Original Article

Claudin-4 immunohistochemistry is a useful pan-carcinoma marker for serous effusion specimens

Melissa Vojtek1,2 | Michael D. Walsh1,2 | David J. Papadimos2 | Paul W. Shield1,2

1School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Brisbane, Qld, Australia
2Sullivan and Nicolaides Pathology, Bowen Hills, Qld, Australia

Correspondence
Paul W. Shield, School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Brisbane, Qld 4001, Australia.
Email: pw.shield@qut.edu.au

Funding information
The authors received no financial support for the research, authorship and publication of this article.

Abstract

Objective: To evaluate the utility of claudin-4 as a pan-carcinoma marker in cell-blocks of effusion specimens and compare results with Ber-Ep4 staining.

Methods: Effusion cell-blocks (n = 284) were stained for claudin-4 and results compared with Ber-Ep4. Cases included 172 metastatic malignancies (137 adenocarcinomas, 20 small cell lung tumours, eight metastatic melanoma, four squamous cell carcinoma, three urothelial cell carcinoma), 49 benign reactive cases and 63 mesotheliomas.

Results: All 49 benign effusions were negative. Only 1/63 (1.6%) mesotheliomas was positive for claudin-4. Claudin-4 staining was positive in 131/137 (95.6%) adenocarcinoma cases. Cases negative for claudin-4 included single cases of metastases from breast, colon, stomach, prostate, kidney and ovary. Claudin-4 outperformed Ber-Ep4. Sensitivity (95.6% vs 85.4%), specificity (99.1% vs 86.6%), negative predictive value (94.9% vs 82.9%) and positive predictive value (99.2% vs 88.6%) were all higher for claudin-4 compared with Ber-Ep4, respectively. Only two cases were claudin-4−/Ber-Ep4+. Significantly (P < .0064) more cases of metastatic adenocarcinoma stained positive for claudin-4 (131/137; 95.6%) than Ber-Ep4 (117/137; 86.2%). Claudin-4 staining was present in 15/20 (75%) of neuroendocrine carcinomas, 3/4 (75%) squamous cell carcinoma and 3/3 (100%) urothelial cell carcinoma. All eight cases of melanoma were negative for both claudin-4 and Ber-Ep4.

Conclusions: Claudin-4 staining is a useful addition to IHC panels for effusions specimens with superior performance to Ber-Ep4.

Keywords
claudin-4, cytology, effusion, immunohistochemistry, mesothelioma, metastatic carcinoma

1 | INTRODUCTION

Immunohistochemistry (IHC) has an established adjunctive role in the diagnosis of serous effusion specimens. It may provide valuable information to confirm diagnoses or to resolve difficult differential diagnoses. A common application is the cytological differential diagnosis of reactive or malignant mesothelial cells vs metastatic carcinoma, where significant morphological overlap may exist. IHC panels that include markers for mesothelial cells and antibodies specific for carcinoma are usually employed. Many antibodies have been investigated in the role of pan-carcinoma markers but none as yet have been described that provide ideal sensitivity or specificity.

Claudins are a family of tight junction proteins mostly recognized as regulators of paracellular ion transport and membrane polarity. They show variable expression in epithelial tissues.1 Preliminary studies have found that claudin-4 is not expressed in...
normal or malignant mesothelium but is frequently overexpressed in tumours of the lung, breast, prostate, kidney and ovary. Several studies have suggested that claudin-4 IHC may be useful in differentiating metastatic carcinoma from mesothelioma and reactive mesothelial cells in serous effusion specimens. However, the reactivity of claudin-4 with mesothelial cells remains unclear, with some studies reporting staining in up to 28% of reactive and malignant mesothelial cases.

This study aimed to evaluate the expression of claudin-4 in a series of benign and malignant effusions and determine its utility as a pan-carcinoma marker for cytological and histological diagnoses. Results were compared with those obtained using Ber-Ep4, a commonly used pan-carcinoma marker. We also examined the staining reaction of claudin-4 with a range of non-glandular tumours less commonly used pan-carcinoma marker. We also examined the staining reaction of claudin-4 with a range of non-glandular tumours less commonly encountered in serous effusions.

### 2 | MATERIALS AND METHODS

Ethics approval was obtained from the Queensland University of Technology (QUT) Human Research Ethics Committee. In total, 284 effusion cell-blocks (200 pleural fluid, 81 peritoneal fluid and three pericardial) were included. Diagnoses were determined by morphological examination and IHC performed on cell-blocks prepared using an agar method. Clinical notes and patient history were reviewed for each case.

A total of 164 metastatic carcinoma cases were selected so as to include a wide variety of primary sites and tumour types. Included were 137 adenocarcinomas: lung (34), digestive tract (23), pancreas (9), gallbladder (1), breast (30), female genital tract (28), kidney (6), and prostate (6). Other malignancies assessed included metastatic neuroendocrine (small cell lung) tumours (20), melanoma (8), squamous cell carcinoma (4) and urothelial cell carcinoma (3). All malignant cases had a known primary site and confirmatory IHC stains. Most cases also had a reported result for Ber-Ep4, the routine pan-carcinoma marker currently used in the laboratory. All 63 mesothelioma cases had confirmatory histology and ancillary studies performed. Forty-nine benign cases had no prior or subsequent known malignancy and supporting IHC (positive for two or more of calretinin, cytokeratin 5, WT1, D2-40; and negative for epithelial markers).

Tumour tissue for 39 cases with a cell-block was also stained with the same claudin-4 protocol to assess correlation of results from cytology and histology specimens. This included 13 mesotheliomas (11 pleural biopsies and two metastases); and 15 breast carcinomas (11 primary and four metastases); nine lung tumours (adenocarcinoma: two primary and four metastases; small cell carcinoma: one primary and two metastases) and two metastatic squamous cell carcinomas.

The staining protocol for claudin-4 IHC was optimised using tissue sections of expected claudin-4 expressers (lung adenocarcinoma, normal colon, kidney, and liver). Normal colon, liver and kidney were selected as the positive control with sections cut and affixed to each case slide. After being baked at 60°C for 15 minutes, slides were loaded onto a BenchMark ULTRA instrument (Ventana Medical Systems, Inc.) for staining. The slides were deparaffinized, and endogenous peroxidase activity blocked by treating with 3% H2O2 for 5 minutes. HIER was performed at 98°C with Cell Conditioning 1 Tris-based buffer pH 8.5 (48 minutes; Ventana Medical Systems Inc.). Slides were incubated at 36°C for 32 minutes with a mouse monoclonal antibody to claudin-4 (clone 3E2C1, 1:200 dilution; Invitrogen). Detection was performed by goat anti-mouse immunoglobulins conjugated with hydroquinone haptens, followed by mouse anti-hydroquinone immunoglobulins conjugated with horseradish peroxidase (OptiView DAB IHC Detection Kit; Ventana Medical Systems, Inc.). The antibody signal was determined by 0.2% 3,3′-diaminobenzidine tetrahydrochloride undergoing a peroxidase reaction with horseradish peroxidase. Slides were counterstained with haematoxylin.

Most of the cases included in the study had Ber-Ep4 staining performed as part of routine reporting. Those with no result had an extra section cut and stained with Ber-Ep4 using an established laboratory protocol on a Benchmark Ultra instrument with HIER at 98°C with Cell Conditioning 1 for 32 minutes. Slides were incubated at 36°C for 32 minutes with antibody to human EpCAM (clone Ber-Ep4, 1:100 dilution; Agilent); detection was performed using the OptiView DAB IHC Detection Kit as above.

All slides were scored by three authors (M.V., M.D., P.S.) on a multi-head microscope. Claudin-4 results were scored for (a) percentage of malignant cells stained (0: Negative; 0%-5%; 1: 5%-25%; 2: 25%-50%; 3: 50%-75%; 4: 75%-100%) and (b) intensity of stain (0: absent; 1+: weak; 2+: moderate; 3+: strong). A positive result was defined as linear membrane staining in any malignant cells. Measures of accuracy were calculated and compared with Fisher’s exact test.

### 3 | RESULTS

Positivity for claudin-4 was identified as linear membrane staining, often accompanied by diffuse cytoplasmic reactivity. Mesothelial cells were often observed to have multiple small cytoplasmic dots that on low power appeared as a pale cytoplasmic blush (Figure 1). This staining pattern was also noted in the tissue samples of mesothelioma and in benign mesothelial cells in many of the cell-block cases stained. It was easily distinguished from membrane staining. Observed positive and negative staining patterns were consistent in both cell-block and matched tissue specimens.

Staining results are summarised in Table 1. Reactive mesothelial cells in all 49 benign effusions were negative for claudin-4. Additionally, many malignant cases contained background mesothelial cells that were uniformly negative. Of the 63 cases of mesothelioma, only one case (1.6%) was found to focally express claudin-4 (Figure 2). This case also stained with Ber-Ep4 in the cell-block specimen but was negative for claudin-4 in the matched pleural biopsy. All stain results and the morphology for this case were reviewed and the claudin-4 staining repeated with similar results.
Claudin-4 staining was positive in 131/137 (95.6%) adenocarcinoma cases. Staining was assessed as moderate to strong in 95% (130/137) of cases and present in more than 50% of malignant cells in 81.8% (112/137). Cases negative for claudin-4 included single cases of metastases from breast, colon, stomach, prostate, kidney and ovary. Positive cells occurred as aggregates with intense staining localized to the membrane between cells; however, moderate to intense membrane staining was also seen in cases with a single-cell pattern (Figure 2).

Claudin-4 was more reliable than Ber-Ep4 for discriminating metastatic adenocarcinoma from benign and malignant mesothelial cells. Sensitivity, specificity, negative predictive value and positive predictive value for the detection of adenocarcinoma were all higher for claudin-4 compared with Ber-Ep4 (Table 2). Significantly ($P < 0.0064$) more cases of metastatic adenocarcinoma stained positive for claudin-4 (131/137; 95.6%) than Ber-Ep4 (117/137; 86.2%). Only two cases were claudin-4−/Ber-Ep4+ (results confirmed on review). Claudin-4 showed similar or superior sensitivity to Ber-Ep4 for all broad primary sites except ovary, and was significantly more sensitive ($P < 0.0025$) for breast metastases (96.7% vs 63.3% respectively). In addition, staining was present in mesothelial cells in significantly ($P < 0.0001$) fewer cases with claudin-4 (1/63 mesothelioma and 0/49 reactive cases) compared with Ber-Ep4 (14/63 mesothelioma and 1/49 reactive cases), resulting in superior positive predictive value for the identification of metastatic adenocarcinoma.

**FIGURE 1** Mesothelioma in a pleural fluid cell-block stained (A) with haematoxylin-eosin ($\times 400$) and (B) for claudin-4 ($\times 400$). Claudin-4 shows multiple fine cytoplasmic dots but is negative for membrane staining.

**TABLE 1** Claudin-4 and Ber-Ep4 staining results for cell-blocks prepared from 284 serous effusion specimens
Claudin-4 staining was present in 15/20 (75%) of neuroendocrine carcinomas, 3/4 (75%) squamous cell carcinoma and 3/3 (100%) urothelial cell carcinoma. All eight cases of melanoma were negative for both claudin-4 and Ber-Ep4.

Of the 39 cases that had both cell-block and matching tumour tissue evaluated for immunoreactivity with claudin-4, all but one showed concordance between the biopsy and effusion cell-block result. All breast and lung adenocarcinomas were positive for claudin-4 in both tissue and cell-block. One of the 13 mesotheliomas was negative in the pleural biopsy but showed focal staining in the matched cell-block and was positive for Ber-Ep4 in both cell-block and tissue.

### DISCUSSION

A wide range of IHC antibodies has been studied as potential pan-carcinoma markers in serous effusion specimens. However, to date none have been found to have ideal sensitivity and specificity. This is due to both the differing immunophenotypes amongst tumours originating from the wide range of primary sites that metastasize to body cavities, and the variable expression of antigens by tumours from the same primary site. For example, Ber-Ep4 staining, which is widely used in this role, is highly sensitive for metastatic lung adenocarcinoma but less so for tumours of non-pulmonary origin, and stains some mesotheliomas. This study investigated the tight junction protein claudin-4 as a potential pan-carcinoma marker in effusion cytology and compared it with Ber-Ep4.

Metastatic adenocarcinomas from a wide range of primary sites were found to express claudin-4, including those from the most common primary sites that spread to body cavities—breast, lung, ovary and digestive system. Only 6/137 (4.4%) metastatic glandular tumours were negative. By contrast, staining was rarely seen in benign or malignant mesothelial cells, with 0/49 reactive cases and 1/63 (1.6%) cases of mesothelioma positive. Claudin-4 was therefore found to have high sensitivity (95.6%) and specificity (99.1%). This diagnostic performance was significantly better than Ber-Ep4, which stained nearly a quarter (14/63) of cases of mesothelioma and fewer cases of metastatic disease. Claudin-4 was significantly more likely than Ber-Ep4 to be expressed in breast metastases, which are the most common cause of malignant pleural effusions in women.

Previous studies have found similarly high sensitivity of claudin-4 for adenocarcinomas from a range of sites. Studies by Jo et al., Lonardi et al. and Kim et al. evaluated the use of claudin-4 in cytology effusion specimens and reported its sensitivity to be 100%, 99% and 99.1%, respectively. Studies based on surgical specimens have also confirmed claudin-4 is expressed by a high proportion of cases in a wide variety of epithelial tumours. The current study found single cases of adenocarcinoma from breast, colon, stomach, prostate, kidney and ovary to be negative. Previous studies of fluid specimens have reported rare negative cases of metastatic ovarian adenocarcinoma. A study of tissue specimens by Ordóñez also found some ovarian serous tumours (2%) and 15% of renal clear cell carcinomas to be negative.

There is less concordance in published results for the expression of claudin-4 by mesothelial cells. Some authors have found no reactivity in mesothelial cells, whereas Kim et al. reported weak membranous staining in 27.5% of reactive mesothelial cases. Soini et al. also reported reactivity in a series of surgical specimens including 24 adenocarcinomas (100% positive) and 35

| TABLE 2 | Accuracy measures for the detection of metastatic adenocarcinoma in effusion specimens for Claudin-4 and Ber-Ep4, based on staining results from 137 adenocarcinoma, 63 mesothelioma and 49 benign cases |
|----------------------------------|----------------------------------|
| | Claudin-4 (%) | Ber-Ep4 (%) |
| Sensitivity | 95.6 | 85.4 |
| Positive predictive value | 99.2 | 88.6 |
| Specificity | 99.1 | 86.6 |
| Negative predictive value | 94.9 | 82.9 |
mesotheliomas (23%). The current study found only one case of mesothelioma to express claudin-4. Interestingly, the pleural biopsy sample on this case was negative, whereas reactivity in all other matched effusion/biopsy samples was concordant. The reasons for this are uncertain but may include factors such as sampling and focal or heterogeneous expression of the antigen, or differences in cell/tissue handling. Adhesion molecules, such as cadherins, have also been shown to have altered expression in fluids compared with solid tumours.11

Interpretation of staining and varying primary antibody dilution may account for some of the variation in reported reactivity of mesothelial cells. All studies cited used the same clone (3E2C1). In the current study, a distinctive cytoplasmic dot pattern of staining was observed in benign and malignant mesothelial cells in both histological and cytological specimens. The degree of intensity and number of these dots were variable; however, the consistent localization of dots to mesothelial cell cytoplasm suggests they are not artefactual. Other authors have noted some degree of staining in mesothelial cells. Lonardi et al3 also described intra-cytoplasmic dot staining in mesothelial and inflammatory cells, which they considered non-specific, while Jo et al2 reported weak cytoplasmic staining in some mesotheliomas. By contrast, others5,6 have described membranous staining with Claudin-4 in around a quarter of cases of reactive mesothelium. We found the pattern of cytoplasmic staining observed in mesothelial cells easy to discriminate from the linear membrane staining observed in the majority of adenocarcinoma cells.

Ber-Ep4 is a commonly used antibody that reacts with epithelial-cell adhesion molecule (Ep-CAM) and has been widely used in IHC panels as a marker for metastatic carcinoma in effusion specimens. A recent meta-analysis of published studies reported sensitivity of 0.8 (95% CI: 0.78-0.82) and specificity 0.94 (95% CI: 0.93-0.96) in effusion cytology.11 A high proportion of carcinomas of lung (squamous and glandular), colon, breast, pancreas and serous carcinomas of ovary express Ber-Ep4. However, it is less sensitive for breast and renal cell carcinomas.10 In addition, 13%-26% of mesotheliomas have been reported to stain positive, often focally.10 Results from the current study are in accordance with this (sensitivity 86%; specificity 85%). Ber-Ep4 was positive in two adenocarcinomas (one colonic, one ovarian) that were claudin-4 negative. Based on our findings, using claudin-4 and Ber-Ep4 together would detect 97.1% of adenocarcinomas compared with 95.6% using claudin-4 alone.

Although the most common cytological differential diagnosis in effusion specimens involves mesothelial cells vs adenocarcinoma, a wide range of other tumour types are encountered. IHC is often applied in these cases to confirm, exclude or help determine a diagnosis. We examined limited numbers of these rare metastases. Claudin-4 was found to be expressed by 3/4 of metastatic squamous cell carcinoma, 15/20 small cell carcinoma of lung and 3/3 urothelial carcinoma. These tumours have not previously been examined for claudin-4 expression in fluid specimens. All eight metastatic melanomas were negative, which confirms the findings from a small number of cases examined in surgical specimens.9

In conclusion, this study confirms the value of claudin-4 as a potential pan-adenocarcinoma marker for the cytological diagnosis of serous effusion carcinoma. The stain offers high sensitivity and specificity, superior to Ber-Ep4. We believe it will make a useful addition to IHC panels for the diagnosis of serous effusion specimens and may be of assistance in histological diagnosis.

CONFLICT OF INTEREST
The authors have no disclosures to make.

AUTHORS’ CONTRIBUTION
All authors meet the criteria for authorship as per the guidelines of the International Committee of Medical Journal Editors (ICMJE), all have participated at: (a) conception or design of the work; or the acquisition, analysis or interpretation of data for the work; (b) drafting the work or revising it critically for important intellectual content; (c) final approval of the version submitted; and (d) agreement to be accountable for all aspects of the work regarding the accuracy or integrity of the research.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Paul W. Shield https://orcid.org/0000-0002-4596-2225

REFERENCES
1. Hewitt KJ, Agarwal R, Morin PJ. The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer. 2006;6:186.
2. Jo VY, Cibas ES, Pinkus GS. Claudin-4 IHC is highly effective in distinguishing AC from malignant MM in effusion cytology. Cancer Cytopathol. 2014;122:299-306.
3. Lonardi S, Manera C, Marucci R, Santoro A, Lorenzi L, Facchetti F. Usefulness of claudin-4 in the cytological diagnosis of serosal effusions. Diagn Cytopathol. 2011;39:313-317.
4. Facchetti F, Lonardi S, Gentili F, et al. Claudin-4 identifies a wide spectrum of epithelial neoplasms and represents a very useful marker for carcinoma versus mesothelioma diagnosis in pleural and peritoneal biopsies and effusions. Virchows Arch. 2007;451:669-680.
5. Kim NI, Kim G-E, Lee JS. Diagnostic usefulness of claudin-3 and claudin-4 for immunocytochemical differentiation between metastatic adenocarcinoma cells and reactive mesothelial cells in effusion cell blocks. Acta Cytol. 2016;60:232-239.
6. Soini Y, Kinnula V, Kahlós K, Paakko P. Claudins in differential diagnosis between mesothelioma and metastatic adenocarcinoma of the pleura. J Clin Pathol. 2006;59:250-254.
7. Pereira TC, Saad RS, Liu Y, Silverman JF. The diagnosis of malignancy in effusion cytology: a pattern recognition approach. Adv Anat Pathol. 2006;13:174-184.
8. Ordonez NG. Role of immunohistochemistry in differentiating epithelial mesothelioma from adenocarcinoma. *Am J Clin Pathol*. 1999;112:75-89.

9. DeMay RM. *The Art and Science of Cytopathology*, 2nd ed. Chicago, IL: American Society for Clinical Pathology, ASCP Press; 2012.

10. Ordóñez NG. Value of claudin-4 immunostaining in the diagnosis of mesothelioma. *Am J Clin Pathol*. 2013;139:611-619.

11. Davidson B, Berner A, Nesland JM, et al. E-cadherin and α-, β- and γ-catenin protein expression is up-regulated in ovarian carcinoma cells in serous effusions. *J Pathol*. 2000;192:460-469.

How to cite this article: Vojtek M, Walsh MD, Papadimos DJ, Shield PW. Claudin-4 immunohistochemistry is a useful pan-carcinoma marker for serous effusion specimens. *Cytopathology*. 2019;30:614–619. [https://doi.org/10.1111/cyt.12765](https://doi.org/10.1111/cyt.12765)