES**

P15，P16，APC-3 和 E-cadherin 抑癌基因在体液中 Epigenetic 變化

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經過起動子的甲基化來不活化抑癌基因，在癌化過程中扮演著重要的角色，因而起動子的甲基化可做為分辨癌細胞的標記。在本研究中，我們探討抑癌基因 P15，P16，APC 及 E-cadherin 在體液細胞的甲基化情形。我們利用甲基化 PCR 分析 31 例惡性及 39 例良性體液。結果顯示，在惡性體液中，P15，P16，APC 及 E-cadherin 起動子甲基化的發生率分別是 0%，25.8%，35.5% 及 6.5%，而良性的體液中則分別為 0%，2.6%，2.6% 及 0%。P16（p = 0.008）及 APC（p = 0.018）的甲基化在惡性體液的發生率有顯著的差異，而 P16，APC 及 E-cadherin 中任何一個基因甲基化在惡性體液（14/31 = 45.2%）及良性體液（2/39 = 5.1%）也有顯著的差異（p = 0.000004）。又同時有兩個以上的基因甲基化在惡性體液為 29%（9/31），在良性體液則為 0%。由此可見，利用甲基化特異性的 PCR 方法來分析抑癌基因甲基化的變化，將可提供另一個可靠的方法來幫助細胞學家分辨體液內是否存有惡性癌細胞。

關鍵詞：體液，細胞學家，惡性癌細胞，甲基化 PCR，抑癌基因

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