Pathology (June 2011) **43(4)**, pp. 313–317

ANATOMICAL PATHOLOGY

Validation of a minimal panel of antibodies for the diagnosis of malignant pleural mesothelioma

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Summary

Aims: We previously established the use of a minimal panel of antibodies as sufficient to diagnose most epithelial malignant mesothelioma (MPM). We aimed to validate this approach and investigate the utility of a D2-40 antibody.

Methods: A series of 80 MPM patients selected for surgery and 21 consecutive patients with pleural metastatic carcinoma were included. A minimal panel of antibodies, consisting of calretinin, BG8 and CD15, and D2-40 was investigated.

Results: There were 61 epithelial and 19 biphasic MPM as well as 12 metastatic lung, six breast (5 ductal adenocarcinomas, 1 mixed ductal/lobular adenocarcinoma), two serous papillary ovarian carcinomas and one moderately differentiated colorectal adenocarcinoma. The sensitivity of positive calretinin labelling to confirm the diagnosis of MPM was 97.5%, while the 'diagnostic sensitivities' of lack of labelling for BG8 and CD15 were 91.3% and 97.5%, respectively. The use of calretinin, BG8 and CD15 resulted in correct classification in 97.5% of all MPMs. All MPM cases investigated showed at least focal positive D2-40 labelling.

Conclusions: We have validated the usefulness of a minimal panel of antibodies with calretinin, BG8 and CD15 as the initial step to the diagnosis of MPM. D2-40 emerged as a helpful diagnostic tool for cases where our initial approach failed to conclusively diagnose MPM.

Key words: Antibody, calretinin, D2-40, malignant pleural mesothelioma.

Received 9 December 2010, revised 18 January, accepted 27 January 2011

INTRODUCTION

The incidence of malignant mesothelioma is expected to rise due to the long latency period after exposure to asbestos and the continued use of asbestos in developing countries.[1] An accurate histological diagnosis not only conveys important prognostic information, but often has medico-legal implications in regard to compensation for the asbestos-exposed victims.

The accurate diagnosis of malignant mesothelioma by biopsy can be problematic and the use of ancillary techniques is mandatory for definitive diagnosis. The most commonly used ancillary technique is immunohistochemistry (IHC).[2] A panel of positive and negative markers for mesothelioma is often employed in line with recommendations from the International Mesothelioma Panel of using at least two mesothelial cell makers and two carcinoma-related markers to diagnose epithelial mesothelioma.[3]

There are several markers advocated to be used for the confirmation of the diagnosis of malignant mesothelioma; including calretinin, cytokeratin 5/6 (CK5/6), Wilms tumour protein 1 (WT-1), thrombomodulin, and HBME-1. More recently D2-40 (podoplanin) has been investigated as a potential mesothelial marker.[4–7] Carcinoma markers that are frequently used to reject the diagnosis malignant mesothelioma include carcinoembryonic antigen (CEA), CD15, B72.3, blood group antigen Lewis (BG8), thyroid transcription factor-1 (TTF-1), and antibodies directed against epithelial cell adhesion molecules such as Ber-EP4. Despite a meta-analysis,[8] little consensus exists about which combination of markers provides the best approach in the diagnosis of malignant mesothelioma.

Using a tree-based regression approach, we have previously shown that a minimal panel of antibodies, consisting of calretinin, BG8 and CD15, is in most cases sufficient to make the diagnosis of epithelial malignant pleural mesothelioma (MPM).[9] The present study aimed to validate this approach, using an independent series of patients with pleural malignancy that has been pathologically confirmed to be either MPM, or metastatic carcinoma. Moreover, we investigated the usefulness of adding the mesothelial marker D2-40 to the previously established minimal panel of antibodies.

MATERIALS AND METHODS

Malignant pleural mesothelioma patient cohort

Eighty-five patients were considered eligible for extrapleural pneumonectomy (EPP) by a team led by one thoracic surgeon (BCM) between October 1994 and October 2009. Pre-operative assessment and operative techniques have been reported previously.[10] The pathological diagnosis of MPM was established pre-operatively by either CT guided needle biopsy, thoracoscopic biopsy or cytological examination from pleural fluid, and confirmed by histological examination of the EPP specimen. The diagnosis of MPM was made according to generally accepted criteria,[2,11] including characteristic radiological appearance (including PET scan in some instances), microscopic features (including presence of invasion into subpleural tissues, mucin histochemistry and typical

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: 10.1097/PAT.0b013e32834642da

Table 1 Antibodies used in this study

	Source	Pattern of labelling	Antigen retrieval	Dilution	Detection system
Markers positive for malignant mesothelioma					
Calretinin	Invitrogen	Accept nuclear labelling only	Citric acid pH6	1:2000	Novocastra Polymer System (Leica)
D2-40	Signet	Accept membrane labelling only	Citric acid pH6	1:100	EnVision+ Dual Link System (Dako)
Markers positive in carcinoma					
BG8	Signet	Membrane labelling	No retrieval	1:800	Novocastra Polymer System
CD15	Dako	Membrane labelling	No retrieval	1:50	EnVision+ Dual Link System
				1:1000	Novocastra Polymer System

immunohistochemistry; if only atypical papillary surface mesothelial proliferation is seen in original sections, this should prompt the cutting of further levels in a search for evidence of invasion) and in those instances in which diagnosis was equivocal, electron microscopy demonstrating long and slender microvilli.

Surgery took place at Royal Prince Alfred Hospital (RPAH) or Strathfield Private Hospital (SPH) and tissues were processed and stored at their respective pathology laboratories. RPAH adopted a conventional fixation protocol using 10% buffered formalin fixation overnight at room temperature (21°C) while SPH used an accelerated protocol where specimens were fixed with 10% buffered formalin for 90 min in heated condition (50°C). Archival formalin fixed, paraffin embedded blocks were able to be retrieved for eighty cases.

This work was conducted as part of a larger study aimed at identifying prognostic factors in MPM and was approved by the Human Research Ethics Committee at Concord Repatriation General Hospital, Sydney, where the Asbestos Diseases Research Institute is based.

Control samples

Samples from a series of 21 patients with confirmed metastatic pleural carcinomas diagnosed at the Department of Anatomical Pathology, Flinders Medical Centre, Adelaide, served as a control series. All tissues were fixed in 10% buffered formalin and underwent standard processing at room temperature prior to being embedded in paraffin wax.

Immunohistochemical analysis

Sections were cut 4 μm thick, deparaffinised and rehydrated prior to quenching with 10% H_2O_2. The panel of antibodies investigated included calretinin, BG8 and CD15, and D2-40. IHC methodology details are summarised in Table 1.

Histological subtype was assessed independently by three investigators (KL, SK and DWH) and IHC labelling was scored on an ordinal scale: positive labelling = 1; equivocal labelling = 0.5; and no labelling = 0. Equivocal labelling was assigned if it was uncertain whether the labelling was genuine or just high background staining, or if there was <2% positive labelling in tumour cells.

In clinical practice, clear positive labelling is required for definitive diagnosis. Therefore equivocal labelling was considered negative in our analysis. This decision was supported by our previous tree-based regression analysis where the outcome did not differ whether equivocal labelling was decided by the algorithm or when equivocal labelling was considered negative.[9]

Statistical analysis

The efficacy of the investigated panel of markers was expressed as sensitivity rates, defined as true positives divided by the sum of true positives and false negatives, and specificity rates defined as true negatives divided by the sum of true negatives and false positives.

In order to validate our previously published model, we obtained the predicted values for the 101 samples in the present study by dropping the data down the original classification tree. Actual classifications were then compared to the tree-based predictions. Analysis was performed in R (http://www.statmethods.net/index.html), using the rpart library.

RESULTS

Malignant pleural mesothelioma patient cohort

A total of 80 MPM patients were included in this study, of which, 61 were of epithelial subtype (76%), and 19 biphasic subtype (24%). In 74 cases, initial diagnostic pathology reports with details of the diagnostic antibodies used were available (see Table 2). The median number of antibodies used at the time of the initial diagnostic biopsy was five (range 0–15). Eighteen patients had less than four antibodies performed (24%), including three patients with no IHC performed at initial diagnosis. The most commonly used mesothelial marker was calretinin (80%), while the most commonly used carcinoma marker was CEA (92%).

Control cases of pleural carcinoma

The controls consisted of 21 patients with confirmed metastatic pleural carcinoma. These included 12 patients with lung cancer, six patients with breast cancer, two patients with ovarian cancer and one patient with colorectal cancer.

Table 2 Antibodies used for initial diagnosis ($n = 74$)

Diagnostic antibody	Numbers used	Percentage used
Mesothelial markers		
Calretinin	59	80
CK5/6	28	38
HBME-1	22	30
Thrombomodulin	8	11
WT-1	6	8
Carcinoma markers		
TTF-1	19	26
CEA	63	92
CD15 (Leu-M1)	19	26
BG8	3	4
B72.3	2	3
MOC31	3	4
Ber-EP4	25	34
E-cadherin	1	1
Tag72	2	3
Markers differentiating between reactive change vs mesothelioma		
p53	2	3
Desmin	3	4
EMA	21	28
Intermediate filament proteins (to highlight invasive nature of the tumour and diagnose sarcomatoid mesothelioma)		
CK7	22	30
CK20	20	27
CK8/18	3	4
Cam5.2	30	41
AEI/III	24	32
Vimentin	1	1
CK 34βE12	1	1
CK MNF116	1	1
Other exclusionary markers		
S100	10	14
Melan A	2	3
HMB45	2	3
PSA	2	3
CD3	1	1
CD20	1	1
Oestrogen receptor	1	1
Progesterone receptor	1	1

Table 3 Sensitivity and specificity of immunohistochemical markers for malignant pleural mesothelioma

Immunohistochemistry markers (total number of tests)	Sensitivity (%)	Specificity (%)
Calretinin+ ($n = 101$)	Overall 97.5	100
Epithelial	98.4	
Biphasic	94.7	
D2-40+ ($n = 101$)	Overall 100	100
Epithelial	100	
Biphasic	100	
BG8− ($n = 100$)	Overall 91.25	65
Epithelial	93.4	
Biphasic	84.2	
CD15− ($n = 100$)	Overall 97.5	35
Epithelial	96.7	
Biphasic	100	

+, positive labelling; −, lack of labelling.

Diagnostic immunohistochemistry

The sensitivity and specificity of the individual single antibodies for the whole cohort and the epithelial/biphasic subtypes are summarised in Table 3, revealing 100% sensitivity and 100% specificity for the mesothelial-related marker, D2-40. The sensitivity of calretinin was 97.5%, while the specificity was 100%.

Table 4 summarises how D2-40 and calretinin labelled for the two components of the biphasic tumours. All 19 biphasic MPMs exhibited 100% positive labelling for calretinin within the epithelial component, while only 21% exhibited positive labelling within the sarcomatoid component. For the assessment of D2-40 in the biphasic MPM, 89% showed positive labelling within the epithelial component, compared to 63% positive labelling within the sarcomatoid component.

The sensitivity of adenocarcinoma marker BG8 for the whole group of MPMs studied was 91.3% (indicating that 8.7% of the MPMs had positive labelling for BG8), while this figure was 97.5% for CD15. The 'overall' specificity of lack of BG8 staining was 65%, while the specificity of negative staining of CD15 was 35%.

Model validation

Using the approach of a minimal panel of antibodies,[9] positive calretinin labelling and negative BG8 labelling was found in 90% of our 80 MPM patients. The addition of negative CD15 labelling in the panel increased the sensitivity to 97.5%. In the two cases (2.5% of the cohort) that showed lack of labelling for calretinin (1 epithelial, and 1 biphasic subtype), both showed convincingly positive labelling for D2-40.

Figure 1 details the classification tree model for the use of a minimal panel of antibodies in the cohort of 101 patients with pleural-based lesions. The classification tree approach calculates for each sample, the probability that it belongs to either the MPM or adenocarcinoma groups and based on this probability, assigns the predicted class. Table 5 shows the predicted classification versus the actual diagnosis. Seventy eight of the 80 MPM were correctly classified as being MPM; both misclassified tumours were negative for calretinin. All adenocarcinomas were correctly classified as such by the minimal panel of antibodies used.

Table 4 Labelling of two components of biphasic malignant mesothelioma ($n = 19$)

	No. (%) of biphasic MPM with positive labelling
Calretinin	
Within the epithelial component	19 (100%)
Within the sarcomatoid component	4 (21%)
D2-40	
Within the epithelial component	17 (89%)
Within the sarcomatoid component	12 (63%)

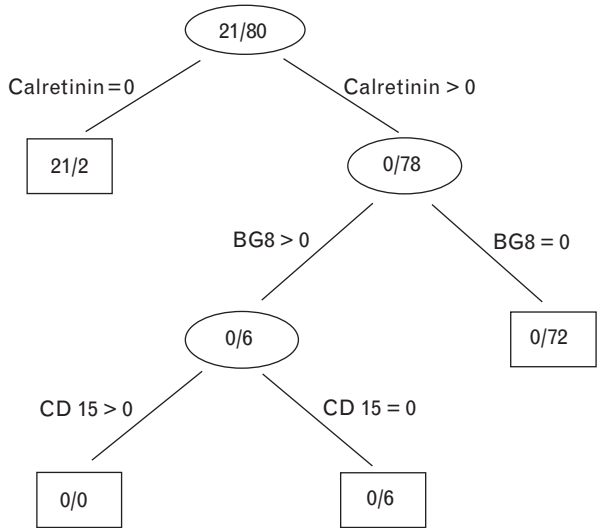

Fig. 1 Classification tree model for the cohort of 101 patients with pleural-based lesions. Each of the following nodes provides the allocation ratio of cases as either tumour (adenocarcinoma/mesothelioma). The inferior nodes (ellipses) reached by those initial distinction are further analysed by left and right splits. The terminal nodes (rectangles) give the final differentiation between the cases.

DISCUSSION

The accurate diagnosis of MPM can be problematic and consequently ancillary techniques such as IHC are required to confirm the diagnosis. Both positive labelling for mesothelial markers and lack of labelling for carcinoma markers are necessary for a definitive diagnosis as recommended by the International Mesothelioma Panel. As such, various panels of antibodies are used by different laboratories for diagnostic purposes. This is reflected in our MPM cohort where a wide range of diagnostic antibodies was employed in community practice setting. The median number of diagnostic antibodies used in the initial workup was five and mostly consisted of at least two mesothelial markers and two carcinoma markers. Eighty-two percent employed calretinin as one of the mesothelial-related markers, while none had D2-40, probably reflecting uncertainty regarding the usefulness of this antibody. However, based on our findings, it appears that D2-40 has

Table 5 Predicted classification (with calretinin/BG8/CD15) versus the actual tumour type for the 101 pleural malignancies

	Actual diagnosis	
Predicted classification	Malignant mesothelioma	Adenocarcinoma
Malignant mesothelioma	78	0
Adenocarcinoma	2	21

higher sensitivity and specificity than the established mesothelial markers, although the number of cases tested is still relatively small. It is possible that D2-40 will emerge as superior marker over calretinin, but currently we would recommend calretinin and D2-40 in parallel.[9] Interestingly, a significant proportion of our patients had less than four diagnostic antibodies used in the initial diagnostic biopsies and in three cases initially no IHC assessment had been performed. This variability in approach to diagnosis may reflect the different clinical settings, and expectations from clinicians, who may be satisfied with a diagnosis of 'metastatic adenocarcinoma' in a case of a known primary, or who may require additional information regarding the likely primary site, for example when there is no known past history or multiple previous malignancies.

Even though several groups of investigators have addressed the sensitivity and specificity of individual antibodies, few have studied what combination of antibodies would lead to the most accurate approach to the diagnosis of MPM.[9,12–14] We previously found that a minimal panel of antibodies consisting of calretinin, BG8 and CD15 was sufficient in most cases to reliably diagnose epithelial mesothelioma.[9] In the current study, using an independent cohort of patients with an established diagnosis of MPM or metastatic pleural carcinoma, we have validated this approach as the primary line of investigation to reliably confirm most MPM cases. Of the 80 MPMs, 72 were correctly classified based on the positive calretinin labelling and negative BG8 labelling. A further six MPMs were correctly classified with the additional use of CD15 antibody. In a period of ever increasing pressure in time and budgetary constraints, the approach of using a minimal panel of antibodies is attractive.

D2-40 is a monoclonal antibody directed against an M2 protein originated from foetal germ cells and germ cell tumours. It has been investigated for the diagnosis of MPM,[4–7] with up to 100% of epithelial MPM cases exhibiting membranous labelling (ranging from 66% to 100%) and up to 75% of sarcomatoid MPM cases (ranging from 0% to 75%). Therefore, the use of D2-40 remains somewhat controversial, particularly with the large discrepant results in the literature regarding its sensitivity in sarcomatoid mesothelioma.

The specificity of D2-40 for MPM has been reported to be less than 100%, as adenocarcinomas can also show membranous labelling, particularly in serous ovarian carcinoma where 65% of cases labelled positively.[15] However, apart from a tissue microarray study from Hinterberger *et al.*, where 341 MPM patient samples were investigated,[5] all the published studies suffered from the inclusion of limited numbers of MPM patients.

In our study, the use of D2-40 antibody appears promising as all MPM cases, regardless of the histological subtypes, demonstrate positive membranous labelling (sensitivity of 100%), albeit only focal in some of the sarcomatoid areas. Importantly, none of the carcinomas (including 2 ovarian carcinomas) labelled positively for D2-40, resulting in 100% specificity in our hands. The high specificity in this study may relate to the different cohort of carcinomas. We have included only secondary adenocarcinomas affecting the pleura (using the tissue blocks containing the tumour in the pleura), unlike published studies that have used tissue from the primary site.[4,6,7] Our approach is more relevant to the clinical perspective. The high specificity observed may be associated with the relatively small number of metastatic pleural carcinomas included, which is a limitation of this study. Also, once a tumour is identified as carcinoma metastatic to pleura, further studies to elucidate the primary site of the tumour and prognostic factors such as hormone receptor status in breast carcinomas will be necessary. Therefore, the proposed panel is suitable and intended for initial classification of tumours as MPM or carcinoma. Depending on the clinical situation, however, additional antibodies, for example for determination of likely primary site if there is no known primary tumour or multiple possible primary tumours, may be necessary.

Although our series did not contain any MPM with a sarcomatoid subtype, there were 19 biphasic MPMs which allowed us to examine the epithelial and sarcomatoid components of the tumour separately. In our series of 19 biphasic tumours, D2-40 appears to be a useful marker for sarcomatoid/spindled component of the tumour, with 63% of the biphasic tumours demonstrating positive membranous labelling in the sarcomatoid component, compared to 21% of biphasic tumours with positive calretinin labelling in the sarcomatoid component. This mirrors the findings of Hinterberger *et al.*,[5] and confirms the usefulness of D2-40 in the diagnosis of sarcomatoid tumours.

The two MPM cases (one epithelial and one biphasic) that failed to label for calretinin showed convincingly positive membranous labelling for D2-40. To the best of our knowledge, this is one of the largest studies examining the immunolabelling of D2-40 in whole sections rather than tissue microarrays in a cohort of MPM patients. D2-40, used in conjunction with negative labelling for carcinomas, appears to have good discriminating power for MPM and metastatic pleural carcinomas.

In conclusion, we have validated the use of a minimal panel of antibodies including calretinin, BG-8 and CD15, as the first step in the diagnostic workup of patients suspected of having malignant mesothelioma. In cases in which uncertainty remained despite this approach, D2-40 appeared to be a sensitive and specific marker. D2-40 should be prospectively evaluated in a larger cohort of patients with malignant pleural disease to validate its potential future role. Such a minimal panel will be useful in making a primary distinction between malignant mesothelioma and metastasis, but depending on the clinical situation additional markers may well be required.

Acknowledgements: We sincerely thank the staff at Vision Pathology, Sydney, for recutting some of the sections for H&E stain. We thank the Department of Tissue Pathology and Diagnostic Oncology at Royal Prince Alfred Hospital and Rosemary Crain from Davies Campbell de Lambert Pathology for the assistance of archival block retrieval. We also thank Lisa Jonavicius and Matthew Hussey for their tireless assistance in the Department of Anatomical Pathology, Flinders Medical Centre.

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