Pathology (December 2016) **48**(7), pp. 660–665

ANATOMICAL PATHOLOGY

Calretinin but not caveolin-1 correlates with tumour histology and survival in malignant mesothelioma

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Summary

Malignant mesothelioma (MM) continues to be a disease with poor prognosis and limited treatment options. Calretinin and caveolin-1 expression by tumour in MM have recently been described to be associated with tumour histology, differentiation and consequently survival. In a large, well annotated cohort, we studied both of these biomarkers and explored their association with clinicopathological parameters and survival.

A retrospective search of patients with MM who underwent surgery at the Austin Hospital in Melbourne, Australia, was conducted. Clinical history and outcome data were retrieved from patient records. Tissue microarrays (TMAs) were constructed and stained for calretinin and caveolin-1. 'H scores' were derived, taking intensity and distribution of staining, and the cohort was dichotomised using median values for both markers.

In the 329 patients evaluated, median age was 67 years. Males outnumbered females by 5:1. Epithelioid histology 202/319 (62.9%) was the most common, followed by biphasic 72/319 (21.8%) and sarcomatoid 45/319 (13.6%); histology could not be confirmed in 10 patients. Calretinin expression was detected in 246 of the 324 (76%) evaluable patients and high expression was associated with epithelioid histology ($p < 0.0001$). Caveolin-1 was expressed in 298 (94%) of 317 evaluable patients which was much higher compared to its expression in a cohort of lung adenocarcinomas (8/58, 13.7%). However, no association with histology was found ($p = 0.409$). When taken as a continuous variable, calretinin expression was found to be an independent predictor of survival, alongside histology, neutrophil-lymphocyte ratio, weight loss and stage. No prognostic value was demonstrable for caveolin-1 expression and calretinin/caveolin-1 ratio. There was no relationship between calretinin and caveolin-1 expression. In MM, increased calretinin expression is associated with epithelioid histology and better survival. Caveolin-1 is a sensitive MM marker and is expressed in a high proportion of cases but lacks association with histology and survival.

Key words: Calretinin; caveolin-1; mesothelioma; immunohistochemistry.

Received 23 June, revised 14 August, accepted 18 August 2016
Available online 22 October 2016

INTRODUCTION

Despite the ban of asbestos use in most industrialised countries, the incidence of malignant mesothelioma (MM) is set to rise in the coming decades.[1,2] The World Health Organization (WHO) mortality database between 1994 and 2008 reported an age-adjusted mortality rate for MM of 4.9 per million.[3] Despite the development of aggressive multimodality treatment, the mainstay of current paradigms remains pemetrexed and cisplatin, based on the results of the EMPHACIS (Evaluation of Mesothelioma in a Phase III Trial of Pemetrexed with Cisplatin) study.[4,5] However, the MAPS study (Mesothelioma Avastin Cisplatin Pemetrexed study) has recently suggested that the addition of bevacizumab further improves overall survival.[6,7]

The use of a panel of immunohistochemistry (IHC) markers is now routine in MM diagnosis. However, the differentiation of MM from other malignancies metastasising to the pleura remains problematic despite the use of several well known mesothelioma markers. Calretinin, which is a calcium binding protein, has been established as a useful marker in distinguishing MM from adenocarcinomas with pleural metastases.[8] However, its expression is also observed in 15% of adenocarcinomas of breast origin[9] and 4–18% of pulmonary adenocarcinomas.[10–12] Caveolin-1, one of a three-member caveolin protein family, is a membrane-associated protein possibly involved in integrin signalling and in cell migration. It is expressed in endothelial and mesothelial cells as well as in alveolar type I pneumocytes.[12] Caveolin-1 has been purported to play a dual and opposite role in tumorigenesis, as it has been implicated in both tumour suppression and oncogenesis. In human cancers, caveolin-1 is a marker of extracellular matrix remodelling[13] and epithelial to mesenchymal transition.[14] It has been associated with aggressive biology and resistance to apoptotic signals in several

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: http://dx.doi.org/10.1016/j.pathol.2016.08.003

malignancies.[15–17] Caveolin-1 has also been described as a marker of dedifferentiation in soft tissue and bone sarcomas.[18,19] It has also been projected as a novel marker for MM. In a study comparing its expression in 80 epithelioid MMs and 80 lung adenocarcinomas, a 100% sensitivity and 93% specificity for caveolin-1 in epithelioid MMs was reported.[12]

Besides their importance in MM diagnosis, both of these IHC markers have also been studied for their association with tumour differentiation, histology and prognosis. Takeshima *et al.* found that higher calretinin scores were seen in more well differentiated tumours, which in turn have more favourable prognosis.[20] The prognostic role of calretinin expression has also been explored by Kao *et al.*, who demonstrated low expression of calretinin to be poorly prognostic in two different patient cohorts.[21–23] They also reported calretinin expression in 828/910 (91%) of all MMs; with 530/545 (97%) of epithelioid and 113/119 (94%) of biphasic but only 92/153 (60%) of sarcomatoid MMs.[23] In a series of 131 MM patients, Righi *et al.* found higher rates of caveolin-1 expression amongst non-epithelioid MM (40/40, 100%) as compared to epithelioid MM (70/91, 77%) and suggested that its expression increases with dedifferentiation from low grade epithelioid to high grade sarcomatoid histology.[24] The same authors also described a poorer outcome for epithelioid MMs in which caveolin-1 expression was detected in stromal cells.

Emerging literature suggests caveolin-1 is a sensitive IHC based diagnostic marker for MM, especially the sarcomatoid subtype, although its specificity is still questionable given its expression in other mesenchymal cells such as endothelial and smooth muscle cells. Using a large cohort of well annotated confirmed MM patients, we studied and compared the expression of these two IHC markers. We also sought to determine if their expression correlated with each other, and with clinico-pathological parameters and survival.

MATERIALS AND METHODS

A retrospective search of the thoracic surgery database of the Austin Hospital in Melbourne, Australia, was carried out for patients who underwent surgery (diagnostic/therapeutic/palliative) for MM between 1988 and 2014. Clinical information on patient demographics, co-morbidities, treatments, follow-up and outcome was retrieved from patient medical records. Archival formalin fixed, paraffin embedded (FFPE) blocks chosen after a review of the original histopathology reports were retrieved from the department of anatomical pathology. Four μm full face sections were stained with haematoxylin and eosin (H&E) and reviewed by a pathologist (KA). The diagnosis was confirmed by histopathological review and IHC. Older samples in which adequate IHC had not been performed at the time of original diagnosis (as judged by the pathologist) were subjected to additional IHC with mesothelial markers: calretinin (clone DAK-Calret1; Dako, Denmark), WT-1 (clone 6F-H2; Dako) and CK5/6 (clone D5/16 B4; Dako); epithelial markers: BER-EP4 (clone Ber-EP4; Dako), CEA (clone IL7; Dako) and MOC31 (clone MOC-31; Dako) before confirming the diagnosis.

Tissue microarrays (TMAs) and IHC analysis

TMAs were created using a tissue arrayer (Tissue Arrayer I; Beecher Instruments, USA) with 1 mm cores in triplicate from each patient's sample placed sequentially. The cores were taken from areas of tumour previously marked by the pathologist. Four μm sections of the TMAs were cut and collected onto charged slides, deparaffinised and rehydrated. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 min. Antigen retrieval for calretinin was performed in 24 min at 95°C in cell conditioning buffer. Monoclonal mouse anti human calretinin antibody (clone DAK-Calret1; Dako) in 1:50 dilution for 32 minwas incubated using the automated Ventana system (Ventana Medical Systems, USA). Optiview DAB detection kit (Ventana Medical Systems) was used for visualisation. For caveolin-1, antigen retrieval was performed by boiling in a microwave for 20 min in target retrieval solution buffer. Polyclonal rabbit anti human caveolin-1 antibody (cat no. 3238; Cell Signalling Technology, USA) was incubated in 1:250 dilutions. Staining was performed manually with slides incubated overnight at 4°C. DAB was used as the chromogen.

The slides were digitally scanned using Aperio Scan-Scope CS (Aperio Technologies, USA), and the scanned images were analysed using Image Scope (Aperio Technologies). Scoring for calretinin and caveolin-1 was performed taking into account both the percentage of neoplastic cells stained and also the intensity of the staining (marked as 1+/2+/3+). Samples staining ≥1% of the malignant cells were considered positive. An 'H score' was calculated for each core separately and an average was taken as the final score based on intensity and percentage of cells stained as follows: (% tumour cells staining 1+ × 1) + (% of tumour cells staining 2+ × 2) + (% tumour cells staining 3+ × 3). Cores with poor tumour content (<20%) were excluded from the analysis. Scoring was performed by BT and pathologists KA and SD independently. Inter-observer reliability was good (Kappa for calretinin = 0.77 and caveolin-1 = 0.74). Disagreements were settled by a combined review. Median H scores were used to stratify patients into high and low expressing groups.

Additionally, we also stained an available TMA of lung adenocarcinomas with 58 cases (sequential 1 mm cores in triplets) with caveolin-1 as described above. We then compared the expression of caveolin-1 in the lung adenocarcinomas with that in MM.

Statistical analyses

Overall survival (OS) was calculated from the time of initial diagnosis to death or last follow-up. Patients who were lost to follow-up or were alive were censored. Clinico-pathological data were described using summary statistics. Categorical variables were compared using the chi-square test or Fisher exact test (as appropriate) and continuous variables using one way ANOVA test. Correlation among continuous variables was estimated using Spearman's test. Survival curves were estimated by means of the Kaplan–Meier method and Cox proportional hazards regression was used to derive hazard ratios (HRs). Statistical analyses were performed using SPSS version software (v22; SPSS, USA).

Ethics approval

Ethics approval was provided by the human research ethics committee of the Austin Hospital, Melbourne, Australia (Local Reference Number: H2006/02394).

RESULTS

Of the 373 patients who underwent surgical procedures for MM between 1988 and 2014, adequate clinical and outcome data were available for 359. A further 30 patients were excluded for various reasons, including lack of adequate archival FFPE tissue blocks and inability to conclusively prove MM despite using a panel of IHC markers. The TMA was constructed from the FFPE tissues of 329 patients with confirmed MM.

In this cohort, males outnumbered females by more than 5:1. The age at presentation ranged from 26 to 89 years (median 67 years). Twenty-six percent of patients had no definite history suggestive of asbestos exposure. Chest pain (50%) and worsening dyspnoea (34%) were the most common presenting features. The demographic parameters and details of treatment received are summarised in Table 1.

Expression of calretinin and caveolin-1 and relationship with tumour histology

Calretinin expression was detected in 246 of the 324 (76%) evaluable patients with median H score of 98 (Table 1). Of