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# Utility of Promoter Hypermethylation for Differentiating Malignant and Benign Effusions in Liquid-Based Cytology Specimens

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**Background :** Making the cytologic differentiation between benign and malignant effusions can be difficult. Because promoter hypermethylation of tumor suppressor genes is a frequent epigenetic event in many human cancers, it could serve as a marker for the diagnosis of cancer. The aim of this study was to investigate the feasibility of detecting promoter hypermethylation as a diagnostic tool with using liquid-based cytology samples for differentiating between malignant and benign effusions. **Methods :** A multiplex, nested, methylation-specific polymerase chain reaction analysis was used to examine promoter methylation of 4 genes (retinoic acid receptor-$\beta$, [*RAR-$\beta$*], adenomatous polyposis coli [*APC*], *Twist* and high in normal-1 [*HIN-1*]) in malignant (n = 85) and benign (n = 31) liquid-based cytology samples. **Results :** The frequencies of hypermethylation of *RAR-$\beta$*, *APC*, *Twist* and *HIN-1* were significantly higher in the malignant effusions than in the benign effusions ($p < 0.001$ for each). On the receiver-operating characteristic analysis, the area under the curve (AUC) for *APC* was the greatest. The AUC for the best two-gene combination (*APC/HIN-1*) was not statistically different from the AUC for the best individual tumor suppressor gene (*APC*). **Conclusions :** This study suggests that promoter methylation analysis on residual liquid-based effusion samples may be a feasible approach to detect malignant effusions, and that *APC* is the best marker for differentiating between malignant and benign effusions.

**Key Words :** Liquid based cytology; Malignancy; Body fluids; Methylation; Polymerase chain reaction

Effusion is the accumulation of fluid in a body cavity and it is caused by the diverse etiologies of benign and malignant diseases. Malignant effusions worsen the quality of life in patients with advanced cancer. These patients have an average life expectancy of 3 to 6 months.[1-3] Therefore, the ability to distinguish between malignant and benign serous effusions is very important for the prognosis and medical management. Serous effusions are usually diagnosed by cytologic assessment. Various studies have shown a sensitivity of 57% and a specificity of 89% by conventional cytology for detecting malignant cells in effusion samples.[4] Of note, reactive mesothelial cells of benign entities occasionally show very atypical features and so they serve as a major pitfall for a false positive diagnosis of malignancy.[5]

The introduction of liquid-based cytology (LBC) has recently allowed for the observation of cells arranged in thin layers on a clear background, resulting in improved specimen quality and a diminished false negative rate.[6] In addition, LBC offers the advantage of providing a residual sample that can be used for ancillary testing. However, identifying malignant cells, which relies on microscopic examination of effusions, is sometimes not sufficient for a definitive diagnosis of malignancy. Therefore, various ancillary techniques such as immunohistochemistry, electron microscopy and flow cytometry have been used to increase the diagnostic accuracy of effusion cytology.[4,7] Nevertheless, there is no single method to diagnose a malignant condition in serous effusions. Accordingly, it is necessary to develop a new diagnostic technique to selectively detect malignant cells in patients with serous effusions.

CpG island methylation is in the spotlight of cancer epigenetics. CpG islands are mostly located in the promoter region. Tumor suppressor gene-silencing by aberrant CpG island hypermethylation is an important mechanism for carcinogenesis and it occurs at an early step in various human cancers.[8] There are studies that have focused on CpG island hypermethylation-based detection of malignant cells in biopsy specimens, blood, sputum, urine and cervicovaginal smears.[9-14] However, only a few studies have attempted using aberrant CpG island hypermethylation in serous effusions to assess the accuracy of diagnosing malignancies.[13-18]

The present study was undertaken to examine using promoter hypermethylation as a diagnostic marker for differentiating between malignant and benign effusions, and also to detect the appropriate genes and gene combinations for differentiating between malignant and benign effusions in LBC samples. A multiplex, nested, methylation-specific polymerase chain reaction (MSP) approach was used to examine promoter methylation of 4 genes (retinoic acid receptor-$\beta$ [*RAR-*$\beta$], adenomatous polyposis coli [*APC*], *Twist* and high in normal-1 [*HIN-1*]) in confirmed malignant (n = 85) and benign (n = 31) LBC samples. These genes were selected because they are frequently methylated in lung, gastrointestinal tract and breast cancers, which are the most common primary tumors that give rise to malignant effusions.[19-21]

## MATERIALS AND METHODS

### Patients and sample collection

Confirmed malignant (n = 85) and benign (n = 31) LBC samples were identified from the pathology archives of Chonnam National University Hwasun Hospital after approval was granted by the Institutional Review Board. These cases were diagnosed with standard cytologic techniques, including Papanicolaou stained ThinPrep slides and hematoxylin and eosin stained cell block slides. We also performed immunohistochemistry with a panel of antibodies against MOC-31 (1 : 50, DAKO, Carpenteria, CA, USA), D2-40 (1 : 50, DAKO) and calretinin (1 : 50, DAKO) for the confirmation of malignancy.

The effusion samples were collected using the Thin Prep Cytology Collection System (CYTYC Corporation, Marlborough, MA, USA) according to the manufacturer's protocol. The sample vials were stored at ambient temperature and used within 30 months of the collection date.

Both pleural (n = 76) and peritoneal (n = 40) effusions were evaluated in the present study. The study group included 67 men and 49 women and their total mean age was 59 years (range, 25 to 84 years). The primary sites for the 85 cases with malignant effusions included 30 gastrointestinal tracts, 6 hepatobiliary tracts, 40 lungs, 6 ovaries and 3 breasts.

### DNA preparation

Genomic DNA was isolated from cells that were spun down from the 5 mL ThinPrep cytology collections. Briefly, the residual samples were spun down in a centrifuge at 2,000 rpm for 5 minutes, and the precipitates were incubated with TNES solution (10 mM Tris [pH 8.0], 150 mM NaCl, 2 mM EDTA and 0.5% SDS) and proteinase K at 52℃ for 4 hours. Bisulfite modification was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) and by following the manufacturer's instructions. The bisulfite-modified DNA was stored at below -20℃ until subsequent MSP.

### Methylation-specific polymerase chain reaction

The methylation status of the 4 genes (*RAR-*$\beta$, *APC*, *Twist*, and *HIN-1*) was analyzed by MSP using a multiplex, nested PCR approach.[22] Step 1 of the nested MSP was performed with primer sets (sense and antisense) for all four individual genes in each reaction. The step 1 primers flanked the CpG-rich promoter regions of their respective targeted genes and the promoter regions contained no CpG dinucleotides. For step 1, a 50-$\mu$L reaction volume was used that contained 10 × PCR buffer, the primers, the bisulfite-treated DNA template (5 $\mu$L), deoxyribonucleoside triphosphates (1.25 mM each) and Platinum Taq polymerase (1 $\mu$L). The reactions were carried out using the following conditions: 95℃ for 5 minutes; then 36 cycles at 95℃ for 30 seconds, 56℃ for 30 seconds and 72℃ for 40 seconds; a final extension step was performed at 72℃ for 5 minutes. A negative control for the assay (sterile water only) and the unmethylated (human sperm DNA) and methylated controls (MDA-MB-231 cells) were included in the step 1 reactions. The PCR products from the step 1 reactions were diluted 1 : 5 in water and 1 $\mu$L of this was used as the DNA template in step 2 of the nested MSP protocol (25 $\mu$L total reaction volume with the components listed above) using the following conditions: 95℃ for 5 minutes, then denature at 95℃ for 30 seconds, annealing at 60℃ for 30 seconds, extension at 72℃ for 40 seconds for 30 cycles, followed by a 5-minutes final extension. All the primer sequences for this nested-MSP approach have been previously published and they are shown in Table 1.[22-24] The PCR products were analyzed by 2% agarose gel electrophore-

Table 1. Primer sets for nested methylation-specific polymerase chain reaction

| Gene | Sense | Antisense | Size (bp) |
|---|---|---|---|
| | External (stage 1) | External (stage 1) | |
| *RAR-β* | GTAGGAGGGTTTATTTTTTGTT | AATTACATTTTCCAAACTTACTC | 239 |
| *APC* | TGGGYGGGGTTTTGTGTTTTATT | TACRCCCACACCCAACCAATC | 136 |
| *Twist* | GAGATGAGATATTATTTATTGTG | CCTCCCAAACCATTCAAAAAC | 273 |
| *HIN-1* | GTTTGTTAAGAGGAAGTTTT | AAACATACAAAACAAAACCAC | 285 |
| | Internal methylated (stage 2) | Internal methylated (stage 2) | |
| *RAR-β* | GAACGCGAGCGATTCGAGT | GACCAATCCAACCGAAACG | 143 |
| *APC* | TATTGCGGAGTGCGGGTC | TCGACGAACTCCCGACGA | 98 |
| *Twist* | TTTCGGATGGGGTTGTTATC | AAACGACCTAACCCGAACG | 200 |
| *HIN-1* | GGTACGGGTTTTTTACGGTTCGTC | AACTTCTTATACCCGATCCTCG | 138 |
| | Internal unmethylated (stage 2) | Internal unmethylated (stage 2) | |
| *RAR-β* | GGATTGGGATGTTGAGAATGT | CAACCAATCCAACCAAAACAA | 159 |
| *APC* | GTGTTTTATTGTGGAGTGTGGGTT | CCAATCAACAAACTCCCAACAA | 108 |
| *Twist* | TTTGGATGGGGTTGTTATTGT | CCTAACCCAAACAACCAACC | 193 |
| *HIN-1* | GGTATGGGTTTTTTATGGTTTGTT | CAAAACTTCTTATACCCAATCCTCA | 140 |

*RAR-β*, retinoic acid receptor-*β*; *APC*, adenomatous polyposis coli; *HIN-1*, high in normal-1.

sis. The methylation status was considered positive if a band was visibly detected after ethidium bromide staining in the lanes with reactions and using methylated primers. Samples were considered noninformative if no band was detected in the lanes using both unmethylated and methylated primers.

### Statistical analysis

The chi-square test was used to determine the association between various categories and the frequency of hypermethylation. The diagnostic accuracy of MSP-based detection of hypermethylation in the serous effusions was assessed by calculating the sensitivity and specificity. Sensitivity was defined as the percentage of malignant cases in which gene methylation was detected. Specificity was defined as the percentage of benign samples in which gene methylation was not detected. The area under the receiver operating characteristic (ROC) curve (AUC) was used as a measure of test performance to determine if gene methylation could distinguish between the malignant and nonmalignant effusion samples. On the ROC analysis, the greater the AUC was, the better the test performance was. A perfect test has an area of 1.0 and a test with no discriminatory value has an area of 0.5.[25] The AUC was then determined for each of the possible 2-gene, 3-gene and 4-gene combinations. We tested whether four genes yielded an improvement over three genes, whether three genes yielded an improvement over two genes, and whether two genes yielded an improvement over one gene.

For all statistical analyses, the SPSS ver. 13.5 (SPSS Inc., Chicago, IL, USA) was used, with p-values < 0.05 being regarded as statistically significant.

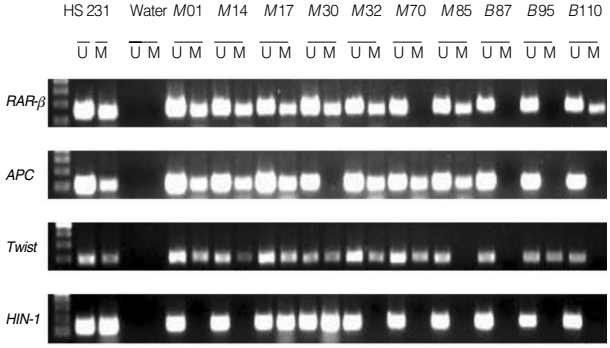


Fig. 1. Representative results of the nested methylation-specific polymerase chain reaction analysis for retinoic acid receptor-*β* (*RAR-β*), adenomatous polyposis coli (*APC*), high in normal-1 (*HIN-1*) and *Twist* in body fluid. Lanes U and M correspond to unmethylated and methylated DNA, respectively. DNA from human sperm (HS) and the MDA-MB-231 breast cancer cell line (231) served as the positive controls for the unmethylated and methylated genes, respectively. Water served as negative control for both the unmethylated and methylated genes.
M, malignant effusion case; B, benign effusion case.

## RESULTS

### Frequency of promoter hypermethylation

We analyzed the promoter methylation status of the *RAR-β*, *APC*, *Twist*, and *HIN-1* genes in the malignant (n = 85) and benign (n = 31) effusions from the LBC samples by employing a multiplex, nested MSP technique. The representative examples of the MSP products obtained from step 2 of the nested MSP analysis for 4 genes are shown in Fig. 1. The frequencies of methylation of each tumor suppressor gene locus in the malignant and

benign effusion samples are shown in Table 2. The promoter hypermethylation frequencies for *RAR-β*, *APC*, *Twist*, and *HIN-1* in the malignant effusions were 87.1% (74/85), 81.2% (69/85), 81.2% (69/85), and 49.4% (42/85), respectively; the frequencies in the benign effusions were 35.5%, 22.6%, 41.9%, and 9.6%, respectively. The differences in the frequencies of methylation between the malignant and benign effusions were statistically significant for all 4 genes ($p < 0.001$ for each).

Next, we investigated whether one gene was preferentially methylated depending on the tumor's site of origin. For all the genes except *Twist*, DNA methylation was not significantly correlated with the primary site of malignancy (Table 3). Aberrant hypermethylation of *Twist* was present in 100% of the hepatobiliary cancers; however, only 33% of breast cancers demonstrated promoter methylation of *Twist* ($p = 0.035$).

Table 2. Frequency of methylation in the benign and malignant effusions

| Gene | No. of methylation (%) | | p-value |
|---|---|---|---|
| | Malignant effusion (n = 85) | Benign effusion (n = 31) | |
| *RAR-β* | 74 (87.1) | 11 (35.5) | < 0.001 |
| *APC* | 69 (81.2) | 7 (22.6) | < 0.001 |
| *Twist* | 69 (81.2) | 13 (41.9) | < 0.001 |
| *HIN-1* | 42 (49.4) | 3 (9.6) | < 0.001 |

*RAR-β*, retinoic acid receptor-β; *APC*, adenomatous polyposis coli; *HIN-1*, high in normal-1.

## Diagnosis of the malignant effusions by MSP

In our population, the sensitivity of hypermethylation of *RAR-β*, *APC, Twist*, and *HIN-1* was 87.1%, 81.2%, 81.2%, and 49.4%, respectively; the specificity was 64.5%, 77.4%, 58.1%, and 90.4%, respectively. The addition of the other genes did

Table 3. Comparison between DNA methylation and the primary site of origin of the malignant effusions

| Gene | No. of methylation (%) | | | | | p-value |
|---|---|---|---|---|---|---|
| | GI tract (n = 30) | Hepatobiliary tract (n = 6) | Lung (n = 40) | Ovary (n = 6) | Breast (n = 3) | |
| *RAR-β* | 26 (86.7) | 6 (100) | 34 (85.0) | 6 (100) | 2 (66.7) | 0.550 |
| *APC* | 25 (83.3) | 4 (66.7) | 32 (80.0) | 5 (5.9) | 3 (100) | 0.796 |
| *Twist* | 26 (86.7) | 6 (100) | 33 (82.5) | 3 (50) | 1 (33.3) | 0.035 |
| *HIN-1* | 17 (56.7) | 3 (50) | 19 (47.5) | 2 (33.3) | 1 (33.3) | 0.805 |

GI, gastrointestinal; *RAR-β*, retinoic acid receptor-β; *APC*, adenomatous polyposis coli; *HIN-1*, high in normal-1.

Table 4. The estimated sensitivity, specificity and area under the curve of the receiver-operating characteristic analysis for a panel of hypermethylated genes to distinguish malignant effusion samples from benign effusion samples

| Genes panel | Sensitivity | Specificity | Area under the ROC curve (SE) | 95% Confidence interval |
|---|---|---|---|---|
| Individual gene | | | | |
| *RAR-β* | 87.1 | 64.5 | 0.75 (0.05) | 0.66-0.85 |
| *APC* | 81.2 | 77.4 | 0.81 (0.04) | 0.72-0.89 |
| *Twist* | 81.2 | 58.1 | 0.71 (0.05) | 0.61-0.80 |
| *HIN-1* | 49.4 | 90.4 | 0.70 (0.04) | 0.62-0.77 |
| Two-gene combination | | | | |
| *RAR-β*/*APC* | 97.6 | 54.8 | 0.77 (0.05) | 0.68-0.86 |
| *Twist*/*HIN-1* | 90.6 | 58.1 | 0.75 (0.05) | 0.66-0.85 |
| *RAR-β*/*Twist* | 95.3 | 64.5 | 0.79 (0.05) | 0.70-0.88 |
| *APC*/*HIN-1* | 90.6 | 71.0 | 0.82 (0.04)[a] | 0.73-0.91 |
| *RAR-β*/*HIN-1* | 92.9 | 58.1 | 0.75 (0.05) | 0.65-0.84 |
| *APC*/*Twist* | 96.5 | 41.9 | 0.70 (0.05) | 0.61-0.79 |
| Three-gene combination | | | | |
| *RAR-β*/*APC*/*Twist* | 98.8 | 35.5 | 0.68 (0.05) | 0.59-0.77 |
| *RAR-β*/*APC*/*HIN-1* | 97.6 | 58.1 | 0.77 (0.05) | 0.68-0.86 |
| *APC*/*Twist*/*HIN-1* | 98.8 | 41.9 | 0.71 (0.05) | 0.62-0.80 |
| All genes | | | | |
| *RAR-β*/*APC*/*Twist*/*HIN-1* | 98.8 | 35.5 | 0.68 (0.05) | 0.59-0.77 |

[a]Not significant for *APC*/*HIN-1* vs *APC*.

ROC curve, receiver operating characteristic curve; SE, standard error; *RAR-β*, retinoic acid receptor-β; *APC*, adenomatous polyposis coli; *HIN-1*, high in normal-1.

not increase the sensitivity and specificity values (Table 4).

To evaluate the predictive utility of the markers, we used ROC analysis and we calculated the AUC for the individual tumor suppressor genes and for the gene combinations (Table 4). ROC curve analyses were first conducted on individual markers and then on combinations to determine whether a panel performed better than any single marker. The AUCs for *RAR-β*, *APC, Twist* and *HIN-1* (0.75, 0.81, 0.71, and 0.70, respectively) were all significantly greater than 0.50. *APC* exhibited the highest AUC. The AUC for the best two-gene combination (*APC*/*HIN-1*) was not statistically different from the best individual tumor suppressor gene (*APC*) for distinguishing malignant effusions from benign effusions.

## DISCUSSION

Aberrant promoter CpG island hypermethylation is a major mechanism of tumor suppressor gene inactivation and it may offer a promising new approach to improve a cytologic diagnosis. In the current study, we used multiplex, nested MSP technology to examine the promoter methylation status of 4 tumor suppressor genes. Aberrant promoter methylation of 4 genes occurred with greater frequency in the malignant effusions than that in the benign effusions. The use of ROC analysis and calculation of the AUC demonstrated that *APC* is the best marker for its ability to distinguish malignant effusions from benign effusions. However, gene combinations did not improve the test performance as compared with the best individual gene.

Although a majority of effusions are reliably diagnosed using standard cytologic criteria, cytomorphological examination of effusion smears is still associated with significant false negative and false positive results.[6] Therefore, more objective techniques based on the specific biomarkers that are expressed by cancer cells, but not by benign cells, are still needed.

The diagnostic and prognostic values of aberrant DNA methylation in cancer have been studied in various types of neoplasia. It has been demonstrated that the presence of promoter methylation of various genes in serum, plasma and sputum is a surrogate for methylation of the same genes in tumor tissue, and moreover, detecting DNA methylation in clinical samples may be useful for accurately detecting cancer cells.[9-14]

Recent studies have demonstrated the presence of promoter hypermethylation of various genes in pleural fluid and peritoneal fluid.[13-18] Brock *et al*.[15] examined the promoter methylation of 8 genes (*p16, MGMT, BRCA1, APC,* RAS association domain family 1A [*RASSF1A*], *RAR-β*, fragile histidine triad [*FHIT*] and cellular retinol binding protein-1 [*CRBP1*]) in malignant pleural effusions. Combined cytology and methylation exhibited 88% sensitivity and 100% specificity. Benlloch *et al*.[13] also examined promoter methylation of 4 genes (death-associated protein kinase [*DAPK*], *RASSF1A, RAR-β,* and *p16/INK4α*) in the pleural fluid and serum of cancer patients with pleural effusion. Methylation was detected in the pleural fluid from 58.5% of the patients with malignant pleural effusions and from 0% of the patients with benign pleural effusions. Katayama *et al*.[16] investigated DNA methylation of 5 tumor suppressor genes (*MGMT, p16/INK4α*, *RASSF1A, DAPK*, and *RAR-β*) in pleural fluid. The sensitivity and specificity of methylation in one or more genes for making the diagnosis of malignant effusions were 56.9% and 78.4%, respectively. Chen *et al*.[18] reported methylation of at least one of three genes (*p16, APC*, and *E-cadherin*) at frequencies of 45.2% and 5.1% in malignant and non-malignant effusions, respectively. These previous studies suggest that aberrant promoter methylation analysis in body fluid samples may serve as a diagnostic tool for malignant effusions, and this can potentially be used alone or in conjunction with cytology.

In our study, aberrant methylation of *RAR-β*, *APC, Twist*, and *HIN-1* was detected at a frequency of 87.1%, 81.2%, 81.2%, and 49.4% of the malignant effusion cases, respectively. The methylation frequency of the malignant effusions was significantly higher than the methylation frequency of the benign effusions. Overall, the frequency of promoter methylation observed in this study was higher than that reported by the previous studies.[13-18] In contrast to most of the previous studies that performed conventional MSP,[14-18] we used nested MSP. A conventional MSP approach can detect 1 copy of methylated DNA in 1,000 unmethylated copies of genomic DNA. Nested MSP is very sensitive and it can detect 1 methylated allele in > 50,000 unmethylated alleles.[12] Brock *et al*.[15] also used nested MSP in 25 malignant and 7 benign pleural effusions. In their study, the prevalence of methylation of *RAR-β*, and *APC* in the malignant pleural effusions was 50.0% and 29.1%, respectively. Differences in the patient population may partially account for this discrepancy between Brock *et al*.'s study[15] and our study. The number of malignant effusions in this study was the highest of all the studies on the methylation profiles in serous effusions.

In the present study, we evaluated the diagnostic efficiency of the methylation status of LBC specimens for differentiating between malignant and benign effusions. On the ROC analysis, the AUC for *RAR-β*, *APC*, *Twist*, and *HIN-1* were all significantly greater than 0.50. We found that *APC* is the best single gene

marker for distinguishing between malignant and benign effusions. The estimated sensitivity of *APC* methylation for detecting malignant effusions was 81.2%, and its specificity was 77.4%. Although the AUC for the best 2-gene combination (*APC*/*HIN-1*, 0.82) was greater compared with the AUC for the *APC* gene (*APC*, 0.81), the difference was not statistically significant. These findings suggest that the addition of *HIN-1* did not significantly improve the test performance compared with *APC* alone for its ability to distinguish malignant effusions from benign effusions. Thus, gene combinations did not improve the test performance as compared with that of individual genes in this study.

We limited our search for useful hypermethylated genes to an assessment of 4 genes that had been previously reported to be associated with cancers. Identification of additional genes that are selectively methylated in malignant effusions will be necessary to construct a panel with higher sensitivity and that maintains high specificity.

Identification of the primary tumor site in malignant effusions has a profound clinical impact. Some evidence has shown that promoter methylation is cancer-type specific.[26] We evaluated the usefulness of promoter hypermethylation for determining the primary tumor site of malignant effusion. Because the DNA methylation of all genes, except *Twist*, was not significantly correlated with the primary site of malignancy in this study, it was difficult to confirm the speculation that methylation profiles may be helpful in suggesting the origin of tumor.

In conclusion, we demonstrated that the application of multiplex, nested MSP technology to residual LBC samples may be a feasible approach to detect malignant effusions. Further studies are needed to identify additional genes that are selectively methylated in malignant effusions and to determine the optimal combination of genes that will provide increased clinical sensitivity while maintaining high specificity.

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