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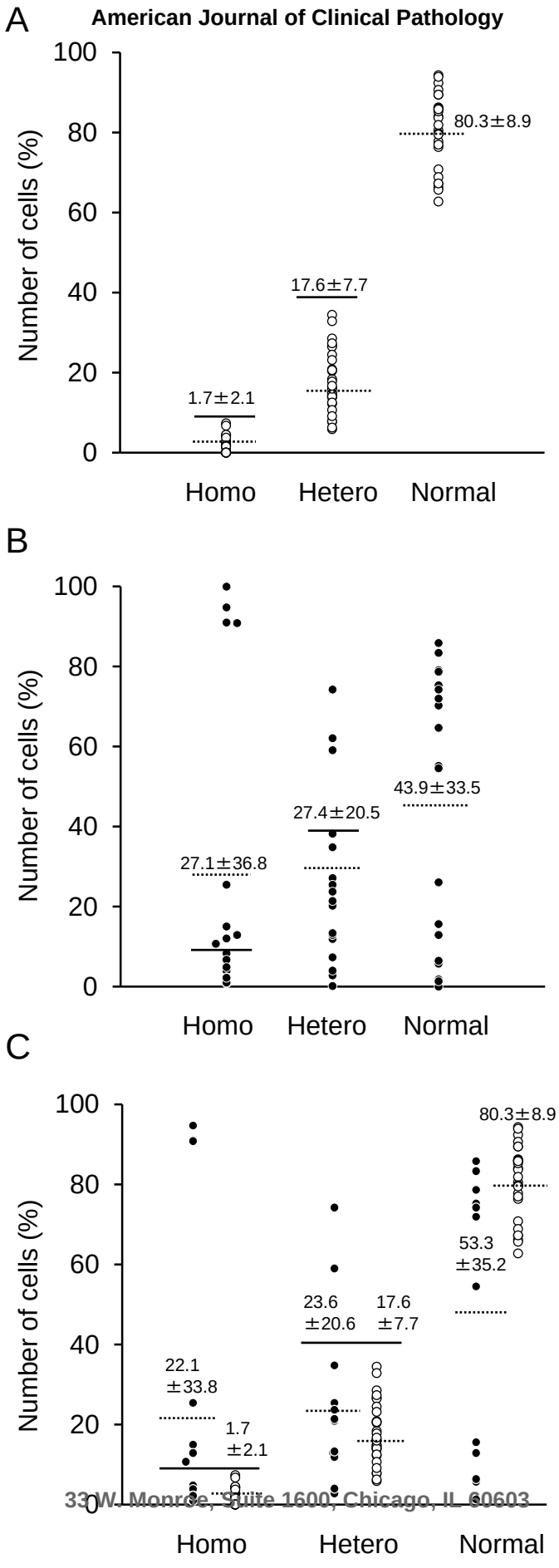
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Number of cells (%)
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80.3±8.9
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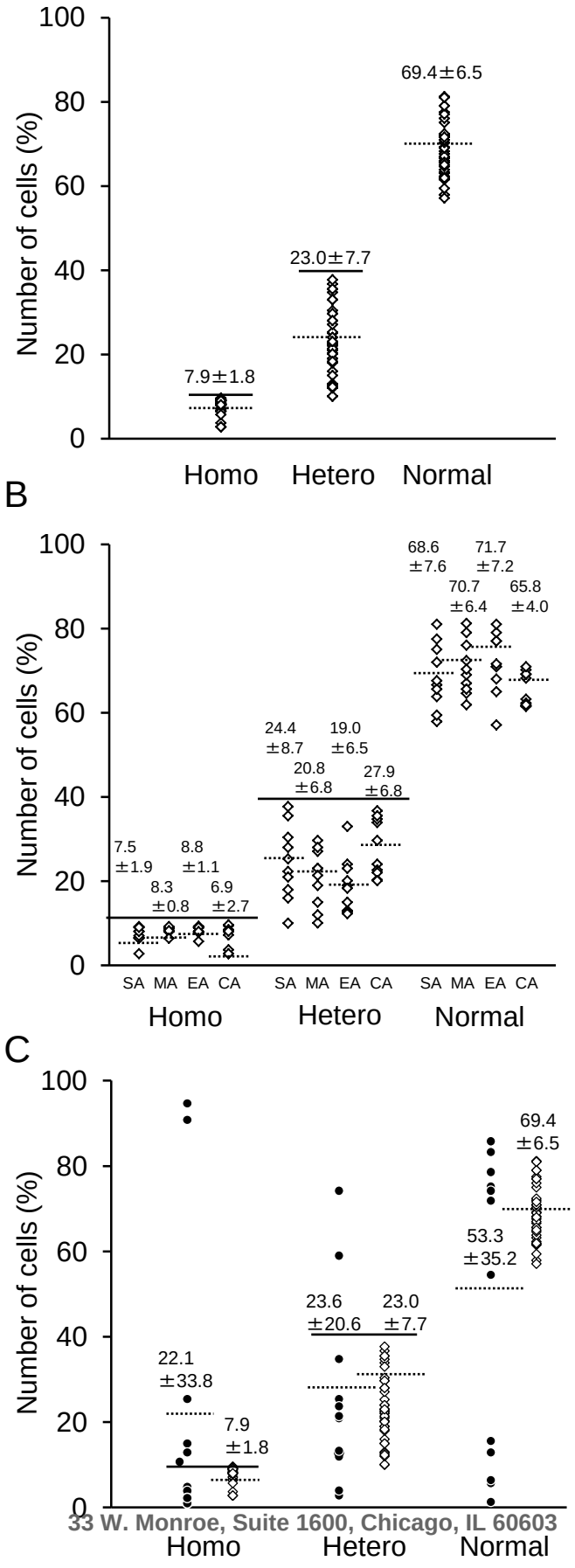
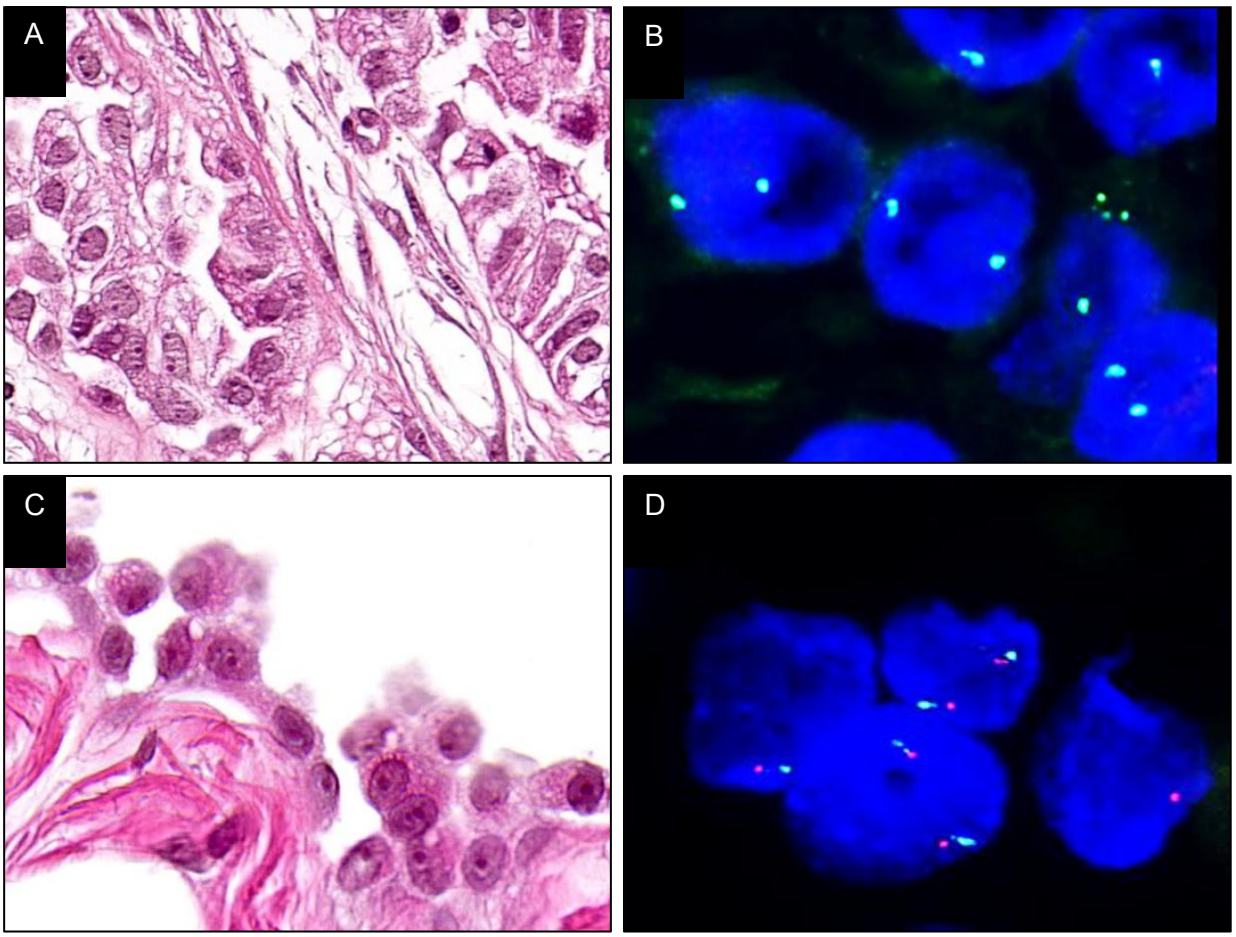
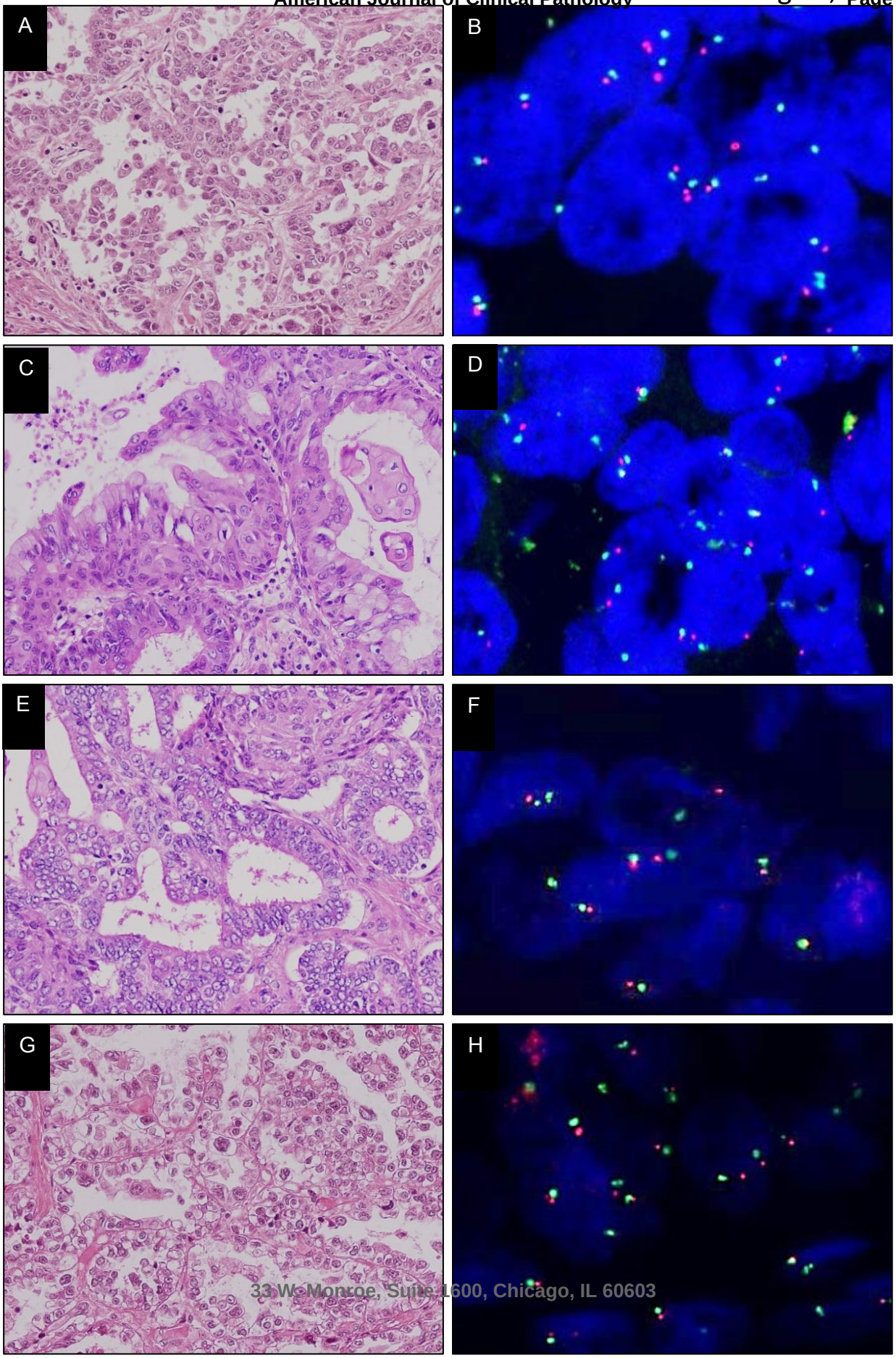


Image 1, Ito et al.

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

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

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

and Hospital, Fukuoka, Japan.

Correspondence to:

Kazuki Nabeshima, MD, PhD, Department of Pathology, Fukuoka University School of Medicine and Hospital, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Tel: +81-92-801-1011, Fax: +81-92-863-8383, Email: kaznabes@fukuoka-u.ac.jp

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Brief title: *p16* FISH in peritoneal mesothelioma

Keywords: 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 petitoneal mesothelioma (DMPM) from reactive mesothelial hyperplasia (RMH) or peritoneal dissemination of gynecological 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 diagnosis of malignant pleural mesothelioma. The aim of this study was to investigate ability of *p16* FISH assay to differentiate DMPM from RMH and EOC.

Methods: *p16* FISH was performed in 28 DMPM (successful in 19), 30 RMH and 40 EOC cases. The cutoff values of *p16* FISH were >10% for homozygous deletion and >40% for heterozygous deletion.

Results: According to the above criteria, 47.4% (9/19) of DMPM cases were homozygous deletion-positive and 15.8% (3/19) were heterozygous deletion-positive, whereas all RMH cases were negative for *p16* deletion. In all four major histological subtypes of EOC, neither *p16* homozygous nor heterozygous deletions were detected. To differentiate DMPM from RMH or EOC, the sensitivity of *p16* homozygous deletion was 47.4% 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.

Introduction

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 histopathological patterns that may potentially mimic a variety of primary and metastatic ovarian tumors.[3] The distinction between reactive mesothelial hyperplasia (RMH) and DMPM is also problematic, because RMH and DMPM have the overlapping morphological findings on cytological and surgical specimens.[5,6] Although combination of several antibodies as positive- and negative-markers for malignant mesothelioma are generally recommended for immunohistochemical support of the diagnosis, no satisfactorily reproducible biomarker has yet been confirmed.[7]

Although no official tumor-node-metastasis (TNM) staging system exists for patients

with DMPM, a new staging system was recently proposed. Patients with T1 (peritoneal cancer index (PCI) 1-10) N0 M0 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.[8] Furthermore, recent studies suggested that a combination of cytoreductive surgery (CRS) and perioperative intraperitoneal chemotherapy (PIC) resulted in improved survival.[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*^{INK4a} (*p16*), *CDKN2B/p15*^{INK4b} and *p14*^{ARF}.[11-15] Deletion of the 9p21 region or *p16* gene has been reported in more than 70 - 80% of mesothelioma by cytogenetic and molecular studies.[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 cytological and surgical specimens, especially in the differentiation of malignant pleural mesothelioma from RMH.[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 25-51% of DMPM cases.[7,22-23]

The aim of this study was to investigate the usefulness and limitations of *p16* FISH

assay in 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 males and 14 females; mean age, 65.1 years; range, 32-72 years), 40 EOC cases (40 females; mean age, 52.9 years; range, 21-74 years) and 30 RMH cases (30 females; mean age, 50.1 years; range, 21-68 years). The data were derived from the peritoneal and gynecological files of the Department of Pathology, Fukuoka University Hospital (FUH), in Fukuoka, Japan, and included both FUH and consultation cases from August 1993 to January 2012. EOC cases were treated at the Department of Obstetrics and Gynecology, FUH from July 2006 to June 2011. RMH lesions were obtained from the greater omentum excised during gynecological tumor resection to rule out metastatic lesions. All cases were histologically diagnosed according to the 2003 WHO classification of tumors of the breast and female genital organs.[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 (CEA), Ber-EP4, MOC-31, thyroid transcription factor-1 (TTF-1)] and others (CAM5.2, CK AE1/AE3, EMA, PAX8). The clinicopathological characteristics of the tumor and reactive cases are summarized in Table 1.

Fluorescence *In Situ* Hybridization (FISH) analysis

p16 FISH was performed on formalin-fixed, paraffin-embedded, 4-μm-thick tissue sections using 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 min and digested with pepsin solution at 37°C for 5 minutes. After refixtation in 10% buffered formalin at room temperature for 3 min, the tissue sections were treated in 2×SSC containing 0.3% Tween 20 at 45°C for 10 min, 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 min 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 two 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 excitors for Spectrum Green, Spectrum Orange, and DAPI. Lymphocytes in each section served as internal controls and showed 2 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.

Statistical Analysis

Statistical comparison of FISH data between DMPM and RMH or EOC was performed using the Mann-Whitney U test. A P-value < 0.05 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 DMPM, RMH, and EOC cases, we first systematically performed histological and FISH analyses on samples from each case. Image 1 shows representative H&E sections and FISH images of epithelioid type DMPM (Image 1A, B) and RMH (Image 1C, D). In DMPM, *p16*FISH analysis was successful in 19 of 28 cases (67.9%). The remaining nine surgical or autopsy samples that failed were collected from 1993 to 1998 pathology files. These samples could not be analyzed because the signal intensity was too low. The 19 successful cases included 7 males (36.8%) and 12 females (63.2%). 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 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 for homozygous and heterozygous deletions were calculated as the mean percentage + 3 standard deviations (SDs), and set >10% for homozygous deletion and >41% for heterozygous deletion, based on the results in RMH. According to these criteria, 9/19 cases (47.4%) of DMPM were homozygous deletion-positive and 4/19 cases (21.0%) of DMPM were heterozygous deletion-positive, whereas all RMH cases were negative for *p16* deletion (Figures 1A and 1B). All of the four heterozygous deletion-positive cases were also homozygous deletion-positive. Analysis of all cases (Figure 1B) and female-only cases (Figure 1C) of DMPM showed significantly more frequent homozygous deletion than RMH cases ($P < 0.05$, Mann-Whitney U test) (Figure 1C). 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 2 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 (Image 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 2).

None of EOC cases (0/40) was *p16* homozygous or heterozygous-deletion positive (Figure 2A). When divided into histological subtypes no single subtype of EOC exceeded the cutoff values for homozygous or heterozygous deletion (Figure 2B). Finally, we compared female cases of DMPM with EOC cases and found that homozygous deletion was significantly more frequent in DMPM than EOC ($P< 0.05$, Mann-Whitney U test) (Figure 2C). Overall, when differentiating DMPM from RMH and EOC, the sensitivity of *p16* homozygous deletion detected by FISH was 47.4%, while the specificity was 100% (Table 2). Based on 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 the best of our knowledge, this is the first report to describe the usefulness and limitations of *p16* FISH analysis in the differentiation of DMPM from RMH and EOC. Based on our study design, *p16* homozygous deletion was found in 47.4% (9/19) of DMPM cases, whereas none of RMH and EOC lesions exhibited the homozygous deletion. Even when considered by their major histological subtypes (serous, mucinous, endometrioid and clear cell

adenocarcinoma), all EOC cases were *p16* deletion-negative. Thus, when homozygous deletion is positive, *p16* FISH can reliably differentiate DMPM from RMH and EOC. Although the sensitivity of *p16* homozygous deletion detected by FISH was 47.4%; its specificity was high (100%), making *p16* FISH a useful ancillary tool in cases where homozygous deletion is positive.

Other studies have shown that *p16* FISH is useful in the differentiation of pleural mesothelioma from RMH; *p16* homozygous deletion was detected in 43-92% of pleural mesothelioma, whereas none of RMH cases were deletion positive.[7,16-25] Correct diagnosis of mesothelioma requires the detection of invasion of stroma and/or adipose tissue, but this is difficult in small biopsy specimens and/or effusion cytology.[27] Moreover, no reliable immunohistochemical markers have been established to differentiate diffuse malignant mesothelioma from benign mesothelial proliferations. The significance of a recently recognized marker of malignancy, GLUT-1, in malignant mesothelial proliferations remains to be validated.[7] In these circumstances, *p16* homozygous deletion was shown to be a very powerful technique; the diagnosis of mesothelioma over reactive mesothelial cells was confirmed in most patients with positive or suspicious cytology.[16] In DMPM, the positive rate of *p16* homozygous

deletion is lower, ranging from 25-51%.[7,22-23] However, all peritoneal RMH cases were deletion negative, the same as pleural RMH cases. Our study confirmed these studies, with a positive rate 47.4% of *p16* homozygous deletion in DMPM and no RMH cases positive for homozygous deletion. This 100% specificity makes *p16* FISH reliable, despite a lower sensitivity.

The presence of malignant ascites is a sign of malignant cells in the peritoneal cavity. DMPM is often associated with massive or bloody malignant ascites. However, the malignant ascites are caused more commonly by secondary peritoneal surface malignancies, which include ovarian, colorectal, pancreatic, uterine and extra-abdominal tumors originating from lymphoma, lung and breast.[28] In the female peritoneum, EOC is one common cause of malignant ascites formation. The distinction between EOC and DMPM is important for proper clinical management and to predict a prognosis. The prognosis of EOC has been improving by use of both neoadjuvant and adjuvant chemotherapy, whereas DMPM remains a radio- and chemo-resistant malignant neoplasm with a poor prognosis.[28,29] Although peritoneal effusion cytology and/or peritoneal biopsy is an universal method for differential diagnosis of peritoneal malignancies, diagnostic distinction only based on morphologies obtained by H&E staining or Papanicolaou staining is often difficult. Recently, combinations of positive and negative

immunohistochemical markers were proposed for the differential diagnosis between EOC and DMPM, but there is still much controversy as to the value of the different immunohistochemical markers and their combinations.[29,30] In this study, *p16* homozygous deletion showed specificity of 100% for the differentiation of DMPM from EOC. Moreover, the specificity was also 100% for distinction of DMPM from RMH as described above. Thus, once a lesion is confirmed to have a *p16* homozygous deletion, it is very useful in the differential diagnosis of DPMM from EOC and RMH.

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.[31] One study suggested that *p16* inactivation by homozygous deletion or mutation was rare in ovarian tissues (in 2/70 and 4/70 EOC, respectively).[32] In that study, the inactivation of *p16*, as detected by loss of *p16* mRNA and protein expression, was a consequence of hypermethylation of the 5'-CpG island, rather than by gene deletion or point mutation.[32] Similarly, neither deletions nor rearrangements of the *p16* gene were detected by Southern blot hybridization in ovarian cancer tissues (0/20), and only 4% of them showed altered migration

(gene alterations) on single-strand conformation polymorphism (SSCP).[33] 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.[32] Our results, which showed no homozygous deletion of *p16* in the 40 tested EOC cases, are in agreement with these known reports and their hypotheses.

The use of *p16* FISH in 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.[34,35] 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.

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Disclosure

There are no conflicts of interest pertinent to this work.

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Image and Figure Legends

Image 1. Histology and *p16* FISH in DMPM and RMH. (**A**), Epithelioid type of DMPM. The cells are arranged in papillotubular structures with fibrovascular stroma. (**B**), *p16* FISH demonstrating homozygous deletions (loss of two red signals per cell). (**C**), An RMH case that shows a mild piling up of reactive mesothelial cells. (**D**), *p16* FISH that shows a normal pattern (two red and two green signals). (**A**) and (**C**): H&E, ×200; (**B**) and (**D**): FISH, ×630. DMPM, diffuse malignant peritoneal mesothelioma; RMH, reactive mesothelial hyperplasia.

Image 2. Subtypes of EOC and their representative *p16* FISH patterns. (**A**), Serous adenocarcinoma showing proliferation of high-grade serous carcinoma cells arranged in irregular papillary structures. (**C**), Mucinous adenocarcinoma, in which atypical mucinous cells are arranged in irregular papillotubular structures. (**E**), Endometrioid adenocarcinoma showing proliferation of atypical endometrial-like cells arranged in irregular fused tubular structures. (**G**), Clear cell adenocarcinoma, in which atypical cells with clear cytoplasm and rounded nuclei proliferate forming irregular papillotubular structures. (**B**), (**D**), (**F**) and (**H**), *p16* FISH, predominantly demonstrating normal pattern with two red and two green signals. (**A**), (**C**), (**E**) and (**G**): H&E, ×200; (**B**), (**D**), (**F**) and (**H**): FISH, ×630. EOC, epithelial ovarian cancer.

Figure 1. *p16* FISH patterns in surgical specimens. Data are given as mean ± standard deviation for RMH cases (**A**), all DMPM cases (**B**) or female DMPM cases (**C**). In (**C**), *p16* FISH patterns in RMH and female cases of DMPM are compared. Data are number of cells exhibiting each

p16 FISH pattern. Dotted lines represent the mean; solid lines represent mean + 3 standard deviations. Based on the results shown in RMH cases (**A**), the cutoff values for homozygous and heterozygous deletions were set at 10% and 40%, respectively. Open circle, RMH cases; solid circle, all (**B**) or female (**C**) cases of DMPM; FISH, fluorescence in situ hybridization; RMH, reactive mesothelial hyperplasia; DMPM, diffuse malignant peritoneal mesothelioma.

Figure 2. *p16* FISH patterns in surgical specimens of EOC cases. Data are given as mean ± standard deviation for all cases (**A**) and each histological subtype (**B**). In (**B**), SA = serous adenocarcinoma; MA = mucinous adenocarcinoma; EA = endometrioid adenocarcinoma; CA = clear cell adenocarcinoma. In (**C**), *p16* FISH patterns in EOC (all cases) and female cases of DMPM are compared. Solid circle, female cases of DMPM; open rhombus, EOC. Data are number of cells exhibiting each *p16* FISH pattern. The mean for each group is denoted with a dotted line. The cutoff values for homozygous and heterozygous deletions were set at 10% and 40%, respectively (solid lines). FISH, fluorescence in situ hybridization; EOC, epithelial ovarian cancer; DMPM, diffuse malignant peritoneal mesothelioma.

Table 1.

Clinicopathological characteristics of 98 cases.

Characteristics	DMPM	EOC	RMH
Number	28	40	30
Sex			
Male/Female	14/14	0/40	0/30
Mean age (range)	65.1 (32-78)	52.9 (21-74)	50.1 (21-68)
Male	66.8 (61-77)		
Female	63.7 (32-78)		
Histological type	Epithelioid, 22 (12/10)	Serous, 10	
	Biphasic, 4 (0/2)	Mucinous, 10	
	Sarcomatoid, 2 (2/2)	Endometrioid, 10	
		Clear cell, 10	
Rate of successful *p16* FISH	19/28 (67.9%)	40/40 (100%)	30/30 (100%)

FISH, fluorescence *in situ* hybridization; DMPM, diffuse malignant peritoneal mesothelioma; EOC, epithelial ovarian cancer; RMH, reactive mesothelial hyperplasia; Serous, serous adenocarcinoma; Mucinous, mucinous adenocarcinoma; Endometrioid, endometrioid adenocarcinoma; Clear cell, clear cell adenocarcinoma.

Table 2.

Sensitivity and specificity of *p16* FISH in differentiation of DMPM from RMH and EOC.

	Homozygous deletion			
	Positive	Negative	Sensitivity	Specificity
DMPM	47.4% (9/19)	52.6% (10/19)	47.4%	100%
RMH	0% (0/30)	100% (30/30)	0%	100%
EOC	0%(0/40)	100% (40/40)	0%	100%

FISH, fluorescence *in situ* hybridization; DMPM, diffuse malignant peritoneal mesothelioma; RMH, reactive mesothelial hyperplasia; EOC, epithelial ovarian cancer.