

# Glypican-1 immunohistochemistry is a novel marker to differentiate epithelioid mesothelioma from lung adenocarcinoma

Vishwa Jeet Amaty<sup>1</sup>, Kei Kushitani<sup>1</sup>, Yuichiro Kai<sup>1,2</sup>, Rui Suzuki<sup>1</sup>, Yoshihiro Miyata<sup>2</sup>, Morihito Okada<sup>2</sup> and Yukio Takeshima<sup>1</sup>

<sup>1</sup>Department of Pathology, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, Japan and <sup>2</sup>Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

**Histological morphology alone is not sufficient for the pathological diagnosis of malignant mesothelioma. Positive and negative immunohistochemical markers are necessary to differentiate it from lung adenocarcinoma. As calretinin and D2-40, the recognized positive markers of mesothelioma, are expressed in lung adenocarcinoma to some extent, novel markers with high specificity are desirable. In this study, we investigated the applicability of glypican-1 immunohistochemistry to differentiate epithelioid mesothelioma from lung adenocarcinoma. We investigated 82 cases of epithelioid mesothelioma and 97 cases of lung adenocarcinoma for glypican-1 expression by immunohistochemistry using a commercially available antibody. All 82 cases of epithelioid mesothelioma showed glypican-1 expression, most with diffuse and strong reactivity. In contrast, only three cases of lung adenocarcinoma showed focal glypican-1 expression. Glypican-1 expression showed 100 sensitivity, 97% specificity, and a 98% accuracy rate to differentiate epithelioid mesothelioma from lung adenocarcinoma. The sensitivity of glypican -1 immunohistochemistry is as high as that of calretinin and D2-40, and its specificity is far better than that of calretinin and D2-40. Therefore, we recommend including glypican -1 immunohistochemistry as a positive marker of epithelioid mesothelioma.**

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Malignant mesothelioma is a highly aggressive asbestos-related malignant neoplasm with unfavorable prognosis. The incidence of mesothelioma is still increasing in Japan and Western countries, with an expected peak in the coming decades.<sup>1</sup> Some of the peripheral lung adenocarcinomas may present as a pseudomesotheliomatous growth pattern and resemble histologically epithelioid mesothelioma.<sup>2</sup> As the prognosis and treatment protocols of epithelioid mesothelioma and lung adenocarcinoma are different, their differential diagnosis is necessary for the proper management of patients.

Mesothelioma is classified into three major histological subtypes: epithelioid, biphasic, and sarcomatoid mesothelioma. The epithelioid mesotheliomas show various histological growth patterns, ranging from tubulopapillary to solid growth.<sup>3</sup> Since there is a

strong resemblance between epithelioid mesothelioma and lung adenocarcinoma, immunohistochemical markers are necessary for their accurate diagnosis. The International Mesothelioma Interest Group guideline recommended various immunohistochemical markers of epithelioid mesothelioma and lung adenocarcinoma. Calretinin, D2-40 (podoplanin), WT1, and cytokeratin 5/6 are positive markers for mesothelioma, and CEA, TTF-1, napsin-A, and claudin 4 are positive markers for lung adenocarcinoma.<sup>4</sup> However, the specificity of calretinin and D2-40 for differential diagnosis of epithelioid mesothelioma is not more than 90%, so caution is required in their interpretation.<sup>4</sup> We have recently reported two other positive immunohistochemical markers of epithelioid mesothelioma, intelectin-1 and DAB2, identified by gene expression analysis of epithelioid mesothelioma and lung adenocarcinoma.<sup>5</sup> Although these two positive immunohistochemical markers showed high specificity, their sensitivities were < 80%. Therefore, the search for the novel positive immunohistochemical markers is still desirable. In the present work, we evaluated the ability of glypican-1 expression to

Correspondence: Professor Y Takeshima, MD, PhD, Department of Pathology, Hiroshima University Graduate School of Biomedical and Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.

E-mail: ykotake@hiroshima-u.ac.jp

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**Table 1** List of Antibodies with their Clone, Commercial Source, and Reaction Conditions

Antibody to	Clone	Source	Dilution	Antigen retrieval
Glypican-1	Polyclonal	Proteintech	1:100	60 min, CC1
Calretinin	SP65	Ventana-Roche	Prediluted	60 min, CC1
Podoplanin	D2-40	Nichirei Bioscience	Prediluted	60 min, CC1
WT1	6 F-H2	Ventana-Roche	1:25	60 min, CC1
CEA	COL-1	Nichirei Bioscience	Prediluted	60 min, CC1
Claudin 4	3E2C1	Life Technologies	1:100	60 min, CC1
TTF-1	8G7G3/1	Dako-Agilent	1:25	60 min, CC1
Napsin-A	MRQ-60	Ventana-Roche	Prediluted	60 min, CC1

Abbreviations: CC1, cell conditioning buffer 1 (Tris-based buffer, pH 8.5 from Ventana-Roche); CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1; WT1, Wilm's tumor 1.

**Table 2** Immunohistochemical Result of Epithelioid Mesothelioma and Lung Adenocarcinoma

Marker	Epithelioid mesothelioma (82 cases)				Lung adenocarcinoma (97 cases)					
	Number of positive cases	Immunohistochemical Score				Number of positive cases	Immunohistochemical score <sup>a</sup>			
		0	1+	2+	3+		0	1+	2+	3+
Glypican-1	82 (100%)	0	2	24	56	3 (3%)	94	3	0	0
Calretinin	81 (99%)	1	8	2	71	18 (19%)	79	11	7	0
D2-40	79 (96%)	3	7	8	64	7 (7%)	90	5	2	0
WT1	65 (79%)	17	18	7	40	0 (0%)	97	0	0	0
CEA	0 (0%)	82	0	0	0	95 (98%)	2	7	14	74
Claudin 4	0 (0%)	82	0	0	0	93 (96%)	4	2	11	80
TTF-1	0 (0%)	82	0	0	0	86 (89%)	11	3	10	73
Napsin-A	0 (0%)	82	0	0	0	81 (84%)	16	12	2	67

Abbreviations: CK, cytokeratin; CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1; WT1, Wilm's tumor 1.

<sup>a</sup>0: 0% or trace; 1+: 1–10%; 2+: 11–50%; 3+: >51% of tumor cells with immunoreactivity.

differentiate epithelioid mesothelioma from lung adenocarcinoma.

linked-anonymized archival specimens, and individual consent was exempted for this research.

## Materials and methods

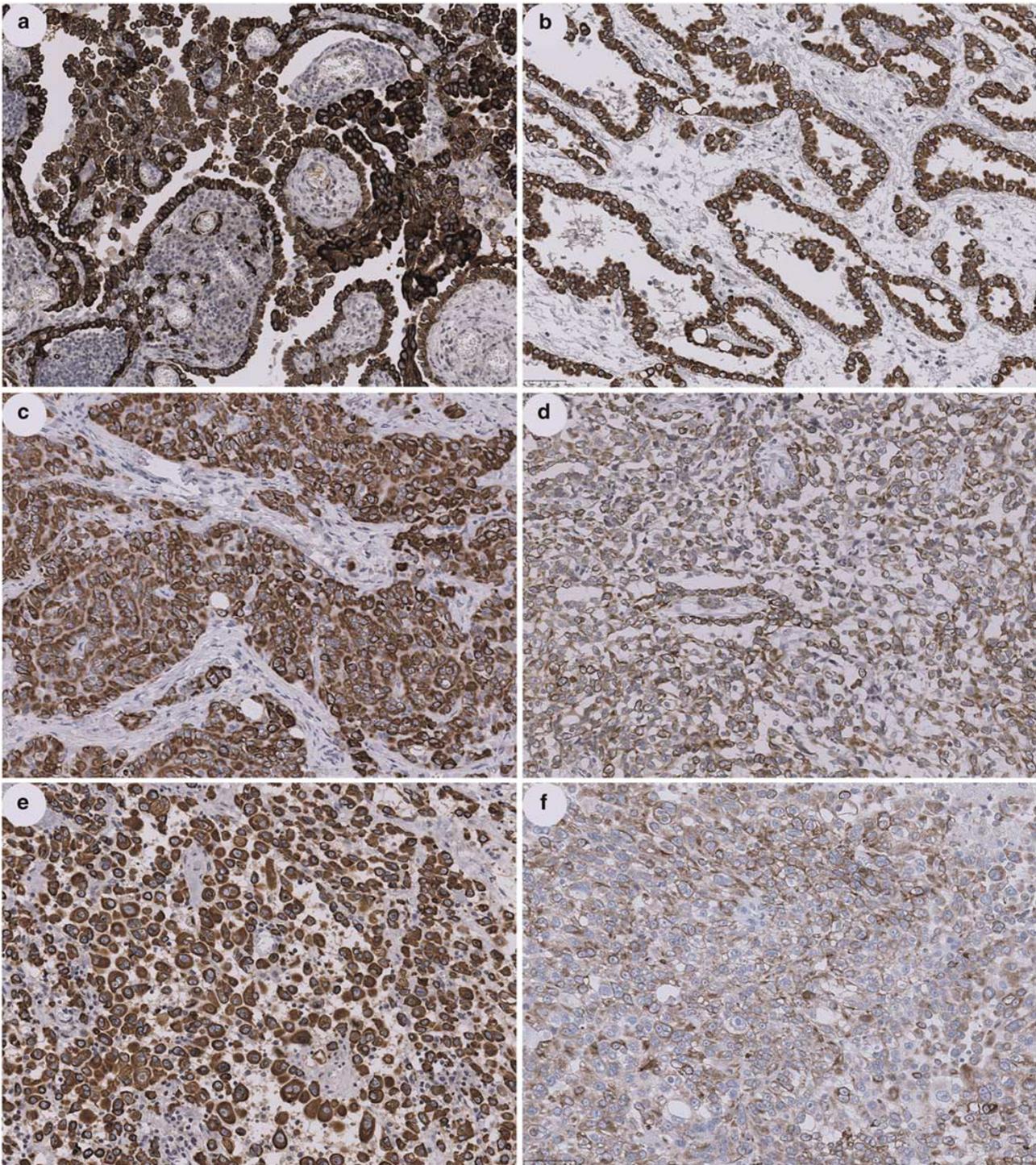
### Patients and Histologic Samples

Histological sections of 82 epithelioid mesotheliomas and 97 lung adenocarcinomas were retrieved from the archives of the Department of Pathology, Hiroshima University. No sarcomatoid mesothelioma was included in this study. The patient record files were reviewed for the clinical details and clinical information, such as chest computed tomography findings, to confirm the tumor localization.

Three pathologists (VJA, KK, and YT) reviewed all histological sections. Pathologic diagnosis of all of these cases was confirmed by histologic findings and an additional immunohistochemical marker panel recommended by the 2012 International Mesothelioma Interest Group Guideline<sup>6</sup> and the 2015 WHO histologic classification.<sup>7</sup> We collected and used the tissue specimens after the approval of our institutional ethics review committee (Hiroshima University E-974) based on 'The Japanese Ethical Guidelines for Medical and Health Research' enacted by the Japanese Government. Samples used were

### Immunohistochemical Procedures and Evaluation of Glypican-1 Expression

Immunohistochemistry was performed using tissue sections prepared from the best representative formalin-fixed, paraffin-embedded blocks of epithelioid mesotheliomas and pulmonary adenocarcinomas. Immunohistochemical staining was performed using the Benchmark GX automated immunohistochemical station (Roche Diagnostics-Ventana, Tokyo, Japan). The antigen retrieval methods and antibodies used in this study are summarized in Table 1. Incubation with the secondary antibody and detection were performed with an ultraView Universal DAB Detection Kit (Roche Diagnostics-Ventana). Cases showing nuclear reactivity for calretinin, WT1, and TTF-1; cytoplasmic reactivity for glypican-1, CEA, and napsin-A; and membranous reactivity for D2-40 and claudin 4 were regarded as positive. This positive immunoreactivity grade was further semiquantified as 0% for 0% positive cells or trace staining; 1+ for up to 10% of tumor cells showing positive immunostaining; 2+ for 10–50% positive tumor cells; and 3+ for >50%



**Figure 1** Glypican-1 expression was present in epithelioid mesothelioma of various histological morphologies. (a) Papillary growth; (b) tubular growth; (c) solid-tubular growth; (d) acinar growth; (e) scattered or discohesive growth; (f) solid growth; (g) microcystic growth; and (h) invasive growth into skeletal muscle.

positive tumor cells. Immunohistochemical staining with the omission of primary antibody is considered as negative control. Bronchial and bronchiolar epithelium for glypican-1, adipose tissue for calretinin, lymphatic vessels for D2-40, and blood vessels for WT1 as internal positive controls were considered as positive controls.

### Statistical Analysis

In this study, the sensitivity was the ability of the marker to correctly identify epithelioid mesothelioma, calculated by the percentage of positive cases (true positive) for the given marker. The specificity was the ability of the immunohistochemical marker

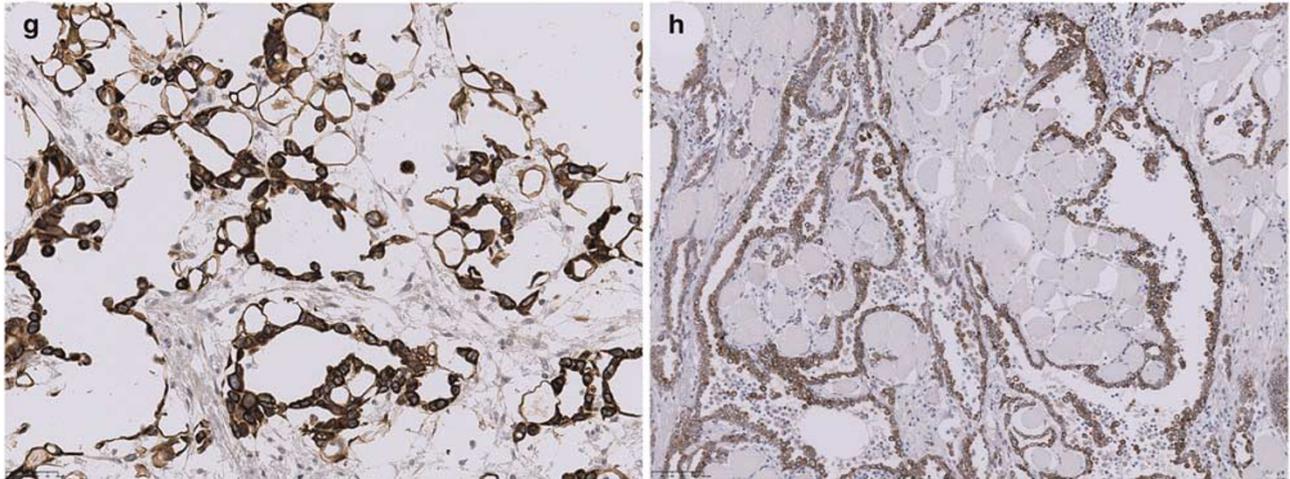


Figure 1 (Continued)

to correctly exclude lung adenocarcinoma, calculated by the percentage of negative cases (true negative) for the given marker. The accuracy rate is expressed as the proportion of cases correctly categorized by the given marker, calculated as a percentage of the sum of true-positive and true-negative cases out of the all cases studied.

## Results

The percentages and immunohistochemical scores of glypican-1 and other immunohistochemical markers in the epithelioid mesothelioma and the lung adenocarcinoma samples are shown in Table 2.

### Glypican-1 Expression

Glypican-1 expression was found in the cytoplasm of tumor cells of epithelioid mesothelioma. It was also observed in basal cells of bronchial and bronchiolar epithelium and normal mesothelial cells, which were considered internal positive controls. Glypican-1 expression was present in all 82 (100%) cases of epithelioid mesotheliomas irrespective of the histological pattern, whether tubulopapillary, cribriform, solid, mucinous, or deciduoid patterns of epithelioid mesothelioma (Figure 1). Among epithelioid mesothelioma cases, 56 cases showed immunohistochemical score 3+, 24 cases showed score 2+, and 2 cases showed score 1+. In contrast, glypican-1 expression was not present in 94 of 97 (97%) cases of lung adenocarcinoma (Figure 2). Only three cases of lung adenocarcinoma showed focal and weak glypican-1 expression (immunohistochemical score: 1+).

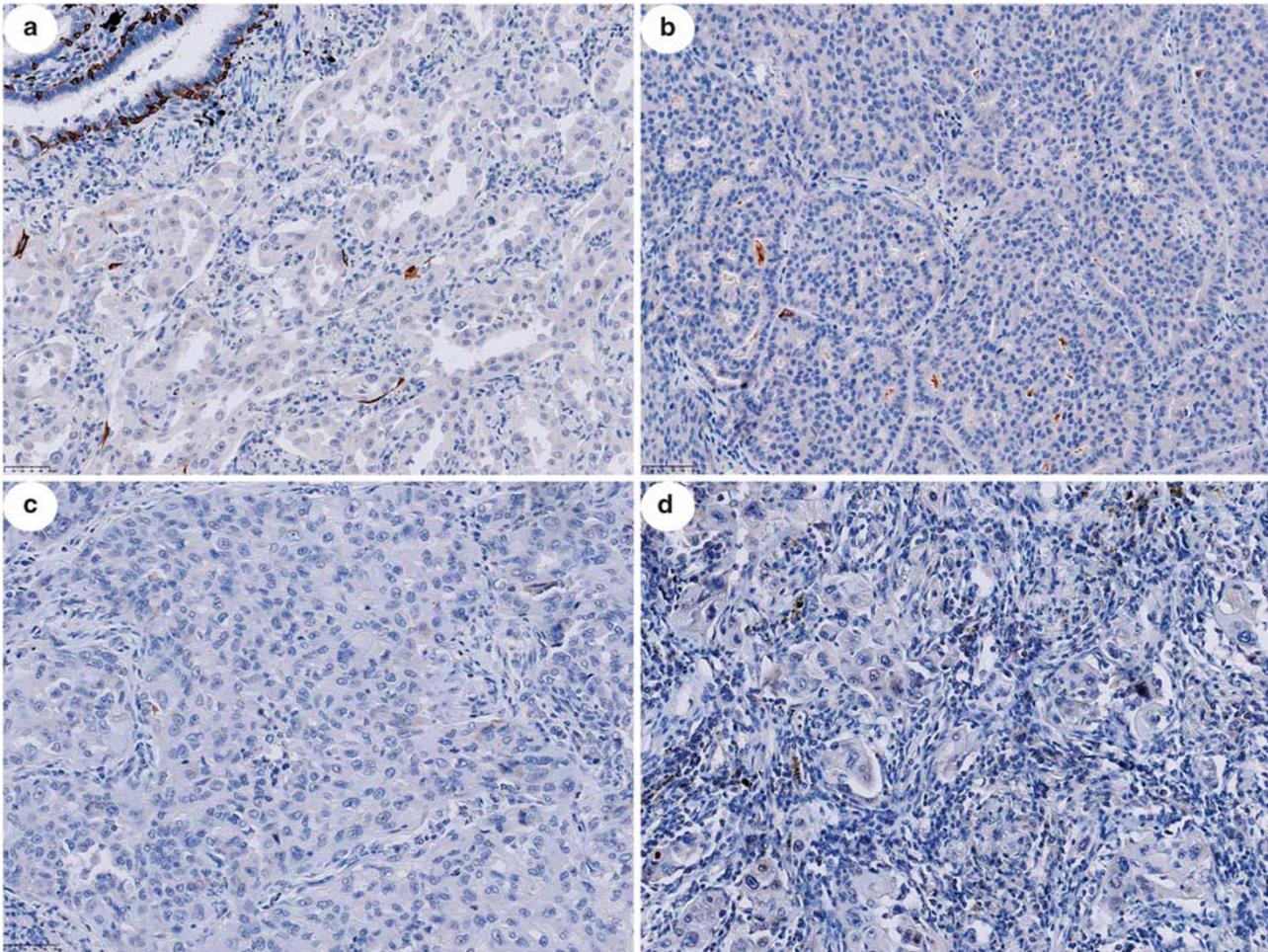
### Calretinin, D2-40, and WT1

Calretinin, D2-40, and WT1 are positive mesothelial cell markers showing nuclear and cytoplasmic positivity

with calretinin, membranous, and cytoplasmic reactivity with D2-40 and nuclear positivity with WT1 in epithelioid mesothelioma. Calretinin was also positive in adipose tissue, D2-40 in lymphatic vessels, and WT1 in blood vessels as positive internal controls, which were considered internal positive controls. Calretinin expression was present in 81 cases (99%) of epithelioid mesothelioma, 71 of these showing the immunohistochemical score of 3+. However, calretinin expression was also present in 18 of 97 (19%) cases of lung adenocarcinoma, with 7 cases showing the immunohistochemical score of 2+ and 11 cases showing the immunohistochemical score of 1+. D2-40 expression was present in 79 cases (96%) of epithelioid mesothelioma, with 64 of these showing the immunohistochemical score of 3+ and 8 cases showing the immunohistochemical score of 2+. D2-40 expression was also present in 7 of 97 (7%) cases of lung adenocarcinoma, with 2 cases showing the immunohistochemical score of 2+ and 5 cases showing the immunohistochemical score of 1+. WT1 expression was present in only 65 cases (79%) of epithelioid mesothelioma, 40 of these showing the immunohistochemical score of 3+, 7 cases showing the immunohistochemical score of 2+, and 18 cases showing the immunohistochemical score of only 1+. WT1 expression was not present in any of 97 cases of lung adenocarcinoma.

### CEA, Claudin 4, TTF-1, and Napsin-A

CEA, claudin 4, TTF-1, and napsin-A expressions were absent from all 82 epithelioid mesotheliomas. CEA and claudin 4 were present in 95 cases (97%) and 93 cases (96%) of lung adenocarcinoma, respectively. The majority of lung adenocarcinomas showed the immunohistochemical score of 3+ for CEA and claudin 4. TTF-1 and napsin-A were present in 86 (89) and 81 (84%) cases of lung adenocarcinoma with 73 and 67 cases showing the immunohistochemical score of 3+, respectively.



**Figure 2** Glypican-1 expression was not detected in lung adenocarcinomas, including papillary (a), acinar (b), solid (c), and discohesive (d). Glypican-1 expression was present in basal cells of the bronchiole (a) and scattered non-neoplastic basal cells (b), as internal positive controls.

**Table 3** Sensitivity, specificity, and accuracy rate of immunohistochemical markers for differentiation of epithelioid mesothelioma from lung adenocarcinoma

Immunohistochemical markers	Sensitivity (%)	Specificity (%)	Accuracy rate (%)
Glypican-1 (+)	100	97	98
Calretinin (+)	99	81	89
D2-40 (+)	97	93	94
WT1 (+)	86	100	91
CEA (-)	100	98	99
Claudin 4 (-)	100	96	98
TTF-1 (-)	100	89	94
Napsin-A (-)	100	84	91

Abbreviations: CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1; WT1, Wilm's tumor 1.

### Sensitivity, Specificity, and the Accuracy Rate of Immunohistochemical Markers

Table 3 shows the sensitivities, specificities, and accuracy rates of the immunohistochemical markers in differentiating epithelioid mesothelioma from

lung adenocarcinoma. Positive expression of glypican-1 showed a sensitivity of 100%, specificity of 97%, and an accuracy of 98%. Among the positive mesothelioma markers, positive expression of WT1 showed the highest specificity of 100%, but its sensitivity was limited to 79%. The sensitivities of positive expression of calretinin and D2-40 were high, at 99 and 96%, respectively, but the specificities were limited to 81% and 93%. Negative expression of CEA, claudin 4, TTF-1, and napsin-A, showed sensitivities of 100% and specificities of 98%, 96%, 89%, and 84%, respectively.

### Discussion

The International Mesothelioma Interest Group guideline has recommended a panel of positive markers of epithelioid mesothelioma, but precaution is needed when interpreting calretinin and D2-40 due to their low specificity and WT1 for its low sensitivity. To improve the accuracy of the diagnosis of epithelioid mesothelioma, we recently reported two positive

immunohistochemical markers of epithelioid mesothelioma, intelectin-1 and DAB2, identified by differential gene expression analysis of epithelioid mesothelioma and lung adenocarcinoma.<sup>5</sup> Although these two new immunohistochemical markers showed high specificity, their sensitivities were not high enough, leading us to search for additional immunohistochemical markers.

Glypican-1 is one of the six glycosylphosphatidylinositol-anchored, cell-surface heparan sulfate proteoglycans that play vital roles in growth factor signaling.<sup>8</sup> Among the six different glypicans, only glypican-3 expression has been studied in mesothelioma, and it is largely silenced due to hypermethylation of its promoter region.<sup>9</sup> There is no available study of glypican-1 in malignant mesothelioma. Increased expression of glypican-1 is reported in breast cancer,<sup>10</sup> pancreatic cancer,<sup>11</sup> esophageal squamous cell carcinoma,<sup>12</sup> and gliomas.<sup>13</sup> However, these reports did not have detailed expression patterns. In the Human Protein Atlas, which shows the expression and localization of human proteins across tissues and organs, based on deep sequencing of RNA (RNA-seq) from 37 major different normal tissue types and immunohistochemistry on tissue microarrays containing 44 different tissue types,<sup>14</sup> most of the human malignancies showed decreased expression of glypican-1 (<https://www.proteinatlas.org/search/GPC1>). Mesothelioma and mesothelial tissues are not included in this database. In the present study, we first investigated glypican-1 expression in epithelioid mesothelioma by immunohistochemistry and were surprised to find its expression in epithelioid mesotheliomas irrespective of their histological heterogeneity. It was also expressed in non-neoplastic mesothelial cells to some extent. These findings prompted us to study the possibility of glypican-1 immunohistochemistry as a novel positive mesothelioma marker. We still do not know the detailed reason of glypican-1 expression in mesothelial cells, but we suppose that the expression of glypican-1 in the non-neoplastic mesothelial cell can be considered valuable for its application as a positive mesothelioma marker. In peripheral lung tissue, glypican-1 was expressed in basal cells of bronchial tissue, and we utilized this as an internal positive control in lung adenocarcinoma cases. The biology of glypican-1 expression was not fully understood except its probable role in development of the various tissue. Glypican-1 expression was related to promote angiogenesis, metastasis, cell division, and perineural invasion of pancreatic cancer cells<sup>15</sup> and has been reported as possible prognostic marker of pancreatic cancer.<sup>16</sup> Recently, Melo *et al.*<sup>17</sup> reported the presence of glypican-1 in exosomes in the circulation of pancreatic cancer patients, suggesting the possibility of glypican-1 as a candidate for detection of exosomes for genetic and proteomic analysis in the early pancreatic cancer. Harada *et al.*<sup>18</sup> reported the increased expression of glypican-1 in esophageal squamous cell carcinoma

and anti-glypican-1 mAb as a therapeutic strategy to decrease tumor growth in patients with glypican-1-positive esophageal squamous cell carcinoma. Further biological and functional analysis are needed to draw conclusions about its importance in malignant mesothelioma. In this study, we did not yet include sarcomatoid mesothelioma or various other human carcinomas and sarcoma. However, we are planning to study the glypican-1 expression pattern in various normal tissues and cancerous tissues of various organs and its functional significance in mesothelioma in the near future.

In conclusion, glypican-1 is a novel positive marker of epithelioid mesothelioma with better sensitivity and specificity than calretinin, D2-40, and WT1 for differentiation from lung adenocarcinoma. Further analysis is necessary to fully understand glypican-1 expression in mesothelioma, including sarcomatoid mesothelioma and other malignancies. We recommend including glypican-1 immunohistochemistry as a positive immunohistochemical marker for differential diagnosis of epithelioid mesothelioma from lung adenocarcinoma.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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