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 p55,4 5 desmin,6 P-glycoprotein,7 platelet-derived growth factor receptor β-chain,8 β-catenin9 and GLUT-112 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...
p16/CDKN2A signals with at least one CEP-9 signal or 1SpO/1SpG (table 1). Therefore, a cut-off for loss of both p16/CDKN2A signals with at least one CEP-9 signal or 1SpO/1SpG (table 1).

**fluence in situ hybridisation**

Dual-colour FISH was performed using a commercially available Spectrum Orange-labeled locus specific p16(9p21) probe and Spectrum Green-labeled chromosome 9 centromere probe (Vysis LSI p16 (9p21) SpectrumOrange/CEP9 SpectrumGreen Probe; Abbott Laboratories, Des Plaines, Illinois, USA). Slides (4 μm sections) were deparaffinized in xylene, followed by rehydration and pretreatment in 10 mM sodium citrate (pH 6.0) at 90°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 associations with survival using the Cox proportional hazards model.

**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. 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).

**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 50%. These three cases also showed hemizygous deletion as defined by the established cut-offs. To mitigate uncertainty from artificial 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, homoygous p16/CDKN2A deletion was detected in 25/54 (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 25 homoygous 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 52% 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 homoygous 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...
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 $\geq 60$ (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, 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 percent 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

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

<table>
<thead>
<tr>
<th>Type</th>
<th>Homozygous deletion</th>
<th>Hemizygous deletion†</th>
<th>Amplification†</th>
<th>Normal diploid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelioid</td>
<td>19</td>
<td>5</td>
<td>2</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>Sarcomatoid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Biphasic</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>23 (43%)</td>
<td>10 (18%)</td>
<td>2 (4%)</td>
<td>19 (35%)</td>
<td>54</td>
</tr>
</tbody>
</table>

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

**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.
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% (55/82) of MPM and 25% (5/20) of peritoneal mesotheliomas. A follow-up multi-institutional TMA study on epithelioid MPM detected homozygous deletion in 60% (21/35) and hemizygous deletion in 6.6% (3/53) 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. Inadequate control samples may influence cut-off values and consequently the sensitivity of the FISH assay. 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. 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.

### REFERENCES


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