p16/CDKN2A FISH in Differentiation of Diffuse Malignant Peritoneal Mesothelioma From Mesothelial Hyperplasia and Epithelial Ovarian Cancer

Tomohiro Ito, MD,1,2 Makoto Hamasaki, MD, PhD,1 Shinji Matsumoto, CT,1 Kenzo Hiroshima, MD, PhD,3 Tohru Tsujimura, MD, PhD,4 Toshiaki Kawai, MD, PhD,5 Yoshiya Shimao, MD, PhD,6 Kousuke Marutsuka, MD, PhD,7 Sayaka Moriguchi, MD, PhD,7 Riruke Maruyama, MD, PhD,8 Shingo Miyamoto, MD, PhD,3 and Kazuki Nabeshima, MD, PhD1

From the 1Department of Pathology and 2Department of Obstetrics and Gynecology, Fukuoka University School of Medicine and Hospital, Fukuoka, Japan; 3Department of Pathology, Tokyo Women’s Medical University Yachiyo Medical Center, Yachiyo, Japan; 4Department of Pathology, Hyogo College of Medicine, Hyogo, Japan; 5Department of Pathology and Laboratory Medicine, National Defense Medical College, Tokorozawa, Japan; 6Department of Pathology, Miyazaki Prefectural Miyazaki Hospital, Miyazaki, Japan; 7Department of Pathology, Miyazaki University School of Medicine, Miyazaki, Japan; and 8Laboratory of Surgical Pathology, Shimane University School of Medicine, Izumo, Japan.

Key Words: Diffuse malignant peritoneal mesothelioma; p16; 9p21; Fluorescence in situ hybridization; Reactive mesothelial hyperplasia; Epithelial ovarian cancer

ABSTRACT

Objectives: It can be difficult to differentiate diffuse malignant peritoneal mesothelioma (DMPM) from reactive mesothelial hyperplasia (RMH) or peritoneal dissemination of gynecologic malignancies, such as epithelial ovarian cancer (EOC), which cause a large amount of ascites. Detection of the homozygous deletion of p16/CDKN2A (p16) by fluorescence in situ hybridization (FISH) is an effective adjunct in the diagnosis of malignant pleural mesothelioma. The aim of this study was to investigate the ability of the p16 FISH assay to differentiate DMPM from RMH and EOC.

Methods: p16 FISH was performed in 28 DMPMs (successful in 19), 30 RMHs, and 40 EOC cases. The cutoff values of p16 FISH were more than 10% for homozygous deletion and more than 40% for heterozygous deletion.

Results: According to the above criteria, nine (47.4%) of 19 successful DMPM cases were homozygous deletion positive, and three (15.8%) of 19 were heterozygous deletion positive, whereas all RMH cases were negative for the p16 deletion. In all four major histologic subtypes of EOC, neither p16 homozygous nor heterozygous deletions were detected. To differentiate DMPM from RMH or EOC, the sensitivity of the p16 homozygous deletion was 32% (9/28), and the specificity was 100%.

Conclusions: Our study suggests that p16 FISH analysis is useful in differentiating DMPM from RMH and EOC when homozygous deletion is detected.

Malignant mesothelioma is an uncommon and aggressive neoplasm that arises from serosal surfaces. In general, these neoplasms have a poor prognosis and short survival.1 After the pleura, the peritoneum is the second most frequent site of origin of mesothelioma.2 In female patients, the diagnosis of diffuse malignant peritoneal mesothelioma (DMPM) is sometimes problematic, because the clinical presentation, diagnostic imaging, and operative findings of DMPM are similar to those of epithelial ovarian cancer (EOC), with widespread disease throughout the peritoneal cavity.3,4 Malignant mesothelioma also exhibits a wide range of histopathologic patterns that may potentially mimic a variety of benign conditions.
of primary and metastatic ovarian tumors.\textsuperscript{3} The distinction between reactive mesothelial hyperplasia (RMH) and DMPM is also problematic, because RMH and DMPM have the overlapping morphologic findings on cytologic and surgical specimens.\textsuperscript{5,6} Although a combination of several antibodies as positive and negative markers for malignant mesothelioma is generally recommended for immunohistochemical support of the diagnosis, no satisfactorily reproducible biomarker has yet been confirmed.\textsuperscript{7}

Although no official TNM staging system exists for patients with DMPM, a new staging system was recently proposed. Patients with T1 (peritoneal cancer index 1-10) N0M0 survived significantly longer than the other patients, and the 5-year survival associated with stage I, II, and III disease was 87%, 53%, and 29%, respectively.\textsuperscript{8} Furthermore, recent studies suggested that a combination of cytoreductive surgery and perioperative intraperitoneal chemotherapy resulted in improved survival.\textsuperscript{9,10} Thus, early and accurate diagnosis of DMPM is critical for improving its clinical outcome.

One of the most common genetic alterations in primary malignant mesothelioma is the homozygous deletion of the 9p21 region, which includes CDKN2A/p16\textsuperscript{INK4a} (p16), CDKN2B/p15\textsuperscript{INK4b}, and p14\textsuperscript{ARF}.\textsuperscript{11-15} Deletion of the 9p21 region or p16 gene has been reported in more than 70% to 80% of mesothelioma by cytogenetic and molecular studies.\textsuperscript{12-14} Detection of the homozygous deletion of p16 by fluorescence in situ hybridization (FISH) was shown to be feasible and helpful in confirming a diagnosis of mesothelioma in cytologic and surgical specimens, especially in the differentiation of malignant pleural mesothelioma from RMH.\textsuperscript{7,16-25} Fewer reports are available for p16 FISH in DMPM. However, some studies have reported that p16 homozygous deletion, detected by FISH, was found in about 25% to 51% of DMPM cases.\textsuperscript{7,22,23}

The aim of this study was to investigate the usefulness and limitations of the p16 FISH assay in the diagnosis of DMPM, especially in terms of its differentiation from RMH and EOC in surgical specimens.

Materials and Methods

Tissue Samples

This study included 28 DMPM cases (14 men and 14 women; mean age, 65.1 years; range, 32-78 years), 40 EOC cases (40 women; mean age, 52.9 years; range, 21-74 years), and 30 RMH cases (30 women; mean age, 50.1 years; range, 21-68 years). The data were derived from the peritoneal and gynecologic files of the Department of Pathology, Fukuoka University Hospital (FUH), in Fukuoka, Japan, and included both FUH and consultation cases from August 1993 through January 2012. EOC cases were treated at the Department of Obstetrics and Gynecology, FUH, from July 2006 through June 2011. RMH lesions were obtained from the greater omentum excised during gynecologic tumor resection to rule out metastatic lesions at the Department of Obstetrics and Gynecology, FUH, from July 2006 through June 2011. The study protocol was approved by the institutional review board of the Fukuoka University School of Medicine. Anonymous use of redundant tissue is part of the standard treatment agreement with patients in our hospital when no objection has been expressed. All cases were histologically diagnosed according to the 2003 World Health Organization classification of tumors of the breast and female genital organs.\textsuperscript{26} The diagnosis of DMPM was confirmed with immunohistochemistry, including mesothelial markers (calretinin, WT-1, D2-40, cytokeratin [CK] 5/6), pan-epithelial markers (carcinoembryonic antigen, Ber-EP4, MOC-31, thyroid transcription factor 1), and others (CAM5.2, CK AE1/AE3, EMA, PAX8). The clinicopathologic characteristics of the tumor and reactive cases are summarized in Table I.

FISH Analysis

p16 FISH was performed on formalin-fixed, paraffin-embedded, 4-μm-thick tissue sections using a DAKO Histology FISH Accessory Kit (DAKO, Carpinteria, CA)
with slight modifications as described previously.²⁵ Briefly, sections were deparaffinized and rehydrated with descending alcohol dilutions. This was followed by treatment with 2× saline-sodium citrate (2× SSC) containing 0.3% Tween 20 (Sigma, St Louis, MO), washed with 2× SSC, and then treated with pretreatment solution (20× dilution) at 95°C for 10 minutes and digested with pepsin solution at 37°C for 5 minutes. After fixation in 10% buffered formalin at room temperature for 3 minutes, the tissue sections were treated in 2× SSC containing 0.3% Tween 20 at 45°C for 10 minutes, dehydrated in ethanol, dried, and exposed to the two probes (p16 and CEP9; Abbott Japan, Tokyo, Japan). Both the probes and tissue sections were denatured at 85°C for 5 minutes in probe solution (Abbott Japan) followed by hybridization at 37°C for 24 hours in ThermoBrite (Abbott Japan). The tissue sections were washed in 2× SSC containing 0.3% Tween 20 at 72°C for 2 minutes and in 2× SSC containing 0.1% Tween 20 at room temperature for 5 minutes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI)/antifade (Vector Laboratories, Burlingame, CA). Analyses were performed using a fluorescence microscope (Axio Imager Z1; Carl Zeiss Microimaging, Jena, Germany) and Isis analysis system (Metasystems, Altlussheim, Germany) equipped with filter sets with single- and dual-band exciters for Spectrum Green, Spectrum Orange, and DAPI. Lymphocytes in each section served as internal controls and showed two signals per FISH probe. Homozygous deletion was defined as lack of both p16 signals in the presence of both CEP9 green signals. Heterozygous deletion was assumed when only one p16 signal was present or when the total number of p16 signals did not exceed half the total number of the centromeric signals. At least 60 cells were scored in each case.

Cutoff Values

The cutoff values for heterozygous and homozygous p16 deletion were determined based on the results in RMH cases according to a previous report.²¹ Specifically, cutoff values were calculated as the mean percentage ±3 SD of nuclei showing one 9p21 signal for heterozygous deletion and a loss of both 9p21 signals for homozygous deletion in RMH cases. Based on the results in RMH cases, a cutoff value of more than 10% was set for homozygous deletion; although the actual value was 8.0% because homozygous deletion in RMH was 1.7 ± 2.1. Figure 1A, more than 10% was used to exclude the possibility of pseudopositives. A cutoff value of more than 41% was set for a heterozygous deletion.

Statistical Analysis

Statistical comparison of FISH data between DMPM and RMH or EOC was performed using the Mann-Whitney U test. P < .05 was considered statistically significant. All statistical evaluations were performed with StatMate IV statistical software for Windows (ATMS Co, Tokyo, Japan).
Results

To determine the rate of p16 deletion in DMPM, RMH, and EOC cases, we first systematically performed histologic and FISH analyses on samples from each case. Figure II shows representative H&E sections and FISH images of epithelioid-type DMPM (Images 1A and 1B) and RMH (Images 1C and 1D). In DMPM, p16 FISH analysis was successful in 19 (67.9%) of 28 cases. Among the 19 cases of DMPM surgical samples, there were 16 (82.4%) successful cases and three (18.6%) failures, including signals that could not be detected in older specimens. Among the nine cases of DMPM autopsy samples, there were three (33.3%) successful cases and six (66.7%) failures. Statistical analysis showed this to be a significant difference (P < .01, $\chi^2$ test). It is reported that more than a 1-hour delay to formalin fixation negatively affected the HER2 FISH results. In autopsy cases, this delay to formalin fixation might be a cause of more failures in detecting FISH signals. These unsuccessful samples could not be analyzed because the signal intensity was too low. The 19 successful cases included seven (36.8%) men and 12 (63.2%) women. Mesothelioma cells with homozygous deletion of p16 showed loss of two red signals (Image 1B), while RMH cells exhibited two red and two green signals (Image 1D). In 30 cases of RMH, p16 homozygous and heterozygous deletions were observed...
in 1.7% ± 2.1% and 17.6% ± 7.7% of cells, respectively, whereas a normal pattern was observed in 80.3% ± 8.9% of cells (Figure 1A).

To determine whether p16 deletion could differentiate between DMPM and RMH, we performed statistical analysis comparing the rates of deletion between the two groups. The cutoff values were set at more than 10% for homozygous deletion and more than 41% for heterozygous deletion, as described in the Materials and Methods section, based on the results in RMH. According to these criteria, nine (32%) of 28 cases of DMPM (9/19 [47.4%] of DMPM cases with successful FISH signals) were homozygous deletion positive and three cases of DMPM were heterozygous deletion positive, whereas all RMH cases were negative for the p16 deletion (Figure 1A). All of the three heterozygous deletion-positive cases were also homozygous deletion positive. Analysis of all cases and female-only cases of DMPM showed significantly more frequent homozygous deletions than RMH cases (P < .05, Mann-Whitney U test) (Figure 1A). These data suggest that homozygous deletion of p16 is indicative of DMPM over RMH.

Finally, we investigated whether p16 homozygous deletion could differentiate between DMPM and EOC. Image 2I shows representative H&E sections of EOC (Image 2A, serous adenocarcinoma; Image 2C, mucinous adenocarcinoma; Image 2E, endometrioid adenocarcinoma; Image 2G,
clear cell adenocarcinoma). These carcinoma cells mostly showed the normal \( p16 \) FISH pattern (Images 2B, 2D, 2F, and 2H). In all cases of EOC (n = 40), the mean rates of homozygous and heterozygous deletions were 7.9% and 15.4%, respectively (Figure 1A). None of the EOC cases (0/40) was \( p16 \) homozygous or heterozygous deletion positive (Figure 1A). When divided into histologic subtypes, no single subtype of EOC exceeded the cutoff values for homozygous or heterozygous deletion \( p16 \) FISH (Figure 1B). Finally, we compared female cases of DMPM with EOC cases and found that homozygous deletion was significantly more frequent in DMPM than in EOC (\( P < .05 \), Mann-Whitney \( U \) test) (Figure 1A). Overall, when differentiating DMPM from RMH and EOC, the sensitivity of \( p16 \) homozygous deletion detected by FISH was 32% (9/28), while the specificity was 100% (Table 1). On the basis of these results, we conclude that \( p16 \) homozygous deletion is a useful tool to confirm that a case is DMPM over RMH or EOC, but in cases where \( p16 \) homozygous deletion is lacking, a diagnosis of DMPM cannot be ruled out.

**Discussion**

To our knowledge, this is the first report to describe the usefulness and limitations of \( p16 \) FISH analysis in the
Although peritoneal effusion cytology and/or peritoneal more resistant malignant neoplasm with a poor prognosis. chemotherapy, whereas DMPM remains a radio- and chemo improving by the use of both neoadjuvant and adjuvant therapy. In this study, p16 homozygous deletion showed a specificity of 100% for the differentiation of DMPM from EOC. Moreover, the specificity was also 100% for the distinction of DMPM from RMH. Despite the lower sensitivity, p16 FISH can reliably differentiate DMPM from RMH and EOC. However, there are also limitations and cautions in the assessment of homozygous deletion in EOC cases. Small numbers of EOC cells showed p16 deletion (7.9% ± 1.8%; range, 2.8%-9.6%), and they were determined negative because they fell below the cutoff of 10%. In the future, however, as the number of evaluated EOC cases increases, homozygous deletions greater than 10% might occur. Thus, further studies with more cases will be needed.

Homozygous deletion of the 9p21 locus, which contains p16, was reported in cell lines derived from many types of human tumors, including lung (59%), breast (10%), brain (35%), bladder (15%), and ovary (29%). Thus, a role of p16 in human tumorigenesis has been suggested. One study suggested that p16 inactivation by homozygous deletion or mutation was rare in ovarian tissues (in 2/70 and 4/70 EOCs, respectively). In this study, the inactivation of p16, as detected by loss of p16 messenger RNA and protein expression, was a consequence of hypermethylation of the 5'-CpG island, rather than by gene deletion or point mutation. Similarly, neither deletions nor rearrangements of the p16 gene were detected by Southern blot hybridization in ovarian cancer tissues (0/20), and only 4% showed altered migration (gene alterations) on single-strand conformation polymorphism. Thus, it seems likely that p16 inactivation by epigenetic mechanisms such as hypermethylation, but not by gene alterations, may play an important role in the formation of human EOC.

The use of p16 FISH in the differentiation of DMPM from other malignancies with peritoneal spreading has some limitations. Both pancreatic ductal adenocarcinoma (PDAC) and cholangiocarcinoma (CCA) of the liver, which may cause malignant ascites, have p16 homozygous deletion in as many as 50% of cases, similar to that of DMPM. Thus, application of p16 FISH is of no use in the differentiation between DMPM and PDAC or DMPM and CCA. p16 FISH can be a useful and reliable adjunct for differentiating DMPM from other malignancies by...
understanding its benefits and limitations. For the differential diagnosis of a widespread abdominal malignancy with “epithelioid” morphology, we provide more information in Table 2, including morphologic and immunohistochemical features and p16 FISH status, as to the diagnostic workup of DMPM cases to definitively establish the diagnosis and rule out misclassification. No reliable immunohistochemical markers are established for differentiation between DMPM and RMH, and thus p16 FISH is useful if homozygous deletion is positive. Although PAX8 is a reliable marker for differentiating ovarian serous adenocarcinoma, p16 FISH can be an additional useful adjunct. For differentiation between PDAC or CCA and DMPM, p16 FISH is useless, while immunohistochemistry is a useful adjunct in addition to morphology.

Table 2
Morphologic and Immunohistochemical Features and p16 Homozygous Deletion of DMPM, RMH, EOC, PDA, and CCA

<table>
<thead>
<tr>
<th>Morphologic Feature</th>
<th>Immunohistochemical Feature</th>
<th>p16 HD FISH, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPM (epithelioid)</td>
<td>Complex papillae; tubules, cellular stratification; stromal invasion usually apparent; dense cellularity, including cells surrounded by stroma</td>
<td>Often positive for EMA, p53, GLUT-1, and IMP3; often negative for desmin; positive mesothelial markers: calretinin, D2-40, CK5/6, WT-1; negative markers: MOC31, Ber-EP4, PAX8, B72.3</td>
</tr>
<tr>
<td>RMH</td>
<td>Simple papillae; single cell layers; loose sheets of cells without stroma; absence of stromal invasion; cellularity may be prominent but is confined to mesothelial surfaces and not in the stroma</td>
<td>Usually negative for EMA, p53, GLUT-1, and IMP3; often positive for desmin; positive mesothelial markers: calretinin, D2-40, CK5/6, WT-1</td>
</tr>
<tr>
<td>EOC (PSC)</td>
<td>Papillary, glandular, solid; papillae are usually irregularly branching and highly cellular; glands are slit-like or irregular</td>
<td>Positive for MOC31, PAX 8, and Ber-EP4; mesothelial markers (calretinin and D2-40) may stain positively (0%-38% and 13%-65%, respectively); WT-1 is positive</td>
</tr>
<tr>
<td>PDA</td>
<td>Well- to moderately developed glandular and duct-like structures that grow in a haphazard pattern, associated with a desmoplastic stroma</td>
<td>Positive for MOC31, CEA, and Ber-EP4; usually negative for mesothelial markers (WT1, D2-40, calretinin)</td>
</tr>
<tr>
<td>CCA</td>
<td>Well- to moderately differentiated; composed of short or long tubular glands lined by cells that vary in height; frequent cytoplasmic and luminal mucin</td>
<td>Positive for MOC31, CEA, Ber-EP4, and B72.3; usually negative for mesothelial markers (WT1, D2-40, calretinin)</td>
</tr>
</tbody>
</table>

CCA, cholangiocarcinoma; CEA, carcinoembryonic antigen; CK5/6, cytokeratin 5/6; DMPM, diffuse malignant peritoneal mesothelioma; EMA, epithelial membrane antigen; EOC, epithelial ovarian cancer; FISH, fluorescence in situ hybridization; GLUT-1, glucose transporter 1; HD, homozygous deletion; IMP3, insulin-like growth factor II messenger RNA-binding protein 3; PDA, pancreatic ductal adenocarcinoma; PSC, papillary serous carcinoma; RMH, reactive mesothelial hyperplasia; WT-1, Wilms tumor 1.

* By Southern blot hybridization.

Address reprint requests to Dr Nabeshima: Dept of Pathology, Fukuoka University School of Medicine and Hospital, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan; kaznabes@fukuoka-u.ac.jp.

This work was supported in part by a grant from the Research Center for Advanced Molecular Medicine, Fukuoka University, and Izumo City Supporting Cancer Research Project.

Acknowledgments: The authors thank K. Yano, M. Onitsuka, and H. Fukagawa for technical assistance in FISH and immunohistochemistry.

References


