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Original contribution

An immunostaining panel of C-reactive protein, N-cadherin, and S100 calcium binding protein P is useful for intrahepatic cholangiocarcinoma subtyping☆

Masayuki Akita MD, PhD [a,b,c], Ryuichiro Sawada MD, PhD [a,d], Masato Komatsu MD, PhD [a], Nadia Suleman BSc [e], Tomoo Itoh MD, PhD [a], Tetsuo Ajiki MD, PhD [b], Nigel Heaton MBBS, FRCS [e], Takumi Fukumoto MD, PhD [b], Yoh Zen MD, PhD, FRCPath [e,*]

[a] *Department of Diagnostic Pathology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan*
[b] *Department of Hepato-Biliary-Pancreatic Surgery, Kobe University Graduate School of Medicine, Kobe, 650-0017, Japan*
[c] *Department of Surgery, Kakogawa Central City Hospital, Kakogawa, 675-8611, Japan*
[d] *Department of Gastroenterological Surgery, Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, 135-8550, Japan*
[e] *Institute of Liver Studies, King's College Hospital & King's College London, SE5 9RS, London, UK*

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Summary This study aimed to establish an immunohistochemical panel useful for subclassification of intrahepatic cholangiocarcinoma (iCCA) into small- and large-duct types. Fifty surgical cases of iCCA consisting of small- (n = 31) and large-duct types (n = 19) were examined. To imitate liver needle biopsies, tissue microarrays were constructed using three tissue cores (2 mm in diameter) obtained from one representative paraffin block of each case. Immunostaining for C-reactive protein (CRP), N-cadherin, tubulin beta-III (TUBB3), neural cell adhesion molecule (NCAM), and S100 calcium binding protein P (S100P) was conducted. Most cases of small-duct iCCA were immunoreactive to CRP and N-cadherin, whereas expressions of these markers were markedly less common in large-duct iCCA (CRP, 97% vs. 5%, $P < 0.001$; N-cadherin, 87% vs. 16%, $P < 0.001$). TUBB3 and NCAM were also more frequently expressed in small-duct iCCA (65% vs. 32%, $P = 0.006$; 58% vs. 5%, $P < 0.001$), but their sensitivities were lower than those of CRP and N-cadherin. S100P was more commonly expressed in large-duct iCCA than in small-duct iCCA (95% vs. 29%, $P < 0.001$), and diffuse expressions were observed in 17 of 19 cases of large-duct iCCA (90%). All cases with

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* Corresponding author. Institute of Liver Studies King's College Hospital London, UK
E-mail address: yoh.1.zen@kcl.ac.uk (Y. Zen).

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a CRP+/S100P− immunophenotype were of small-duct type, whereas all but one case with a CRP −/S100P+ immunophenotype were of large-duct type. Of 10 cases with a double-positive or double-negative immunophenotype, 7 were appropriately classified based on immunoreactivity to N-cadherin. In conclusion, CRP, N-cadherin, and S100P form a useful immunohistochemical panel for iCCA subclassification, and correct subclassification was possible in 92% of cases based on a proposed, simple algorithm.

1. Introduction

Intrahepatic cholangiocarcinoma (iCCA) accounts for approximately 5% of primary liver cancers. A recent pathological achievement of iCCA is the development of a dichotomous classification scheme [1], which was endorsed by the newest edition of the World Health Organization classification of digestive system tumors. Small-duct−type iCCA is histologically characterized by ductule-like tubular adenocarcinoma with ambiguous lumens, whereas large-duct−type iCCA consists of ductal carcinomas with mucus production, and the microscopic appearance is similar to perihilar cholangiocarcinoma [1]. Unlike small-duct iCCA, which is suspected to arise from the septal/interlobular ducts or ductules, large-duct iCCA supposedly originates from the second branches of the biliary tree [2].

Clinical features differ between the two types. Patients with small-duct iCCA often have a history of chronic liver disease or cirrhosis and typically present with a mass-forming tumor in the liver [1,3,4]. In contrast, potential preceding conditions of large-duct iCCA include primary sclerosing cholangitis and hepatolithiasis [5,6]. Similar to perihilar cholangiocarcinoma, large-duct iCCA often shows periductal infiltration or intraductal polypoid growth [1]. Five-year postoperative survival rates of small- and large-duct iCCA are approximately 60% and 20%, respectively [1]. This morphology-based classification standard is also correlated with genetic features, with alterations in *IDH1/2*, *BAP1,* and *FGFR2* almost restricted to small-duct iCCA, whereas mutations in *KRAS* and *SMAD4* and amplification of *MDM2* are more common in large-duct cancers [1,3,6,7].

A caveat is that large-duct iCCA may display small-duct−type morphology at the invasive front, particularly the interface between the tumor and liver parenchyma [1]. Similarly, small-duct iCCA may have small foci of large-duct−type morphology [1]. Potential hybrid morphology in either type makes iCCA subclassification by biopsy specimens challenging and requires ancillary markers. In the present study, we tested five immunohistochemical markers of cholangiocarcinoma to establish a panel of antibodies that are useful for iCCA subtyping.

2. Materials and methods

2.1. Case selection

This study was approved by the Ethics Committee at Kobe University Graduate School of Medicine. Of 55 patients with iCCA who underwent surgical resection in Kobe University Hospital between 2000 and 2016, 50 cases with paraffin blocks available for tissue microarray (TMA) were used in the present study. No patients had neoadjuvant chemotherapy before the surgery.

2.2. Tissue microarray

TMAs were constructed by 3 tissue cores (2 mm in diameter) obtained from one representative formalin-fixed paraffin-embedded block of each case, aiming to imitate liver biopsy specimens. The tissue cores were randomly obtained from the tumor masses.

2.3. iCCA subclassification

Histology slides of surgically resected specimens were reviewed, and cases of iCCA were classified into small- and large-duct types as per the criteria described previously by Akita et al [1]. In brief, large-duct iCCA was defined as an adenocarcinoma with a predominantly ductal morphology and minor tubular components, if present, restricted to the tumor-liver interface. Small-duct iCCA consisted of predominantly tubular adenocarcinoma with the ambiguous lumen, and the characteristic morphological features were confirmed in central parts of the tumor. In cases of poorly differentiated adenocarcinomas, subtypes were determined on more differentiated areas. Based on these criteria, two investigators (M.A. and Y.Z.) read the original heamatoxylin and eosin−stained slides of the surgically resected specimens and independently classified each case.

Concordant diagnoses were achieved in 47 of 50 cases. For 3 cases with discrepant interpretations, a consensus opinion was reached after discussions using a multiheader microscope. Pathological features (eg, gross appearance, tumor size, degrees of differentiation, lymphovascular invasion, lymph node metastasis, and tumor stage) were compared between the two groups.

2.4. Immunohistochemistry

Previous studies suggested that N-cadherin may serve as a marker for small-duct iCCA [3], whereas S100 calcium binding protein P (S100P) was commonly expressed in large-duct iCCA [3,4]. Our proteomic study also demonstrated that tubulin beta-III (TUBB3) is more commonly expressed in iCCA with small-duct morphology than in perihilar cholangiocarcinoma [8]. Neural cell adhesion molecule (NCAM) is known to be expressed in iCCA with ductular morphology, in keeping with the small-duct type [9]. C-reactive protein (CRP) was recently reported as a useful marker for discriminating iCCA (subtype not specified) from metastatic liver cancers including metastatic pancreatic ductal carcinoma [10]. Based on these findings, CRP, N-cadherin, TUBB3, NCAM, and S100P were tested in the present study.

Immunohistochemistry for these five markers was performed using a Bond Max autostainer (for CRP, TUBB3, and S100P; Leica Microsystems, Wetzlar, Germany) or Ventana Benchmark GX (for N-cadherin and NCAM; Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocols. Three antibodies for S100P were tested because frequencies of S100P expression in iCCA varied widely from 9% to 79% in previous studies with different antibodies used [5,6,11]. The antibodies and staining conditions were as follows: CRP (rabbit monoclonal; clone Y284; 1:300; heat pretreatment in ethylenediaminetetraacetic acid (EDTA) buffer; Abcam, Cambridge, UK), N-cadherin (mouse monoclonal; clone IAR06; dilution 1:100; heat treatment in citrate buffer; Leica Microsystems), TUBB3 (mouse monoclonal; clone TU20; dilution 1:500; heat pretreatment in EDTA buffer; Abcam), NCAM (rabbit monoclonal; clone MRQ-42; prediluted; heat treatment in citrate buffer; Novocastra Laboratories, Newcastle, UK), S100P (goat polyclonal; clone AF2957; dilution 1:500; heat treatment in citrate buffer; R&D systems, Minneapolis, MI, USA), S100P (mouse monoclonal; clone 16/S100P; dilution 1:100; pretreatment with proteinase; BD Biosciences, San Jose, CA, USA), and S100P (mouse monoclonal; clone 357,517; dilution 1:500, R&D systems).

To correlate the immunohistochemical results between TMA and whole-section staining, one representative block selected from each case was also applied for immunohistochemistry of CRP, N-cadherin, TUBB3, NCAM, and S100P (clone 16/S100P). The results were correlated with those of TMA staining.

Expression levels were evaluated semiquantitatively based on the percentages of positive cells: negative (score = 0; no expression), focally positive (score = 1+; <50% of tumor cells), and widely positive (score = 2+; ≥50% of tumor cells).

2.5. Statistical analyses

Analyses were performed using JMP statistical software (version 12; SAS Institute, Cary, NC, USA). Continuous variables not showing a bell-shaped distribution were assessed using the unpaired t-test or Mann-Whitney U-test, whereas categorical variables in each group were compared using the chi-square test or Fisher's exact test.

3. Results

3.1. Postoperative prognosis and pathological findings

Based on the microscopic morphological appearance of the surgically resected specimens, 31 cases of iCCA were classified into the small-duct type and 19 were categorized as the large-duct type. Table 1 summarizes the pathological features of both types. All cases of small-duct iCCA were grossly mass-forming tumors, whereas 13 large-duct iCCA cases (68%) showed at least focal periductal infiltration. No significant difference was observed in tumor size, degrees of microscopic differentiation, lymph node metastasis, and staging between the two types. Large-duct iCCA more frequently had microscopic vascular and perineural invasion.

3.2. Immunohistochemistry

The results of immunohistochemistry are summarized in Fig. 1, and the representative pictures are shown in Fig. 2. CRP was expressed in the cytoplasm of cancer cells. Positive CRP expression was confirmed in 30 of 31 cases of small-duct iCCA (97%), and two-thirds of these cases showed wide (2+) immunoreactivity. In contrast, a single case of large-duct iCCA (5%) had focal (1+) positivity for CRP ($P < 0.001$). Similarly, N-cadherin was positive in 27 of 31 cases of small-duct iCCA (87%) and only 3 of 19 cases of large-duct iCCA (16%; $P < 0.001$). TUBB3 and NCAM were also more commonly expressed in small-duct iCCA (65% and 58%) than in large-duct iCCA (32% and 5%, respectively), but their expressions in small-duct iCCA were less common and less extensive than those of CRP and N-cadherin. S100P was positive in the nuclei and cytoplasm of cancer cells. It was more commonly expressed in large-duct iCCA than in small-duct iCCA, using any of the three antibodies, but positive-case ratios varied widely (Fig. 1). The monoclonal antibody (clone 16/S100P) showed the best performance, with 95% positivity

Table 1 Clinicopathological features of small- and large-duct iCCA.

Variables	Small-duct iCCA (n = 31)	Large-duct iCCA (n = 19)	*P* value
Age (median, range)	76 (63−80)	70 (62−75)	0.109
Gender (M/F)	16 (52%)/15 (48%)	16 (84%)/3 (16%)	0.016
Chronic liver disease	14 (45%)	3 (16%)	0.033
Gross appearance (%)			
Mass forming	31 (100%)	6 (31%)	<0.001
Periductal infiltration	0	11 (68%)	
Mixed	0	2 (11%)	
Size (median, range), mm	45 (35−81)	55 (30−76)	0.496
Degree of differentiation			
Well	20 (65%)	15 (79%)	0.503
Moderately	7 (22%)	2 (11%)	
Poorly	4 (13%)	2 (11%)	
Lymphatic invasion	12 (39%)	10 (52%)	0.341
Vascular invasion	18 (58%)	17 (89%)	0.018
Perineural invasion	8 (24%)	18 (95%)	<0.001
Lymph node metastasis	7 (22%)	7 (37%)	0.282
pT stage			
pT1	6 (19%)	1 (5%)	0.382
pT2	15 (48%)	8 (42%)	
pT3	2 (6%)	0	
pT4	8 (27%)	10 (53%)	
Recurrence	22 (29%)	5 (26%)	0.748

Abbreviation: iCCA, intrahepatic cholangiocarcinoma.

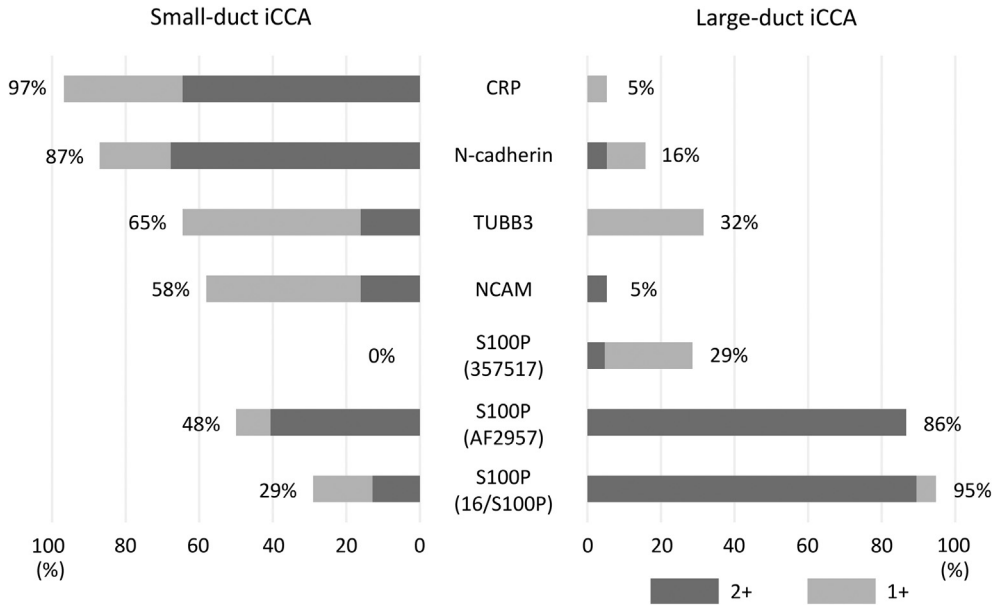


Fig. 1 Results of immunohistochemistry. CRP, N-cadherin, TUBB3, and NCAM are more commonly expressed in small-duct iCCA than in large-duct cancers, whereas the expression of S100P was more frequent in large-duct iCCA than in small-duct cases. P-values: CRP, $P < 0.001$; N-cadherin, $P < 0.001$; TUBB3, $P = 0.006$; NCAM, $P < 0.001$; S100P (357,517), $P = 0.004$; S100P (AF2957), $P = 0.021$; S100P (16/S100P), $P = 0.001$. CRP, C-reactive protein; TUBB3, tubulin beta-III; NCAM, neural cell adhesion molecule; iCCA, intrahepatic cholangiocarcinoma; S100P, S100 calcium binding protein P.

in large-duct iCCA (mostly wide expression) and 71% negativity in small-duct iCCA ($P < 0.001$).

Sensitivity, specificity, positive predictive value, and negative predictive value of individual markers are summarized in Table 2. CRP and N-cadherin appeared to be highly sensitive and specific markers for small-duct iCCA, whereas S100P (16/S100P) was a highly sensitive and moderately specific marker for large-duct

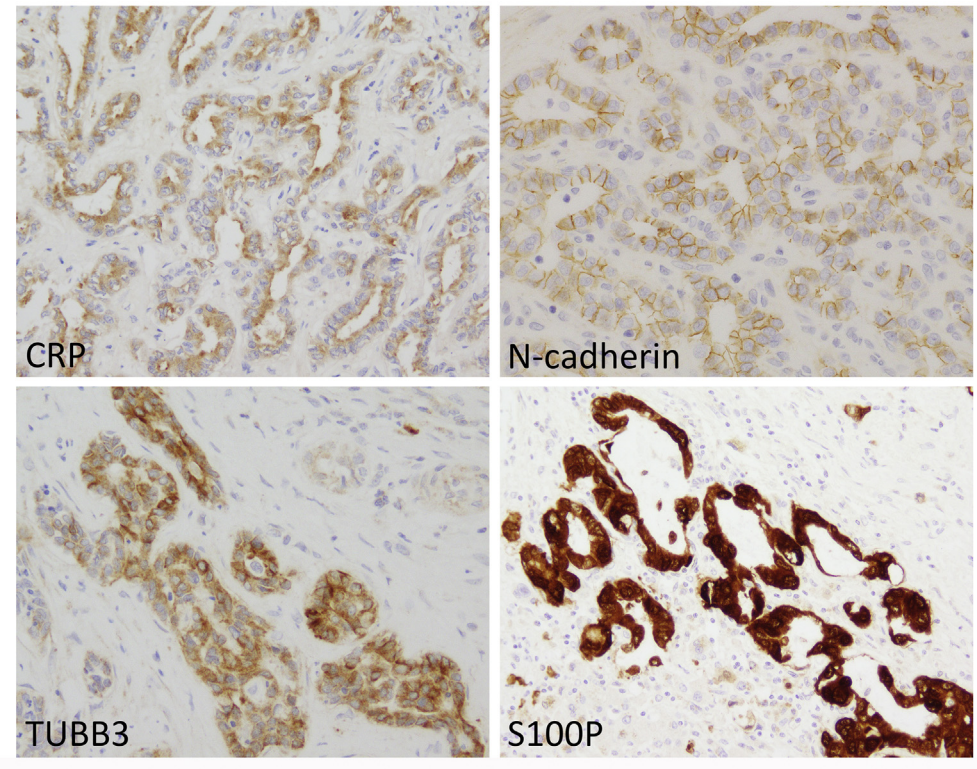


Fig. 2 Expressions of CRP, N-cadherin, and TUBB3 are observed in small-duct iCCA, whereas strong immunoreactivity to S100P (16/S100P) is noted in large-duct iCCA. CRP, C-reactive protein; TUBB3, tubulin beta-III; iCCA, intrahepatic cholangiocarcinoma; S100P, S100 calcium binding protein P.

Table 2 Values of immunohistochemical markers for subclassification of iCCA.

Immunohistochemical markers	Sensitivity	Specificity	PPV	NPV
For small-duct iCCA				
CRP	97%	95%	97%	95%
N-cadherin	87%	84%	90%	80%
TUBB3	65%	68%	77%	54%
NCAM	58%	95%	95%	58%
At least one of CRP or N-cadherin	97%	80%	88%	94%
At least two of CRP, N-cadherin, TUBB3	84%	95%	96%	78%
At least two of CRP, N-cadherin, NCAM	84%	95%	96%	78%
Proposed criteria[a]	90%	95%	97%	86%
For large-duct iCCA				
S100P (16/S00P)	95%	71%	67%	96%
S100P (AF2957)	86%	48%	45%	88%
S100P (357,517)	29%	100%	100%	67%
Proposed criteria[a]	95%	90%	86%	97%

Abbreviations: NCAM, neural cell adhesion molecule; CRP, C-reactive protein; TUBB3, tubulin beta-III; iCCA, intrahepatic cholangiocarcinoma; S100P, S100 calcium binding protein P; PPV, positive predictive value; NPV, negative predictive value.
[a] Criteria shown in Table 3.

iCCA. For the diagnosis of small-duct iCCA, CRP and N-cadherin were reliable discriminators, and addition of TUBB3 or NCAM did not increase the diagnostic accuracy.

3.3. Algorithm for immunophenotyping

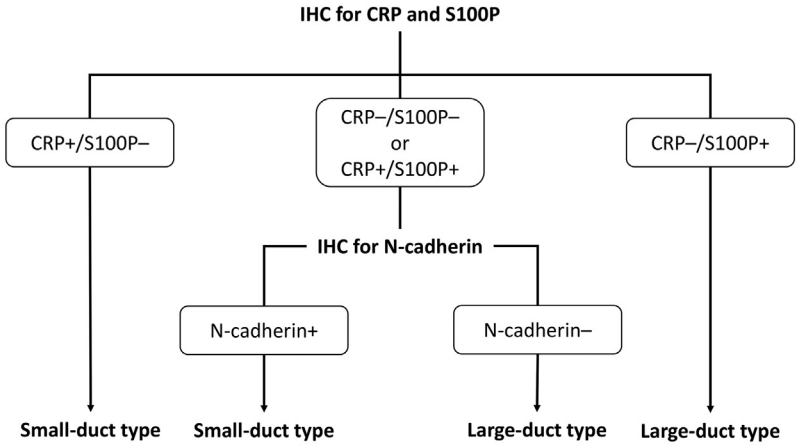
Immunohistochemistry-based criteria for iCCA subclassification were created using three markers: CRP, N-cadherin,

Table 3 Proposed criteria for immunohistochemistry-based classification of iCCA.

Small-duct iCCA
- CRP positive and S100P negative, irrespective of N-cadherin results
- N-cadherin positive, in cases with a CRP+/S100P+ or CRP−/S100P− immunophenotype

Large-duct iCCA
- CRP negative and S100P positive, irrespective of N-cadherin results
- N-cadherin negative, in cases with a CRP+/S100P+ or CRP−/S100P− immunophenotype

Abbreviations: iCCA, intrahepatic cholangiocarcinoma; CRP, C-reactive protein; S100P, S100 calcium binding protein P.



Small-duct iCCA	22 (71%)	6 (19%)	2 (7%)	1 (3%)
Large-duct iCCA	0	1 (5%)	1 (5%)	17 (90%)

Fig. 3 A proposed subclassification algorithm for iCCA based on three immunohistochemical markers. Cases are classified based on CRP and S100P immunoreactivity. N-cadherin is applied to cases with a double-positive or double-negative immunophenotype. Based on that standard, 28 of 31 cases (90%) of small-duct iCCA and 18 of 19 cases (95%) of large-duct iCCA are appropriately subclassified. IHC, immunohistochemistry; CRP, C-reactive protein; iCCA, intrahepatic cholangiocarcinoma; S100P, S100 calcium binding protein P.

and S100P (16/S100P) (Table 3), and the evaluation algorithm based on that standard is shown in Fig. 3. Applying this standard to our cohort, all CRP+/S100P− cases were of small-duct type, and 17 of 18 cases with a CRP−/S100P+ phenotype were of large-duct type (Fig. 3). Of 8 cases of small-duct iCCA with a double-positive or double-negative immunophenotype, 6 were positive for N-cadherin. In summary, the proposed algorithm made correct subclassification in 46 of 50 cases (92%). Classification performance of the proposed criteria is also summarized in Table 2.

3.4. Correlation of immunohistochemical results between TMA and whole-section staining

As shown in Table 4, a good correlation was observed for all markers examined. CRP showed the best correlation. A couple of cases that were negative for S100P (16/S100P) (n = 1), N-cadherin (n = 2), NCAM (n = 3), or TUBB (n = 2) on TMA staining showed immunoreactivity on whole-section staining. Concordant results were confirmed in the remaining cases.

4. Discussion

Subclassification has become an important part of the histological diagnosis of iCCA. Given the biological differences between the two types, this classification is expected to be more important and clinically relevant in the future. For surgical candidates, preoperative subtyping may affect surgical procedures. Similar to perihilar cholangiocarcinoma, large-duct iCCA has a high risk of lymph node metastasis, necessitating systematic lymphadenectomy, whereas routine lymph node dissection is still controversial for small-duct iCCA [12−14]. For nonsurgical cases, the subclassification may become important for selection of chemotherapeutic drugs. Currently, both types of iCCA are treated on the same clinical guideline; however, given the distinct biological natures, response to standard cytotoxic chemotherapy may

Table 4 Comparison of results of TMA and whole-section immunohistochemistry.

Markers	Small-duct iCCA				Large-duct iCCA			
CRP			Whole section				Whole section	
			0	1+/2+			0	1+/2+
	TMA	0	1	0	TMA	0	18	0
		1+/2+	0	30		1+/2+	0	1
S100P (16/S100P)			Whole section				Whole section	
			0	1+/2+			0	1+/2+
	TMA	0	21	1	TMA	0	1	0
		1+/2+	0	9		1+/2+	0	18
N-cadherin			Whole section				Whole section	
			0	1+/2+			0	1+/2+
	TMA	0	3	1	TMA	0	15	1
		1+/2+	0	27		1+/2+	0	3
NCAM			Whole section				Whole section	
			0	1+/2+			0	1+/2+
	TMA	0	10	3	TMA	0	18	0
		1+/2+	0	18		1+/2+	0	1
TUBB3			Whole section				Whole section	
			0	1+/2+			0	1+/2+
	TMA	0	9	2	TMA	0	13	0
		1+/2+	0	20		1+/2+	0	6

Abbreviations: TMA, tissue microarray; iCCA, intrahepatic cholangiocarcinoma; TUBB3, tubulin beta-III; CRP, C-reactive protein; S100P, S100 calcium binding protein P; NCAM, neural cell adhesion molecule.

differ between the two types. Drugs specific to either type may become available. Some molecular abnormalities in small-duct iCCA (eg, *IDH1/2* mutations and *FGFR2* fusions) are known drug targets, and recent clinical trials demonstrated promising results [15−17].

Previous studies used different criteria for iCCA subtyping (eg, gross appearance [4], microscopic morphology [1,5], and a combination of morphology and immunohistochemistry [3]). However, types of some cases were indeterminate based on the proposed standard [5,6]. Another caveat is that most studies used surgically resected specimens. Potential occurrence of hybrid features and poorly differentiated morphology necessitate the use of ancillary markers for iCCA subclassification particularly in biopsy specimens.

In the present study, 5 markers were tested. One unexpected result was that CRP appeared to be a highly sensitive and specific marker for small-duct iCCA. A single study has previously examined the expression of CRP in iCCA [10]. The expression of CRP in iCCA was confirmed in 76% of TMA samples and 93% of whole sections, whereas CRP immunoreactivity was identified in only 9% of adenocarcinomas of extrahepatic origin [9]. In the same cohort, N-cadherin was also positive in 54% (TMA) and 80% (whole sections) cases of iCCA, suggesting that most cases examined in that study were of the small-duct type [2]. As CRP is a marker for inflammatory hepatocellular adenoma and is also expressed in 54% of hepatocellular carcinoma cases, it cannot be used for differentiating iCCA from hepatocellular carcinoma [18].

The expression of S100P in iCCA was examined in several studies, but positive-case ratios varied widely. One study demonstrated 79% positivity in large-duct iCCA [6], whereas another study showed only 9% immunoreactivity in large-duct iCCA [5]. Other studies had intermediate results [4]. We suspected that these discrepant results might have been due to the use of different antibodies; therefore, three types of antibodies were tested in the present study. Two antibodies (clones 16/S100P and 357,517) were monoclonal, and the third (clone AF2957) was polyclonal. The best performance was obtained with clone 16/S100P. Notably, a majority of large-duct iCCA showed diffuse, strong immunoreactivity to this antibody. We also noticed that immunoreactivity to this antibody was stronger with a clearer contrast with protease pretreatment than with heat treatment. When S100P is used as a marker for large-duct iCCA, selection of the antibody and pretreatment method should be carefully determined. Similar to CRP, S100P can be expressed in 57% of hepatocellular carcinoma cases; therefore, it cannot be used for discriminating iCCA from hepatocellular carcinoma [19]. In addition, S100P can be positive in a variety of carcinomas including pancreatic ductal carcinomas [20,21]; therefore, diagnosis of iCCA cannot be made solely based on immunohistochemical expression patterns in particular by needle biopsy, requiring clinicopathological correlation to rule out the possibility of metastasis.

Expressions of TUBB3 and NCAM were also significantly higher in small-duct iCCA than in large-duct cancers. However, because of high sensitivity and specificity of CRP and N-cadherin, addition of TUBB or NCAM did not increase

diagnostic performance. However, these markers may still be valuable for cases with an intermediate CRP/S100P immunophenotype. Of 10 cases with either a CRP+/S100P+ or CRP-/S100P− immunophenotype, 8 were of small-duct type, but N-cadherin was negative in two cases. Use of additional markers for the small-duct type may better characterize such cases.

In conclusion, CRP, N-cadherin, and S100P form a useful immunohistochemical panel for iCCA subclassification, and the proposed, simple algorithm made correct subclassification in 92% of cases.

Author contributions

M.A. contributed to study design, histological analysis, review of clinical records, and drafting the manuscript. R.S., M.K., N.S., and T.I. contributed to histological analysis and editing the manuscript. T.A. and T.F. contributed to clinical evaluation and editing the manuscript. N.H. contributed to study design, selection of antibodies, and editing the manuscript. Y.Z. contributed to study design, histological analysis, interpretation of data, supervising the study, and drafting the manuscript.

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