EDITOR'S CHOICE

FISH assay development for the detection of *p16/CDKN2A* deletion in malignant pleural mesothelioma

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ABSTRACT

Aims To develop a fluorescence in-situ hybridisation (FISH) assay for detecting *p16/CDKN2A* deletion on paraffin tissue sections for use as an ancillary test to distinguish reactive from malignant mesothelial proliferations.

Method Dual-colour FISH for *p16/CDKN2A* and chromosome 9 (CEP-9) was performed on 11 benign mesothelial proliferations and 54 malignant pleural mesothelioma (MPM) cases to establish cut-off values for *p16/CDKN2A* deletion. A third MYC probe was used to verify cases showing homozygous deletion. Eight equivocal biopsies were used for assay testing.

Results Cut-off values for *p16/CDKN2A* deletion were calculated based on FISH signalling patterns obtained from the benign controls (mean percent nuclei plus three standard deviations). Hemizygous deletion was defined as >44% of nuclei showing the hemizygous (one *p16/CDKN2A*, two CEP-9 signals) or >15% of nuclei showing the monosomy (one *p16/CDKN2A*, one CEP-9 signal) deletion patterns. None of the benign cases showed a homozygous deletion pattern (no *p16/CDKN2A*, at least one CEP-9 signal). In the malignant cases, the percentage of nuclei showing homozygous deletion ranged from 1% to 87%. Therefore, the cut-off value for homozygous deletion was defined as >10%. *P16/CDKN2A* deletion was detected in 61% (33/54) of MPM cases. Among the equivocal biopsies, four showed homozygous and one showed hemizygous *p16/CDKN2A* deletion. Age over 60 years, asbestos exposure and *p16/CDKN2A* deletion were associated with a worse prognosis.

Conclusion Distinction between benign and malignant mesothelial proliferations can be diagnostically challenging. FISH for *p16/CDKN2A* deletion is a useful test for confirming the diagnosis of MPM.

INTRODUCTION

Differentiating benign pleural mesothelial proliferations from malignant pleural mesothelioma (MPM) on routine biopsies with limited tissue can pose a diagnostic challenge.[1] If the biopsy fails to capture invasion into fat or skeletal muscle, mesothelioma can be difficult to differentiate from reactive mesothelial hyperplasia.[1] Many epithelioid MPM have monotonous-appearing cells that are deceptively bland. Similarly, the desmoplastic variant of mesothelioma is difficult to distinguish from fibrous pleuritis in the absence of frankly sarcomatous foci or invasion into adjacent structures.[1] Conversely, reactive mesothelial proliferations can exhibit cytological and architectural features that mimic malignancy. Given the prognostic and treatment implications, accurate distinction between benign and malignant mesothelial proliferations is imperative.

Immunohistochemical markers can readily identify cells of mesothelial origin, but to date, no markers reliably distinguish benign from malignant mesothelial proliferations.[1 2] The use of epithelial membrane antigen,[3] p53,[4 5] desmin,[6] P-glycoprotein,[7] platelet-derived growth factor receptor β-chain,[8] β-catenin[9–11] and GLUT-1[12] have been reported as markers of malignancy; nevertheless, all lack sufficient sensitivity and specificity for clinical adoption and implementation.[1 6 13 14]

Deletion involving the 9p21 locus, the site of the cyclin-dependent kinase inhibitor 2A/p16 gene (*p16/CDKN2A*), frequently occurs in mesothelioma.[15] Homozygous deletion has been reported in 22–74% of mesotheliomas.[16–21] However, previous studies lacked details on assay development. In fluorescence in situ hybridisation (FISH) it has been shown that for each probe a cut-off value needs to be determined based on normal nuclei of specific target nuclei and from different subjects.[22] A critical factor affecting the accurate interpretation of FISH signals, particularly when looking for deletions, is the establishment of cut-off values for all signal patterns that might appear with a given assay.[23] The objectives of this study were to: (a) develop a FISH assay for clinical use in the diagnosis of MPM, by establishing cut-off values for the detection of *p16/CDKN2A* deletion on formalin-fixed paraffin-embedded (FFPE) material; and (b) test the assay on tissue sections from equivocal cases with known clinical outcome.

MATERIALS AND METHODS

Samples for assay development

With institutional research ethics board approval, we collected 54 archival biopsy and resection specimens with clinically, radiologically and histopathologically confirmed diagnosis of MPM (42 epithelioid, 11 biphasic and 1 sarcomatoid variant) and 11 cases of reactive mesothelial proliferations as negative controls, to establish the cut-off values. Clinical data collected included age, sex, smoking history, previous asbestos exposure and clinical outcome.

Samples for assay testing

We collected prospectively eight biopsy samples in which the distinction between a benign mesothelial

proliferation from MPM could not be made definitively by histology and immunohistochemistry alone, but were proven malignant clinically or by repeat biopsy. The samples were typically small biopsies lacking evidence of true stromal invasion, such as invasion into fat or muscle, thereby making it difficult to distinguish from reactive mesothelial cell entrapment. Immunohistochemistry confirmed their mesothelial cell origin by positive staining for calretinin or CK5/6 and negative staining for markers of metastatic adenocarcinoma (eg, BerEp4, CEA, TTF-1).

Fluorescence in situ hybridisation

Dual-colour FISH was performed using a commercially available Spectrum Orange-labelled locus specific p16(9p21) probe and Spectrum Green-labelled chromosome 9 centromeric probe (Vysis LSI p16 (9p21) SpectrumOrange/CEP9 SpectrumGreen Probe; Abbott Laboratories, Des Plaines, Illinois, USA). Slides (4 μm sections) were deparaffinised in xylene, followed by rehydration and pretreatment in 10 mM sodium citrate (pH 6.0) at 80°C for 45 min. After washing in 2× standard saline citrate for 5 min, sections were digested with pepsin (37 500 U in 0.1 N HCl) (Sigma, St Louis, Missouri, USA) at 37°C for 10–25 min. Slides were co-denatured with the probes, allowed to hybridise and washed according to the manufacturer's protocol.

With tissue sections potential loss of target sequences by nuclear truncation can be problematic for analysis of deletions. To verify that samples showing a deletion pattern are not due to nuclear truncation or differences in hybridisation efficiency between the *p16/CDKN2A* and CEP-9 probes, FISH was repeated in homozygous deleted cases with a third in-house MYC probe (BAC RP11-367L7), which is known to be not deleted in mesothelioma. The MYC probe was labelled with DEAC-dUTP (PerkinElmer, Waltham, Massachusetts, USA) using the Vysis nick translation kit (Abbott Laboratories) according to the manufacturer's protocol. Slides were analysed, blinded from the clinical data, using a Zeiss microscope (Axioplan 2, Jena, Germany) equipped with the appropriate filters. A minimum of 100 non-overlapped intact (uniform DAPI staining with intact nuclear contours) interphase nuclei of consecutive cells in at least two different areas of the section were scored. An H&E stained section was used to verify the presence of tumour.

Statistics

Survival was defined as time between date of surgery and date of death or last follow-up. Each biomarker was tested for association with survival using the Cox proportional hazards model. The percent survival at every point in time was estimated using the Kaplan–Meier method. Associations between *p16/CDKN2A* status and the clinical parameters were performed using the Wilcoxon rank-sum test.

RESULTS

Cut-off values for normal *p16/CDKN2A* FISH signalling

FISH was performed on 11 cases of reactive mesothelial proliferations. Cut-off levels were calculated as the mean percentage + three standard deviations (SD) of nuclei showing one signal as previously described.[22] None of the reactive cases showed the *p16/CDKN2A* homozygous deletion pattern, which was defined as loss of both *p16/CDKN2A* signals with at least one CEP-9 signal or 0SpO/1-2SpG (table 1). Therefore, a cut-off for homozygous deletion could not be established using the benign controls. The hemizygous loss pattern (one *p16/CDKN2A* signal with two CEP-9 signals or 1SpO/2SpG) was noted in 1–10% (mean 6.3%) of nuclei. The monosomy pattern (one *p16/CDKN2A* signal with one CEP-9 signal or 1SpO/1SpG) was present in 6–34% (mean 19.9%) of nuclei. As monosomy pattern could be interpreted as hemizygous deletion, hemizygous *p16/CDKN2A* deletion was defined as >44% of nuclei showing 1SpO/1SpG or >15% of nuclei showing 1SpO/2SpG (table 2).

Table 1 Copy number for *p16/CDKN2A* gene in benign mesothelial cells

FISH signalling in benign mesothelial cells	Mean (%)*±SD	Range (%)
2SpO/2SpG	63±8	44–75
0SpO/2SpG	0	0
0SpO/1SpG	0	0
1SpO/2SpG	6±3	1–10
1SpO/1SpG	20±8	6–34

*Percentage of nuclei showing the FISH signal pattern.
FISH, fluorescence in situ hybridisation.

Malignant pleural mesothelioma

Among MPM cases, the mean percentage of nuclei with homozygous deletion pattern was 61% (range 1–87%), with only three cases showing a percentage less than 30%. These three cases also showed hemizygous deletion as defined by the established cut-offs. To mitigate uncertainty from artifactual loss of signals due to nuclear sectioning, a cut-off of >10% for 0SpO/1-2SpG was defined for homozygous deletion (table 2). Using this cut-off, homozygous *p16/CDKN2A* deletion was detected in 23/54 (43%) of MPM cases (table 3, figure 1). Homozygous loss was confirmed by repeating the FISH with the addition of the MYC probe. Eleven of the 23 homozygous cases showed concurrent hemizygous (1SpO/2SpG) deletion.

Hemizygous-only deletion was seen in 10/54 (18%) cases, three (5%) of which had the 1SpO/1SpG deletion pattern and seven (13%) had the 1SpO/2SpG deletion pattern. Two cases showed *p16/CDKN2A* amplification, defined as at least 10% of nuclei with a SpO:SpG ratio of 2 or higher, in 32% and 20% of nuclei, respectively (figure 1D). PCR-direct sequencing of the *p16/CDKN2A* gene on both cases did not identify mutations (data not shown). *P16/CDKN2A* deletion shows 61% sensitivity and 100% specificity for MPM.

Testing samples

Among the eight equivocal biopsies, seven had sufficient tissue for evaluation. Four cases showed homozygous *p16/CDKN2A* deletion (mean 49%, range 16–75%) and one showed hemizygous (1SpO/2SpG) deletion. The pauci-cellular and cytologically bland nature of the two desmoplastic mesothelioma cases

Table 2 Definitions for homozygous and hemizygous *p16/CDKN2A* deletion determined by fluorescence in situ hybridisation (FISH)

p16/CDKN2A deletion pattern	FISH signal pattern		Cut-off value for % nuclei with FISH signal pattern
	p16/CDKN2A (SpO)	CEP-9 (SpG)	
Homozygous	No *p16/CDKN2A* signal 0SpO	At least one CEP-9 signal 1-2SpG	>10%
Hemizygous	One *p16/CDKN2A* signal 1SpO	Two CEP-9 signals 2SpG	>15%
Hemizygous (monosomy)	One *p16/CDKN2A* signal 1SpO	One CEP-9 signal 1SpG	>44%

SpO, Spectrum Orange; SpG, Spectrum Green.

Table 3 Fluorescence in situ hybridisation for *p16/CDKN2A* gene copy number in malignant mesothelioma

	Homozygous deletion*	Hemizygous deletion‡	Amplification†	Normal diploid	Total
Epithelioid	19	5	2	16	42
Sarcomatoid	0	0	0	1	1
Biphasic	4	5	0	2	11
Total	23 (43%)	10 (18%)	2 (4%)	19 (35%)	54

*>10% of tumour cells show 0SpO/2SpG.
†At least 10% of tumour cell nuclei show SpO/SpG ratio ≥2.
‡>15% of tumour cells show 1SpO/2SpG or >44% show 1SpO/1SpG.

made it difficult to distinguish under fluorescence microscopy normal from neoplastic spindle cells. All eight equivocal cases were subsequently confirmed to be MPM; seven patients died of their disease and one showed advanced disease on imaging.

Prognostic significance of *p16/CDKN2A* deletion

The mean age of patients at the time of surgery was 58 years and 17% were female. Asbestos exposure and a smoking history were documented in 50% and 46% of patients, respectively. Correlation of patient characteristics and *p16/CDKN2A* FISH status with clinical outcome showed that age ≥60 (35% vs 20% survival at 2 years; p=0.026), asbestos exposure (39% vs 17% survival at 2 years; p=0.039) and *p16/CDKN2A* deletion by FISH (50% vs 17% survival at 2 years; p=0.039) were significantly associated with reduced survival on univariate analysis (figure 2). There was no association between *p16/CDKN2A* deletion status and any of the clinical parameters.

DISCUSSION

We demonstrate that FISH for *p16/CDKN2A* deletion can be a clinically useful marker to confirm a diagnosis of malignancy, particularly in biopsies with limited material that lack definitive evidence of invasion. Moreover, we determined cut-off values for homozygous and hemizygous *p16/CDKN2A* deletion by FISH on routine FFPE tissue sections and tested our assay using equivocal cases. To confirm the accuracy of the assay, a third MYC probe was used to verify samples showing a homozygous deletion pattern, thereby avoiding potential false positive results. Using the established criteria, we show that hemizygous deletion alone may occur in mesothelioma and is, in and of itself, sufficient for the diagnosis of malignancy.

The establishment of cut-off values for detecting deletions in FFPE material requires special consideration because sectioning creates truncation artifacts; therefore cut-offs need to be higher than in samples containing intact nuclei,[23] and criteria for deletion must account for this artifactual loss of signals. We showed in our study that in reactive mesothelial cells, monosomy was identified in up to 34% of nuclei. Our overall rate of *p16/CDKN2A* deletion in MPM was 61%; 43% homozygous and 18% hemizygous. Half of the homozygous deletion samples included a concurrent hemizygous pattern suggesting genetic tumour progression. Importantly, all MPM cases with low per cent nuclei showing homozygous deletion pattern also showed hemizygous deletion as defined by our cut-offs. In cases with hemizygous-only deletion, the second allele may be inactivated by other mechanisms such as promoter methylation and/or point mutations. Alternatively, haploid deficiency by hemizygous

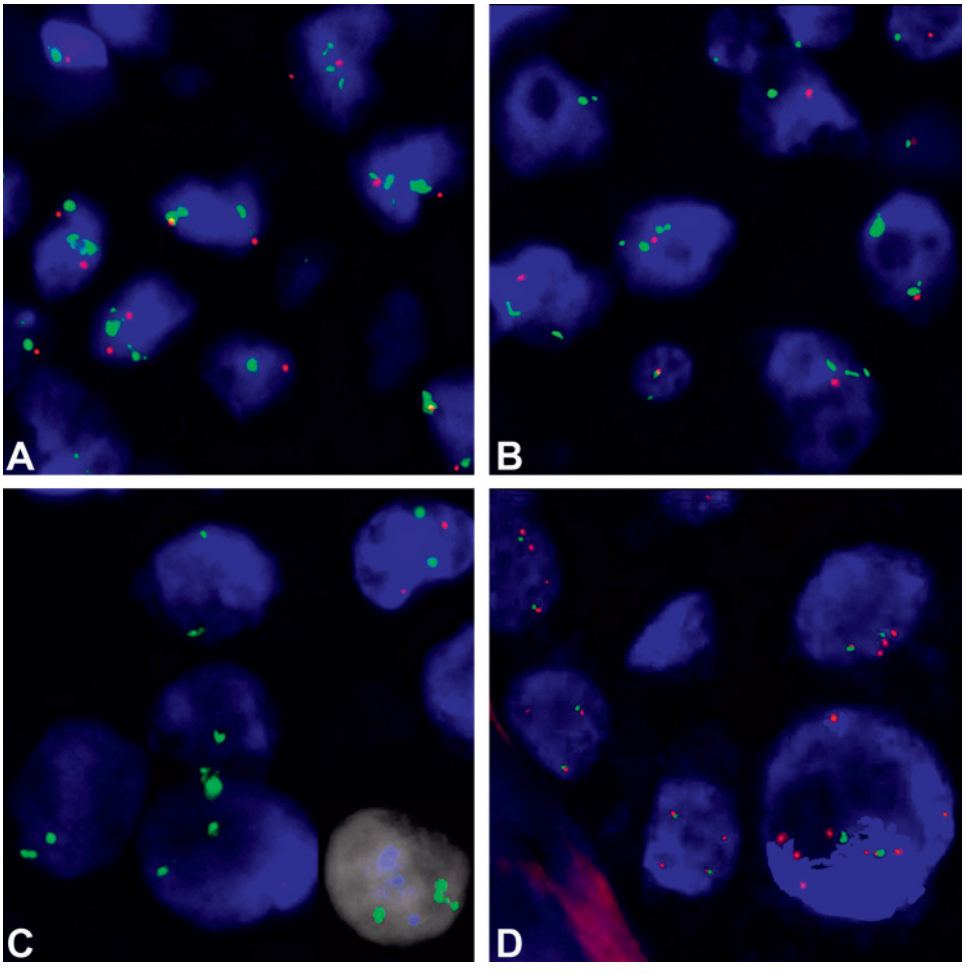

Figure 1 Fluorescence in situ hybridisation showing: (A) normal *p16/CDKN2A* signalling in a benign reactive mesothelial case; (B) hemizygous (loss of one red signal); and (C) homozygous (loss of both red signals) loss of *p16/CDKN2A* in two mesothelioma cases, respectively. Inset: presence of MYC (blue signals) probe in the absence of p16 signalling confirms the homozygous loss of p16. (D) Amplification of *p16/CDKN2A* in a mesothelioma case.

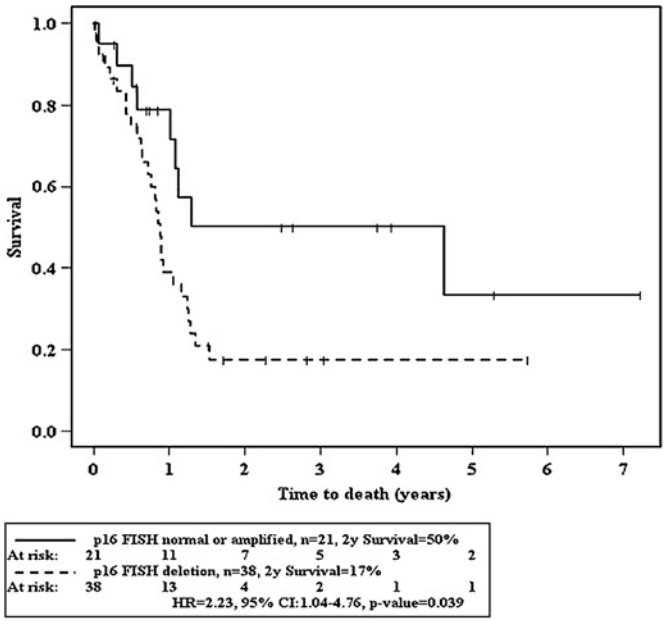

Figure 2 The Kaplan–Meier curve for survival in malignant mesotheliomas with *p16/CDKN2A* deletion versus no *p16/CDKN2A* deletion by fluorescence in situ hybridisation.

deletion in combination with other oncogenic events could be sufficient for malignant transformation.

Although 9p21 locus deletion by FISH on cytological preparations and tissue sections has been suggested as a clinical assay for diagnosing MPM,[17 24 25] none of the previous reports have provided details on the establishment of cut-offs to score the FISH results on FFPE sections. Using FISH on 32 cytological specimens (whole cell nuclei), Illei *et al* identified homozygous *p16/CDKN2A* deletion in 85.7% (6/7) of cases with malignant cytology and 100% (6/6) with suspicious cytology; no deletion was detected in 19 cytologically negative specimens.[25] They subsequently reported FISH on freshly imprinted tumour cells of 95 MPM and showed homozygous *p16/CDKN2A* deletion in 74% of cases and hemizygous loss in five cases (5%).[19] Their cut-off for homozygous deletion was defined as >20% of nuclei with loss of both *p16/CDKN2A* signals with at least one CEP-9 signal. More recently, a study that combined FFPE whole sections and tissue microarray (TMA) cores identified homozygous *p16/CDKN2A* deletion in 67% (35/52) of MPM and 25% (5/20) of peritoneal mesotheliomas.[17] A follow-up multi-institutional TMA study on epithelioid MPM detected homozygous deletion in 60% (21/35) and hemizygous deletion in 8.6% (3/35) of cases, but the criteria for hemizygosity was not clearly defined. In these latter two studies, homozygous *p16/CDKN2A* deletion was defined by the same cut-off used by Illei *et al*, but without the rationale to justify the use of this cut-off in tissue sections. Cut-off values for monosomy and trisomy depend on the type of probe and target nuclei; the lack of proper controls can result in misdiagnosis of chromosomal abnormalities.[22] Inadequate control samples may influence cut-off values and consequently the sensitivity of the FISH assay.[22]

P16/CDKN2A deletion was associated with a worse outcome, with a 50% two-year survival for lack of *p16/CDKN2A* deletion versus 17% survival for patients with the deletion. This is consistent with previous studies identifying loss of *p16/CDKN2A* as a poor prognostic indicator.[24 26 27] Outcome analyses showed that age over 60 years and previous asbestos exposure were associated with a significantly worse survival.

In conclusion, FISH for *p16/CDKN2A* deletion on FFPE sections is a clinically relevant confirmatory test for diagnosing MPM. In equivocal cases, the identification of deletion using the established cut-offs (table 2) may prevent delay in diagnosis and allow earlier management for this fatal disease. The test also provides important prognostic information and can be implemented in clinical laboratories with routine FISH service.

Take-home messages

- Establishing cut-off values for *p16/CDKN2A* deletion using appropriate controls is essential to fluorescence in situ hybridisation (FISH) assay development on paraffin sections.
- Detection of hemizygous *p16/CDKN2A* deletion alone is supportive of the diagnosis of malignant pleural mesothelioma.
- Presence of *p16/CDKN2A* deletion by FISH is associated with a worse prognosis in malignant pleural mesothelioma (MPM).
- The following *p16/CDKN2A* deletion patterns were defined by our FISH assay:
  - Homozygous deletion: no *p16/CDKN2A* (0Sp0) and at least one CEP-9 signal (1-2SpG) in >10% of scored nuclei.
  - Hemizygous deletion: one *p16/CDKN2A* signal (1Sp0) and two CEP-9 (2SpG) in >15% of nuclei.
  - Hemizygous deletion: one *p16/CDKN2A* signal (1Sp0) and one CEP-9 (1SpG) in >44% of nuclei.

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Competing interests None.

Ethics approval This study was conducted with the approval of the University Health Network.

Provenance and peer review Not commissioned; externally peer reviewed.

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