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Tumor Markers and Signatures

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N-cadherin: A diagnostic marker to help discriminate primary liver carcinomas from extrahepatic carcinomas

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Abstract

Distinguishing primary liver cancer (PLC), namely hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), from liver metastases is of crucial clinical importance. Histopathology remains the gold standard, but differential diagnosis may be challenging. While absent in most epithelial, the expression of the adherens junction glycoprotein N-cadherin is commonly restricted to neural and mesenchymal cells, or carcinoma cells that undergo the phenomenon of epithelial-to-mesenchymal transition (EMT). However, we recently established N- and E-cadherin expression as hallmarks of normal hepatocytes and cholangiocytes, which are also preserved in HCC and iCCA. Therefore, we hypothesized that E- and/or N-cadherin may distinguish between carcinoma derived from the liver vs carcinoma of other origins. We comprehensively evaluated E- and N-cadherin in 3359 different tumors in a multicenter study using immunohistochemistry and compared our results with previously published 882 cases of PLC, including 570 HCC and 312 iCCA. Most carcinomas showed strong positivity for E-cadherin. Strong N-cadherin positivity was present in HCC and iCCA. However, except for clear cell renal cell carcinoma (23.6% of cases) and thyroid cancer (29.2%), N-cadherin was only in some instances faintly expressed in adenocarcinomas of the gastrointestinal tract (0%–0.5%), lung (7.1%), pancreas (3.9%), gynecological organs (0%–7.4%), breast (2.2%) as well as in urothelial (9.4%) and squamous cell carcinoma (0%–5.6%). As expected, N-cadherin was detected in neuroendocrine tumors (25%–75%), malignant melanoma (46.2%) and malignant mesothelioma (41%). In conclusion, N-cadherin is a useful marker for the distinction of PLC vs liver metastases of extrahepatic carcinomas ($P < .01$).

KEYWORDS
adherens junctions, cadherin, carcinoma of unknown primary, immunohistochemistry, primary liver cancer

What's new?

A diagnostic marker capable of distinguishing primary liver cancer from liver metastases of extrahepatic origin remains elusive. Of particular interest as a marker, however, is N-cadherin, an adherens junction transmembrane glycoprotein normally expressed by hepatocytes and cholangiocytes. Here, the authors investigated N-cadherin expression as a possible marker for distinguishing liver-derived carcinoma from carcinoma of other origins. Immunohistochemical analysis reveals strong N-cadherin expression in hepatocellular carcinoma and intrahepatic cholangiocarcinoma, with faint expression in adenocarcinomas in extrahepatic tissues. The findings suggest that N-cadherin is a sensitive and specific marker capable of discriminating primary liver carcinoma from liver metastases of extrahepatic origin.

1 | INTRODUCTION

Primary liver cancer (PLC) is the fourth leading cause of cancer death and the fifth or eighth most common type of cancer worldwide in men or women, respectively.[1,2] Hepatocellular carcinoma (HCC) accounts for 80% and intrahepatic cholangiocarcinoma (iCCA) for 15% of PLC.[3] Synchronous liver metastases are reported in 5% of cancer patients without underlying liver disease or liver cirrhosis.[4] Metastases typically occur late during the course of the disease and thus may frequently remain unreported. With this respect, an autopsy-based study detected liver metastases in up to 36% of cancer patients.[5] Although radiologic diagnosis is essential for the assessment of liver masses, the gold standard of tumor diagnosis remains histopathology, including immunohistochemistry for lineage differentiation. From morphology alone, HCC and iCCA may not be reliably discriminated from liver metastases. Concerning HCC, specific immunohistochemical markers such as HepPar1, Arginase 1 and Alpha-fetoprotein (AFP) and also in addition albumin in situ hybridization are available, yet, these markers may be negative in advanced HCC. In addition, liver metastases of extrahepatic primaries such as gastric carcinomas may stain positive for HepPar1 too.[6] For pancreatobiliary carcinomas, CA19-9 represents a rather unspecific marker, as it is commonly expressed also in diverse other cancers.

Cell–cell contacts are not only essential for embryogenesis and organogenesis of multicellular organisms but also for maintaining structure and organ-specific functions.[7] In addition, cell–cell contacts play a major role in tumorigenesis, especially in the invasion and metastasis of malignant tumors.[8,9] Besides gap junctions that enable cell communication and tight junctions that seal membranes to provide barrier functions, adhering junctions, namely desmosomes and adherens junctions, mechanically connect cells.[10] Adherens junctions contain a Ca^{2+}-dependent transmembrane domain formed by cell-type specific proteins of the cadherin family that interact on the cytoplasmic side with the specific plaque proteins α- and β-catenin as well as plakoglobin and mediate the connection to the actin cytoskeleton.[7,11] The expression of E- and N-cadherin is closely intertwined with embryological development. Cadherins play a crucial role in cell adhesion, cell sorting, segregation, and polarity establishment in complex epithelial cell assemblies and tissue morphogenesis. Whereas E-cadherin is characteristic of epithelia, N-cadherin has been described in mesenchymal and neural cells and is vital for the development of mesodermal and neuroectodermal structures, as well as the formation of neural tissues.[12,13] During gastrulation and neural crest delamination, cell sorting is orchestrated by cadherin switches, for example, from E-cadherin to N-cadherin, a highly evolutionarily conserved process referred to as epithelial-to-mesenchymal transition (EMT).[14] Moreover, changes in the expression pattern of adherens junction proteins have been implicated in the development and progression of malignant tumors. According to the literature, EMT, which is characterized by loss of E-cadherin expression and increased expression of N-cadherin, has been correlated with tumor invasion and metastasis.[8,15] In previous work, we showed that E- and N-cadherin form cis-E:N-cadherin heterodimers in adherens junctions at the basolateral membrane of hepatocytes and cholangiocytes,[16] and that E- and N-cadherin-expression is retained in iCCA and HCC.[17,18] In fact, regardless of tumor grading, HCC show high N-cadherin expression in 92% of cases, and co-expression of E-cadherin.[17,18] Interestingly, N-cadherin expression gradually declines from HCC and small duct iCCA to large duct iCCA, carcinomas of the extrahepatic bile ducts including perihilar cholangiocarcinomas (pCCAs), and gallbladder carcinomas (GBCs) to pancreatic ductal adenocarcinomas (PDACs). Thus, N-cadherin immunohistochemistry could be used to distinguish between iCCA and PDAC.[17,19–21] Furthermore, we hypothesized that E- and/or N-cadherin may serve as a valuable marker in differentiating PLC from liver metastases originating from an extrahepatic primary tumor.

Immunohistochemistry is a useful tool to analyze E- and N-cadherin expression, which has been vastly studied in the context of EMT and in different carcinomas (Table S1). However, immunohistochemical scoring varies widely between previously performed studies. For instance, the definition of N-cadherin positivity ranges from any staining reaction[22] or to a threshold of 1%–5% to 20% of stained tumor cells.[23] Although cadherins are transmembrane proteins, immunohistochemical scoring was often based on cytoplasmic or nuclear staining patterns in the absence of membranous staining, although the

significance of nuclear or cytoplasmic staining has not been plainly elucidated.[7,11]

The strong potential as a diagnostic marker urges the need for a systematic re-evaluation of N-cadherin expression in carcinoma of hepatic and extrahepatic origin using a uniform and stringent immunohistochemical scoring system. The objective of the present multicenter study, therefore, was to comprehensively compare E- and N-cadherin expression in PLC to various extrahepatic carcinomas. Our approach aims to streamline the routine use of E- and N-cadherin immunohistochemistry and to implement a uniform scoring system to assist pathologists in their differential diagnostic considerations.

2 | MATERIALS AND METHODS

2.1 | FFPE and cryopreserved human tissues

Paraffin-embedded formalin-fixed (FFPE) tissue samples of patients diagnosed at the Institutes of Pathology, University Medical Center Mainz, between 2006 and 2020 and the University of Heidelberg were collected. Tissue microarrays (TMA) were constructed in accordance with the regulations of the Tissue Biobank of the University Medical Center Mainz and the National Center for Tumor Diseases Heidelberg, respectively. The patients were fully informed about the surgical procedures, including the intended research purposes, and provided informed consent for the utilization of materials for research. Besides primary tumors of diverse carcinomas, liver metastases of extrapulmonary neuroendocrine tumors and carcinomas (NET and NEC; $n = 47$) and of colorectal adenocarcinomas (CRC, $n = 204$, each with their primaries) were analyzed.[24] Histologically representative tumor areas were identified, and cores (diameter 1–2 mm) were punched out and embedded in the final TMAs. Immunohistochemistry (IHC) was performed on 4-µm thick histological sections of the TMAs. Cores that did not contain tumor tissue were excluded. In addition, cryopreserved normal human stomach, kidney, colon, thymus, and pancreas tissues, as well as their respective tumors, were used in cooperation with the 2nd Institute of Pathology, Semmelweis-University Budapest.

2.2 | Antibodies

Mouse monoclonal antibodies were against E-cadherin (Clone 36, Bioscientia Healthcare GmbH, Ingelheim, Germany) and N-cadherin (Clone 32, Bioscientia Healthcare GmbH, Ingelheim, Germany). Rabbit antibodies were against E-cadherin (clone EP700Y, monoclonal, from Merck Millipore, Burlington, MA) as well as N-cadherin (clone 42031, polyclonal, from Sigma-Aldrich, St. Louis, MO).

2.3 | Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described before.[16] Cryosections were cut to a thickness of 5 µm, air dried for 1 h, and fixed with acetone at −20°C for 10 min. After permeabilization for 4 min in 0.1% Triton-X-100 and two washing steps in phosphate-buffered saline (PBS), the primary antibodies E- and N-cadherin were applied for 45 min, which was followed by two washing steps for 5 min in PBS and 30 min incubation with the respective secondary antibodies (cy 3, rabbit, Dianova; Alexa 488 anti-mouse, MoBiTec GmbH, Göttingen, Germany) in a humidity chamber. Subsequently, after two washing steps for 5 min in PBS and a short washing step in distilled water, the slides were dehydrated with 100% ethanol for 5 min and mounted with a DAPI-containing embedding medium. A confocal laser scanning immunofluorescence microscope (LSM 510 Meta; Carl Zeiss AG, Oberkochen, Germany) equipped with Plan Apochromat ×63/1.40 NA oil and Plan Neofluar ×40/1.30 NA oil objectives was used. AxioVision release 4.6.3.0 software, and LSM Image browser 3.2.0.115 (Carl Zeiss AG) were used for image processing.

2.4 | Semiquantitative evaluation and image analysis

Slides were digitalized by a whole slide scanner at ×40, with a pixel size of 0.2278 × 0.2278 µm (Nanozoomer, Hamamatsu Photonics, Japan). E- and N-cadherin IHC stains were manually evaluated by scoring the intensity of membranous staining from 0 to 3 (no, faint, moderate to strong staining). We assigned points in 0.5 increments and, if there were several scores for the same entity, calculated the average. A rating of 1.5 was exclusively attributed to full circumferential membrane staining in 10% or more tumor cells, irrespective of intensity. Further score increases were contingent upon staining intensity. Tumors with a mean manual score of ≥1.5 were assigned to the E- or N-cadherin high group. Primary tumors, lymph node metastases, and distant metastases were each separately evaluated. This method, which we have applied in previous studies[17,18] has proven to reflect the most precise gradation of staining intensity for E- and N-cadherin in our experience. Sensitivity and specificity were calculated using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA). To evaluate the discrimination of the dichotomous variables (high vs low N-cadherin expression), we used McNemar's chi-squared test.[25] This analysis was performed using IBM SPSS Statistics Version 27 (IBM Corp., Armonk, NY). A P-value ≤.05 was considered statistically significant.

3 | RESULTS

3.1 | E-cadherin is a universally, N-cadherin a differentially expressed marker for tumors of epithelial lineage

We previously demonstrated that the co-expression of E- and N-cadherin is a characteristic of hepatocytes, cholangiocytes, and their derived tumors.[17,18] To test the potential of E- and N-cadherin

TABLE 1 Immunohistochemical E- and N-cadherin expression in carcinomas.

System	Organ	Entity	*n*	High E-cadherin (%)	High N-cadherin (%)	Specificity[a] (%)
Digestive system	Liver	PLC	882	97.3	81.3	
		HCC[b]	570	96.4	92.6	
		iCCA, small duct type[c]	196	99.0	67.0	
		iCCA, large duct type[c]	116	99.1	50.3	
	Biliary	pCCA[c]	328	99.1	17.6	82.5
		GBC[c]	228	97.3	12.7	87.3
		dCCA[c]	185	100.0	8.7	91.3
	Esophagus	SCC	3	100.0	0.0	100.0
		AEG	3	100.0	0.0	100.0
	Stomach	Adenocarcinoma	8	87.5	0.0	100.0
	Intestine	Ampulla of Vater	2	100.0	0.0	100.0
		CRC	204	99.0	0.5	99.5
	Pancreas	PDAC[c]	131	100.0	3.9	96.2
Thoracic	Lung	NSCLC	47	97.9	6.5	93.5
		Adenocarcinoma	28	96.4	7.1	92.9
		SCC	19	100.0	5.6	94.4
Breast	Breast	Overall	240	89.6	2.2	97.8
		IBC-NST	199	97.5	2.7	97.3
		IBC-LC	23	17.4	0.0	100.0
		Medullary carcinoma	3	100.0	0.0	100.0
		Mucinous carcinoma	4	100.0	0.0	100.0
		Mixed carcinoma	12	91.7	0.0	100.0
Female genital tract	Tubo-ovarian	Overall	134	88.8	6.8	93.2
		HGSC	83	89.2	7.4	92.6
		LGSC	4	100.0	0.0	100.0
		Borderline tumor	14	100.0	7.7	92.3
		Mucinous carcinoma	13	76.9	0.0	100.0
		Clear cell carcinoma	4	75.0	0.0	100.0
		Malignant Brenner tumor	4	100.0	0.0	100.0
	Cervix/vulva	SCC	123	100.0	0.8	99.2
	Uterus	Overall	8	100.0	0.0	100.0
		Endometrioid carcinoma	4	100.0	0.0	100.0
		Serous carcinoma	4	100.0	0.0	100.0
Head and neck	Oropharynx	SCC	177	100.0	2.3	97.7
	Salivary	ACC	4	75.0	0.0	100.0
Urinary system	Kidney	Overall	296	33.4	23.6	76.4
		ccRCC	288	32.3	23.6	76.4
		pRCC	4	50.0	50.0	50.0
		chRCC	4	100.0	0.0	100.0
	Bladder	UCA	32	96.9	9.4	90.6
Male genital tract	Prostate	PCA	35	100.0	2.8	97.2

Abbreviations: ACC, adenoid cystic carcinoma; AEG, adenocarcinomas of the esophagogastric junction; ccRCC, clear cell renal carcinoma; chRCC, chromophobe renal cell carcinoma; CRC, colorectal adenocarcinoma; GBC, gallbladder carcinoma; HCC, hepatocellular carcinoma; HGSC, high-grade serous carcinoma of the ovary; IBC-NST, invasive breast carcinoma of no special type; iCCA, intrahepatic cholangiocarcinoma; ILC, invasive lobular carcinoma; LGSC, low-grade serous carcinoma of the ovary; NSCLC, non-small-cell lung cancer; PCA, prostatic acinar adenocarcinoma; pCCA, perihilar cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma; PLC, primary liver cancer; pRCC, papillary renal cell carcinoma; SCC, squamous cell carcinoma; UCA, urothelial carcinoma.

[a]Specificity of N-cadherin compared to primary liver cancer overall.

[b]Data were previously published (Gerber et al. 2022).[18]

[c]Data were previously published (Gerber et al.,2022).[17]

TABLE 2 E- and N-cadherin expression in epithelioid tumors including germ cell and (neuro-) endocrine tumors.

System	Organ	Entity	*n*	High E-cadherin (%)	High N-cadherin (%)	Specificity[a] (%)
Endocrine/neuro-endocrine	Thyroid	Overall	65	75.4	29.5	70.5
		WDTC	23	100.0	21.1	78.9
		PTC	20	100.0	18.8	81.3
		FTC	3	100.0	33.3	66.7
		PDTC	27	88.9	48.1	51.9
		ATC	15	13.3	6.7	93.3
	Lung	SCLC	4	50.0	75.0	25.0
	Intestinal	NET G1	17	100.0	35.3	64.7
		NET G2	15	100.0	60.0	40.0
		NET/NEC G3	4	100.0	25.0	75.0
	Pancreas	NET/NEC	11	90.9	72.7	27.3
Neuroectodermal	Skin/mucosa	Melanoma	26	65.4	46.2	53.9
Mesothelial	Mesothelium	Mesothelioma	131	67.9	41.0	59.0
Male genital tract	Testis	Overall	12	50.0	16.7	83.3
		Seminoma	4	25.0	50.0	50.0
		Non-seminoma	8	62.5	0.0	100.0
Female genital tract	Ovary	Granulosa cell tumor	4	25.0	0.0	100.0

Abbreviations: ATC, anaplastic thyroid carcinoma; FTC, follicular thyroid carcinoma; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumor; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; SCLC, small cell lung carcinoma; WDTC, well-differentiated thyroid carcinoma.
[a]Specificity of N-cadherin compared to overall primary liver cancers.

as markers in the differential diagnosis of PLC vs extrahepatic carcinomas, their expression was assessed in a total of 13,295 individual TMA cores comprised of 3359 different tumors (Tables 1 and 2), and compared to previously published analyses of 882 cases of PLC, including HCC and iCCA.[17,18] In a first step, we investigated E-cadherin (Figure S1) and N-cadherin (Figure 1) expression via immunohistochemistry (Tables 1, S2, and S3). Membranous staining intensity was relatively quantified from 0 to 3 (no, faint, moderate to strong staining). Isolated cytoplasmic or nuclear E- or N-cadherin staining was not evaluated due to faint staining. To establish a cut-off for positive staining, a mean score of 1.5 was determined, as it effectively differentiated negative or insignificant staining from clearly positive staining (compare Tables 1, S2, and S3). Additionally, expression of E- and N-cadherin in each tumor was also correlated to normal human tissues from which the tumor originated. In the next step, we used double-label laser scanning immunofluorescence microscopy on tissue specimens to specify the subcellular localization of E- and N-cadherin at adherens junctions and evaluate their possible involvement in tumor-stroma interaction (Figure 2). In all analyzed samples, the tumor stroma was positive for N-cadherin, but not E-cadherin.

E- and N-cadherin expression was found in the majority (97.3% and 81.3%, respectively) of PLC cases in a homogenous membranous staining pattern (Table 1). As expected, immunofluorescence microscopy revealed complete colocalization of E- and N-cadherin at the basolateral cell membranes in normal hepatocytes, cells of hepatocellular adenoma (HCA) and HCC, as well as bile duct epithelial cells and cells of iCCA (Figures 1A, B, and 2A–A‴). Perihilar cholangiocarcinoma showed only faint N-cadherin positivity (Figure 1C). With regards to extrahepatic tissues, E-cadherin was consistently positive in all carcinomas with the exception of lobular invasive carcinomas of the breast and diffuse gastric cancer as well as in their respective normal epithelia (Figure S1), whereas N-cadherin was mostly negative or faintly expressed. Specifically, we found colocalization of E- and N-cadherin in the pancreas, larger pancreatic ducts, and some endocrine cells of pancreatic islets and their derived endocrine pancreas tumors, whereas PDAC were mostly negative or only faintly positive for N-cadherin in few cases (Figures 1D and 2B–B″).[14] In the stomach, overall faint N-cadherin staining was observed in both normal stomach mucosa and gastric cancer, which remained under the established threshold for positive staining (Figure 2C–C′, Table 1). In normal epithelium of the colon, colorectal adenocarcinomas (CRC; Figure 2D–D′) as well as CRC liver metastases (Figure 1E), no significant N-cadherin expression was observed. Amongst lung carcinomas, N-cadherin expression was detected in 6.5% of non-small cell lung cancers (NSCLCs), including 7.1% of adenocarcinomas (Figures 1F and 2F) and 5.6% of squamous cell carcinomas (SCCs). In addition, N-cadherin staining was absent in SCCs of the cervix, vulva (Figure 1I), thymus (Figure 2H″), and oropharynx (Table 1). Moderate N-cadherin expression was found in 2.2% of breast carcinomas (Figure 1G) and 6.8% of tubo-ovarian carcinomas, with higher levels observed in high-grade serous carcinomas (HGSCs) at 7.4% (Figure 1H). In contrast, only isolated cases of prostatic acinar adenocarcinomas (PCAs) exhibited relevant, albeit faint, N-cadherin staining (Figure 1L). In renal cell carcinomas (RCCs), high N-cadherin

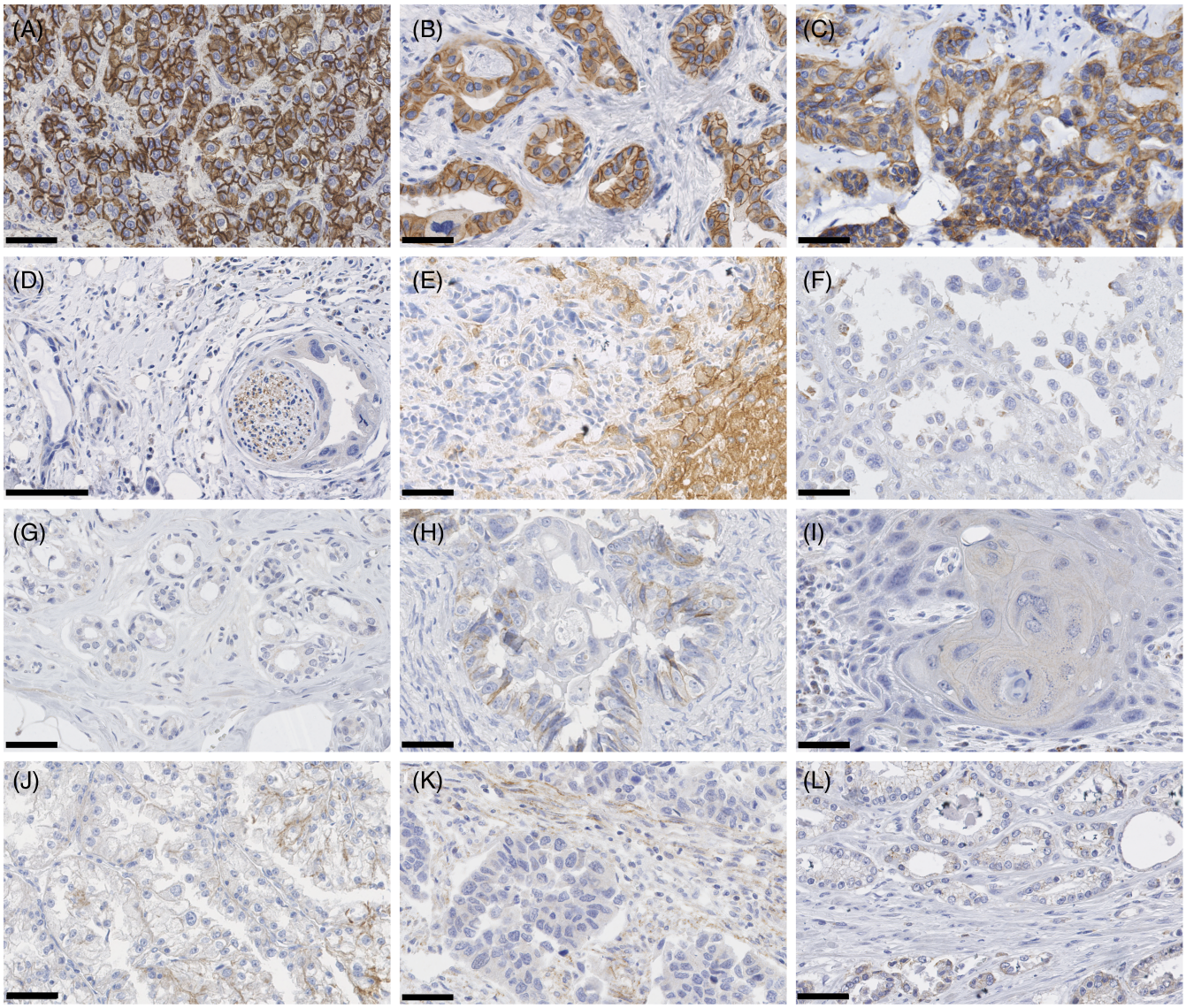

FIGURE 1 Immunohistochemical analysis of N-cadherin expression in carcinomas. N-cadherin is positive in hepatocellular carcinoma (HCC; A) and intrahepatic cholangiocarcinoma (iCCA), small duct type (B), whereas perihilar cholangiocarcinomas (pCCAs) are only faintly positive (C) while other gastrointestinal carcinomas such as pancreatic ductal adenocarcinomas (PDACs; D; with infiltration of an N-cadherin-positive nerve) and colorectal carcinomas (CRCs; E; colorectal liver metastasis with adjacent N-cadherin positive hepatocytes on the right side) are negative, as are adenocarcinomas of the lung (F), invasive breast carcinomas of no special type (IBCs-NST; G), high-grade serous carcinomas of the ovary (HGSCs; H) and vulvar squamous cell carcinomas (vulvar SCCs; I). Clear cell renal cell carcinomas (ccRCCs) may show a moderate to strong staining reaction to N-cadherin (J; shown focally in the bottom right). Other urogenital carcinomas such as urothelial carcinomas (UCAs; K; note N-cadherin-positive stromal cells) and acinar adenocarcinomas of the prostate (PCAs; L) are usually negative. Bars: 50 µm (A–C, E–L) and 100 µm (D). Semiquantitative N-cadherin scores: (A) 3, (B) 3, (C) 2.5, (D) 0, (E) 1, (F) 0.5, (G) 0, (H) 1, (I) 0, (J) 1, (K) 0, (L) 0.5.

expression was observed in 23.6% (Figures 1J and 2E′), compared to urothelial carcinomas (UCAs) with 9.4% (Figure 1K). Using immunofluorescence microscopy, proximal tubules of the human kidney exhibited positive N-cadherin staining (Figure 2E) as did respective clear cell RCCs (ccRCC; Figure 2E′). E-cadherin was expressed at high levels in the majority (97.3%) of PLC cases as in other carcinomas of whatever primary.

To summarize, N-cadherin exhibited characteristic positivity in PLC as well as ccRCC, while other carcinomas showed either negativity or only low levels of N-cadherin expression.

3.2 | E- and N-cadherin are both found in epithelioid tumors

Since not only classical adenocarcinomas and squamous cell carcinomas, but also neuroendocrine tumors, and epithelioid tumors such as malignant mesothelioma and melanoma have the capacity to metastasize to the liver and therefore need to be taken into account, we aimed to comprehensively analyze E- and N-cadherin in malignant melanoma, mesothelioma and endocrine as well as neuroendocrine tumors (Figure 3, Table 2), which were expected to express both

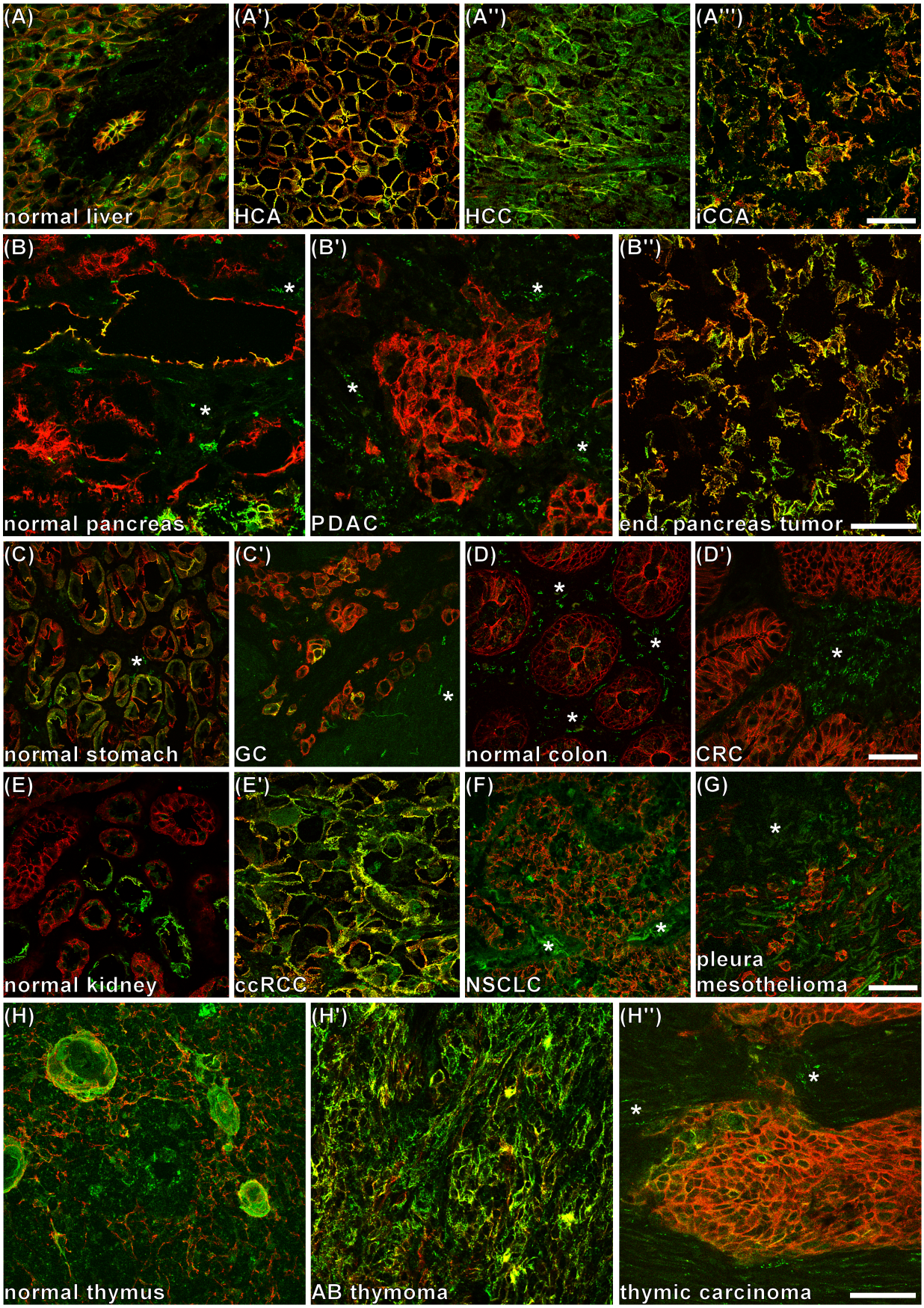

FIGURE 2 Legend on next page.

N-cadherin and E-cadherin.[13] Besides PLC and ccRCC, we observed strong E- and N-cadherin expression in the endoderm-derived thyroid carcinomas (Figure 3A–A′). Interestingly, also non-neoplastic thyroid tissue, that is, multinodular goiter showed high expression of N-cadherin in 10.9% of cases, while N-cadherin was expressed in 21.1% of well-differentiated thyroid carcinomas (WDTCs) and 48.1% of poorly differentiated thyroid carcinomas (PDTCs). In contrast, anaplastic thyroid carcinomas (ATCs) rarely showed high expression of N-cadherin (6.7%). In addition, high E- and N-cadherin positivity was noted in neuroendocrine tumors (NETs) and carcinomas (NECs), for example, in small cell lung cancer (SCLC, Figure 3B–B′; NET, Figure 3C–C′) and malignant melanomas (Figure 3D–D′), malignant mesotheliomas (Figures 2G and 3E–E′) and germ cell tumors. Concerning thymus, in immunofluorescence microscopy, differential expression of E- and N-cadherin was also observed in normal thymus (Figure 2H), with distinct patterns noted in lymphocytes and the epithelial Hassall bodies. Similar expression patterns were also observed in thymoma (Figure 2H′). SCC of the thymus behaved like SCCs of other origins with positive staining for E-cadherin, but not N-cadherin (Figure 2H″).

To summarize, epithelioid tumors such as (neuro)endocrine tumors, malignant melanoma, malignant mesothelioma, germ cell tumors, and thymoma typically exhibit staining for both E- and N-cadherin. Therefore, the differential diagnosis of PLC and liver metastasis of epithelioid tumors would require additional markers for cell lineage tracking beyond cadherin staining.

Overall, N-cadherin demonstrates strong potential in distinguishing PLC and liver metastases of extrahepatic carcinomas, exhibiting a specificity of 89.2% ($P < .01$). The sensitivity of N-cadherin in correctly identifying PLC was 81.3%.

4 | DISCUSSION

With our study, we propose N-cadherin as a marker to differentiate PLC from liver metastases of an extrahepatic primary. Furthermore, our findings demonstrate that E-cadherin reliably marks cells and derived tumors of epithelial lineage. Our data confirm that N-cadherin is not absent from epithelia and epithelial tumors as it has been widely postulated, but that it is in fact a hallmark of certain epithelia, most prominently hepatocytes, cholangiocytes and proximal tubular epithelia of the kidney as well as their derived tumors. Our finding that E- and N-cadherin expression in normal cells and their derived tumors were correlated suggests that cadherins are relatively stable differentiation markers, which are preserved in tumorigenesis. Moreover, primary cells and tumors of neuroectodermal or mesenchymal origin consistently retain N-cadherin staining.

Hepatocytes and cholangiocytes both emerge from hepatoblasts. These bipotential progenitors develop from the endodermal-derived liver bud of the ventral gut, which is positive for E- but not for N-cadherin (ie, up to E9.5 of mouse development). In further development (from E10.5 onwards), however, there is a gradual increase in positivity towards N-cadherin, depending on the localization within the liver lobule.[26] The maturation of hepatoblasts into hepatocytes and the biliary epithelium begins in E13.5 of mouse development.[27] Besides PLC, ccRCC showed the second-highest N-cadherin expression among carcinomas. ccRCC are adenocarcinomas derived from proximal tubular cells.[28] The proximal tubule itself originates from the mesoderm-derived metanephros by *NOTCH2*-mediated mesenchymal to epithelial differentiation,[29] which could explain preserved N-cadherin-positivity. Other entities also show N-cadherin positivity depending on their tissue of origin.[30] For example, neuroectoderm-derived malignant melanomas, as well as mesenchyme-derived mesotheliomas, show high N-cadherin expression.[31–33] For the thyroid gland, endodermal origin has been postulated and thyroid carcinomas as well as normal thyroid gland were shown positive for both E- and N-cadherin. In the same line, also in clear cell renal cell carcinoma originating from the E- and N-cadherin-positive proximal tubular epithelium, coexpression of E- and N-cadherin is noted. Neuroendocrine tumors may express other markers such as synaptophysin and chromogranin A that may be used to determine their origin.[34,35]

When evaluating immunohistochemical staining reactions against cadherins, pathologists should be aware of certain pitfalls. First, a dot-like N-cadherin staining in mesenchymal cells of desmoplastic stroma should not be mistaken for tumor positivity. In cases of N-cadherin

FIGURE 2 Expression of E- and N-cadherin in malignant tumors recapitulate their expression in their normal tissue counterparts. Double-label laser scanning immunofluorescence microscopy of E-cadherin (red) and N-cadherin (green). Colocalization of E- and N-cadherin is detected in hepatocytes and biliary epithelial cells in normal human liver (A), in hepatocellular adenoma (HCA, A′), hepatocellular carcinoma (HCC, A″) and intrahepatic cholangiocarcinoma (iCCA, A‴). Colocalization of E- and N-cadherin is observed in larger pancreatic ducts, whereas acini are positive for E-cadherin, but not N-cadherin (B), and pancreatic islet cells show expression of either E-cadherin or N-cadherin. Ductal adenocarcinomas of the pancreas display exclusive positivity for E-cadherin and no significant N-cadherin expression. (B′). In endocrine tumors of the pancreas (B″), both E- and N-cadherin are detected. In normal stomach corpus mucosa, E-cadherin is positive, whereas N-cadherin is faintly expressed in parietal cells (C). Only faint N-cadherin and strong E-cadherin expression is seen in gastric cancer (C′). Positivity for E-cadherin in normal colon crypts and colon adenocarcinoma, with N-cadherin negativity (D–D′). Differential E- and N-cadherin expression in tubules of the normal kidney are seen with colocalization of E- and N-cadherin in proximal tubules and E-cadherin positivity but N-cadherin negativity in distal tubules and collecting ducts (E). Positivity of both E- and N-cadherin in clear cell renal cell carcinoma (ccRCC, E′). Positivity of E-cadherin and negativity of N-cadherin in pulmonary adenocarcinoma (F), whereas faint N-cadherin expression was noted in pleura mesothelioma (G). Differential E- and N-cadherin expression in the normal thymus (H, note Hassall's bodies), with partial colocalization in thymoma (H′, an example of a type AB thymoma) and E-cadherin positivity but N-cadherin negativity in thymic squamous cell carcinoma (H″). Note N-cadherin positive fibroblasts (asterisks) in desmoplastic tumor stroma. Bars: 50 μm.

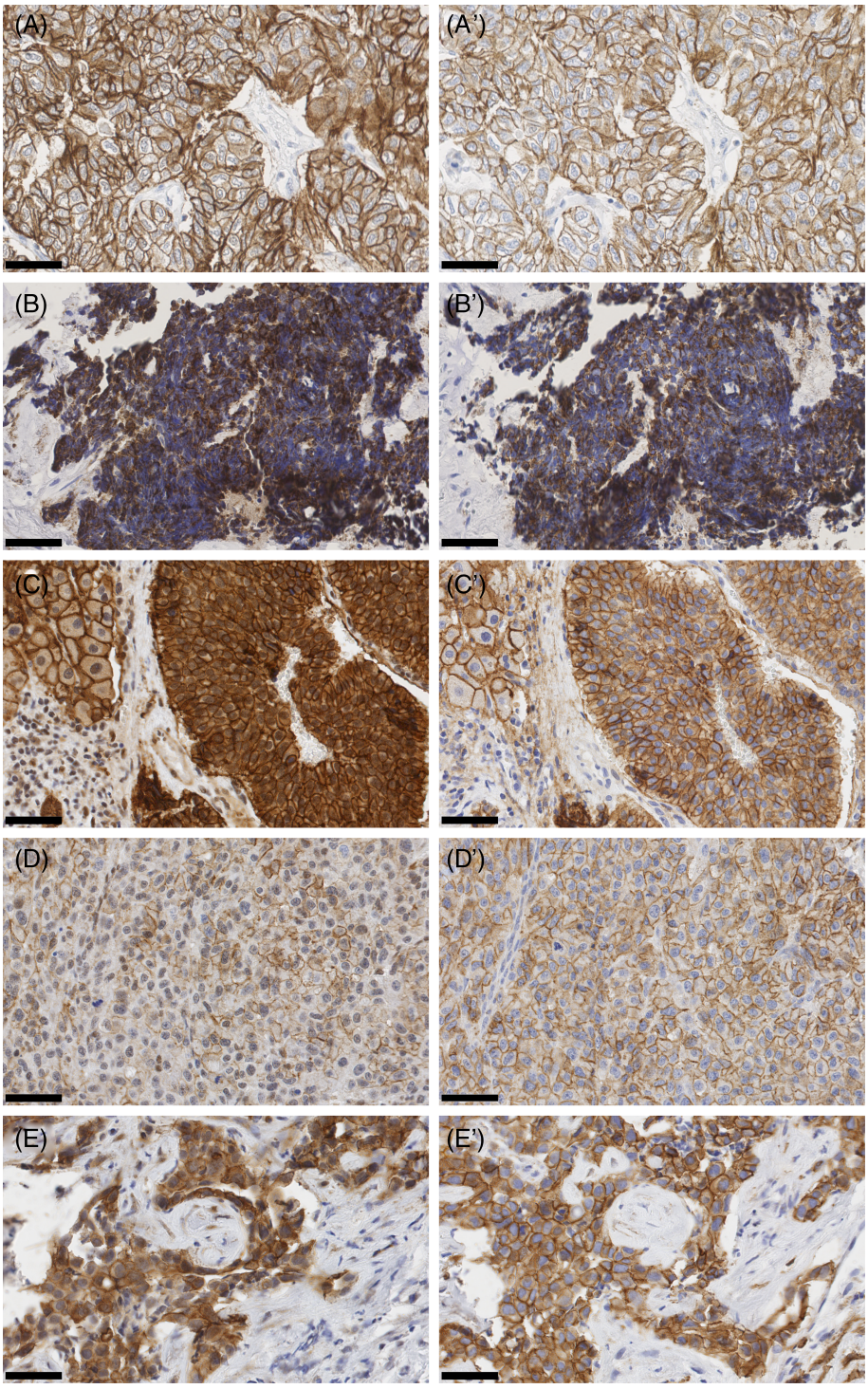

FIGURE 3 Immunohistochemical analysis of E- and N-cadherin in epithelioid tumors. E-cadherin (left) and N-cadherin (right) are positive in poorly differentiated thyroid carcinoma (A–A′), small cell lung cancer (SCLC; B–B′), moderately differentiated NET of the small intestine with adjacent liver parenchyma (C–C′) as well as in malignant melanoma (D–D′), and malignant mesothelioma (E–E′). Bar: 50 μm. Semiquantitative E- and N-cadherin scores: (A) 3, (A′) 2.5, (B) 2, (B′) 2, (C) 3, (C′) 3, (D) 2, (D′) 2.5, (E) 2.5, and (E′) 3.

positivity in tumor cells, tumor–stroma interaction has also been described.[16,36] Second, N-cadherin-positivity in residual hepatocytes may lead to potential confusion with tumor cells, especially in cases of diffuse-infiltrative liver metastases. Third, meticulous care should be taken in only evaluating membranous staining reaction, and not cytoplasmic or nuclear staining, which may be present in tumor necrosis and/or suboptimal immunohistochemical staining procedures. In our hands, only very faint if any cytoplasmic or nuclear staining was present. This point necessitates a close examination, since the staining pattern may be heterogeneous, and incomplete staining may be present. Complete circumferential N-cadherin staining indicates positivity, even in smaller tumor volumes. Fourth, as is the case with other immunohistochemical stains, laboratory-specific and material-specific factors should be considered. This implies that a liver biopsy generally requires lower antibody concentrations than a resected specimen, and the respective staining should be standardized using a comparative sample. For optimal evaluation, we suggest using an on-slide normal liver control.

Immunohistochemical staining of E- and N-cadherin has already been proposed for some tumor entities. High N-cadherin expression in adenocarcinomas with pancreatobiliary morphology favors the diagnosis of iCCA over the diagnosis of PDAC.[17] In pleural masses, N-cadherin has also been proposed to help distinguish malignant mesothelioma from lung carcinoma.[37] N-cadherin may be helpful in the differential diagnosis of primary and metastatic testicular germ cell cancer.[38] Moreover, tumors with *CDH1* mutations show aberrant E-cadherin expression. In this context, E-cadherin-immunohistochemistry has been used to distinguish invasive lobular breast carcinomas from those of no special type (NST), and poorly cohesive gastric adenocarcinomas from conventional and other gastric carcinomas.[39–41] Most interestingly, we detected negativity or only marginal N-cadherin expression in invasive lobular carcinoma of the breast, without upregulation despite E-cadherin- negativity or only cytoplasmic aberrant localization of E-cadherin.

Furthermore, downregulation of E-cadherin expression and upregulation of N-cadherin (so-called cadherin-switch) may be characteristics of EMT, as it has been discussed for various entities such as PDAC, melanoma, breast and gastric carcinomas.[42,43] However, previous studies use alternate definitions of EMT and also consider cytoplasmic staining as positive (Table S1). Thus, determining EMT should be based on several markers.[44] Several authors have described intratumoral heterogeneity associated with EMT, involving the expression of a wide variety of EMT markers, such as E- and N-cadherin.[45] In our cohort, consistent with our previous studies, we observed relatively minor intratumoral heterogeneity in the immunohistochemical staining of E- and N-cadherin. While we observed certain variations in tumor staining, especially in poorly differentiated tumors, it is essential to acknowledge that the TMA approach has inherent limitations in capturing the entirety of diversity present in larger tumor sections, as well as the dynamic and transient changes in phenotypes frequently associated with EMT.

In our comprehensive analysis, we could recapitulate the finding that amongst carcinomas, N-cadherin is vastly negative. N-cadherin negativity especially accounts for tumors that frequently metastasize to the liver, such as lung, breast, colorectal, and urinary bladder carcinomas.[4] As an exception, tumors of the kidney show relatively high levels of N-cadherin in 23.6% of cases, which may make it difficult to distinguish ccRCC from clear cell HCC. In such cases, in situ hybridization (ISH) for albumin mRNA may be more specific and sensitive than using HepPar1 for immunohistochemical analysis.[46] Liver metastases from NETs and NECs are common, with 82% of patients developing metastases at this site.[47] In our study, N-cadherin was often present in SCLC, NET, and NEC. However, the distinct morphology of most neuroendocrine tumors, and the availability of specific markers like synaptophysin, chromogranin A, and CD56 facilitate differential diagnosis in this respect. Liver metastases of malignant melanoma, which may also be E- and N-cadherin positive, may be differentiated from PLC by the presence of melanin pigment or immunohistochemically by the ancillary markers S100, MelanA, and HMB45.[48] For the liver, several more markers are available and may be combined with N-cadherin enhancing the strength of security of PLC diagnosis. For example, HepPar1 demonstrates a high specificity for hepatocytes and HCCs, yet also other tumors may stain positive with antibodies against HepPar1, as in gastric carcinomas, especially of the poorly cohesive type, iCCA, and more rarely carcinomas of the colon, endocervix, esophagus, prostate, lung, and endometrium.[6]

Although our comprehensive study comprises a large number of different tumor entities, naturally, certain limitations need to be acknowledged. First, the availability of resection specimens constrained our study to mostly primary tumors and liver metastases of only some tumor entities. In these cases, we did not discover significant differences between the staining patterns of primary and secondary tumors. Second, despite our efforts to include a comprehensive selection of cases, our study's scope inherently covered only a subset of all possible liver tumor scenarios typically encountered. Third, it is essential to note that immunohistochemistry of N-cadherin may show certain variations with respect to different laboratory procedures such as in staining intensity due to the use of different immunohistochemistry procedures, antibodies and different fixation, which may need to be taken into account. To summarize, N-cadherin is a valuable marker of primary liver origin. Prospective studies may be helpful in determining its value in liver biopsies of carcinomas of unknown primary in combination with other immunohistochemical markers.

5 | CONCLUSIONS

In the present study, we conducted a comprehensive evaluation of E- and N-cadherin expression in a large cohort of PLC and extrahepatic malignancies. Our findings suggest that N-cadherin may serve as a valuable marker to distinguish PLC from liver metastases of extrahepatic carcinoma. However, strong N-cadherin expression is not sufficient to exclude liver metastases of neuroendocrine tumors, germ cell tumors, melanoma, mesothelioma, and RCC during evaluation of a strong N-cadherin reaction. Therefore, with these exceptions, N-cadherin positivity predominantly favors the diagnosis of PLC.

AUTHOR CONTRIBUTIONS

Tiemo S. Gerber: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Validation; Visualization; Writing – original draft; Writing – review & editing. **Dirk A. Ridder:** Data curation; Investigation. **Benjamin Goeppert:** Resources. **Alexander Brobeil:** Resources. **Philipp Stenzel:** Data curation; Resources. **Stefanie Zimmer:** Data curation; Resources. **Jörg Jäkel:** Data curation; Resources. **Marie Oliver Metzig:** Resources. **Roxana Schwab:** Resources. **Steve Z. Martin:** Data curation; Resources. **András Kiss:** Resources. **Frank Bergmann:** Resources. **Peter Schirmacher:** Supervision. **Peter R. Galle:** Data curation. **Hauke Lang:** Data curation; Resources. **Wilfried Roth:** Supervision. **Beate K. Straub:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing –

original draft; Writing – review & editing. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures performed in studies involving human tissue were in accordance with the ethical standards of the institutional and regional ethics committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all subjects involved in the study. The study was approved by the ethics committee of the State Medical Council of Rhineland-Palatinate (837.280.17 (11114), 837.146.17 (10980) with addendum 2018-13857_1, and 2021-15819) and by the ethics committee of the University of Heidelberg (S-206/2005, S-207/2005, and S-519/2019).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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