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Original Article

# Diagnostic Utility of Claudin-4 Immunohistochemistry in Distinguishing Adenocarcinoma Cells from Reactive Mesothelial Cells in Effusion Cytology

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## Abstract

**Background**: Differentiating adenocarcinoma cells from reactive mesothelial cells in cases of effusion remains a diagnostic challenge due to overlapping morphological features. Claudin-4, a tight junction protein expressed in epithelial but absent in mesothelial cells, has emerged as a potential immunohistochemical (IHC) marker for this distinguishing between these cell types.

**Objective**: to evaluate the diagnostic utility of Claudin-4 immunohistochemistry in differentiating adenocarcinoma cells from reactive mesothelial cells in effusion cytology.

**Methods**: This prospective study was conducted at the Department of Pathology, Nizam's Institute of Medical Sciences, Hyderabad, India, between June 2023 and December 2024. A total of 77 formalin-fixed paraffin-embedded cell block samples from effusion fluids (pleural, ascitic, and peritoneal) were analyzed. Inclusion criteria comprised cases diagnosed as malignant or reactive on cytology. Claudin-4 immunostaining was performed by using EP417 clone. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Claudine for differentiating malignant versus non-malignant effusion was calculated.

**Results**: Out of the 77 studied cases, 57 were malignant and 20 were non-malignant. Claudin-4 showed positive membranous staining in 50/57 malignant cases (87.72%) and in none of the non-malignant cases (100% specificity). The PPV was 100%, and NPV was 74.10%. Pulmonary adenocarcinoma was the most common malignancy showing Claudin-4 positivity. Claudin demonstrated excellent specificity for diagnosis of malignant effusions.

**Conclusion**: Claudin-4 immunohistochemistry is highly specific as a reliable marker for differentiating malignant cells from reactive mesothelial cells. Its high specificity and positive predictive value make it a valuable diagnostic tool. However, a negative Claudin-4 immunohistochemistry results should be interpreted cautiously, particularly in clinically suspicious cases.

**Keywords:** Adenocarcinoma, claudin-4, effusion cytology, immunohistochemistry, mesothelial cells

## Introduction

Effusions can be defined as abnormal accumulation of fluid within body cavities such as pleural, pericardial, and peritoneal cavities. These effusions can arise from a wide range of benign and malignant conditions. Accurate diagnosis of the nature of these effusions is

important for determining the underlying cause and appropriate treatment. Various methods can be used for distinguishing malignant cells from reactive mesothelial cells. Immunohistochemistry (IHC) is one of such widely used method.[1]

Distinguishing malignant adenocarcinoma cells from reactive mesothelial cells is a major challenge in effusion cytology because the cytological characteristics of the two cell types often overlap. Mesothelial cells which line the serous cavities can undergo reactive changes in response to benign conditions. These changes may cause cytological features that may closely mimic that of malignant cells. This makes the accurate identification of malignancy difficult using traditional cytological methods alone.[2]

Claudin-4 is a member of the claudin family of tight junction proteins that has emerged as a promising biomarker in this context.[3] Tight junctions are essential components of epithelial and endothelial barriers and claudins play a crucial role in maintaining the integrity as well as function of these junctions.[4] Claudin-4 is expressed exclusively in cells of epithelial origin including malignant epithelial cells however it is absent in mesothelial cells. This classically distinct expression pattern makes Claudin-4 a valuable marker for differentiating between malignant and reactive cells in patients presenting with various effusions.[5]

The application of Claudin-4 immunohistochemistry in effusion cytology provides a reliable method for identifying malignant adenocarcinoma cells. By specifically staining malignant cells and not reactive mesothelial cells Claudin-4 helps pathologists make more accurate diagnoses. This is particularly important in cases where the cytomorphological distinction between reactive mesothelial cells and malignant cells is unclear.[6]

Recent studies have reported high sensitivity and specificity of Claudin-4 in identifying malignant cells in effusions. For instance, Claudin-4 immunostaining has been reported to effectively differentiate metastatic adenocarcinoma cells from reactive mesothelial cells in pleural, pericardial and peritoneal effusions. This characteristic of Claudin-4 is important for diagnosis and also for guiding the clinical management of patients with effusions.[7]

Many studies have reported use of Claudin-4 in immunohistochemical staining provides a highly accurate method for distinguishing malignant adenocarcinoma cells from reactive mesothelial cells, thereby improving diagnostic accuracy and aiding in the effective management of patients with effusions.[8] This study was undertaken to evaluate the diagnostic utility of Claudin-4 immunohistochemistry in differentiating malignant adenocarcinoma cells from reactive mesothelial cells in effusion cytology, and to assess its sensitivity, specificity, and predictive values in an Indian tertiary care setting.

## Methods

This prospective observational study was conducted in the Department of Pathology, Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India, on formalin-fixed paraffin-embedded (FFPE) cell blocks prepared from serous effusion samples received between June 2023 and December 2024. Institutional ethic committee of Nizam's Institute of Medical Sciences clearance was obtained (vide letter ref 2366/2023). A total of 77 cases were included in this study on the basis of a predefined inclusion and exclusion criteria. The sample size was calculated using a prevalence-based formula assuming a Claudin-4 sensitivity of approximately 90% and absolute precision of 10%, and a 95% confidence interval. The minimum required number of cases was estimated to be 70. Therefore available 77 cases provided sufficient statistical power for meaningful analysis.

All cases of malignant effusions and those with reactive mesothelial cells diagnosed on cytology during the study period were included. Exclusion criteria were cases with insufficient cellularity in the effusion sample, poorly preserved material unsuitable for cell block preparation, and cases in which complete demographic or clinical data were not available from hospital records.

Demographic data, clinical history, and relevant laboratory findings were obtained from hospital and departmental records. In cases where morphology raised concern for atypia or borderline features, slides were independently reviewed by a second senior cytopathologist. Cell block preparations were performed in all included cases, particularly when cytology alone did not permit a confident distinction between malignant adenocarcinoma cells and reactive mesothelial cells.

Cell blocks were prepared using the fixed sediment method. Effusion fluid was centrifuged at 2500 rpm for 5–10 minutes, and the supernatant was discarded. The sediment

was fixed in 95% ethanol and centrifuged again at 3000 rpm for 10–15 minutes. The resultant cell button was carefully transferred into a labelled tissue cassette, fixed in 10% neutral buffered formalin, and processed by routine histopathological protocols.

Sections of 4 µm thickness were cut from the paraffin blocks and mounted on poly-L-lysine–coated slides. The slides were incubated overnight at 50°C, deparaffinized, and rehydrated. Antigen retrieval was done using CC1 buffer (pH 8.5) for 60–75 minutes in the Ventana Benchmark automated system. The Ultra view universal DAB inhibitor was applied for a duration of 4 minutes. It was followed by incubation of cell blocks with the primary antibody Claudin-4 (clone SP27, rabbit monoclonal antibody) for 30 minutes. Slides were treated with Ultra view universal HRP multimer for a duration of 8 minutes followed subsequently by Ultra view universal copper for approximately 4 minutes. Final visualization was done using diaminobenzidine (DAB) chromogen with hydrogen peroxide for approximately 10 minutes. This was followed by counterstaining with hematoxylin and then slides were subsequently mounted for evaluation.

Claudin-4 immunoreactivity was considered to be positive when membranous staining was seen in at least 10% of cells. This cutoff value is supported by the review by Kleinaki *et al*.[9] who noted that Claudin-4 positivity thresholds in published studies range from ≥1% to ≥10%, with ≥10% among the most frequently used definitions. The sensitivity, specificity, positive predictive value, and negative predictive value of Claudin-4 immunostaining in distinguishing malignant adenocarcinoma cells from reactive mesothelial cells were calculated.

Reactive mesothelial cases were confirmed by dual cytopathologist review and clinico-radiologic correlation. Sensitivity, specificity, PPV and NPV of Claudin-4 were calculated using a 2×2 contingency table in SPSS version 23. Categorical variables (Claudin-4 vs. diagnosis) were analyzed using the Chi-square test. Continuous variables were compared using the independent samples t-test after confirming normality.

## Results

The analysis of the gender distribution of the studied cases showed that out of a total of 77 individuals, 47 (61.04%) were females and 30 (38.96%) were males. There was a female preponderance with M:F ratio of 1:1.56 (Table 1).

The analysis of the age group distribution among the studied cases showed that the most frequently affected age group in both males and females was 50–59 years (15.58% and 20.78%), followed by the 60–69 age

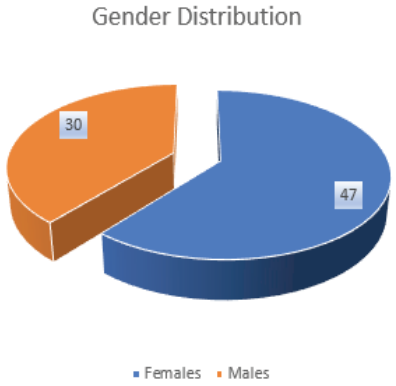


**Fig. 1 Gender Distribution of Studied Cases**

**Table 1 Age Distribution of Studied Cases**

| Age Group | Male (n=30) | | Female (n=47) | | p-value |
|---|---|---|---|---|---|
| | Number of Cases | % | Number of Cases | % | |
| <40 | 0 | 0.00 | 6 | 7.79 | 0.1126 (Not Significant) |
| 40–49 | 5 | 6.49 | 8 | 10.39 | |
| 50–59 | 12 | 15.58 | 16 | 20.78 | |
| 60–69 | 9 | 11.69 | 11 | 14.29 | |
| 70–79 | 4 | 5.19 | 7 | 9.09 | |
| Total | 30 | 38.96 | 47 | 61.04 | |
| Mean Age | 61.4 +/- 10.95 | | 56.67 +/- 13.67 | | |

**Table 2 IHC- CL-4 status of studied cases**

| Diagnosis | Claudin 4 IHC | | Total | p-value |
|---|---|---|---|---|
| | Positive | Negative | | |
| Malignant Effusion | 50 | 7 | 57 | p<0.001 (highly significant) |
| Non-Malignant Effusion | 0 | 20 | 20 | |
| Total | 50 | 27 | 77 | |

**Table 3 Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of Claudin-4 for Differentiating Malignant and Non-Malignant Effusion**

| Parameter | Value (%) |
|---|---|
| Sensitivity | 87.72 |
| Specificity | 100 |
| PPV | 100 |
| NPV | 74.10 |

group with 9 males (11.69%) and 11 females (14.29%). Notably, there were no males in the <40 age group, while 6 females (7.79%) were observed in this category. The mean age was slightly higher among males (61.4±10.95 years) compared to females (56.67±13.67 years). However, this difference in mean age was not statistically significant (p=0.1126) (Table 1).

The analysis of the type of fluid collected from the studied cases showed that pleural fluid was the most common (66.2%), followed by ascitic fluid with (24.6%). Peritoneal fluid constituted 7 cases (9.1%). The analysis of the disease distribution among the studied cases showed that malignant effusions were significantly more common, comprising 57 cases (74.03%) of the total, while non-malignant effusions accounted for only 20 cases (25.97%). The analysis of the IHC-CL-4 expression in relation to diagnosis showed that majority of Malignant effusion cases (87.72%) were IHC-CL-4 positive , while only 7 cases (12.28%%) were negative. In contrast, all 20 cases of non-malignant effusion were found to be negative for IHC-CL-4 expression (100%). This indicated a strongly positive association between IHC-CL-4 positivity and malignant effusions. The observed difference was statistically highly significant (p<0.001 (Table 2) (Figure 2).

The analysis of Claudin-4 as a diagnostic marker showed that it had a high sensitivity of 87.72% and specificity of 100%. The positive predictive value (PPV) was found to be 100%. However, the negative predictive value (NPV) was found to be 74.70%. This was reflective of a moderate ability to correctly identify true negatives (Table 3).

Out of a total of 77 effusion samples, 57 (74.0%) were malignant and 20 (26.0%) were non-malignant. Claudin-4 immunopositivity was observed in 50 cases (64.9%), all of which were malignant. Among malignant effusions most common malignant diseases included pulmonary adenocarcinoma (26.0%), mixed adenocarcinomas (26.0%) and ovarian

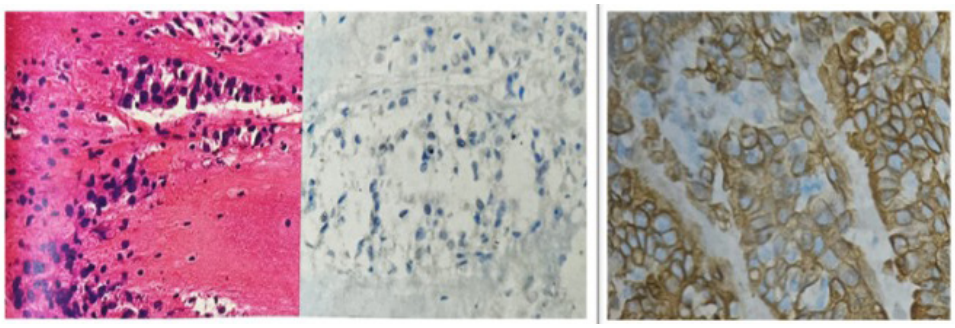

**Fig 2. Reactive Mesothelial Cells Showing Typical Morphology on H&E (left) with Negative Claudin-4 IHC (middle), Contrasted With Strong Membranous Claudin-4 Positivity in Malignant Epithelial Cells (right)**

**Table 4 Distribution of Diagnoses Across Different Fluid Types and Claudin-4 Expression**

| Diagnosis | Claudin-4 IHC | | Total n (%) |
|---|---|---|---|
| | Positive n (%) | Negative n (%) | |
| Malignant Effusion | 50 (64.9) | 7 (9.1) | 57 (74.0) |
| Pulmonary adenocarcinoma | 18 (23.4) | 2 (2.6) | 20 (26.0) |
| Ovarian carcinoma | 5 (6.5) | 1 (1.3) | 6 (7.8) |
| Breast carcinoma | 3 (3.9) | 0 (0.0) | 3 (3.9) |
| Colonic adenocarcinoma | 1 (1.3) | 0 (0.0) | 1 (1.3) |
| Cholangiocarcinoma | 2 (2.6) | 0 (0.0) | 2 (2.6) |
| Papillary thyroid ca. | 1 (1.3) | 0 (0.0) | 1 (1.3) |
| GI primary adenocarcinoma | 1 (1.3) | 0 (0.0) | 1 (1.3) |
| Cervical carcinoma | 1 (1.3) | 0 (0.0) | 1 (1.3) |
| Papillary urothelial ca. | 1 (1.3) | 0 (0.0) | 1 (1.3) |
| Signet-ring rectal ca. | 1 (1.3) | 0 (0.0) | 1 (1.3) |
| Others (mixed adenocarcinoma) | 16 (20.8) | 4 (5.2) | 20 (26.0) |
| Non-Malignant Effusion | 0 (0.0) | 20 (26.0) | 20 (26.0) |
| Reactive mesothelial | 0 (0.0) | 12 (15.6) | 12 (15.6) |
| Lymphocytic effusion | 0 (0.0) | 6 (7.8) | 6 (7.8) |
| Suppurative inflammation | 0 (0.0) | 2 (2.6) | 2 (2.6) |

carcinoma (7.8%). Claudin-4 negativity was seen in 27 cases (35.1%), comprising 7 malignant effusions (9.1%) and all 20 non-malignant effusions (26.0%). The association between Claudin-4 expression and malignant effusion was statistically highly significant ($p<0.001$).

## Discussion

In this study of 77 effusion cell-block cases Claudin4 immunohistochemistry (IHC) had high specificity (100%) and good sensitivity (87.72%) for distinguishing malignant adenocarcinoma cells from reactive mesothelial cells. Claudin-4 reactivity was found to have a PPV of 100% and NPV 74.10% for differentiating malignant from non-malignant effusions.

Sensitivity of claudin 4 for differentiating malignant from non-malignant effusion was somewhat lower (87.7%) than that reported in recent meta-analyses and large series, though specificity in this study matches or comes close. A meta-analysis by Kleinaki *et al.* of 14 observational studies reported pooled sensitivity of 98.02% (95% CI, 93.9699.37%) and specificity of 99.72% (95% CI, 97.3699.97%) for Claudin4 in serous effusion cytology.[9] 100% specificity in this study is consistent with the high end of that range but sensitivity of 87.7% as seen in this study is lower. Possible reasons could include differences in the cell block preparation the antigen retrieval or staining protocols. For example, in some studies, when the threshold for positivity is more permissive (≥1% or any positive cells), sensitivity tends to be higher. Similarly in a study by Najjar *et al.*, Claudin4 had 93.7% sensitivity and 100% specificity, with no benign or mesothelioma cases positive in a cohort of 229 cytology specimens.[10] Specificity and positive predictive value were both 100%, in line with the present study, while sensitivity was slightly higher. This difference may be explained by variations in case distribution or diagnostic cutoffs. In that study, none of the mesotheliomas (0/39) or benign effusions (0/47) showed positive staining for Claudin4. This parallels the finding that all non-malignant effusions were negative in this study. Elhosainy *et al* studied claudin4 plus EZH2 in 80 serous effusions (48 metastatic adenocarcinomas vs. 32 reactive mesothelial cells. The study reported sensitivity of 95.8% and specificity 96.9% for Claudin4 alone. However, when combined with EZH2, sensitivity increased to 100%,

although specificity decreased slightly.[11] Claudin 4 continued to demonstrate strong performance. In comparison with the 87.7% sensitivity achieved in the present study, the sensitivity reported in that investigation was higher.

Earlier smaller studies have shown greater variability. For example, Moghaddam *et al.* evaluated Claudin 4 in pleural and peritoneal fluid samples and reported a sensitivity of 85% and a specificity of 100% for differentiating metastatic carcinoma from reactive mesothelium.[12] The sensitivity reported in that study was similar to the value observed in the present analysis. Similarly, in some of the component studies in the meta-analysis, there are reports of sensitivity in the 8595% range, and specificity almost uniformly high (close to 100%) when reactive mesothelium and benign effusions were compared. These are quite consistent with findings of this study.

One of the key strengths of this study was the absolute specificity. No nonmalignant effusion showed Claudin4 positivity. This is critical clinically because a false positive in benign or reactive effusions can lead to significant misdiagnoses. However, the NPV (74–75%) being moderate indicates that a negative Claudin4 does *not* reliably exclude malignancy. Some cases will be false negatives — malignant cases that lack expression or are below the cutoff. In this data 7 of 57 malignant cases were negative. The reason for such negative results includes poor differentiation or mucinous/signet-ring features which are known to lose Claudin-4 expression. In the remaining cases low tumour cell content or focal staining below the 10% cut-off likely explains the negativity.

Several factors may contribute to false negatives that may include low tumor cell content in cell blocks, weak or focal expression below threshold, antigen retrieval issues or certain tumor types which may express Claudin4 less robustly. In the study by Elhosainy *et al.* also noted one reactive case showing partial Claudin4 positivity.[11] In some series, thresholds vary (e.g., ≥5%, ≥10% or any positive cells) leading to variation in sensitivity. Another possibility, as reported by Tessier-Cloutier *et al*, is the tumor differentiation poorly differentiated adenocarcinomas might lose Claudin4 expression in some cases.[13]

Thus, in practice Claudin4 can be used perhaps as a single marker in many cases or part of a small panel such as pan-carcinoma marker (Claudin4) plus a mesothelial marker (e.g., calretinin, WT1) to help increase specificity. Results of this study reinforce what Najjar *et al.* reported that Claudin4 has specificity superior to or matching commonly used epithelial markers like MOC31 or BerEP4.[10] Similar findings have also been reposted by the authors such as Naso *et al*[14] and Haque *et al*.[15]

Limitations of this study include the sample size (though reasonably powered). Also, tumor subtype or grade breakdown was not deeply analyzed in relation to Claudin4 negativity. It would be useful to examine whether certain adenocarcinoma primaries have lower Claudin4 expression more often, or whether poorly differentiated tumors lose expression. In this study clone EP417 with ≥10% cutoff was considered as Claudin-4 positive. This requirement of 10% may reduce sensitivity. Furthermore, inclusion of additional markers may improve sensitivity when Claudin4 is negative, at some cost of specificity.

Claudin4 is a highly specific IHC marker with excellent PPV for detecting malignant adenocarcinoma cells in effusion cytology, though its sensitivity is somewhat below the highest values reported in the literature. As such, Claudin4 is valuable in confirming malignancy but negative staining alone cannot rule it out. This aligns with the broader literature and suggests that workflow strategies that incorporate Claudin4 especially with mesothelial markers or adjuncts are likely to yield optimal diagnostic accuracy.

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