MPM cells with BAP1 loss

Original Article

Morphological Difference between Pleural Mesothelioma Cells in Effusion Smears with either BAP1 Loss or 9p21 Homozygous Deletion and Reactive Mesothelial Cells without the Gene Alterations

Running Title: MPM cells with BAP1 loss

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Abbreviations:


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Abstract

We previously characterized the morphological characteristics of MPM cells with 9p21 homozygous deletion (HD) using a combination of the virtual microscopy and fluorescence *in situ* hybridization (FISH). In this study, we investigated whether MPM cells with BRCA1-associated protein 1 (BAP1) loss show the same morphological characteristics identified in MPM cells with 9p21 HD. MPM cells with either BAP1 loss detected by immunocytochemistry (ICC) or 9p21 HD detected by FISH were identified via virtual microscopy prior to ICC or FISH, followed by analysis and quantification of their morphological characteristics. MPM cells with BAP1 loss or 9p21 HD exhibited significantly more frequent cell-in-cell engulfment, multinucleation, and larger multicellular clusters composed of more than 10 cells than reactive mesothelial cells. In conclusion, MPM cells with BAP1 loss or 9p21 HD share similar cytological features, indicating that the same morphological criteria can be used to detect MPM cells harboring such genetic aberrations.

Key words:

Pleural mesothelioma, effusion smear, morphological characteristics, BAP1 loss, 9p21 homozygous deletion
**Introduction**

Malignant Pleural Mesothelioma (MPM) is a highly aggressive malignancy associated with asbestos exposure. As a result of heavy industrial use of asbestos in the recent past, the disease incidence in Japan is expected to rise over the next one or two decades.\(^1\) Poor prognosis associated with the disease is related, at least in part, to difficulties in diagnosis at early stages. Because 54–89% of MPM patients present initially with pleural effusion, cytologic analysis is the primary diagnostic approach for most patients.\(^2,3\) Detection of mesothelioma cells in effusion smear cytology is thus critical for early diagnosis.\(^1\)

Immunohistochemistry (IHC) is useful in distinguishing MPM from metastatic tumor cells in the pleura. However, distinguishing mesothelioma cells from reactive mesothelial cells (RMC) remains challenging. A variety of markers, including desmin, epithelial membrane antigen (EMA), glucose transporter-1 (GLUT-1), and CD146, have been evaluated in both tissue and cytological samples to discriminate MPM cells from RMC, but these are not useful in an individual case.\(^4-8\)

Recent studies have revealed the usefulness of two new markers: 1) homozygous deletion (HD) of the 9p21 region, including the \(p16\) gene, detected by fluorescence in situ hybridization (FISH)\(^3,9-20\), and 2) loss of nuclear expression of BRCA1-associated protein 1 (BAP1), detected by IHC.\(^18,20-31\) While sensitivity for detecting these markers is not high, they do provide high specificity (100%). Detection of BAP1 using IHC and 9p21 HD using FISH provide candidate approaches for discriminating between MPM cells and RMC, as these techniques can be applied to effusion smears.\(^21,23,24,28,30,31\)

Previously, we analyzed and reported the morphological characteristics of mesothelioma cells with 9p21 HD using a combination of virtual microscopy and 9p21 FISH.\(^15,17\) Compared with RMC that retained the 9p21 region, MPM cells with 9p21 HD exhibited significantly more frequent cell-in-cell engulfment, multinucleation (with 2 or more
nuclei), and larger multicellular, berry-like clusters composed of more than 10 cells. These characteristics were the same as those included in the cytological criteria proposed for diagnosing MPM by the International Mesothelioma Interest Group (IMIG).\textsuperscript{5-7} It remains unclear, however, whether loss of BAP1 in MPM cells is also associated with these characteristic morphological changes. The objective of the current study was to determine whether the same morphological criteria could be used to identify MPM cells with BAP1 loss in addition to MPM cells with 9p21 HD. For this purpose, we investigated and compared morphological changes in MPM cells associated with BAP1 loss with those associated with 9p21 HD, using our previously developed methodology.

**Materials and Methods**

All study procedures were approved by the Fukuoka University Hospital Institutional Review Board, which approved the study protocol (approval number 11-7-11) and allowed a waiver of informed consent for the study. Use of redundant tissues and cells is allowed under the standard patient treatment agreement at Fukuoka University Hospital, provided no patient objections are expressed.

**Case Selection and Smear Preparations**

This study included cytological preparations obtained from pleural effusions in 43 patients with MPM (32 males and 11 females; age range: 55-95 years [mean, 72.1 years]) and in 45 patients with RMC associated with tuberculosis, pneumonia, cardiovascular disease, lung cancer, and bullae (30 males and 15 females; age range: 36-95 years [mean, 68.6 years]). The MPM cases included 40 epithelioid and 3 biphasic mesotheliomas. All 88 cases were derived from the pleural lesion file of the Department of Pathology, Fukuoka University Hospital, and included consultation cases between 2005 and 2016. The diagnosis of MPM was confirmed in tissue samples, and the histologic tumor subtypes were identified using the
World Health Organization classification. Mesothelial origin of tumors was determined by IHC, using a combination of calretinin, podoplanin (D2-40), cytokeratin 5/6, and Wilms tumor 1 (WT-1) staining as positive mesothelial markers, and thyroid transcription factor 1 (TTF-1), claudin-4, and carcinoembryonic antigen (CEA) as negative markers. All smear preparations were fixed in 95% ethanol, stained according to Papanicolaou staining protocol, digitized using a virtual microscope system (NanoZoomer 2.0-HT, Hamamatsu Photonics, Hamamatsu, Japan), and then subjected to BAP1 immunocytochemistry (ICC) and 9p21 FISH analyses.

Immunocytochemistry (ICC) of BAP1

ICC for BAP1 was performed on an automated Dako Autostainer Link 48 platform using the DAKO EnVision Plus Kit (Agilent Technologies Company, Glostrup, Denmark). Briefly, Papanicolaou-stained smear preparations were treated in xylene overnight to remove the mounting medium, followed by rehydration in descending alcohol dilutions and heating at 95 °C in pH 9.0 Tris-EDTA buffer for 10 minutes using water bath to retrieve epitopes. After blocking the endogenous peroxidase activity using a blocking reagent (Dako EnVision Plus Kit) for five minutes at room temperature (RT), slides were incubated with mouse monoclonal anti-human BAP1 antibody (clone C-4; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilution) at RT for 30 minutes. Immunoreacted cells were visualized with 3,3’-diaminobenzidine, and nuclei were counterstained with Mayer’s hematoxylin. BAP1 staining was evaluated by a cytologist and a pathologist and nuclear BAP1 staining was scored as loss or retained. Cytoplasmic staining was considered nonspecific, and positive nuclear staining in non-neoplastic cells was noted as an internal positive control. The cutoff value for loss of BAP1 was set at 50%, as described previously.18, 29, 31
FPISH Assay

9p21 FISH was performed on smears of decoversliped Papanicolaou-stained slides using Vysis LSI CDKN2A SpectrumOrange/CEP9 SpectrumGreen Probes (Abbott Japan, Tokyo, Japan). Briefly, smear slides were treated with 2 × saline-sodium citrate buffer (SSC) containing 0.3% Tween 20 (SSC-0.3T; Sigma-Aldrich), incubated in pretreatment solution (0.01M citrate buffer, pH 6.0) at 80 °C for 15 minutes, and then digested using pepsin solution (0.3%-pepsin/0.01N-HCl; Sigma-Aldrich, P-6887) at 37 °C for 15 minutes. After refixation in 10% neutral buffered formalin at RT for five minutes, the preparations were treated in 2 × SSC-0.3T at 45 °C for 30 minutes, dehydrated in ethanol, dried, and exposed to the probes. The probes and preparations were denatured at 80 °C for 10 minutes in the probe solution provided (Abbott Japan), followed by hybridization at 37 °C for 24 hours in a ThermoBrite unit (Abbott Japan). The preparations were washed in 2 × SSC-0.3T at 72 °C for 3–5 minutes and in 2 × SSC containing 0.1% Tween 20 at RT for 3–5 minutes. The nuclei were counterstained using 4’, 6-diamidino-2-phenylindole (DAPI) in the antifade reagent (Vector Laboratories, Burlingame, CA, USA). Analyses were performed using a fluorescence microscope (AxioImager Z1; Carl Zeiss Microimaging, Jena, Germany) and the Isis analysis system (Metasystems, Altlussheim, Germany) equipped with filter sets with single- and dual-band excitors for SpectrumGreen, SpectrumOrange, and DAPI (UV, 360 nm).

Lymphocytes or histiocytes in each smear served as internal controls. HD was defined as a lack of both 9p21 signals. The percentage of cells with 9p21 HD detected by FISH was determined by evaluating at least 200 mesothelial cells per sample. The cutoff value for HD of 9p21 as detected by FISH was set at 10%, as described previously.15-17

Morphological analysis

MPM cells with BAP1 loss (detected by ICC) and MPM cells with 9p21 HD (detected by FISH) were identified on the Papanicolaou-stained smears of MPM that had been recorded
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using a virtual microscope system. Similarly, RMC without BAP1 loss and RMC retaining the 9p21 locus were identified in Papanicolaou-stained smears of non-mesothelioma cases. We analyzed the morphological characteristics of MPM cells with BAP1 loss and/or 9p21 HD-positive MPM cells. We compared these characteristics to those in RMC without BAP1 loss or 9p21 deletion. The frequency of cell-in-cell engulfment (with or without cytoplasmic protrusion [i.e., hump formation]), multinucleate cells, and berry-like or papillary cell aggregates composed of >10 cells (i.e., cellular spheres or morules) were quantified. At least 200 mesothelial cells per slide were evaluated. We set the cutoff values which were calculated as the mean percentage + 3 standard deviations (SDs) of frequency in RMC without BAP1 loss or 9p21 HD as previously described.15

**Statistical Analysis**

Statistical comparison of cytological features between MPM cells and RMC was performed using the Wilcoxon test. A p value < 0.05 was considered statistically significant. All statistical evaluations were performed with JUMP 10 software (SAS Institute, Cary, NC).

**Results**

*Results of BAP1 ICC and 9p21 FISH in effusion smears*

Table 1 shows the results of BAP1 ICC and 9p21 FISH in effusion smears. Twenty-one of the 43 MPM samples (48.8%) showed a loss of nuclear BAP1 expression. All RMC (non-mesothelioma samples) in which BAP1 ICC was performed showed retention of nuclear BAP1 expression (17/17 samples). Of the 43 MPM samples that were analyzed by FISH, 21 cases (48.8%) showed 9p21 HD. None of the 28 RMC cases in which 9p21 FISH was performed, showed 9p21 HD. Seven of the 43 MPM cases (16.2%) showed a loss of nuclear BAP1 expression as well as 9p21 HD, and eight cases (18.6%) had neither BAP1 loss nor 9p21 HD. Fourteen MPM cases (32.6%) showed either BAP1 loss or 9p21 HD. Based on
these results, ICC for BAP1 detection and FISH for detection of 9p21 HD were each characterized by a specificity of 100% and a sensitivity of 48.8% for differentiation of MPM from RMC. The combination of the two approaches yielded an increased sensitivity of 81.4%.

**Morphology of BAP1 loss or p16 HD positive mesothelioma cells**

We analyzed cellular morphology in MPM cases with BAP1 loss alone (n=14, 12 males and 2 females; age range: 57-82 years [mean, 73.3 years]) or in those with 9p21 HD alone (n=14, 10 males and 4 females; age range: 58-95 years [mean, 74.1 years]). As a control comparison, morphological characteristics were examined in smears of non-mesothelioma cases, in which ICC was used to detect BAP1 expression (n=17, 10 males and 7 females; age range: 44-89 years [mean, 67.7 years]) or FISH was used to detect the 9p21 locus (n=28, 19 males and 9 females; age range: 36-95 years [mean, 69 years]). The cytologic features of mesothelioma cells with BAP1 loss and those with 9p21 HD are shown in Figure 1 and 2, respectively.

MPM cells with BAP1 loss demonstrated significantly more frequent cell-in-cell engulfment (with or without a hump-like appearance) than RMC in which BAP1 expression was retained (Figure 1A-B and 3A). This feature was seen in 13.0 ± 7.1% of MPM cells with BAP1 loss in contrast to 3.3 ± 1.4% of RMC in which BAP1 expression was retained.

Ten cases of MPM with BAP1 loss (10/14, 71.4%) showed cell-in-cell engulfment at a rate that was higher than a cutoff value (7.5%) calculated as the mean frequency of cell-in-cell engulfment found in RMC + 3SDs. Whereas all RMC vases remained below the cutoff values. (100% specificity). Similarly, MPM cells with 9p21 HD also exhibited significantly more frequent cell-in-cell engulfment (17.4 ± 10.6% of cells) than RMC that retained the 9p21 locus (3.5 ± 2.5% of cells; Figure 2A-B and 3B). Moreover, ten cases of MPM with 9p21 HD (10/14, 71.4%) showed cell-in-cell engulfment at a rate that was higher than the calculated cutoff value (11%). Whereas all RMC vases remained below the cutoff values.
Malignant pleural mesothelioma (MPM) cells with BAP1 loss (100% specificity).

Binucleation and multinucleation (> 2 nuclei) were also significantly more frequent in MPM cells with BAP1 loss or MPM cells with 9p21 HD relative to control RMC. Binucleation was observed in 27.5 ± 6.7% vs 10.5 ± 3.1% of MPM cells with BAP1 loss vs RMC, respectively. Similarly, multinucleation was observed in 7.3 ± 2.9% vs 1.3 ± 1.1% of cells, respectively. A similar difference was noted in comparing MPM cells with 9p21 HD vs RMC. Binucleation was present in 26.6 ± 9.2% vs 9.8 ± 3.9% of cells, and multinucleation was present in 11.0 ± 7.0% vs 1.0 ± 0.9% of cells, respectively (Figure 1C-D, 2C-D and 4). The cutoff values for multinucleation were much lower than those for binucleation for both BAP1 loss and 9p21 HD (19.8% vs 4.6% for BAP1 loss; 21.5% vs 3.7% for 9p21 HD). Twelve MPM cases with BAP1 loss (12/14, 86%) showed a rate of multinucleation higher than the cutoff value (4.6%), while 11 cases of MPM with 9p21 HD (11/14, 78%) showed a rate of multinucleation higher than the cutoff value (3.7%). All RMC cases were below the cutoff values.

Multicellular ball or berry-like clusters of mesothelial cells were also more often found in MPM cells (Figure 1E-F, 2E-F and 5). These multicellular clusters were three-dimensional, with many overlapping cells. BAP1 loss could be evaluated by ICC even in these cell clusters. However, determination of 9p21 HD by FISH could be achieved only at the periphery of the clusters. Based on this limitation, statistical analysis of 9p21 HD in multicellular clusters was performed only in 9p21 HD-predominant MPM cases, in which more than 90% of cells were 9p21 HD positive (n=12). Multicellular, three-dimensional cell clusters composed of 10-99 cells were found significantly more frequently in MPM cells with loss of BAP1 expression (11.2 ± 10.1%) and MPM cells with 9p21 HD (16.1 ± 12.4%) relative to RMC in which BAP1 expression was retained (0.7 ± 1.1%) and RMC in which the 9p21 locus was retained (1.2 ± 1.5%). In analyzing multicellular clusters composed of 10-99 cells, ten MPM cases...
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with BAP1 loss (10/14, 71%) and nine cases of MPM with 9p21 HD (9/12, 75%) showed cluster rates over the cutoff values of 4.0% and 5.7%, respectively. All RMC cases were below the cutoff values. In addition, clusters of more than 100 cells were found in 11 MPM cases with BAP1 loss (11/14, 78.6%) and ten MPM cases with 9p21 HD (10/12, 83%). In contrast, these clusters were not found in any RMC cases in which BAP1 expression was retained, and were found in only two RMC cases in which the 9p21 locus was retained (2/28, 7.1%). Moreover, clusters seen in RMC cases were frequently composed of flat, sheet-like monolayers, rather than the three-dimensional, overlapping clusters typically formed by MPM cells.

Discussion

In the current study, we demonstrated that loss of BAP1 in MPM results in the similar morphological characteristics as found in MPM cells with the 9p21 HD. Our findings indicate that MPM cells with either BAP1 loss or 9p21 HD can be detected using the same morphological criteria. Furthermore, these findings suggest that distinct genetic alterations lead to convergent morphological changes. These results show that a number of cellular morphological features, including cell-in-cell engulfment, multinucleation, and berry-like clusters, are not exclusively dependent on either BAP1 loss or 9p21 HD.

BAP1 is a nuclear ubiquitin hydrolase that regulates gene expression, transcription, and DNA repair, and functions as a classical tumor suppressor. BAP1 mutations are thought to be driver mutations for the development of MPM.\textsuperscript{27, 33, 34} Mutations in the \textit{BAP1} gene is observed in 56–81% of epithelioid mesotheliomas,\textsuperscript{17} which is a higher frequency than that observed in either biphasic (45–60%) or sarcomatoid mesothelioma (15–63%)\textsuperscript{20, 27}. Mutations in the \textit{BAP1} gene lead to loss of BAP1 expression in cell nuclei detected by IHC/ICC in 88-100% of mesotheliomas.\textsuperscript{35} This enabled us to investigate and analyze the cellular morphological
characteristics associated with BAP1 loss.

A number of studies (including a publication from our group) in which tissue and cell block samples were analyzed, have shown that assaying for both loss of BAP1 and 9p21 HD substantially increased test sensitivity, leading to increased confidence in MPM diagnosis and in differentiation of MPM from reactive mesothelial hyperplasia.\textsuperscript{18, 20, 21, 24, 28, 29} In the present study, combining the two assays yielded a sensitivity of 81.4%, which was higher than that achieved with either BAP1 IHC alone (48.8%) or 9p21 FISH alone (48.8%).

In this study, we demonstrated that BAP1 ICC can be applied to Papanicolaou-stained effusion smears as proposed previously.\textsuperscript{30} Relative to FISH, use of ICC to detect BAP1 allows target cells to be identified more easily, because cells can be easily observed using a light microscope. Moreover, the evaluation of BAP1 loss using ICC is possible even in overlapping clusters of cells. In contrast, the limitations of fluorescence microscopy make identification of target cells within such clusters difficult using FISH-based assays, in which identification of 9p21 HD can generally be achieved only at the periphery of overlapping cell clusters. Caution is warranted, however, in using ICC to assay for loss of BAP1 nuclear staining. In particular, good internal controls are necessary, to ensure that robust staining is achieved when BAP1 protein is present. In our studies, we assessed BAP1 loss in tumor cells only when clear staining in background lymphocytes or histiocytes was present and could serve as an internal positive control.

Cytomorphological features of MPM cells have been characterized in numerous studies, but the cytologic diagnosis of MPM can still present significant challenges. The cytomorphological features found in MPM cells, RMCs, and metastatic carcinoma cells sometimes exhibit substantial overlap. Moreover, MPM cells and RMC are typically both present in effusion smears, making it difficult to detect morphological characteristics essential for the diagnosis of MPM. IMIG guidelines, which provide specific guidance for the
cytopathologic diagnosis of epithelioid and mixed-type malignant mesothelioma, have also
been endorsed by the International Academy of Cytology and the Papanicolaou Society of
Cytopathology.\textsuperscript{5-7} The guidelines specify ten cytomorphological criteria for identifying
malignant mesothelioma, including the presence of: 1) a highly cellular sample;
2) mesothelial cells that are significantly larger than RMC; 3) papillary clusters with a smooth
surface or berry-like clusters with a scalloped surface; 4) acidophilic extracellular matrix
cores; 5) macro nucleoli; 6) protrusions from the cell membrane or blebbing; 7) a prominent
degree of cell-within-cell arrangement; 8) multinucleated giant cells and pyknotic eosinophilic
cells; and 10) vacuoles overlapping the nuclei of May-Grünwald Giemsa (MGG) stained cells.

The morphological characteristics of MPM cells with BAP1 loss or 9p21 HD observed in our
study meet the criteria for mesothelioma diagnosis proposed by the guidelines.

The frequency with which morphological characteristics suggestive of MPM occur can
provide additional important information that strengthens confidence in a diagnosis of
mesothelioma. In the present study, we analyzed the frequency of occurrence of
morphological characteristics associated with MPM cells, and calculated cutoff values for
each characteristic associated with BAP1 loss or 9p21 HD. These cutoff values may be useful
for daily practice of MPM diagnosis.

The current study has a few limitations. First, we selected the cellular characteristics
that had been frequently found in 9p21 HD-positive MPM cells\textsuperscript{15} as study items. because we
intended to know the difference or similarity between MPM cells with BAP1 loss or 9p21 HD.
Therefore, other morphological criteria described in the IMIG guidelines were not examined.
Further studies about these criteria will be needed in the future. Second, it is important to
compare the cytomorphological parameters between BAP1 loss and BAP1 retained cases or
between 9p21 HD-positive and negative cases. However, it was technically difficult to pick up
BAP1 retained or 9p21 HD-negative MPM cells accurately, since smears almost always
MPM cells with BAP1 loss include both MPM and RMC. Thus, in this study, we identified MPM cells by detecting BAP1 loss or 9p21 HD using ICC or FISH, respectively, and performed cytomorphological analysis between the MPM cells with loss/deletion and RMC without loss/deletion.

In the present study, we distinguished MPM cells from RMC by detecting either loss of BAP1 or 9p21 HD. In addition, we examined morphological characteristics of MPM cells that had either loss of BAP1 expression, or 9p21 HD, and found that their morphological characteristics were similar. These findings suggest that the same morphological criteria can be applied for screening or detecting MPM cells with either loss of BAP1 or 9p21 HD. The three morphological characteristics observed in our study, cell-in-cell engulfment, multinucleation and larger multicellular clusters, are included in the IMIG cytomorphological criteria for the diagnosis of malignant mesothelioma. Based on the cutoff values to favor diagnosis of MPM in the current study, sensitivities for detecting MPM cells with either BAP1 loss or 9p21 HD using the three morphological characteristics were 71.4%, 78-86% and 71-83%, respectively. A combination of these characteristics may increase the sensitivities. Moreover, their specificities were all 100% since values for RMC were below the cutoff value. Thereby, these lines of evidence support the usefulness of the guideline criteria to detect MPM cells with either of the two genetic changes. To our knowledge, this study is the first to characterize the morphological characteristics of MPM cells that have lost nuclear expression of BAP1.
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Author contributions

SM: methodology, investigation, data curation, writing–original draft, writing–review and editing, and visualization. YK: methodology, validation, investigation, and writing–review and editing. MH: conceptualization, methodology, investigation, and writing–review and editing. TK: writing–review and editing. KK: writing–review and editing. KN: conceptualization, investigation, writing–original draft, writing–review and editing, supervision, project administration, and funding acquisition.
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References


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**Figure Legends**

**Figure 1.**
Micrographs show cytologic features of mesothelioma cells with BAP1 loss after immunocytochemistry (A, C, and E) and Papanicolaou-stained smears (B, D, and F). The same cells are shown in image pairs: (A) and (B), (C) and (D), and (E) and (F). Note the cell-in-cell engulfment with or without hump formation (A and B), multinucleation (C and D), and berry-like multicellular clusters (E and F). Nuclear staining for BAP1 was negative in mesothelioma cells but positive in cell nuclei of adjacent inflammatory cells.

**Figure 2.**
Micrographs show cytologic features of mesothelioma cells with 9p21 HD after FISH (A, C, and E) and Papanicolaou-stained smears (B, D, and F). The same cells are shown in image pairs: (A) and (B), (C) and (D), and (E) and (F). Note the cell-in-cell engulfment with or without hump formation (A and B), multinucleation (C and D), and berry-like multicellular clusters (E and F).

**Figure 3.**
Frequency of cell-in-cell engulfment in MPM cells with BAP1 loss or 9p21 HD vs. RMC with BAP1 expression or retention of the 9p21 locus.

(A) Comparison of MPM cells with BAP1 loss (14 cases) vs. RMC with nuclear BAP1 expression (17 cases).

(B) Comparison of MPM cells with 9p21 HD (14 cases) vs. RMC in which the 9p21 locus was retained (28 cases).

HD; homozygous deletion, RMC; reactive mesothelial cell.
**Figure 4.**

Frequency of multinucleated cells in MPM cells with BAP1 loss or 9p21 HD vs. RMC with BAP1 expression or retention of the 9p21 locus.

(A) Comparison of MPM cells with BAP1 loss (14 cases) vs. RMC with nuclear BAP1 expression (17 cases).

(B) Comparison of MPM cells with 9p21 HD (14 cases) vs. RMC in which the 9p21 locus was retained (28 cases).

HD; homozygous deletion, RMC; reactive mesothelial cell.

**Figure 5.**

Frequency of multicellular clusters in MPM cells with BAP1 loss or 9p21 HD vs. RMC with BAP1 expression or retention of the 9p21 locus.

(A) Comparison of MPM cells with BAP1 loss (n=14) vs. RMC with nuclear BAP1 expression (n=17).

(B) The number of multicellular clusters was classified according to their size in smears from mesothelioma cases (n=12) in which 9p21 HD predominated (> 90%) vs. RMC with normal 9p21 pattern (n=28).

HD; homozygous deletion, RMC; reactive mesothelial cell.
Figure 1.
Figure 2.
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Figure 3.
Figure 4.
Figure 5.
TABLE 1. Results of BAP1 loss and 9p21 homozygous deletion on effusion smears

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Total</th>
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<th>9p21 HD (%)</th>
<th>BAP1 loss / 9p21 HD (%)</th>
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<td></td>
<td>- / -</td>
<td>+ / -</td>
<td>- / +</td>
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<tr>
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<td>21/43 (48.8)</td>
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<td>0/28 (0)</td>
<td>17/28 (100) *</td>
</tr>
</tbody>
</table>

BAP1 loss indicates loss of BAP1 expression in nuclei detected by immunocytochemistry. 9p21 HD indicates homozygous deletion (HD) of the 9p21 region detected by fluorescence in situ hybridization (FISH).

* All RMC in which BAP1 immunocytochemistry was performed retained nuclear BAP1 expression (17/17 samples) and none of the 28 RMC cases in which 9p21 FISH was performed, showed 9p21 HD (28/28 samples).

MPM, malignant pleural mesothelioma cases; RMC, reactive mesothelial cells cases