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ARTICLE

Hemizygous loss of *NF2* detected by fluorescence in situ hybridization is useful for the diagnosis of malignant pleural mesothelioma

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Abstract

Neurofibromatosis type 2 (NF2) gene, a tumor suppressor gene located on chromosome 22q12.2, is frequently abnormal in mesothelioma. Recent studies have revealed the effectiveness of diagnostic assays for differentiating malignant pleural mesothelioma from reactive mesothelial hyperplasia. These include detection of homozygous deletion of the 9p21 locus by fluorescence in situ hybridization (FISH) (9p21 FISH), loss of expression of BAP1 as detected by immunohistochemistry, and loss of expression of methylthioadenosine phosphorylase (MTAP) as detected by immunohistochemistry. However, the application of FISH detection of *NF2* gene deletion (*NF2* FISH) in differentiation of malignant pleural mesothelioma from reactive mesothelial hyperplasia has not been fully evaluated. In this study, we investigated whether *NF2* FISH, either alone or in a combination with other diagnostic assays (9p21 FISH, MTAP immunohistochemistry, and BAP1 immunohistochemistry), is effective for distinguishing malignant pleural mesothelioma from reactive mesothelial hyperplasia. This study cohort included malignant pleural mesothelioma ($n = 47$) and reactive mesothelial hyperplasia cases ($n = 27$) from a period between 2001 and 2017. We used FISH to examine deletion status of *NF2* and 9p21 and immunohistochemistry to examine expression of MTAP and BAP1 in malignant pleural mesothelioma and in reactive mesothelial hyperplasia. Hemizygous *NF2* loss (chromosome 22 monosomy or hemizygous deletion) was detected in 25 of 47 (53.2%) mesothelioma cases. None of the mesothelioma cases showed homozygous *NF2* deletion. Hemizygous *NF2* loss showed 53.2% sensitivity and 100% specificity in differentiating malignant pleural mesothelioma from reactive mesothelial hyperplasia. A combination of *NF2* FISH, 9p21 FISH, and BAP1 immunohistochemistry yielded greater sensitivity (100%) than that detected for either diagnostic assay alone (53.2% for *NF2* FISH, 78.7% for 9p21 FISH, 70.2% for MTAP immunohistochemistry, or 57.4% for BAP1 immunohistochemistry). Thus, *NF2* FISH in combination with other diagnostic assays is effective for distinguishing malignant pleural mesothelioma from reactive mesothelial hyperplasia.

Introduction

Malignant pleural mesothelioma is the most common primary neoplasm of the pleura [1]. Its incidence is closely linked with exposure to asbestos fibers [1]. The prognosis of malignant pleural mesothelioma is generally poor with a median survival period of 9.2 to 14 months [2, 3]. Although histologic diagnosis of malignant pleural mesothelioma is not always straightforward [1], diagnosis at an early stage may lead to a better prognosis [4, 5].

Mesothelioma has frequent genetic alterations in *Cyclin-dependent kinase inhibitor 2A* (*CDKN2A)/p16* found in the 9p21 locus (35–62%), *BRCA1-associated protein 1* (*BAP1*) (21–63%), and *neurofibromatosis type 2* (*NF2*) (14–50%) [6, 7]. Recent studies have revealed the usefulness of assays

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detecting changes in the 9p21 locus and BAP1 expression for mesothelioma diagnosis—homozygous deletion of the 9p21 locus as detected by fluorescence in situ hybridization (FISH) (9p21 FISH) [8–17] and expression loss of BAP1 as detected by immunohistochemistry [12, 15–21]. In addition, we have previously reported that immunohistochemical detection of methylthioadenosine phosphorylase (MTAP) is a reliable surrogate assay for 9p21 FISH [14, 15, 17, 22–24]. These aforementioned assays can provide a definitive diagnosis of mesothelioma and can facilitate differentiation of malignant mesothelial proliferations from benign proliferations with 100% specificity [1, 15–17, 23, 25]. Although the sensitivity of either of these assays on their own is insufficient, the combined use of 9p21 FISH (or MTAP immunohistochemistry) and BAP1 immunohistochemistry enhances the sensitivity [12, 14–17, 21, 23, 25].

The *NF2* is a tumor suppressor gene which is located on chromosome 22q12.2 and encodes moesin-ezrin-radixin-like protein (merlin) [26]. This protein modulates signal transduction pathways including Hippo pathways and mammalian target of rapamycin (mTOR) pathways, which regulate cell proliferation, growth, and apoptosis [26]. Inactivation of the *NF2* gene has been observed in certain benign and malignant tumors including neurofibromatosis type 2, sporadic schwannoma, meningioma, and mesothelioma [26].

Genetic mutations in *NF2* are frequently observed in mesothelioma [6, 7, 27]. Singhi et al. [28] showed the prevalence of *NF2* hemizygosity in 35% of peritoneal mesotheliomas cases using FISH. However, the application of *NF2* FISH for differentiating malignant pleural mesothelioma from reactive mesothelial hyperplasia has not been established in pleural mesotheliomas. In this study, we investigated whether *NF2* FISH, either alone or in a combination with other diagnostic assays, is effective for distinguishing malignant pleural mesothelioma from reactive mesothelial hyperplasia.

Materials and methods

Case selection

This study included malignant pleural mesothelioma and reactive mesothelial hyperplasia cases identified from the pleural lesion files of the Department of Pathology, Fukuoka University Hospital, which includes pleural diseases such as malignant pleural mesothelioma, reactive mesothelial hyperplasia, various pleuritis, metastatic carcinomas, and primary thoracic sarcomas, between the years of 2001 and 2017. Histological diagnosis and classification of mesothelioma were performed according to the 2015 World Health Organization guidelines [29]. The mesothelial nature of each tumor was confirmed using immunohistochemical assays. Calretinin, podoplanin (D2–40), and Wilms' tumor-1 (WT-1) were used as positive mesothelial markers, while thyroid transcription factor-1 (TTF-1), Ber-EP4, claudin-4, and carcinoembryonic antigen (CEA) were used as negative markers.

Each mesothelioma case was classified into three histologic types (epithelioid, biphasic, or sarcomatoid). Classification of either epithelioid or sarcomatoid mesothelioma required at least 90% of the tumor to be composed of this morphologic pattern [1, 29]. Biphasic mesothelioma classification required both components to represent at least 10% of the tumor [1, 29].

Anonymous use of redundant tissues is part of the standard treatment agreement with patients at our hospital, provided no objection is expressed. The Fukuoka University Hospital Institutional Review Board approved the study protocol (approval number: 11-7-11).

FISH assay

A representative tissue specimen from each case was selected for FISH analysis. The FISH studies were carried out on 4-μm-thick tissue sections as previously described [11, 13, 15, 23]. Sections were deparaffinized and rehydrated using descending alcohol dilutions, washed with 2x saline-sodium citrate (SSC), exposed to pretreatment solution at 80 °C for 30 min (PathVysion HER2 DNA probe kit; Vysis, Downers Grove, IL, USA), and digested with pepsin solution (Sigma-Aldrich, Tokyo, Japan) at 37 °C for 90 min. After refixation in 10% buffered formalin at room temperature (RT) for 10 min, the sections were treated with 2x SSC, dehydrated in ethanol, dried, and exposed to Vysis LSI *p16*/CEP 9 probe (Abbott Japan, Tokyo, Japan) and *NF2*/CEN 22q probe (Abnova, Walnut, CA, USA). Tissue sections were denatured at 80 °C for 10 min in the probe solution provided (Abbott Japan), followed by hybridization at 37 °C for 20 h in ThermoBrite (Abbott Japan). Finally, the tissue sections were washed with 2× SSC containing 0.3% Tween 20 (Sigma, St. Louis, MO, USA) at 72 °C for 2 min and then with 2× SSC containing 0.1% Tween 20 at RT for 5 min. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) in Antifade reagent (Vector Laboratories, Burlingame, CA, USA). 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 (UV 360 nm).

Lymphocytes in each preparation served as internal controls and showed two signals per FISH probe. The use of internal controls helped to confirm that loss of FISH signals was not due to preanalytical factors such as fixation or processing. We evaluated the FISH signal patterns in at least 100 mesothelial cells per sample. A cutoff value of 10% was set for homozygous deletion of 9p21 FISH as described previously [11, 14–16, 30].

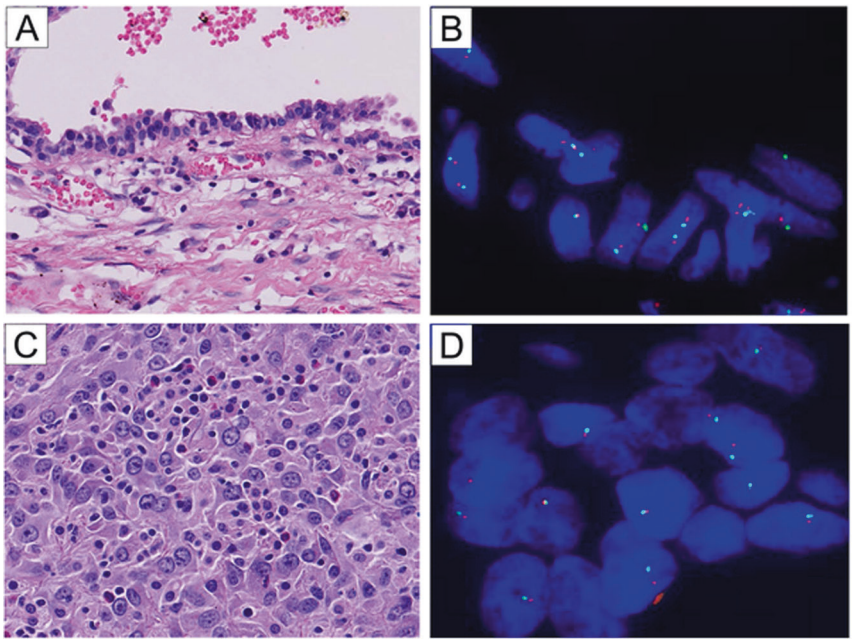

Fig. 1 Hematoxylin-eosin staining and *NF2* dual-color fluorescence in situ hybridization in reactive mesothelial hyperplasia (**a**, **b**) and malignant pleural mesothelioma (**c**, **d**). **a**, **b** mesothelial cells with a normal copy number showing 2 spectrum red and 2 spectrum green signals. **c**, **d** mesothelioma cells with hemizygous *NF2* loss showing 1 spectrum red and 1 spectrum green signals (pattern of chromosome 22 monosomy)

As for *NF2* FISH, signal cutoff values for each deletion pattern [hemizygous deletion, chromosome 22 monosomy, homozygous deletion, and one fluorescein isothiocyanate (FITC) signal were established according to the mean frequency (%) + 3 standard deviations (SDs) for each of the patterns in the reactive mesothelial hyperplasia cases [11]. We defined one Spectrum Red (SpR) and two Spectrum Green (SpG) signals (1SpR/2SpG) as hemizygous deletion, 1SpR/1SpG as chromosome 22 monosomy, 0SpR/2SpG as homozygous deletion, and 0SpR/1SpG as one FITC signal. In this study, hemizygous *NF2* loss indicates hemizygous *NF2* deletion (1SpR/2SpG) or chromosome 22 monosomy (1SpR/1SpG), as described previously [28].

Immunohistochemistry of MTAP and BAP1

A representative tissue specimen from each case was selected for immunohistochemical analysis. Immunostaining was performed on 4-μm-thick sections which were mounted on a glass microscope slide. After blocking the endogenous peroxidase activity using blocking reagent (included in the Dako Envision Kit, Dako, California, USA) for 5 min at RT, epitopes were retrieved using pH 9.0 Tris-EDTA buffer for 40 min at 95 °C followed by incubation with mouse monoclonal antibody MTAP clone 2G4 (Abnova, Taipei, Taiwan; 1:100 dilution; RT 30 min) or mouse monoclonal anti-human BAP-1 clone C-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50 dilution; RT 45 min). The sections were then washed and incubated with ChemMate EnVision kit (Dako). Immunoreacted cells were visualized using diaminobenzidine (Dako, California, USA) followed by a hematoxylin counterstain.

Non-mesothelial cells that were immunoreactive to BAP1 and MTAP (e.g., inflammatory cells including histiocytes and lymphocytes, fibroblasts, pneumocytes, and endothelial cells) served as internal positive controls in each staining protocol. BAP1 immunohistochemistry revealed staining in the nucleus, and BAP1 loss in tumor cells was defined as complete nuclear loss [14, 15, 23]. Cytoplasmic staining was interpreted as a nonspecific reaction. MTAP immunohistochemistry revealed cytoplasmic as well as nuclear staining, and MTAP loss as detected by immunohistochemistry in tumor cells was defined as cytoplasmic staining at an intensity lower than the internal positive control [14, 15, 23, 31]. We set the cutoff value at 50% for MTAP immunohistochemistry and BAP1 immunohistochemistry, as described previously [14, 15, 19, 23].

Statistical analysis

Continuous data are shown as the group means (±SD), and categorical data are shown as a percentage of the group. Fisher's exact test was used to compare categorical variables. The differences in the mean values of continuous data were assessed using Student's *t*-test for unpaired data. We performed survival analysis using overall survival as the endpoint, beginning at the time of resection. The survival curves were plotted via the Kaplan-Meier method, and the differences between survival curves were analyzed using the log-rank test. A p-value < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using R (version 3.2.2; R Foundation for Statistical Computing, Vienna, Austria).

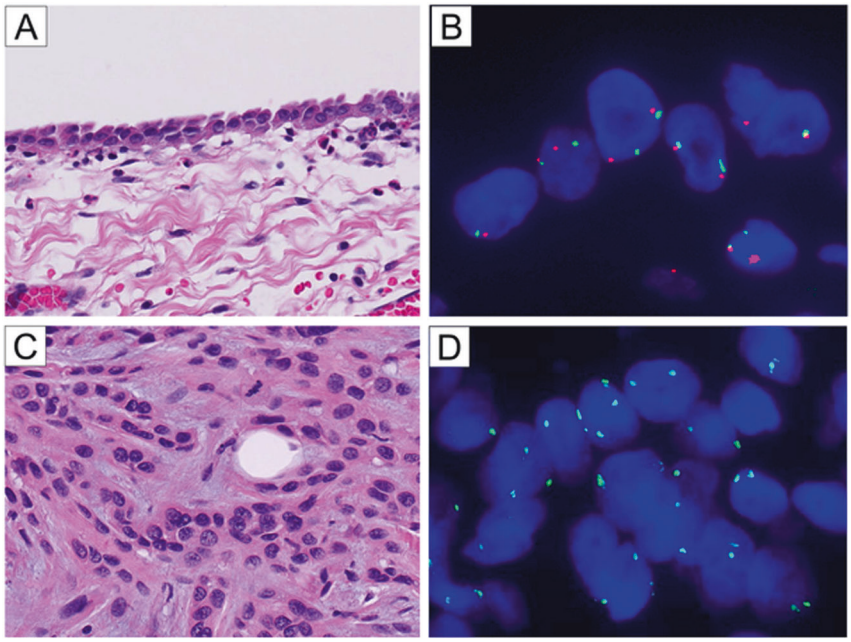

Fig. 2 Hematoxylin-eosin staining and 9p21 dual-color fluorescence in situ hybridization in reactive mesothelial hyperplasia (**a**, **b**) and malignant pleural mesothelioma (**c**, **d**). **a**, **b** mesothelial cells with a normal copy number showing 2 spectrum red and 2 spectrum green signals. **c**, **d** mesothelioma cells with homozygous 9p21 deletion showing the pattern of 0 spectrum red and 2 spectrum green signals

Results

Clinicopathological characteristics

Forty-seven cases with malignant pleural mesothelioma and 27 cases with reactive mesothelial hyperplasia were included in this study. Mesothelioma cases comprised 41 males and 6 females with a mean age of 67.3 years (range: 45–86 years) and included 32 epithelioid mesothelioma, 5 sarcomatoid mesothelioma, and 10 biphasic mesothelioma cases. Reactive mesothelial hyperplasia cases included 26 males and 1 female with a mean age of 33.0 years (range: 18–78 years).

NF2 and 9p21 FISH

Mesothelial cells with a normal copy number for 9p21 or *NF2* have two red signals (9p21 probe or *NF2* probe) and two green signals (chromosome 9 or chromosome 22 centromeric probes). In mesothelioma, *NF2* deletion as detected by FISH was characterized by hemizygous loss (hemizygous deletion: 1SpR/2SpG or chromosome 22 monosomy: 1SpR/1SpG). Mesothelioma cells with homozygous 9p21 deletion showed 0SpR/2SpG signal profile. Representative examples of *NF2* FISH and 9p21 FISH in reactive mesothelial hyperplasia and malignant pleural mesothelioma cases are shown in Figs. 1 and 2, respectively.

Determination of the cutoff values for *NF2* FISH

The signal cutoff values (calculated as indicated in the Materials and Methods section) for *NF2* FISH were determined to be 18.2% for hemizygous deletion (1SpR/2SpG), 48.4% for chromosome 22 monosomy (1SpR/1SpG), 5.5%. for homozygous deletion (0SpR/2SpG), and 12.9% for one FITC signal (0SpR/1SpG) (Table 1).

Table 1 The cutoff values for *NF2* FISH

NF2 FISH signal	Cutoff values (mean + 3 SD in RMH) (%)
Hemizygous loss	
Hemizygous deletion (1SpR/2SpG)	18.2
Chromosome 22 monosomy (1SpR/1SpG)	48.4
Homozygous deletion (0SpR/2SpG)	5.5
One FITC signal (0SpR/1SpG)	12.9

FISH fluorescence in situ hybridization, *SD* standard deviation, *RMH* reactive mesothelial hyperplasia, *SpR* spectrum red, *SpG* Spectrum Green, *FITC* fluorescein isothiocyanate

MTAP and BAP1 immunostaining

BAP1 immunohistochemistry shows staining in the nucleus. BAP1 loss in mesothelioma showed nuclear staining at an intensity lower than that in the internal positive controls. MTAP immunohistochemistry showed cytoplasmic staining as well as nuclear staining and MTAP loss in mesothelioma showed cytoplasmic staining at an intensity lower than that in the internal positive controls. BAP1 loss was detected in 27 of the 47 mesothelioma cases (57.4%), whereas none of the reactive mesothelial hyperplasia cases showed BAP1 loss. Meanwhile, MTAP loss was detected in 33 of the 47

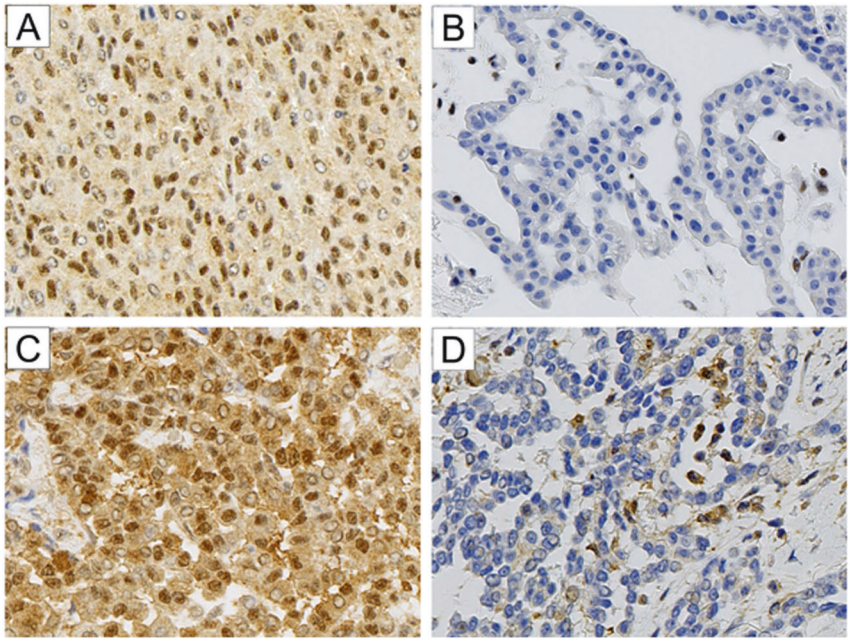

Fig. 3 Representative examples of MTAP and BAP1 immunohistochemistry in malignant pleural mesothelioma. Non-mesothelial cells that were reactive to BAP1 and MTAP served as internal positive controls in each staining protocol. Preserved BAP1 immunohistochemistry (nuclear staining) (**a**), loss of BAP1 immunohistochemistry (**b**), preserved MTAP immunohistochemistry (cytoplasmic staining) (**c**), and loss of MTAP immunohistochemistry (**d**) are shown

mesothelioma cases (70.2%), while none of the reactive mesothelial hyperplasia cases showed MTAP loss. Representative examples of MTAP and BAP1 immunostaining in mesothelioma cases are shown in Fig. 3.

Deletion status of *NF2* using FISH

Hemizygous *NF2* loss was detected in 25 of the 47 mesothelioma cases (53.2%; 4.3% for 1SpR/2SpG and 48.9% for 1SpR/1SpG), while homozygous *NF2* deletion was not detected in mesothelioma cases. Neither hemizygous loss nor homozygous deletion of *NF2* was observed in reactive mesothelial hyperplasia cases.

The correlation between hemizygous *NF2* loss and other parameters in malignant pleural mesothelioma is summarized in Table 2. Hemizygous *NF2* loss was not associated with age ($p = 0.39$), gender ($p = 0.67$), or histologic subtype ($p = 0.32$), statistically. It is noteworthy that hemizygous *NF2* loss was also not associated with homozygous 9p21 deletion ($p = 0.73$), MTAP loss as detected by immunohistochemistry ($p = 0.52$), or BAP1 loss as detected by immunohistochemistry ($p = 0.77$), statistically.

Table 2 The correlation between hemizygous *NF2* loss and other parameters in malignant pleural mesothelioma

Factor	Group	Hemizygous loss ($n = 25$)	Wild type ($n = 22$)	p-value
Age	≥70	9 (36.0)	11 (50.0)	0.39
	<70	16 (64.0)	11 (50.0)	
Gender	Female	4 (16.0)	2 (9.1)	0.67
	Male	21 (84.0)	20 (90.9)	
Histologic subtype	Epithelioid	18 (72.0)	14 (63.6)	0.32
	Sarcomatoid	1 (4.0)	4 (18.2)	
	Biphasic	6 (24.0)	4 (18.2)	
9p21 FISH	Homozygous deletion	19 (76.0)	18 (81.8)	0.73
	Wild type	6 (24.0)	4 (18.2)	
MTAP IHC	Loss	16 (68.0)	17 (77.3)	0.52
	Retained	8 (32.0)	5 (22.7)	
BAP1 IHC	Loss	15 (60.0)	12 (54.5)	0.77
	Retained	10 (40.0)	10 (45.5)	

FISH fluorescence in situ hybridization, *IHC* immunohistochemistry

Sensitivity and specificity of detection assays for discriminating malignant pleural mesothelioma from reactive mesothelial hyperplasia

The results for each detection assay in the 47 mesothelioma cases are shown in Fig. 4. The sensitivity and specificity of each detection assay for discriminating malignant pleural mesothelioma from reactive mesothelial hyperplasia are summarized in Table 3. None of the reactive mesothelial hyperplasia cases showed loss of expression of BAP1 or MTAP as detected by immunohistochemistry or deletion of *NF2* or 9p21 as detected by FISH. Thus, each detection assay was characterized by a 100% specificity. Assay sensitivities were 53.2% for *NF2* FISH, 78.7% for 9p21 FISH, 70.2% for MTAP immunohistochemistry, and 57.4% for BAP1 immunohistochemistry. The assay sensitivity was increased to 93.6% for the combination of 9p21 FISH and

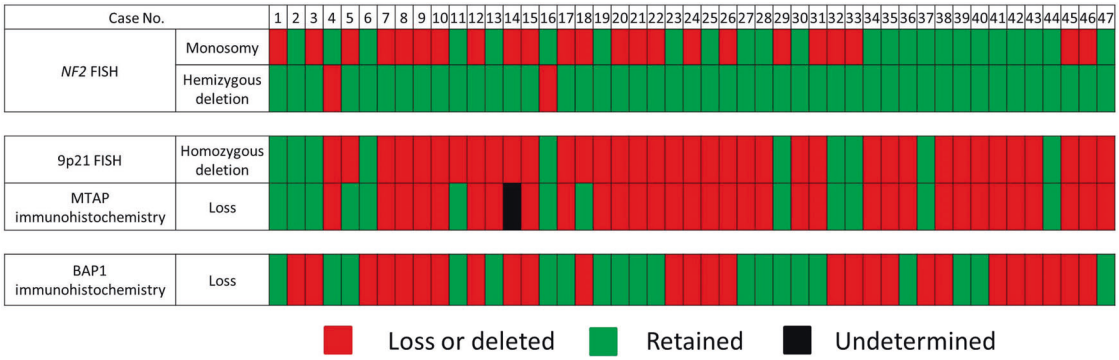

Fig. 4 The results of each detection assay in the 47 cases of malignant pleural mesothelioma. Green represent retained expression as determined by immunohistochemistry or fluorescence in situ hybridization (FISH); red represents loss or deleted expression as detected by immunohistochemistry or FISH; black represents undetermined expression as detected by immunohistochemistry. Note: For 9p21 FISH, deleted indicates homozygous deletion; for *NF2* FISH, deleted indicates hemizygous loss

Table 3 Sensitivity and specificity of each detection assay for discriminating malignant pleural mesothelioma from reactive mesothelial hyperplasia

	MPM ($n = 47$)		RMH ($n = 27$)		Sensitivity (%)	Specificity (%)
	Loss or deleted[b]	Retained	Loss or deleted[b]	Retained		
NF2 FISH	25	22	0	27	53.2	100
9p21 FISH	37	10	0	27	78.7	100
MTAP IHC[a]	33	13	0	27	70.2	100
BAP1 IHC	27	20	0	27	57.4	100
BAP1 IHC/9p21 FISH	44	3	0	27	93.6	100
BAP1/MTAP IHC	42	5	0	27	89.4	100
NF2 FISH/ BAP1 IHC/9p21 FISH	47	0	0	27	100	100
NF2 FISH/ BAP1 IHC/MTAP IHC	46	1	0	27	97.9	100

MPM malignant pleural mesothelioma, *RMH* reactive mesothelial hyperplasia, *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry

[a]One case was excluded from sensitivity and specificity calculation for MTAP IHC due to data interpretation issues

[b]For 9p21 FISH, deleted indicates homozygous deletion; for *NF2* FISH, deleted indicates hemizygous loss

BAP1 immunohistochemistry and to 89.4% for the combination of MTAP immunohistochemistry and BAP1 immunohistochemistry. A triple combination of *NF2* FISH, 9p21 FISH, and BAP1 immunohistochemistry yielded greater sensitivity (100%) than that detected for either diagnostic assay alone or for the combination of either two of these assays.

Survival analysis

Overall survival curves for patients with malignant pleural mesothelioma are shown in Fig. 5. As expected, there was a significant difference in overall survival based on the histologic type ($p = 0.001$) (Fig. 5a). Similarly, 9p21 homozygous deletion detected by FISH and MTAP loss detected by immunohistochemistry were clearly associated with a shorter overall survival ($p < 0.001$ and $p < 0.001$, respectively) (Fig. 5b, c). However, BAP1 loss detected by immunohistochemistry or hemizygous *NF2* loss detected by FISH were not associated with a shorter overall survival (Fig. 5d, e).

Discussion

In this study, we revealed that *NF2* loss as detected by FISH was characterized by hemizygous loss. Hemizygous *NF2* loss was not associated with FISH detection of homozygous 9p21 deletion, immunohistochemical detection of MTAP loss, or immunohistochemical detection of BAP1 loss.

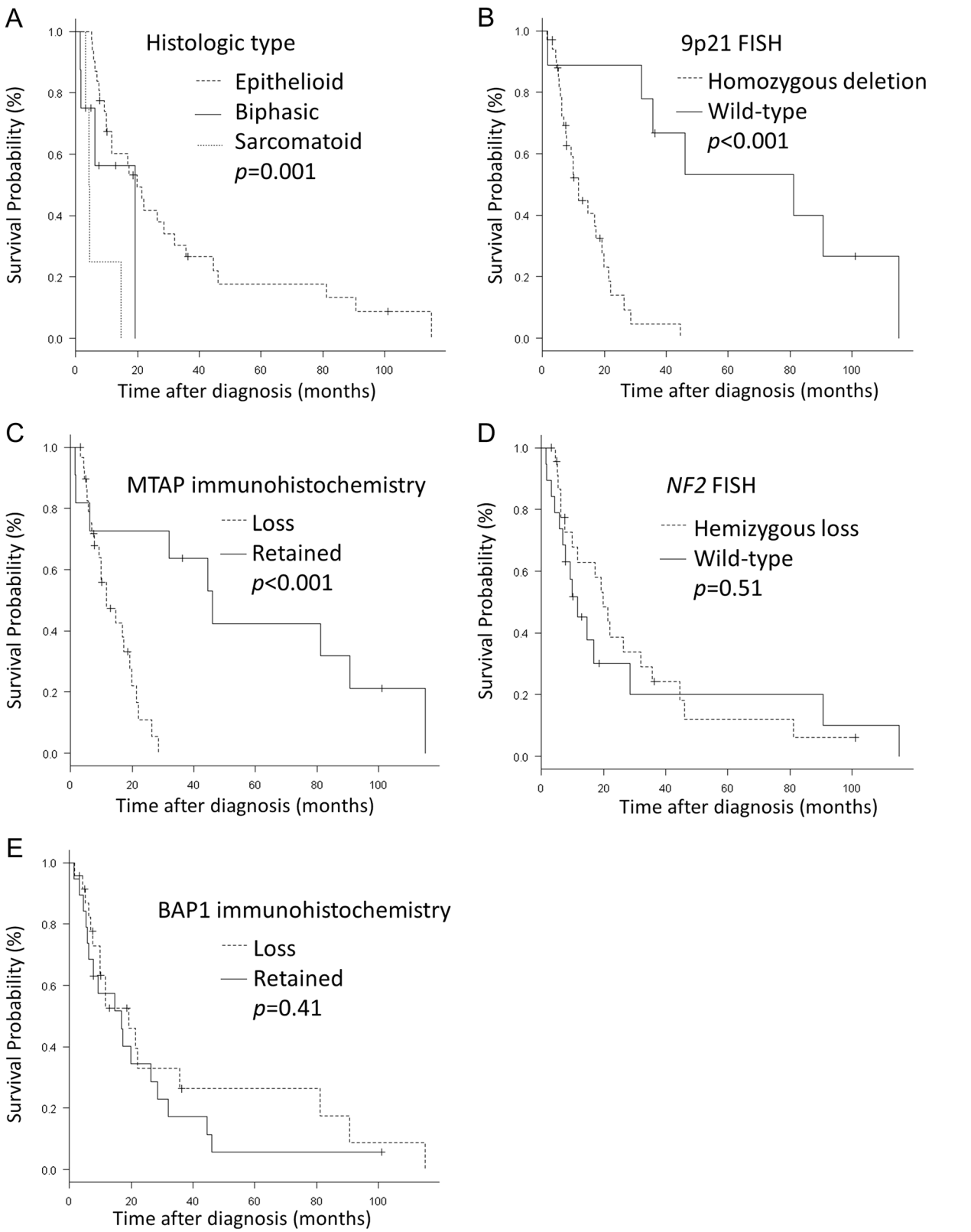

Fig. 5 Overall survival curves for patients with malignant pleural mesothelioma. The effect of histologic type (**a**), the status of 9p21 FISH (**b**), MTAP immunohistochemistry (**c**), *NF2* FISH (**d**), and BAP1 immunohistochemistry (**e**) on overall survival curves is shown

Hemizygous *NF2* loss showed 53.2% sensitivity and 100% specificity in differentiating malignant pleural mesothelioma from reactive mesothelial hyperplasia. In this cohort, *NF2* FISH in a combination with diagnostic assays for 9p21 homozygous deletion (FISH) and BAP1 expression loss (immunohistochemistry) is effective for distinguishing malignant pleural mesothelioma from reactive mesothelial hyperplasia at 100% specificity and sensitivity.

Karyotypic studies have previously shown that clonal abnormalities in chromosome 22 are the most frequent site of chromosomal aberrations in mesothelioma (39.2–65.0%) [32, 33]. A limited number of studies have investigated the frequency of *NF2* deletion in mesothelioma using FISH [28, 34, 35]. Vivero et al. [34] reported that the frequency of *NF2* deletion detected by FISH was 72.5% (37/51 cases) in malignant pleural mesothelioma (cutoff values: 4% for monosomy and 8% for hemizygous deletion). Takeda et al. [35]. reported that the frequency of chromosomal loss at 22q12 detected by FISH was 38% in malignant pleural mesothelioma cases (cutoff values: not described). Singhi et al. [28] showed that *NF2* loss as detected by FISH was identified in 35% of peritoneal mesotheliomas (cutoff values: not described). The frequency of *NF2* deletion detected by FISH is highly influenced by the cutoff value used for the analysis of FISH signal. Using procedure described in Materials and Methods section, the cutoff values calculated in our study were 48.4% for monosomy and 18.2% for hemizygous deletion. Applying these criteria, hemizygous *NF2* loss detected by FISH could differentiate malignant pleural mesothelioma from reactive mesothelial hyperplasia with 53.2% sensitivity and 100% specificity.

In the above-mentioned peritoneal mesothelioma study, *NF2* loss as detected by FISH was characterized by hemizygous loss (35%), and chromosome 22 monosomy (30%) was more frequent than hemizygous *NF2* deletion (5%) [28]. In our study, the frequency of hemizygous *NF2* loss in malignant pleural mesothelioma (53.2%) was higher than that in peritoneal mesothelioma. Meanwhile, similar to the peritoneal mesothelioma study, chromosome 22 monosomy (48.9%) was more frequent than hemizygous *NF2* deletion (4.3%). In contrast to 9p21 FISH, homozygous *NF2* deletion was not detected in any of the mesothelioma cases in our study as well as in other previous reports [17, 28]. Thus, *NF2* loss as detected by FISH appears to be characterized by hemizygous loss in mesothelioma with chromosome 22 monosomy being more frequent than hemizygous *NF2* deletion.

Clinically, 9p21 FISH and BAP1 immunohistochemistry have 100% specificity for differentiating malignant mesothelial proliferations from benign proliferations [1, 14–17, 23, 25]. Chiosea et al. [9] first reported the usefulness of 9p21 FISH for detecting mesothelioma cells in tissue sections. For differentiating malignant pleural mesothelioma from reactive mesothelial hyperplasia, 9p21 FISH shows a sensitivity of 45–85% in epithelioid mesothelioma [8–12, 14, 17] and 67–100% in sarcomatoid mesothelioma [21, 23]. The role of BAP1 in mesothelioma was first suggested in reports of BAP1 somatic mutations by Bott et al. [27] and of germline mutations in BAP1 by Testa et al. [36]. For differentiation between malignant pleural mesothelioma from reactive mesothelial hyperplasia, BAP1 immunohistochemistry shows a sensitivity of 56–81% in epithelioid mesothelioma [12, 17, 18] and 0–63% in sarcomatoid mesothelioma [12, 18, 21, 23]. In addition, we have previously reported that MTAP immunohistochemistry is an acceptable surrogate assay for 9p21 FISH, a finding that was also supported by Berg et al. [14, 15, 17, 23]. In our previous study, 65–75% of the malignant pleural mesothelioma cases harboring 9p21 homozygous deletion (detected by FISH), demonstrated MTAP loss detected by immunohistochemistry [14, 15, 23]. In addition, MTAP immunohistochemistry distinguished malignant mesothelial proliferations from benign proliferations with a specificity of 100% and a sensitivity of 45.1% in epithelioid mesothelioma and 80% in sarcomatoid mesothelioma [14, 15, 23].

In the current study, hemizygous *NF2* loss as detected by FISH was not correlated with FISH detection of 9p21 homozygous deletion, immunohistochemical detection of MTAP loss, or immunohistochemical detection of BAP1 loss. Therefore, the combined use of *NF2* FISH and these three diagnostic assays can enhance the sensitivity of malignant pleural mesothelioma differentiation from reactive mesothelial hyperplasia. Indeed, a triple combination of *NF2* FISH, 9p21 FISH, and BAP1 immunohistochemistry yielded greater sensitivity (100%) than that detected for either diagnostic assay alone or either combination of two of these assays.

Technical challenges and cost can limit FISH assays from widespread adoption. Unfortunately, immunohistochemical assays for the *NF2* gene have not shown efficacy in the differentiation of malignant pleural mesothelioma from reactive mesothelial hyperplasia, to date. In their study examining the usefulness of merlin immunohistochemistry for the differentiation of mesothelioma from a benign proliferation, Sheffield et al. [37] concluded that merlin immunohistochemistry is unsuitable due to its low sensitivity (4%) and the discordance between *NF2* genetic changes and immunohistochemical detection of merlin expression. Recent studies have identified alterations in the components of the Hippo signaling cascade in mesothelioma cells, including large tumor suppressor homolog 2 (LATS2) and their downstream effectors Yes-associated protein (YAP) and transcriptional coactivator with the PDZ-binding motif (TAZ) complex [37, 38]. The application of immunohistochemical detection of LATS and YAP/TAZ immunohistochemistry for the differentiation of

mesothelioma from a benign proliferation was also investigated; however, these immunohistochemical assays were also found to be unsuitable due to their low sensitivity or specificity [37]. Nevertheless, further investigation may be needed to fully elucidate whether immunohistochemical detection of NF2 has a role in mesothelioma diagnosis.

In mesothelioma, functional inactivation of *NF2*/merlin is known to be associated with mesothelial oncogenesis, invasiveness, spreading, and migration [39, 40]. A recent study showed that low merlin expression as detected by immunohistochemistry is related to the poor prognosis of malignant pleural mesothelioma [41]. In peritoneal mesothelioma, Singhi et al. [28] showed that hemizygous *NF2* deletion as detected by FISH was significantly associated with a poor prognosis. However, in our current study, there was no significant prognostic implications of hemizygous *NF2* loss in malignant pleural mesothelioma. Merlin is inactivated by not only genetic alteration but also by epigenetic events (e.g., phosphorylation) [39]. Thurneysen et al. [39] revealed that merlin is rendered inactive upon phosphorylation in all malignant pleural mesothelioma cases wherein no *NF2* truncation could be detected. The lack of association between hemizygous *NF2* loss and survival observed in our study might be because merlin is frequently inactivated even in mesothelioma cases harboring wild-type *NF2* gene.

Our study has a few limitations. First, this study is a single-center retrospective study, and the number of patients is relatively small due to the rarity of the disease. Validation studies are preferred to confirm these results. Second, several old samples were utilized in our study with some cases dating back to 2001. However, for FISH analysis, we obtained two red and green FISH signals in the internal control cells as described in the "Materials and methods" section; this validated the reactivity of FISH analysis. Third, inactivation of *NF2* gene in the remaining allele of chromosome 22 showing hemizygous loss could not be examined. This is relevant in light of the fact that inactivation of *NF2* gene occurs via a two-hit mechanism [40, 42]. However, previous studies have shown that more than half of mesothelioma cases harboring allelic loss of 22q also showed *NF2* gene mutation in the remaining allele [43, 44]. More studies are warranted to confirm these findings.

In conclusion, FISH assay shows that the most common molecular alteration of *NF2* in malignant pleural mesothelioma is hemizygous loss. *NF2* FISH in a combination with other diagnostic assays was effective for distinguishing malignant pleural mesothelioma from reactive mesothelial hyperplasia.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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