Short 57 kb *CDKN2A* FISH probe effectively detects short homozygous deletion of the 9p21 locus in malignant pleural mesothelioma

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Abstract. Homozygous deletion (homo-d) of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene is frequently found in malignant pleural mesothelioma (MPM). Fluorescence *in situ* hybridization (FISH) is commonly used to detect chromosomal deletion, and sometimes reveals more frequent heterozygous deletion (hetero-d) compared with homo-d. In clinical practice, such *CDKN2A* FISH results belong to the 'borderline' homo-d rate, which makes it difficult to definitively diagnose MPM. Microdeletion, [<200 kilobase (kb)], can induce a 'pseudo' hetero-d signal in FISH assays with long probes owing to redundant probe reactivity. Thus, the present study hypothesized that shorter FISH probes can effectively detect the small deletion status of the *CDKN2A* gene and increase homo-d rate in MPM, which has high hetero-d and low homo-d status. The present study aimed to evaluate the effectiveness of a shorter *CDKN2A* FISH probe in diagnosing MPM. *CDKN2A* FISH with either a 222 kb long probe (L-probe) or a 57 kb short probe (S-probe) was performed in four MPM cases with high hetero-d and low homo-d patterns. Furthermore, immunohistochemistry for methylthioadenosine phosphorylase (MTAP) and quantitative (q)PCR analyses were performed to confirm the microdeletion of the 9p21 locus. The results demonstrated that all four MPM cases retained MTAP protein expression. *CDKN2A* FISH with L-probe revealed high hetero-d (cases 1-4; 73.3, 37.1, 59.2 and 64.8%, respectively) and low homo-d (cases 1-4; 12.1, 12.4, 25.4 and 22.2%, respectively). *CDKN2A* FISH with S-probe revealed high homo-d (cases 1-4; 96.8, 90.0, 87.5 and 82.6%, respectively), with low hetero-d (cases 1-4; 0.0, 1.2, 1.2 and 4.3%, respectively). qPCR analysis demonstrated no allele deletions of the *MTAP* gene and two-allele deletions of the *CDKN2A* gene in 3/4 cases. Taken together, these results suggest that the S-probe detects the short homo-d of the 9p21 locus more effectively than the L-probe in MPM. This can assist in solving diagnostic difficulties in cases involving high hetero-d with low homo-d.

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Abbreviations: MPM, malignant pleural mesothelioma; FISH, fluorescence *in situ* hybridization; homo-d, homozygous deletion; IHC, immunohistochemistry; BAP1, BRCA1-associated protein 1; MTAP, methylthioadenosine phosphorylase; CDKN2A, cyclin-dependent kinase inhibitor 2A; kb, kilobase; hetero-d, heterozygous deletion; qPCR, quantitative PCR; TBLB, transbronchial lung biopsy

Key words: malignant mesothelioma, CDKN2A, fluorescence in situ hybridization, microdeletion, MTAP, qPCR

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive neoplasm that arises from mesothelial cells that line the serosal membrane of the pleura (1). MPM requires accurate and early diagnosis as early treatment improves patient prognosis (2). However, it is difficult to distinguish MPM from benign mesothelial proliferative disorders in routine practice due to overlaps in morphology (3). Thus, genomic-based ancillary assays, such as fluorescence *in situ* hybridization (FISH) to detect homozygous deletion (homo-d) of the 9p21 locus and immunohistochemistry (IHC) analysis to detect loss of BRCA1-associated protein 1 (BAP1) expression and methylthioadenosine phosphorylase [MTAP; the protein product of the *MTAP* gene located in the telomere side of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) in the 9p21 locus (3)] proteins, are effective tools for detecting characteristic genetic abnormalities that are essential in the diagnosis of MPM (3,4).

The *CDKN2A* gene, located at the 9p21 locus (5), is a tumor suppressor gene which encodes the p16 protein (5). Homo-d of *CDKN2A* is one of the most frequent genetic abnormalities in malignant mesothelioma and is found in 50-65.8% of MPM cases (6,7). *CDKN2A* FISH is a common assay used to detect this deletion, and a 222 kilobase (kb) *CDKN2A* probe (long probe, L-probe) is a commercially available and the most frequently used FISH probe, which covers an area of 222 kb,

including the *CDKN2A*, *CDKN2B* and *MTAP* genes. Another commercially available probe is the 57 kb *CDKN2A* probe (short probe, S-probe) that covers an area of 57 kb, confined to the *CDKN2A* and *CDKN2B* genes (6,7). FISH reveals heterozygous deletion (hetero-d) signals in addition to homo-d and normal signals (8). Recently, *CDKN2A* FISH with L-probe revealed high proportions of hetero-d, while S-probe yielded high proportions of homo-d, with low hetero-d (8).

Microdeletion, defined as a deletion <200 kb, can induce false negative results of signal deletion in FISH assays, owing to redundant probe reactivity (9-13). Some of the high hetero-d signals may result from microdeletions in MPM (8). It was hypothesized that an S-probe can effectively detect microdeletion status of the *CDKN2A* gene, and thus increase homo-d rate in MPM, which has a high hetero-d and low homo-d status with L-probe (8).

The present study aimed to evaluate the effectiveness of S-probe in diagnosing MPM. In routine practice, it is important to confirm homo-d of the *CDKN2A* gene for an accurate diagnosis of MPM, to start treatment and predict prognosis. Borderline cases are infrequently found in which FISH with L-probe reveals high hetero-d and low homo-d; however, it is worth performing FISH with S-probe if homo-d of *CDKN2A* is detected (2). Thus, the aforementioned hypothesis was investigated in four cases of MPM, in which *CDKN2A* FISH with L-probe yielded high hetero-d and low homo-d. The results of FISH with S-probe were assessed in association with the deletion range evaluated via quantitative (q)PCR analysis.

Materials and methods

Case selection. CDKN2A FISH results were reviewed in 106 MPM cases that were diagnosed at the Department of Pathology of Fukuoka University Hospital (Fukuoka, Japan) between January 2017 and December 2019, including consultation cases. A total of five cases of MPM exhibited high hetero-d and low homo-d pattern in *CDKN2A* FISH. A total of four cases were used in the present study, owing to the presence of redundant tissue, for further investigation. All four samples were obtained from male patients, the mean age at diagnosis was 68.5 years (age range, 63-80 years). Pathological diagnoses were performed according to the 2021 World Health Organization classification of thoracic tumors (1). The mesothelial origin was confirmed by positive reactivity for Wilms tumor 1, calretinin or podoplanin (D2-40) and negative reactivity for epithelial cell adhesion molecule (BerEP4), thyroid transcription factor-1, claudin-4 or carcinoembryonic antigen. The present study was approved by the Institutional Review Board of Fukuoka University (approval no. 11-7-11). The requirement for informed consent was waived as the anonymous use of redundant tissues is part of the standard treatment agreement with patients, when no objection is expressed.

IHC staining. For immunostaining, 4-μm-thick sections of formalin-fixed paraffin embedded (FFPE) tissues were used. Sections were deparaffinized and rehydrated with xylene, alcohol and tap water. IHC analysis was performed on an automated DAKO Omnis platform using the DAKO EnVision FLEX, High pH (Omnis https://www.agilent.com/ja-jp/product/dako-omnis-solution-for-ihc-ish/dako-omnis/dako-omnis-75902, code GV800), according to the manufacturer's instructions. Heat-epitope retrieval was performed using a high pH for 30 min at 97˚C. Protein blocking was performed using Protein block Serum-Free (Agilent Technologies, Inc.) for 20 min at 25˚C. The slides were subsequently incubated using one of the following antibodies: Mouse monoclonal anti-calretinin antibody (clone Calret1, cat. no. M7245, Dako; Agilent Technologies, Inc., ready to use; room temperature for 40 min), mouse monoclonal anti-BAP1 antibody (clone C-4, cat. no. sc-28383, Santa Cruz Biotechnology, Inc.; 1:100 dilution; room temperature for 1 h) or mouse monoclonal anti-MTAP antibody (clone 2G4, cat. no. H00004507-M01J, Abnova; 1:100 dilution; room temperature for 45 min). Endogenous peroxidase was inactivated using peroxidase blocking reagent (Dako; Agilent Technologies, Inc.) for 3 min at room temperature. Subsequently, slides were incubated with a peroxidase-labeled polymer (EnVision Flex/HRP, cat. no. DM842; Agilent Technologies, Inc.), at room temperature for 20 min). The slides were visualized with 3,3'-diaminobenzidine at room temperature for 5 min and counterstained with hematoxylin at room temperature for 5 min. Analysis was performed using an Olympus BX 53 light microscope (magnification, x400). Each specimen was evaluated by two authors (YO and KN).

BAP1 and MTAP staining results were scored as loss or retained. BAP1 loss in tumor cells was defined as complete loss of nuclear staining. MTAP loss in tumor cells was defined as complete loss of cytoplasmic staining in the area where internal positive control cells (lymphocytes, histiocytes, fibroblasts, pneumocytes or endothelial cells) exhibited cytoplasmic and nuclear MTAP staining (14).

FISH assay. CDKN2A FISH was performed using 4-μm-thick FFPE tissue sections. Sections were deparaffinized, rehydrated in 100, 90 and 70% ethanol, washed with 2x saline sodium citrate (SSC) (UltraPure™ 20X SSC, Invitrogen; Thermo Fisher Scientific, Inc.), incubated in pretreatment solution (PathVysion HER2 DNA probe kit; 30 min at 80˚C), and digested with pepsin solution (Sigma-Aldrich; Merck KGaA, for 3 h at 37˚C). Refixation was performed using 10% formalin (10 min at room temperature); sections were subsequently washed with 2x SSC, dehydrated in ethanol, dried and exposed to either of two DNA probe: 222 kb Vysis LSI CDKN2A/CEP9 probe alone (Abbott Japan LLC) or 57 kb SureFISH 9p21.3 CDKN2A (Agilent Technologies, Inc.) probe and 367 kb SureFISH Chr9 CEP FISH probe (Agilent Technologies, Inc,). Denaturation (10 min at 80˚C) and hybridization (24 h at 37˚C) were performed in ThermoBrite (Abbott Japan LLC.). After 24 h, the specimens were treated with 2x SSC containing 0.3% NP 40 (Nonidet P-40, nacalai tesque) (2 min at 72˚C), 2x SSC containing 0.1% NP 40 (5 min at room temperature) and 2x SSC (5 min at room temperature). Nuclei were counterstained with DAPI using antifade reagent for 24 h at 4˚C (VECTASHIELD Mounting Medium, Vector Laboratories, Inc.). Analysis was performed using a fluorescence microscope (magnification, x1,000) (Axio imager Z1; Carl Zeiss AG) and Isis FISH imaging system (Metasystems, https://metasystems-international.com/en/products/isis). FISH signal was classified under three signal patterns: Normal (two red and two green signals), hetero-d (one red and two green signals) and homo-d (no red and two green signals), and >100 tumor cells were assessed for each case.

Table I. Primer sets.

Set	Primer	Sequence (5'-3')	Target region (gene)	Product size (bp)
A	F	GGGCACTTTGTGACTCTTCTTAACC	*MTAP*	105
	R	GGAGACTTTGGGGTATGGTCCTC		
B	F	TACAGGTAGGGAAGCAGGCAATC	Non-coding region between *MTAP* and *CDKN2A*	90
	R	TATGGGTATGCTCTGGTCTTCTGC		
C	F	GCGATTGCTGCCAAGATACCC	Non-coding region between *MTAP* and *CDKN2A*	89
	R	TCCACAGGCCTTTGTCTCCAG		
D	F	TTCCGGACACACTGGGTCAC	Non-coding region between *MTAP* and *CDKN2A*	99
	R	GTTTACAGGCAAGTGAGGTTGTCC		
E	F	GCCTGTTTTCTTTCTGCCCTCTGC	*CDKN2A*	89
	R	GACTGATGATCTAAGTTTCCCGAGGTTTCTC		
F	F	CCACATCTTCACGCCTTCGC	*CDKN2A*	83
	R	AGGGTTGCAAGAAGAAAACGAGTG		
RPS6	F	CTACTGAGTAAGGGGCATTCCTGT	The reference gene	102
	R	GAGAACGCTCAGATTTGCATCCAC		

F, forward; R, reverse; bp, base pair.

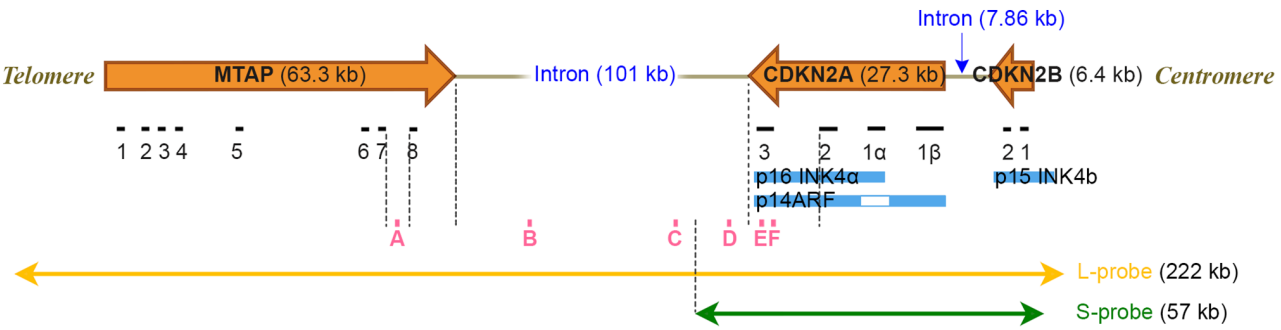

Figure 1. Chromosome map of the 9p21 region and CDKN2A FISH probe targeting areas. The L-probe covers areas up to 222 kb, which includes the *CDKN2A*, *CDKN2B* and *MTAP* genes. The S-probe only covers areas up to 57 kb, which includes the *CDKN2A* and *CDKN2B* genes. A, B, C, D, E and F are the locations of each PCR product used in quantitative PCR analysis. *CDKN*, cyclin-dependent kinase inhibitor; MTAP, methylthioadenosine phosphorylase; L-probe, long probe; S-probe, short probe.

DNA extraction. A total of 6/7 sections of 10-μm FFPE-cut specimens were used for each case. Before extracting DNA, macrodissection was performed to purify the tumor samples, with the exception of case 4 where the sample was too small. Genomic DNA was extracted using a RecoverAllTM Total Nucleic Acid Isolation kit (Ambion; Thermo Fisher Scientific, Inc.; cat. no. AM1975), according to the manufacturer's instructions, with one modification: Protease digestion was performed for 20-24 h. To confirm the quality of extracted DNA samples, the positive control (β-globin) primer (forward, 5'-ACACAACTGTGTTCACTAGC-3' and reverse, 5'-CAACTTCATCCACGTTCACC-3') region was amplified via PCR using KOD FX Neo (Toyobo, Ltd., cat. no. KFX-201). The PCR reaction protocol was as follows: 94˚C for 15 min, followed by 35 cycles of denaturation at 98˚C for 10 sec, annealing at 55˚C for 30 sec, and extension at 68˚C for 30 sec. The PCR products were detected by electrophoresis of 3% agarose gel with GelRed™ Nucleic Acid Gel Stain (10,000x) in DMSO (Biotium, Inc.), visualized by ultraviolet light.

qPCR. The extracted DNA from tumor tissue samples was used for qPCR. The chromosome map of the 9p21 region and the targeting regions of the two commercially available *CDKN2A* FISH probes are presented in Fig. 1. The seven primer sets for analyzing the deleted region and the reference gene region were designed by custom oligo primers (Sigma-Aldrich; Merck KGaA) (Table I). Primer A was designed for the *MTAP* gene, primers B-D were designed for the non-coding region between the *CDKN2A* and *MTAP* genes and primers E and F were designed for the *CDKN2A* gene. The copy number of the regions was quantified via qPCR analysis of genomic DNA using KOD SYBR® qPCR Mix (Toyobo Life Science). The PCR reaction protocol was as follows: 98˚C for 15 min, followed by 40 cycles of denaturation at 98˚C for 10 sec, annealing at 60˚C (primer A, primer F, RPS6) or 62˚C (primers B, C, D and E) for 10 sec, and extension at 68˚C for 30 sec. Melting curve analysis was also performed for non-specific products. The copy number was determined by the relative standard curve method using the RPS6 gene (9p22) (15) as the reference gene according to the previous reports (9,15), and the calculated

Table II. Clinicopathological characteristics of the four MPM cases assessed in the present study.

Characteristic	Case 1	Case 2	Case 3	Case 4
Age, years	62	78	68	66
Sex	Male	Male	Male	Male
Histology	Epithelioid	Epithelioid	Epithelioid	Epithelioid
MTAP IHC	Retained	Retained	Retained	Retained
BAP1 IHC	Loss	Retained	Loss	Loss
Source of tissue	EPP	Pleural biopsy	Pleural biopsy	TBLB
Treatment	EPP + radiation; CDDP + PEM; GEM + VNR	CBDCA + PEM	Chemotherapy	None
Time from diagnosis to mortality	7 years and 6 months	2 years and 2 months	5 months	3 months

MPM, malignant pleural mesothelioma; MTAP, methylthioadenosine phosphorylase; BAP1, BRCA1-associated protein 1; IHC, immunohistochemistry; EPP, extrapleural pneumonectomy; TBLB, transbronchial lung biopsy; CDDP, cisplatin; PEM, pemetrexed sodium hydrate; GEM, gemcitabine hydrochloride; VNR, vinorelbine detartrate; CBDCA, carboplatin.

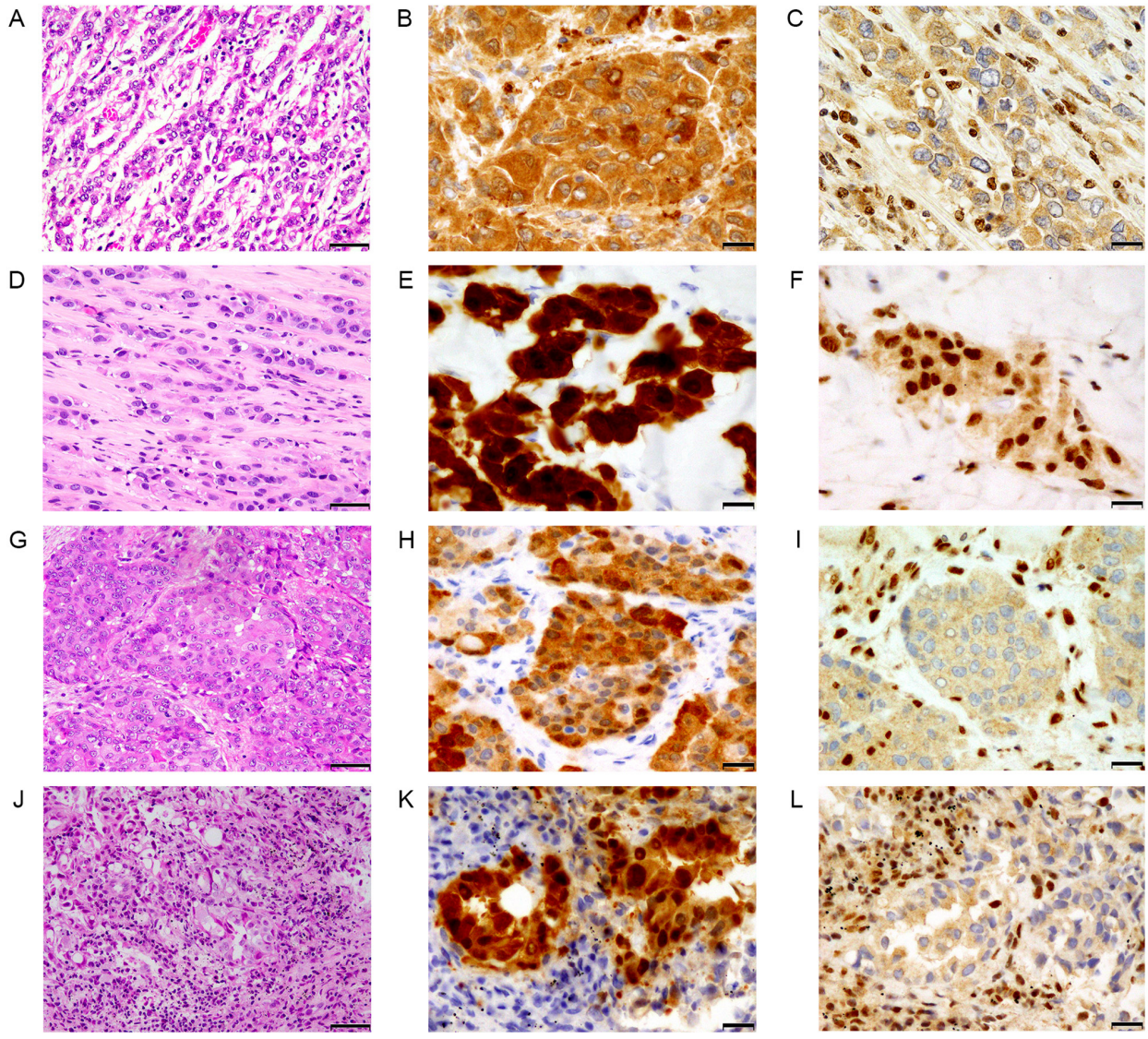

Figure 2. Histology of malignant pleural mesothelioma (A-C, case 1; D-F, case 2; G-I, case 3 and J-L, case 4). (A, D, G and J) The tumor exhibited proliferation of atypical epithelioid tumor cells with eosinophilic cytoplasm, arranged in cords or small nests. (B, E, H and K) Tumor cells were positive for calretinin. (C, I and L) Cases 1, 3 and 4 exhibited BAP1 loss, (F) whereas case 2 retained BAP1 expression. Olympus BX 53 light microscope. (A, D, G and J) Magnifications: x200 scale bar, 50 μm). (B, C, E, F, H, I, K and L) Magnification: x400 scale bar, 20 μm. BAP1, BRCA1-associated protein 1.

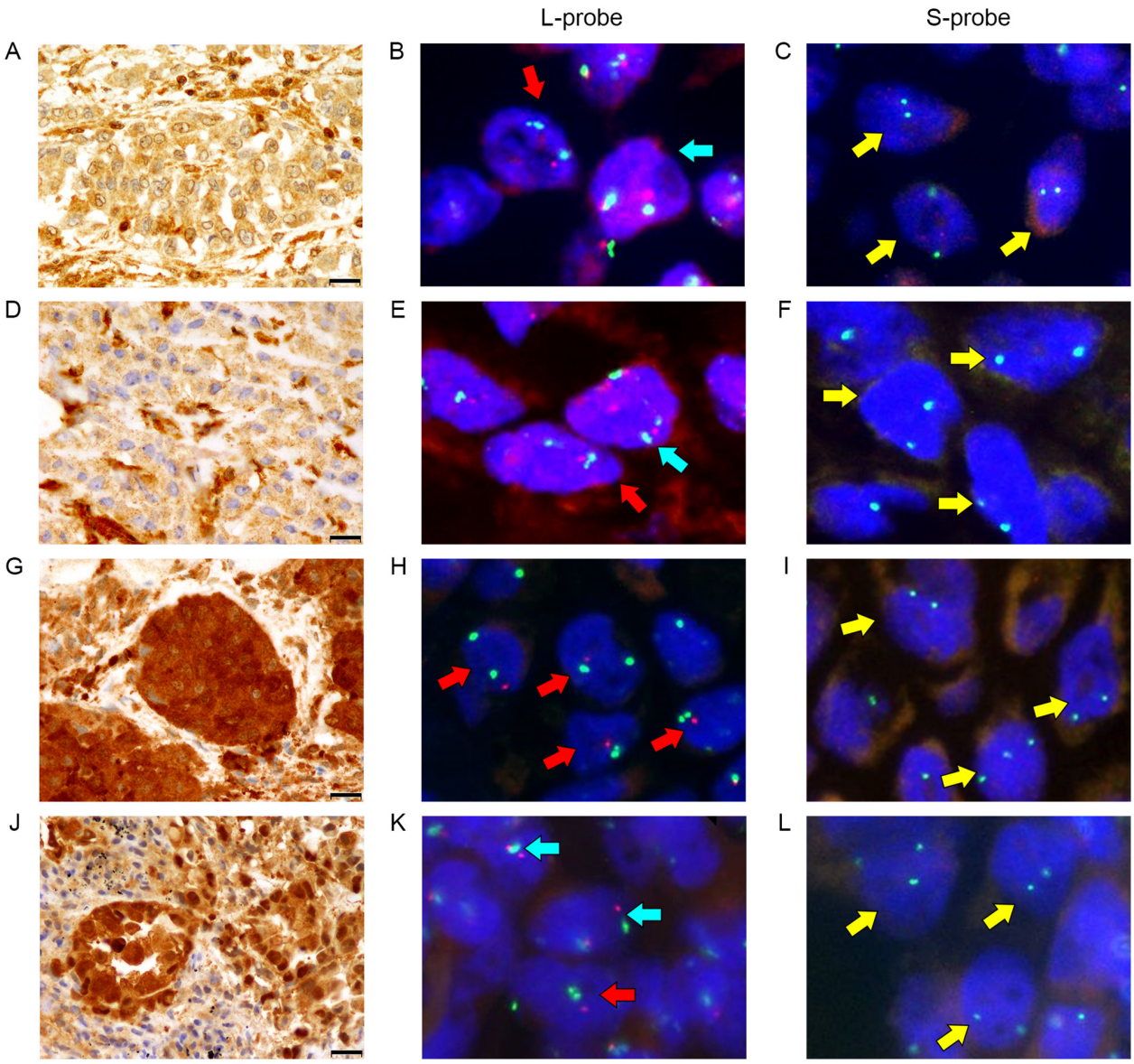

Figure 3. MTAP immunostain and two cyclin-dependent kinase inhibitor 2A fluorescence *in situ* hybridization probes (L-probe and S-probe). Summary of the four cases (A-C, case 1; D-F, case 2; G-I, case 3 and J-L, case 4) are presented. (A, G and J) Cases 1, 3, 4 exhibited strong MTAP expression in both the nuclei and cytoplasm, (D) while case 2 exhibited weak cytoplasmic positivity for MTAP protein. (B, E, H and K) All cases exhibited a high hetero-d signal (red arrow) compared with normal signal (blue arrow) and homo-d signal (yellow arrow) in L-probe. (C, F, I and L) All cases exhibited a high rate of homo-d signal in S-probe. (A, D, G and J) Olympus BX 53 light microscope (magnification, x400; scale bar, 20 μm). (B, C, E, F, H, I, K and L) Axioplan2 imaging fluorescence microscope. MTAP, methylthioadenosine phosphorylase; L-probe, long probe; S-probe, short probe.

numbers were evaluated as follows: ≥0.8 as no deletion, >0.5 and <0.8 as a one-allele deletion, and ≤0.5 as a two-allele deletion.

Results

Clinicopathological characteristics. The clinicopathological characteristics are summarized in Table II. All four samples were obtained from male patients, the mean age at diagnosis was 68.5 years (age range, 63-80 years). A total of two samples were obtained following pleural biopsy, one following extrapleural pneumonectomy and one following transbronchial lung biopsy (TBLB) from a metastatic lesion. All cases were epithelioid MPM in terms of histological subtype (Fig. 2A, D, G and J). Mesothelial phenotype was confirmed by positive immunostaining for calretinin (Fig. 2B, E, H and K). IHC analysis of BAP1 protein (Fig. 2C, F, I and L) was as follows: Three cases with BAP1 loss and one with BAP1 retained. The mean period from diagnosis to mortality was 2.6 years (3-90 months).

MTAP IHC and CDKN2A FISH results. A total of 3/4 cases exhibited strong nuclear and cytoplasmic or cytoplasmic only expression for MTAP protein (Fig. 3A, G and J), while case 2 exhibited relatively weak cytoplasmic reactivity for MTAP (Fig. 3D). With the L-probe, *CDKN2A* FISH revealed high hetero-d signals in all cases (cases 1-4; 73.3, 37.1, 59.2 and 64.8%, respectively) (Fig. 3B, E, H and K) compared with homo-d signals (cases 1-4; 12.1, 12.4, 25.4 and 22.2%, respectively). In the hetero-d signals with L-probe, one red signal was slightly smaller than two CEP9 green signals.

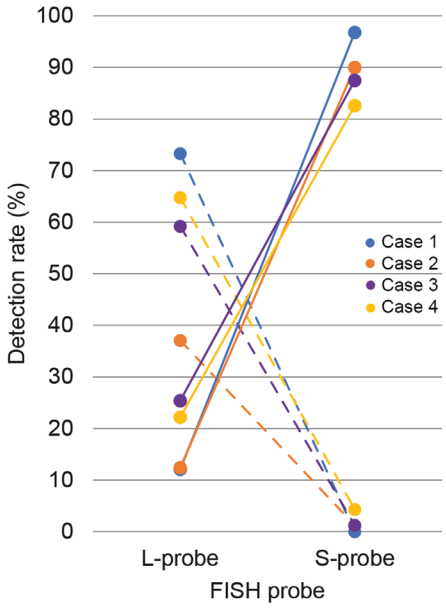

Figure 4. Comparative line graph of homo- and hetero-d rates between the L- and S-probes. The vertical line represents the detection rate of homo-d (solid line) and hetero-d (dashed line) signals, while the horizontal line represents the corresponding results of L- and S-probes. For the L-probe, the hetero-d rate was high compared with the homo-d signal for each case. However, the homo-d rate increased by the S-probe. L-probe, long probe; S-probe, short probe; homo-d, homozygous deletion; hetero-d; heterozygous deletion; FISH, fluorescence *in situ* hybridization.

However, the *CDKN2A* FISH with S-probe exhibited high homo-d in all cases (cases 1-4; 96.8, 90.0, 87.5 and 82.6%, respectively) (Fig. 3C, F, I and L) compared with low hetero-d (cases 1-4; 0.0%, 1.2, 1.2 and 4.3%, respectively). In the control tissues, *CDKN2A* FISH with S-probe exhibited normal signals (Fig. S1). The comparative line graph of homo-d (solid line) and hetero-d (dotted line) demonstrated the increased proportions of homo-d and decreased proportions of hetero-d in FISH with S-probe compared with L-probe (Fig. 4).

qPCR. qPCR analysis revealed no allele deletions in the *MTAP* gene in all cases, whereas two-allele deletions were detected in the *CDKN2A* gene in 3/4 cases (Fig. 5). Case 4 exhibited one-allele deletion in the *CDKN2A* gene. Deletion status of the non-coding region between the *CDKN2A* and *MTAP* genes varied, two-allele deletions for case 2 and one-allele deletion for cases 1, 3 and 4, respectively.

Discussion

To the best of our knowledge, the preset study was the first to demonstrate that *CDKN2A* FISH with S-probe is more effective in elucidating short homo-d in the 9p21 locus compared with L-probe in MPM. *CDKN2A* FISH with conventional L-probe revealed high hetero-d (range 37.1-73.3%) and low homo-d (range 12.1-25.4%) in all four cases with microdeletions, whereas that with S-probe yielded high proportions of homo-d (82.6-96.8%). Furthermore, qPCR analysis in these four cases demonstrated that 3/4 cases exhibited homo-d in the coding region of the *CDKN2A* gene, while case 4 exhibited hetero-d, according to the calculated value. In case 4, two-allele deletions were not confirmed via qPCR analysis

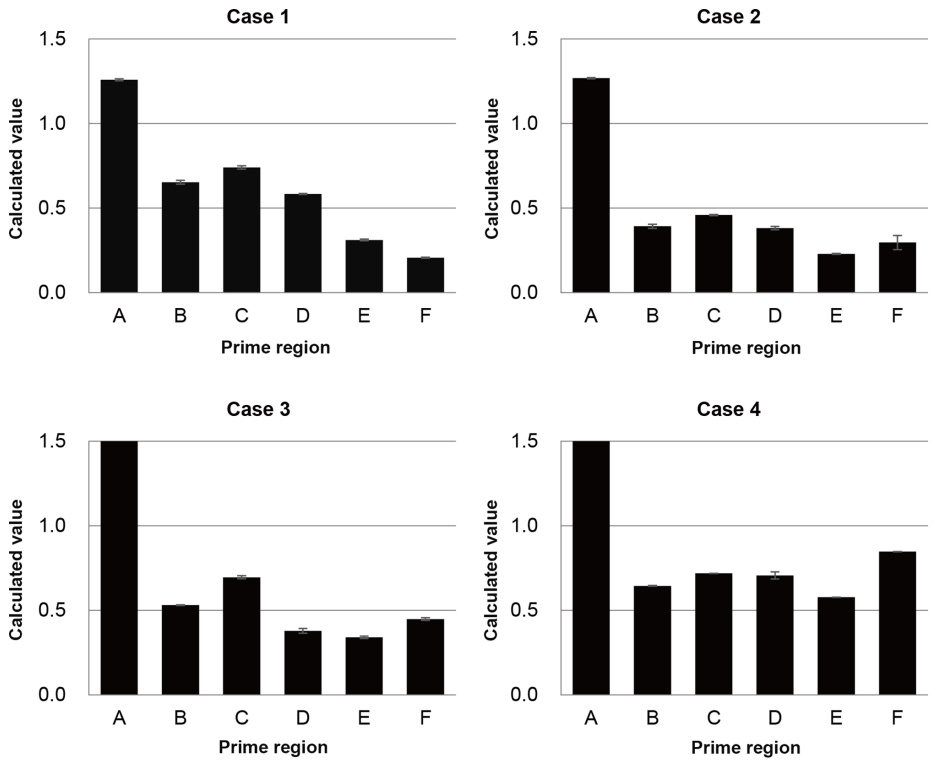

Figure 5. qPCR analysis in the 9p21 locus. qPCR analysis was performed to detect the deletion status of the 9p21 regions. The *MTAP* gene (primer A) had no allele deletion, while two-allele deletions were observed for the *CDKN2A* gene (primer E and F) in all cases except for case 4. Deletion status of the non-coding region between the *CDKN2A* and *MTAP* genes (primer B-D) was two- or one-allele deletion for each case. qPCR, quantitative PCR; MTAP, methylthioadenosine phosphorylase; *CDKN2A*, cyclin-dependent kinase inhibitor 2A.

despite the clear evidence of homo-d exhibited by FISH as the TBLB section was too small to allow macrodissection prior to qPCR. Thus, it was speculated that contamination of normal cells would affect the qPCR results. Based on these results, the size of deletions was ~27.3-142.5 kb at most. The results of the present study suggest it is worth performing *CDKN2A* FISH with S-probe when *CDKN2A* FISH with conventional L-probe results in high hetero-d and low homo-d.

The deletion size of the 9p21 locus found in MPM is usually 2-3 megabase in in vitro studies using cell lines; this range is large enough to accommodate the *MTAP* gene, as well as the *CDKN2A* and *CDKN2B* genes (16). However, in tumor specimens, the deleted region varies in size and range (9,10). Microdeletion in 9p21.3 is found in 32% of cases of acute lymphoblastic leukemia in adolescents and 10% of Ewing sarcoma (9,10). The smallest deletion is only 25 kb on the *CDKN2A* gene (10). Cases with *CDKN2A* microdeletion fail to exhibit high proportions of homo-d by FISH with L-probe, as the L-probe, which is designed to cover three genes in the 9p21 locus (*CDKN2A*, *CDKN2B* and *MTAP*) (10), is large enough to hybridize the non-deleted area next to the microdeleted *CDKN2A* gene, resulting in 'pseudo' hetero-d signals (9,13). In the present study, the false red signals were smaller compared with normal CEP9 green signals. Given the design of the L-probe and respective gene sizes, it is understandable that microdeletion cases exhibited high hetero-d and low homo-d with L-probe in *CDKN2A* FISH.

In the present study, MTAP expression was retained in all four cases. MTAP IHC is a useful surrogate assay for *CDKN2A* FISH as homo-d of *MTAP* always occurs in association with homo-d of *CDKN2A* (6,14). However, it would be favorable to perform *CDKN2A* FISH in cases with retained MTAP expression determined by IHC as retained MTAP protein expression does not necessarily rule out a microdeletion status of the *CDKN2A* gene (8,14).

Detection of *CDKN2A* deletion is important in routine practice as it effectively discriminates MPM from reactive mesothelial proliferations, and is associated with poor prognosis in MPM (3-6). There are several approaches used to circumvent the false negative results in detecting chromosomal microdeletion, including use of smaller probes (as used in the present study), enhancement of FISH signals (13), multiple ligation-dependent probe amplification (MLPA) assay (11) and array comparative genomic hybridization (CGH) (10). Practical use of S-probe can be somewhat challenging as the target red signal is small owing to the short probe length (9); thus, lower hybridization efficiency can result in false positive results for *CDKN2A* deletion (9). Therefore, careful establishment of an accurate FISH assay, which always reveals two red signals and two green signals in normal cells, is critical.

The present study is not without limitations. First, the sample size was too small. Prospective studies including S-probe performance in several MPM cases, that have high hetero-d and low homo-d status with L-probe, are warranted to verify the effectiveness of S-probe. Secondly, the S-probe can cause false negative results of signal deletion if the deletion range is too small (9-13). On such occasions, enhancement of FISH signals, MLPA assay and array CGH can be alternative approaches to detect *CDKN2A* gene deletion if MPM is highly suspected.

In the single institutional cohort assessed, 4.7% of MPM cases exhibited microdeletion of the *CDKN2A* gene, with high hetero-d and low homo-d detected by FISH with the conventional L-probe. The use of *CDKN2A* FISH with a short 57 kb probe can be an effective approach to detect a small homo-d in MPM. Furthermore, S-probe can increase the homo-d rate in MPM cases with high hetero-d with L-probe, and thus assist in solving diagnostic difficulties in cases involving high hetero-d with low homo-d.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YO performed the experiments, drafted and revised the manuscript and analyzed the data. MH conceptualized and supervised the present study, and drafted and revised the manuscript. SM curated the data, acquired the data, analyzed and interpreted the data, and drafted and revised the manuscript. AS and TT acquired the resources, curated the data, acquired the data, and analyzed and interpreted the data, and drafted and revised the manuscript. KN conceptualized and supervised the present study, was project administrator, acquired funding, and drafted and revised the manuscript. YO, MH, and KN confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Fukuoka University (Fukuoka, Japan; approval no. 11-7-11). The requirement for informed consent was waived as the anonymous use of redundant tissues is part of the standard treatment agreement with patients, when no objection is expressed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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