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ORIGINAL ARTICLE

Diagnostic and prognostic value of immunohistochemical expression of S100P and IMP3 in transpapillary biliary forceps biopsy samples of extrahepatic bile duct carcinoma

Hiroki Kawashima · Akihiro Itoh · Eizaburo Ohno · Ryoji Miyahara · Naoki Ohmiya · Tsutomu Tanaka · Yoshie Shimoyama · Shigeo Nakamura · Tomoki Ebata · Masato Nagino · Hidemi Goto · Yoshiki Hirooka

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Abstract
Background Because the biopsy specimen of extrahepatic bile duct carcinoma (EHBDC) is small and shows reactive changes, the histological distinction between malignant and benign tissue can be difficult. Recent studies reported that S100P and insulin-like growth factor II mRNA-binding protein 3 (IMP3) were not only diagnostic molecules but also prognostic biomarkers in several organs. The objective of this study is to clarify the diagnostic and prognostic value of immunohistochemical expression of S100P and IMP3 in transpapillary biliary forceps biopsy (TBFB) samples.
Methods The TBFB samples were collected from 80 patients (EHBDC, 68 patients; benign, 12 patients), retrospectively.
Results When using cytoplasmic-positive staining for IMP3 as a marker of malignancy, the sensitivity and specificity reached 79.4 and 91.7 %, respectively. The sensitivity, specificity and accuracy achieved 89.7, 91.7 and 90.0 %, respectively, when using positive staining for IMP3 and/or positive histology as a maker of malignancy. While univariate ($P = 0.033$) and multivariate ($P = 0.039$) analysis revealed that S100P-positive EHBDC patients showed significantly shorter survival.
Conclusions The results of this study suggest that immunohistochemical staining for IMP3 is useful in the diagnosis of EHBDC and that of S100P is useful as a prognostic marker for EHBDC.

Keywords S100P · IMP3 · Biopsy · Cholangiocarcinoma · Immunohistochemistry

H. Kawashima · A. Itoh · R. Miyahara · N. Ohmiya · T. Tanaka · H. Goto
Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Nagoya, Japan

E. Ohno · Y. Hirooka (✉)
Department of Endoscopy, Nagoya University Hospital, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan
e-mail: hirooka@med.nagoya-u.ac.jp

Y. Shimoyama · S. Nakamura
Department of Pathology and Clinical Laboratories, Nagoya University Hospital, Nagoya, Japan

T. Ebata · M. Nagino
Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

Introduction

Because surgical therapy of extrahepatic bile duct carcinoma (EHBDC) is invasive (i.e., extended hepatectomy and/or pancreatoduodenectomy) [1–4], preoperative confirmation of the malignant diagnosis is important. Although the usefulness of transpapillary biliary forceps biopsy (TBFB) in the diagnosis of biliary strictures has been reported [5–7], the sensitivity of detection of malignancy ranges from 43 to 81 % [8, 9]. This variability indicates that there are false negatives in more than 20 % of biopsied patients. The false negatives occur because biliary tissue samples, gained via forceps biopsy, are small, show crush artifacts and can show reactive changes, particularly following biliary stent placement. Therefore, immunohistochemical markers that differentiate between benign and malignant tissue would be beneficial.

S100P protein is a small isoform of the S100 protein family that is present in human placenta [10]. Insulin-like growth factor II mRNA-binding protein 3 (IMP3) is an oncofetal protein. IMP3 is a member of the IMP family, which comprises IMP1, IMP2, and IMP3 [11]. Overexpression of S100P or IMP3 has been detected in several

tumors, including those of the bile duct [12–15]. It has been reported that immunohistochemical staining for S100P and IMP3 differentiates between benign and malignant processes in different organs [12–21]. Furthermore, in surgically resected samples, S100P expression correlates with patient prognosis in intrahepatic cholangiocarcinoma [12] and IMP3 expression correlates with patient prognosis in bile duct carcinoma [15]. If the prediction of prognosis is possible using preoperative biopsy samples without the requirement for surgically resected samples, it will greatly impact the clinical setting.

Thus, we studied retrospectively the diagnostic and prognostic value of S100P and IMP3 expression in tissues obtained by TBFB of patients with suspected EHBDC.

Methods

Tissue samples

Forceps biopsy samples were collected from 80 patients who underwent diagnostic endoscopic retrograde cholangiography and TBFB for biliary stricture or irregularities of the bile duct wall and were ultimately diagnosed with EHBDC or benign biliary stricture during the period of April 2005–March 2010 at Nagoya University Hospital [5, 22]. The ages of the patients ranged from 29 to 88 years (median, 67 years). Fifty-five patients were male, and 25 were female. The final diagnosis was based on the surgical specimen or was made after more than 12 months of follow-up. Sixty-eight patients were ultimately diagnosed with EHBDC (33 patients with perihilar bile duct carcinoma and 35 patients with distal bile duct carcinoma), and 12 patients were ultimately diagnosed with benign biliary stricture [primary sclerosing cholangitis (PSC), 5 patients; IgG4 related cholangitis, 2 patients; and inflammatory change, 5 patients). A pancreatoduodenectomy, a hemihepatectomy, or a more extended resection was performed in 55/68 bile duct carcinoma patients, and the remaining 13 patients underwent either laparotomy alone or palliative surgery due to peritoneal dissemination, liver metastasis and/or periaortic lymph node metastasis, which were detected during the laparotomy.

The clinical and pathological findings of EHBDC were classified based on the American Joint Committee on Cancer (AJCC) cancer staging manual [23]. Based on the previous study [24], the gross tumor type was assessed as papillary, nodular or diffusely infiltrating. This study was approved by the Institutional Review Board at Nagoya University Hospital.

Immunohistochemical staining and evaluation

Immunohistochemical staining for IMP3 and S100P was performed using 4-μm-thick formalin-fixed, paraffin-embedded tissue sections, as previously described [14, 19, 20]. In brief, the tissue sections were deparaffinized and then incubated in 0.3 % H_2O_2/methanol for 20 min at room temperature to block endogenous peroxidase activity. Antigen retrieval for IMP3 was performed by heating samples in a microwave oven for 10 min in 0.01 mol/L citrate buffer, pH 9.0, and was performed for S100P by incubating samples with proteinase K for 10 min at room temperature. The tissue sections were then incubated with a mouse monoclonal antibody specific for IMP3 (Clone 69.1; Dako; dilution 1:100) and S100P (Clone 16; BD Biosciences Pharmingen; dilution 1:100) for 1 h at room temperature and then incubated with biotinylated secondary antibodies, followed by incubation with ABC complex (Vectastain Elite ABC kit: Vector laboratories Inc., Burlingame, CA, USA). Staining was visualized using diaminobenzidine, and sections were counterstained with hematoxylin.

All hematoxylin and eosin slides were independently reviewed by two observers (T.T. and Y.S.) to verify the diagnoses of forceps biopsy tissue samples, which were based on criteria described by the World Health Organization [25]. Samples showing significant discrepancy in interpretations were resolved by a rereview by two observers. In general, an immunostain was considered positive if 1 % or more of the cells of interest exhibited immunoreactivity. Positive stains were graded as weak, intermediate or strong for staining intensity; focal if 1–50 % of the cells stained; and diffuse if more than 50 % of the cells were positively stained. Cytoplasmic staining was considered positive for IMP3. Nuclear or nuclear/cytoplasmic staining was considered positive for S100P, but if only cytoplasmic staining was detected, the sample was considered negative.

Statistical analysis

Statistical analyses were performed using IBM SPSS, version 19 (Chicago, IL, USA). Statistical analyses of group differences were performed using the Chi-squared test, the Fisher exact test or the Mann–Whitney *U* test. The Kaplan–Meier method was used for univariate survival analysis, and a comparison was made based on the log-rank test. Multivariate analyses were calculated based on the Cox's proportional hazards model. $P < 0.05$ was considered to be statistically significant.

Results

S100P and IMP3 expression in samples of transpapillary biliary forceps biopsies

Nuclear or nuclear/cytoplasmic immunostaining for S100P was detected in 52 (76.5 %) of 68 biopsy samples of

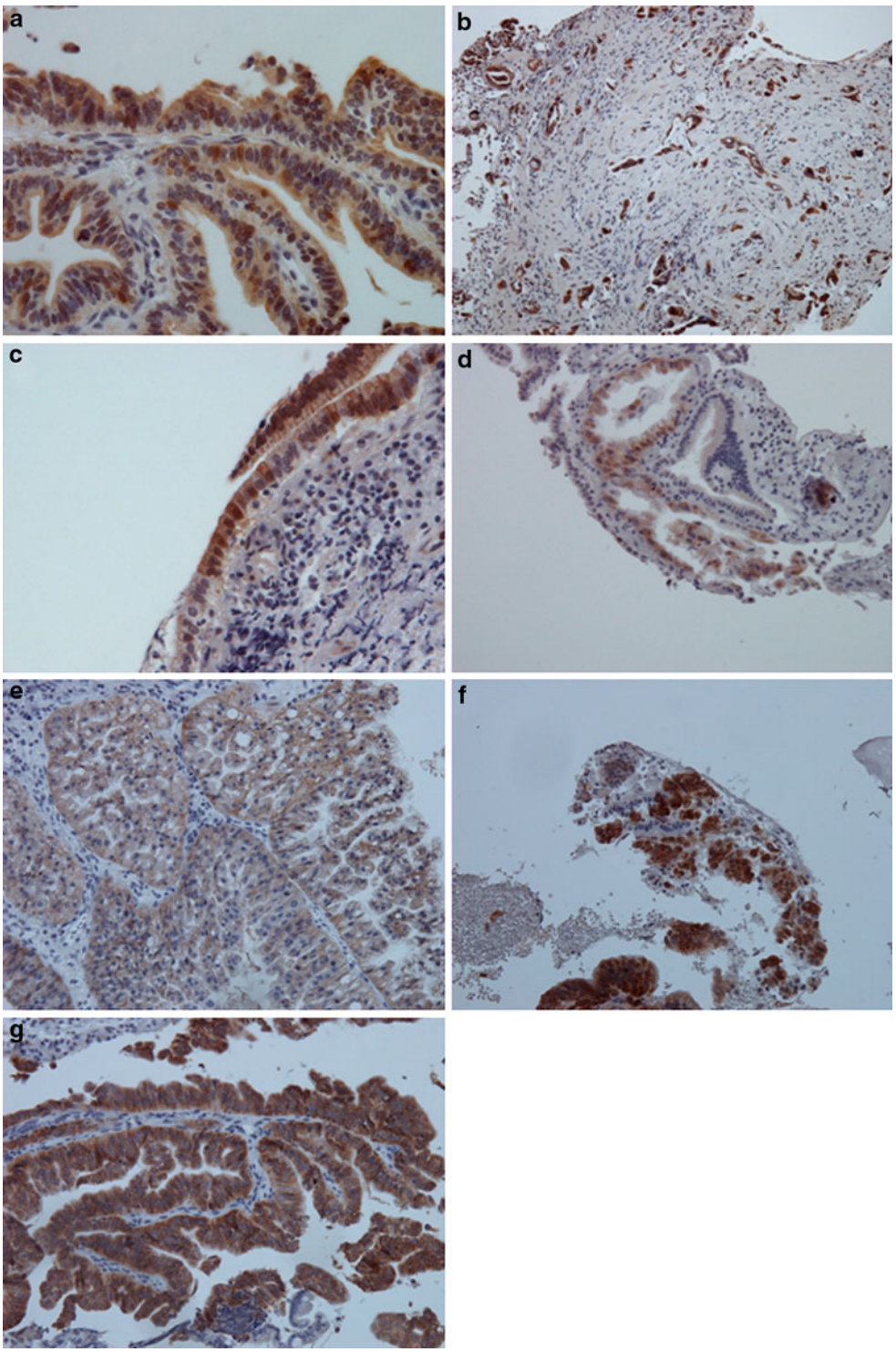

Fig. 1 S100P and IMP3 expression in transpapillary biliary forceps biopsy samples. **a** Focal and intermediate nuclear/cytoplasmic staining for S100P observed in extrahepatic bile duct carcinoma (EHBDC) (original magnification 200×). **b** Diffuse and strong nuclear/cytoplasmic staining for S100P observed in EHBDC (original magnification 100×). **c** Focal and intermediate nuclear/cytoplasmic staining for S100P observed in benign biliary stricture (IgG4 related cholangitis, original magnification 400×). **d** Focal and weak cytoplasmic staining for IMP3 observed in EHBDC (original magnification 200×). **e** Diffuse and weak cytoplasmic staining for IMP3 observed in EHBDC (original magnification 200×). **f** Focal and strong cytoplasmic staining for IMP3 observed in EHBDC (original magnification 200×). **g** Diffuse and strong cytoplasmic staining for IMP3 observed in EHBDC (original magnification 200×)

Table 1 Immunohistochemical detection of S100P expression in malignant and benign bile duct biopsy samples

Final diagnosis	Pathological diagnosis of biopsy sample	Number of patient samples showing positive staining (%)			Number of samples showing each type of staining intensity (%)		
		Diffuse	Focal	Negative	Strong	Intermediate	Weak
Malignant ($n = 68$)	Positive ($n = 49$)	26 (53.1)	10 (20.4)	13 (26.5)	20 (40.8)	16 (32.7)	0 (0)
	False negative ($n = 19$)	9 (47.4)	7 (36.8)	3 (15.8)	9 (47.4)	7 (36.8)	0 (0)
Benign ($n = 12$)	Negative ($n = 12$)	1 (8.3)	5 (41.7)	6 (50.0)	3 (25.0)	3 (25.0)	0 (0)

Table 2 Immunohistochemical detection of IMP3 expression in malignant and benign bile duct biopsy samples

Final diagnosis	Pathological diagnosis of biopsy sample	Number of patient samples showing positive staining (%)			Number of samples showing each type of staining intensity (%)		
		Diffuse	Focal	Negative	Strong	Intermediate	Weak
Malignant ($n = 68$)	Positive ($n = 49$)	31 (63.1)	11 (22.4)	7 (14.3)	25 (51.0)	12 (24.5)	5 (10.2)
	False negative ($n = 19$)	3 (15.8)	9 (47.4)	7 (36.8)	2 (10.5)	4 (21.1)	6 (31.6)
Benign ($n = 12$)	Negative ($n = 12$)	0 (0)	1 (8.3)	11 (91.7)	0 (0)	0 (0)	1 (8.3)

Table 3 The sensitivity, specificity and accuracy of each diagnostic strategy

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Histology	72.1	100	100	38.7	76.3
S100P	76.5	50.0	89.7	27.3	72.5
IMP3	79.4	91.7	98.2	44.0	81.3
Histology + S100P	95.6	50.0	91.5	33.3	88.8
Histology + IMP3	89.7	91.7	98.4	61.1	90.0

PPV positive predictive value, *NPV* negative predictive value

patients who were ultimately diagnosed with malignant tumors (Fig. 1), and in 6 (50 %) of 12 samples of patients diagnosed with benign tumors (Table 1). When using positive immunochemical staining for S100P as indicative of EHBDC, the sensitivity and specificity were 76.5 and 50.0 %, respectively (Table 3).

Cytoplasmic immunostaining for IMP3 was detected in 54 (79.4 %) of 68 biopsy samples of patients who were ultimately diagnosed with malignancies (Fig. 1), and it was detected in only 1 (8.3 %) of 12 samples of patients diagnosed with benign strictures (Table 2). This benign patient was ultimately diagnosed with PSC, and IMP3 expression in the biopsy from this patient was only focal and weak. When using positive immunochemical staining for IMP3 as indicative of EHBDC, the sensitivity and specificity reached 79.4 and 91.7 %, respectively. Moreover, the sensitivity, specificity and accuracy reached 89.7, 91.7 and 90.0 %, respectively, when positive histology and/or positive immunochemical staining for IMP3 was used to indicate EHBDC (Table 3).

S100P and IMP3 expression and clinicopathologic correlations in EHBDC

Of the 68 patients who were ultimately diagnosed with malignant strictures, S100P expression was significantly lower in patients with papillary growth type ($P = 0.024$). There was no statistically significant association between S100P or IMP3 expression and any of the other clinical characteristics (Table 4).

Of the 55 EHBDC patients who underwent surgical resection, although IMP3 expression was significantly lower in females ($P = 0.024$), there was no statistically significant association between S100P or IMP3 expression and any other clinical or pathological characteristic.

S100P and IMP3 expression and outcomes in EHBDC patients who underwent surgical resection

The survival of the 55 patients who underwent surgical resection was evaluated over a mean period of 865 days. In univariate and multivariate analyses, the EHBDC patients from the S100P-positive expression group showed significantly shorter survival times than those in the S100P-negative expression group. There was no significant association between IMP3 expression and survival. Of the other clinicopathologic factors, the EHBDC patients who underwent R1 operations showed significantly shorter survival times than patients who underwent R0 operations. And T3- and 4-pathological-grade patients showed significantly shorter survival times than patients carrying

Table 4 Clinical characteristics of S100P- and IMP3-positive and negative tumors in all EHBDC patients ($n = 68$)

	S100P		*P* value	IMP3		*P* value
	Positive 52	Negative 16		Positive 54	Negative 14	
Age	67.1 ± 10.0	66.8 ± 7.9	0.977	66.2 ± 9.4	70.3 ± 9.7	0.151
Gender						
Male	37	13	0.326	42	8	0.113
Female	15	3		12	6	
Location						
Perihilar	28	5	0.114	24	9	0.186
Distal	24	11		30	5	
Primary tumor						
cT1,2	25	10	0.324	28	7	0.902
cT3,4	27	6		26	7	
Regional lymph node						
cN0	29	11	0.356	32	8	0.886
cN1,2	23	5		22	6	
Gross type						
Papillary	8	7	0.024	13	2	0.349
Others	44	9		41	12	
Surgical resection						
Possible	37	13	0.326	39	11	0.458
Impossible	15	3		15	3	

T1- and 2-grade patients. There was no significant association between any other clinical or pathological characteristic and patient survival (Table 5).

Discussion

Recent studies have reported the diagnostic value of immunohistochemical staining for S100P and IMP3 using tissue samples obtained from endoscopic transpapillary bile duct biopsy. Levy et al. [14] reported that a combination of immunohistochemical staining for S100P, von Hipple–Lindau gene product and IMP3 were useful in the diagnosis of cholangiocarcinoma. Furthermore, Hamada et al. [13] reported that a combination of S100P immunohistochemical staining and conventional histology improved diagnostic sensitivity. In this study, S100P staining provided high sensitivity (76.5 %), but the specificity was low (50.0 %) due to many false-positive samples. Focal S100P-positive staining has been detected in normal intrahepatic bile duct [19]. In this study, focal expression of S100P was detected in 5/12 (41.7 %) of the extrahepatic bile duct samples that were ultimately diagnosed as benign and 17/68 (25.0 %) of the samples ultimately diagnosed as malignant. Because false positive results should be avoided as much as possible in the clinical setting, S100P immunohistochemical staining is not useful for the diagnosis of EHBDC. In contrast, IMP3 immunohistochemical staining showed high sensitivity and specificity (79.4 and 91.7 %, respectively). However, there were 7 (14.3 %) IMP3-negative samples in 49 histologically positive samples. These results suggested that the combination of IMP3 immunohistochemistry with conventional histology was straightforward and that it was the most useful test for the diagnosis of EHBDC when using TBFB samples. This result could contribute to the preoperative examination of bile duct strictures.

The reason underlying the significant association between IMP3 expression and gender in EHBDC patients was unclear.

There was a significant association between S100P expression and the outcome of EHBDC patients who underwent surgical resection in this study. In addition to T factor and residual-tumor factor, the multivariate analysis revealed that S100P expression was a significant independent prognostic factor. It was reported that peripheral intrahepatic cholangiocarcinoma with positive S100P showed poorer prognosis after surgery than those with negative S100P [12]. Because there was no significant difference between S100P expression and clinicopathologic features in patients who underwent surgical resection in this study, the reason why S100P-positive EHBDC patients showed poorer prognosis remained unclear. The molecular studies which investigate the correlation

Table 5 Univariate and multivariate analysis of poor prognostic factor in EHBDC patients who underwent surgical resection ($n = 55$)

	n	Univariate Log-rank test	Multivariate Cox's proportional hazards model		
		P value	Hazard ratio	95 % confidence interval	*P* value
Age		0.84			
<65 years	21				
≥65 years	34				
Gender		0.592			
Male	42				
Female	13				
Location		0.819			
Perihilar	26				
Distal	29				
Primary tumor		0.01			0.047
pT1,2	33		1		
pT3,4	22		2.99	1.01–8.83	
Regional lymph node		0.072			
pN0	36				
pN1,2	19				
Gross type		0.557			
Papillary	15				
Others	40				
Residual tumor		0.002			0.006
R0	43		1		
R1	12		4.30	1.52–12.2	
Histological grade		0.564			
G1	11				
G2	30				
G3	14				
Microscopic lymphatic invasion		0.38			
Absent	15				
Present	40				
Microscopic venous invasion		0.245			
Absent	36				
Present	19				
Microscopic perineural invasion		0.07			
Absent	12				
Present	43				
S100P		0.033			0.039
Negative	15		1		
Positive	40		8.51	1.18–64.8	
IMP3		0.578			
Negative	12				
Positive	43				

between S100P expression and outcome of EHBDC patients are required. But of the 68 patients who were ultimately diagnosed with malignant strictures, S100P expression was significant lower in patients with papillary growth type in this study. Papillary growth type EHBDC has been reported to show unique features, such as a tendency to result in percutaneous transhepatic biliary drainage catheter tract recurrence [26], and to spread superficially [24] when compared with other types of EHBDC. Typical papillary type EHBDC was thought not

to be invasive. These features of papillary type EHBDC may contribute to the significant association between S100P expression and patient outcome.

In conclusion, the results of this study suggest that IMP3 immunohistochemical staining is useful for the diagnosis of EHBDC, and S100P immunohistochemical staining is useful as a prognostic marker for EHBDC even in a small endoscopic transpapillary biopsy sample.

This study is limited by its design because it is a single-center based retrospective study. Further confirmation of the usefulness of S100P and IMP3 immunohistochemical staining is required.

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