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

Catherine T-S Chung,1,2 Gilda Da Cunha Santos,2,3 David M Hwang,2,3 Olga Ludkovski,4 Melania Pintilie,5 Jeremy A Squire,2,3,4,6 Ming-Sound Tsao2,3,4,6

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 desmin,5 P-glycoprotein,6 platelet-derived growth factor receptor β-chain,7 β-catenin8 9 10 and GLUT-11 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 p921 locus, the site of the cyclin-dependent kinase inhibitor 2A/p16 gene (p16/CDKN2A), frequently occurs in mesothelioma.13 Homozygous deletion has been reported in 22–74% of mesotheliomas.14–21 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 entrainment. 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 centromere 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 rehybridisation 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 homozygous deleted cases with a third in-house MYC probe. Eleven of the 23 homozygous cases showed concurrent hemizygous (1SpO/2SpG) deletion. The pauci-cellular and cytological pattern of 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 entrainment. 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).

The percent survival at every point in time was estimated using the Kaplan–Meier method. Associations between status and the clinical parameters were performed using the Wilcoxon rank-sum test.

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).

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 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, homozgyous p16/CDKN2A deletion was detected in 23/54 (43%) of MPM cases (table 3, figure 1). Homozgyous loss was confirmed by repeating the FISH with the addition of the MYC probe. Eleven of the 23 homozgyous cases showed concurrent hemizygous (1SpO/2SpG) deletion.

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Hemizygous-only deletion was seen in 10/54 (18%) cases, three (5%) of which had the 1SpO/15SpG deletion pattern and seven (15%) 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 homozgyous 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 1
Copy number for p16/CDKN2A gene in benign mesothelial cells

<table>
<thead>
<tr>
<th>FISH signalling in benign mesothelial cells</th>
<th>Mean (%) ± SD</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2SpO/2SpG</td>
<td>63 ± 8</td>
<td>44–75</td>
</tr>
<tr>
<td>0SpO/2SpG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0SpO/1SpG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1SpO/2SpG</td>
<td>6 ± 3</td>
<td>1–10</td>
</tr>
<tr>
<td>1SpO/1SpG</td>
<td>20 ± 8</td>
<td>6–34</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>p16/CDKN2A deletion pattern</th>
<th>FISH signal pattern</th>
<th>CEP-9 (SpG)</th>
<th>Cut-off value for % nuclei with FISH signal pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozgyous</td>
<td>No p16/CDKN2A signal</td>
<td>At least one CEP-9 signal</td>
<td>&gt;10%</td>
</tr>
<tr>
<td>0Sp0</td>
<td>1-2SpG</td>
<td>Two CEP-9 signals</td>
<td>&gt;15%</td>
</tr>
<tr>
<td>1SpO</td>
<td>2SpG</td>
<td>One CEP-9 signal</td>
<td>&gt;44%</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th></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 ≥2.
‡ >15% of tumour cells show 1SpO/2SpG or >44% show 1SpO/1SpG.

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**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 artificial loss of signals. We showed in our study that in reactive mesothelial cells, monosomy was identified in up to 54% 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
74% of cases and hemizygous loss in 95 MPM and showed homozygous subsequently reported FISH on freshly imprinted tumour cells of was detected in 19 cytologically negative specimens.25 They deletion in 85.7% (6/7) of cases with malignant p16/CDKN2A fi cut-off for homozygous deletion was de FISH results on FFPE sections. Using FISH on 32 cytological provided details on the establishment of cut-offs to score the deletion in combination with other oncogenic events could be sufficient for malignant transformation. Although 9p21 locus deletion by FISH on cytological prepa- rations 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

<table>
<thead>
<tr>
<th>Time to death (years)</th>
<th>Survival</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>1</td>
<td>0.86</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
</tr>
<tr>
<td>3</td>
<td>0.59</td>
</tr>
<tr>
<td>4</td>
<td>0.47</td>
</tr>
<tr>
<td>5</td>
<td>0.37</td>
</tr>
<tr>
<td>6</td>
<td>0.29</td>
</tr>
<tr>
<td>7</td>
<td>0.22</td>
</tr>
</tbody>
</table>

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.

- 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 (0SpO) and at least one CEP-9 signal (1-2SpG) in >10% of scored nuclei.
  - Hemizygous deletion: one p16/CDKN2A signal (1SpO) and two CEP-9 (2SpG) in >15% of nuclei.
  - Hemizygous deletion: one p16/CDKN2A signal (1SpO) and one CEP-9 (1SpG) in >44% of nuclei.

REFERENCES


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