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Original contribution

Details of human epidermal growth factor receptor 2 status in 454 cases of biliary tract cancer☆

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Summary Human epidermal growth factor receptor 2 (HER2)-targeted therapy has improved clinical outcomes in patients with HER2-positive breast and gastric cancers, although ineffective or recurrent cases are present. One reason for this is the heterogeneity of HER2 expression in cancer cells. The aim of this study was to investigate the clinicopathological characteristics and HER2 status of patients with biliary tract cancers (BTCs). We examined HER2 protein expression by immunohistochemistry, *HER2* gene amplification by fluorescence *in situ* hybridization, and both HER2 protein and gene levels simultaneously by gene-protein assay. Samples were collected from 454 patients who underwent surgical resection for BTCs (110 intrahepatic cholangiocarcinomas [ICC], 67 perihilar extrahepatic cholangiocarcinomas [ECC-Bp], 119 distal extrahepatic cholangiocarcinomas [ECC-Bd], 80 gallbladder carcinomas [GBC], and 79 ampullary carcinomas [AVC]). HER2 status was assessed according to the guidelines for HER2 testing in gastroesophageal adenocarcinoma. HER2-positive status was detected in 14.5% of BTCs (3.7% of ICC, 3.0% of ECC-Bp, 18.5% of ECC-Bd, 31.3% of GBC, and 16.4% of AVC). Furthermore, HER2-positivity tended to correlate with low histological grade, tumor histology,

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and macroscopic features in certain tumors. HER2 heterogeneity was common and highly frequent (83%) in BTC cases. Reduced HER2 protein expression in the deeper invasive areas with simultaneous dedifferentiation was frequently observed in HER2-positive cancer cells. The findings of this study suggest that a large subgroup of HER2-positive BTC cases can be considered for HER2-targeted therapy. Moreover, the HER2 status in BTCs should be determined carefully using a sensitive approach toward larger cancer tissues.

1. Introduction

Biliary tract cancers (BTCs) are a series of tumors that develop in the biliary tract, which include intrahepatic cholangiocarcinoma (ICC), extrahepatic cholangiocarcinoma (ECC), gallbladder carcinoma (GBC), and ampullary cancer (AVC). They are rare cancers, but most of them show geographic variation in their incidence [1−4]. BTCs have different clinicopathological features, biological characteristics, and carcinogenic pathways [1−4]. All BTCs show aggressive characteristics with poor outcomes, and currently the only available treatment for long-term survival is surgery [1−4]. Therefore, it is necessary to develop effective treatments, especially for recurrent or unresectable BTCs.

Human epidermal growth factor receptor 2 (HER2), a transmembrane tyrosine kinase receptor, has been shown to have roles in the development of various types of cancer [5]. HER2-targeted therapy has been explored in clinical settings and demonstrated an improvement of clinical outcomes in patients with HER2-positive breast and gastric cancers [6,7]. The predictive biomarker for the HER2-targeted therapy is HER2 protein overexpression in cancer cells, called HER2 status, which is identified by immunohistochemistry (IHC) and supported by the presence of *HER2* gene amplification [8]; both HER2 overexpression and *HER2* gene amplification are closely correlated. The frequency of BTCs with HER2-positive status has been reported to vary, which is not lower than that of breast and gastric cancers. In several case reports, the efficacy of HER2-targeted therapy has been explored in BTCs [9−11]. Two phase II studies reported objective response rates of 64% and 22% in HER2-positive BTC patients treated with HER2-targeted therapies [12,13]. These findings suggest that overexpression of HER2 could be a promising therapeutic approach for BTC. It has been known that ineffective or recurrent cases also exist in patients with breast and gastric cancers treated with HER2-target therapies, potentially because of the heterogeneity of HER2 expression in cancer cells [14−17].

Several studies have shown the frequency of HER2 positivity in BTCs, although the data vary strongly, as follows: 0−82% in ICC [18,19], 0−21.4% in ECC [18−22], 0−23% in GBC [20,22−25], and 0−13% in AVC [26,27]. No details of clinicopathological findings were provided, and no studies evaluating HER2 heterogeneity have been reported.

The aim of this study was to systematically investigate the clinicopathological characteristics and HER2 status of patients with BTCs. HER2 heterogeneity in BTCs was also examined.

2. Materials and methods

2.1. Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the National Cancer Center, Japan (#2018−182). Informed consent was obtained from all participants involved in the study, and all clinical investigations were conducted in line with the principles of the Declaration of Helsinki.

2.2. Study population

A total of 454 patients with BTCs (109 ICC, 67 perihilar ECC [ECC-Bp], 119 distal ECC [ECC-Bd], 80 GBC, and 79 AVC) underwent surgical resections between 2004 and 2016 at the National Cancer Center Hospital. Patients who received any therapy before surgery were excluded. All patients included in this study underwent macroscopic curative resection. The clinicopathological characteristics of the patients are summarized in Table 1. Recurrence was diagnosed when a new local or distant metastatic lesion was detected in imaging studies or when an increase in tumor marker levels with deterioration of the patient's condition was observed. The median follow-up period was 32.5 (0.6−167.6) months. Overall, 257 patients were alive, 162 died because of BTC, and 35 died of other causes.

2.3. Pathological examination

All of the BTCs were examined pathologically and classified according to the World Health Organization classification [1−4,28] and the International Union against Cancer tumor-node-metastasis (TNM) classification [29]. All patients with stage IV disease were diagnosed on the basis of para-aortic lymph node involvement. Surgically

resected specimens were fixed in 10% formalin and cut into serial slices 5 mm thick. All the sections were stained with hematoxylin and eosin for pathological examination. The maximum cut surface of the tumor was used for examination of the tissue specimen. The "nodular" macroscopic type is defined as a solid tumor mass formed in the wall of the bile duct or the gallbladder.

2.4. IHC, fluorescence *in situ* hybridization, and a gene-protein assay

IHC was performed on formalin-fixed, paraffin-embedded tissue sections as described previously [30]. The BenchMark XT System (Roche Tissue Diagnostics, Tucson, AZ) and anti-HER2 antibody (clone 4B5, Roche Tissue Diagnostics) were used. For a negative control, the same protocol was carried out without the primary antibody. *HER2* gene amplification was examined using the PathVysion HER2 DNA probe Kit II (Abbott Laboratories, Abbott Park, IL), and both HER2 protein and gene levels were analyzed simultaneously using the gene-protein assay (GPA) similar to previously described [31,32]. HER2 status was assessed according to the guideline for HER2 testing in gastroesophageal adenocarcinoma [8].

2.5. Evaluation of HER2 heterogeneity

"HER2 heterogeneity" is defined as the presence of $\geq$5% of cancer cells with a HER2 status different from those of other cancer cells in the same case. For evaluating HER2 heterogeneity, we additionally assessed whether 5%−10% ($\geq$5% and <10%) of the cancer cells were HER2 IHC3+ and IHC2+ with *HER2* gene amplification in each HER2-negative case judged according to the guideline [8]. To elucidate the details of heterogeneity analysis, each case was categorized into 6 types/subtypes based on the findings of HER2 protein expression and *HER2* gene amplification in cancer cells as shown in Supplementary Fig. S1. HER2-positive cancer was categorized into types 1, 3a, 4a, and 5a. Homogeneous HER2-positive cancer was categorized into type 1, and heterogeneous HER2-positive cancer was categorized into types 3a, 4a, and 5a. Types 3b, 4b, and 5b were also heterogenous HER2-positive cancers, and they accounted for 5−10% of HER2-positive cancer cases. Next, we combined all heterogeneous HER2-positive cancer cases (types 3a, 3b, 4a, 4b, 5a, and 5b) and analyzed them for HER2 heterogeneity.

2.6. Statistical analysis

The χ^2 analysis or Fisher's exact test was used to compare categorical data. The postoperative overall survival (OS) rate and disease-free survival (DFS) rates were calculated using the Kaplan-Meier method. Univariate analysis was performed for prognostic factors using the log-rank test. Factors found to be significant in univariate analysis were incorporated into the multivariate analysis using the Cox proportional hazards model (backward elimination method). Differences at $P < 0.05$ were considered statistically significant. Statistical analyses were performed using StatView-J 5.0 software (Abacus Concepts, Berkeley, CA).

3. Results

3.1. HER2 status in BTCs

The HER2 status in BTCs was evaluated by IHC, fluorescence *in situ* hybridization (FISH), and GPA (Table 2), according to the guideline for HER2 testing in gastroesophageal adenocarcinoma [8]. The results of GPA were similar to those of IHC and FISH. HER2 positivity was detected in 3.7% of ICC cases, including 1 case of IHC3+ and 3 cases of IHC2+ with *HER2* gene amplification. In addition, HER2 positivity was found in 3.0% of ECC-Bp cases, including 1 case of IHC3+ and 1 case of IHC2+ with *HER2* gene amplification, and in 18.5% of ECC-Bd cases, including 9 cases of IHC3+ and 13 cases of IHC2+ with *HER2* gene amplification. Furthermore, HER2 positivity was detected in 31.3% of GBC cases, including 15 cases of IHC3+ and 10 cases of IHC2+ with *HER2* gene amplification and in 16.4% of AVC cases, including 5 cases of IHC3+ and 8 cases of IHC2+ with *HER2* gene amplification. *HER2* gene amplification without HER2 overexpression was also detected in some BTC cases: 1 (0.9%) of ICC, 1 (1.5%) of ECC-Bp, 8 (6.7%) of ECC-Bd, 8 (10%) of GBC, and 3 (3.8%) of AVC.

3.2. Correlation of HER2 positivity with other clinicopathological variables

The correlation between HER2 positivity and various clinicopathological factors was analyzed (Table 1). Results showed that HER2-positive cancers showed a strong correlation or tendencies of correlation with positive tumor margin status in ICC ($P = 0.037$); low histological grade in ECC-Bd ($P = 0.072$) and GBC ($P = 0.076$); histology of papillary adenocarcinoma in ECC-Bd ($P = 0.049$) and GBC ($P = 0.084$); and macroscopic papillary tumor in ECC-Bd ($P = 0.040$). Papillary adenocarcinoma cases in ECC-Bd, GBC, and AVC were classified (Table 3) according to the definition of precursor lesions provided in the WHO classification [2−4]: intraductal papillary neoplasm of the bile duct (IPNB) (corresponding to intracholecystic papillary neoplasm in the gallbladder and intraampullary papillary-tubular neoplasm in the ampullary region) and biliary intraepithelial neoplasia (BilIN). Similar ratios of HER2-positive cases were found in papillary adenocarcinomas arising from BilIN and IPNB type 2, although no HER2-positive case was present among cases of papillary adenocarcinoma arising from IPNB type 1.

Table 1 Relationship between clinicopathological characteristics and HER2 status in biliary tract cancers.

Variables	ICC				Variables	ECC-BP				Variables	ECC-Bd	
	HER2 status					HER2 status					HER2 status	
	Total	Positive	Negative	*P*		Total	Positive	Negative	*P*		Total	Positive
Age, years				N.S.					N.S.			
<70	62	2	60		<70	39	1	38		<70	48	10
≥70	47	2	45		≥70	28	1	27		≥70	71	12
Sex				N.S.					N.S.			
Male	72	3	69		Male	51	1	50		Male	99	19
Female	37	1	36		Female	16	1	15		Female	20	3
Pathologic tumor status				N.S.					N.S.			
T1a	6	0	6		Tis	6	0	6		Tis	3	0
T1b	6	0	6		T1	1	0	1		T1	38	12
T2	79	2	77		T2a	20	1	19		T2	62	6
T3	11	2	9		T2b	16	0	16		T3	16	4
T4	7	0	7		T3	16	1	15		T4	0	0
					T4	8	0	8				
Pathologic node status				N.S.					N.S.			
N0	38	1	37		N0	38	1	37		N0	61	11
N1, N2	29	1	28		N1, N2	29	1	28		N1, N2	58	11
Pathologic metastasis status				N.S.					N.S.			
M0	105	3	102		M0	64	2	62		M0	111	19
M1	4	1	3		M1	3	0	3		M1	8	3
Tumor histology									N.S.			
					pap	10	0	10		pap	17	6
					tub	51	2	49		tub	80	15
					por	6	0	6		por	22	1
Tumor histological grade				N.S.					N.S.			
G1, G2	97	4	93		G1, G2	61	2	59		G1, G2	97	21
G3, G4	12	0	12		G3, G4	6	0	6		G3, G4	22	1
Tumor margin status				**0.037**					N.S.			
Negative	84	1	83		Negative	33	1	32		Negative	70	12
Positive	25	3	22		Positive	34	1	33		Positive	49	10
Lymphatic invasion				N.S.					N.S.			
0, 1	67	0	67		0, 1	40	1	39		0, 1	58	10
2, 3	42	4	38		2, 3	27	1	26		2, 3	61	12
Venous invasion				N.S.					N.S.			
0, 1	46	1	45		0, 1	22	1	21		0, 1	70	14
2, 3	63	3	60		2, 3	45	1	44		2, 3	49	8
Perineural invasion				N.S.					N.S.			
0, 1	46	0	46		0, 1	20	0	20		0, 1	27	4
2, 3	63	4	59		2, 3	47	2	45		2, 3	92	18
Macroscopic type				N.S.					N.S.			
MF	52	1	51		nodular type	45	2	43		nodular type	79	14
MF + PI	26	2	24		papillary type	11	0	11		papillary type	25	8
MF + IG	10	0	10		flat type	11	0	11		flat type	15	0
PI	8	0	8									
PI + MF	7	1	6									
IG/IG + PI	6	0	6									
Total	109	4	105			67	2	65			119	22

Abbreviations: ICC, intrahepatic cholangiocarcinoma; ECC: extrahepatic cholangiocarcinoma, Bp: perihilar type, Bd: distal type, GBC: gallbladder cancer, AVC: ampullary cancer, MF: mass-forming type, PI: periductal-infiltrating type, IG: intraductal growth type; HER2, human epidermal growth factor receptor 2.*P*: Tendency is indicated and bold number indicates statistically significant. N.S.: not significant ($P > 1.0$).

ECC-Bd		Variables	GBC				Variables	AVC			
HER2 status			HER2 status					HER2 status			
Negative	*P*		Total	Positive	Negative	*P*		Total	Positive	Negative	*P*
	N.S.					N.S.					N.S.
38		<70	39	9	30		<70	41	5	36	
59		≥70	41	16	25		≥70	38	8	30	
	N.S.					N.S.					N.S.
80		Male	45	12	23		Male	47	7	40	
17		Female	35	13	22		Female	32	6	26	
	N.S.					N.S.					N.S.
3		T1a	4	0	4		Tis	19	1	18	
26		T1b	3	2	1		T1a	7	1	6	
56		T2a	22	10	12		T1b	12	1	11	
12		T2b	11	3	8		T2	16	5	11	
0		T3	20	7	13		T3a	18	3	15	
		T4	20	3	17		T3b	7	2	5	
	N.S.					N.S.					N.S.
50		N0	42	15	27		N0	52	7	45	
47		N1, N2	38	10	28		N1, N2	27	6	21	
	N.S.					N.S.					N.S.
92		M0	75	25	50		M0	75	11	64	
5		M1	5	0	5		M1	4	2	2	
	0.049					0.084					N.S.
11		pap	33	14	19		pap	23	5	18	
65		tub	30	9	21		tub	49	8	41	
21		por/sq/sig/ec	17	2	15		por/sq/sig/ec	7	0	7	
	0.072					0.076					N.S.
76		G1, G2	63	23	40		G1, G2	72	13	59	
21		G3, G4	17	2	15		G3, G4	7	0	7	
	N.S.					N.S.					N.S.
58		Negative	65	21	44		Negative	79	13	66	
39		Positive	15	4	11		Positive	0	0	0	
	N.S.					N.S.					0.096
48		0, 1	38	13	25		0, 1	58	7	51	
49		2, 3	42	12	30		2, 3	21	6	15	
	N.S.					N.S.					N.S.
56		0, 1	48	15	33		0, 1	66	9	57	
41		2, 3	32	10	22		2, 3	13	4	9	
	N.S.					0.078					N.S.
23		0, 1	52	20	32		0, 1	75	12	63	
74		2, 3	28	5	23		2, 3	4	1	3	
	0.040					N.S.					N.S.
65		nodular type	33	10	23		protruded type	59	10	49	
17		papillary type	33	13	20		mixed type	14	3	11	
15		flat type	3	1	2		ulcerative type	1	0	1	
		solid type	11	1	10		polyp type/flat type	5	0	5	
97			80	25	55			79	13	66	

Table 2 HER2 status in biliary tract cancers.

Type	Total number of case	HER2 Status [IHC (%)/gene amplification]				
		0	1+	2+	3+	Positive
ICC	109	59 (54)/0	44 (40)/1	5 (4.6)/3	1 (0.9)/1	4 (3.7)
ECC-Bp	67	25 (37)/1	28 (42)/0	13 (19)/1	1 (1.5)/1	2 (3.0)
ECC-Bd	119	33 (28)/2	49 (41)/6	28 (24)/13	9 (7.6)/9	22 (18.5)
GBC	80	15 (19)/1	31 (39)/7	19 (24)/10	15 (19)/15	25 (31.3)
AVC	79	22 (28)/0	35 (44)/3	17 (22)/8	5 (6.3)/5	13 (16.4)

Abbreviations: ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; Bp, perihilar type; Bd, distal type; GBC, gallbladder cancer; AVC, ampullary cancer; HER2, human epidermal growth factor receptor 2.

3.3. Association of HER2 status in BTCs with patient outcomes

Because patients with ICC and ECC-Bp rarely displayed HER2 positivity, we analyzed association of HER2 status in ECC-Bd, GBC, or AVC. Results showed that HER2 positivity in ECC-Bd, GBC, or AVC was not significantly associated with patient outcome (OS or DFS) (Fig. 1). HER2 status was not prognostic in the entire patients with BTCs for OS and DFS (Fig. 1).

3.4. HER2 heterogeneity in BTCs

We defined "HER2 heterogeneity" as the presence of ≥5% of cancer cells with a HER2 status different from those of other cancer cells in the same case. For evaluating HER2 heterogeneity, we additionally assessed whether 5%−10% (≥5% and <10%) of the cancer cells in each case were HER2 IHC3+ and IHC2+ with *HER2* gene amplification. HER2 heterogeneity was observed in BTCs with HER2 protein overexpression at the following frequencies: 83% (65/78) in total BTCs, 100% (4/4) in ICC, 100% (3/3) in ECC-Bp, 89% (25/28) in ECC-Bd, 78% (21/27) in GBC, and 75% (12/16) in AVC (Table 4). On subclassification of the cases based on HER2 heterogeneity, 2% (1/65), 26% (17/65), and 72% (47/65) of the cases were of types 3, 4, and 5, respectively (Supplementary Fig. S1). There was 18% of HER2 heterogeneity in type b BTCs. A few cases were detected that exhibited *HER2* gene amplification without HER2 protein overexpression; these were categorized as types 2 and 6.

In heterogeneous BTCs except ICC, HER2 overexpression was commonly detected in cancer cells in the mucosal layer and tended to be close to the mucosal layer (Supplementary Fig. S2). The staining intensity of the HER2 protein in cancer cells with *HER2* gene amplification was found to decrease according to the direction of tumor invasion: 86% (37/43) of cases with reduced HER2 intensity showed a reduction in HER2 expression in the direction of deeper invasion, 42% (18/43) in the horizontal direction, 8% (3/43) in a shallower direction, and 2% (1/43) in irregular directions. As the reduction in HER2 protein expression was

Table 3 HER2 status in papillary adenocarcinoma in ECC-Bd, GBC, and AVC.

Type	Precursor lesion that papillary adenocarcinoma arising from	Total	HER2-positive	HER2-negative	*P*
ECC-Bd	BilIN	8	4	4	N.S.
	IPNB type 1	5	0	5	
	IPNB type 2	4	2	2	
	Total	17	6	11	
GBC	BilIN	14	7	7	0.066
	IPNB type 1	6	0	6	
	IPNB type 2	13	7	6	
	Total	33	14	19	
AVC	BilIN	5	1	4	N.S.
	IPNB type 1	4	0	4	
	IPNB type 2	14	4	10	
	Total	23	3	20	
ECC-Bd + GBC + AVC	BilIN	27	12	15	**0.007**
	IPNB type 1	15	0	15	
	IPNB type 2	31	13	18	
	Total	73	25	48	

Note: All papillary adenocarcinomas are classified according to the definition of intraductal papillary neoplasm of the bile duct (IPNB) and biliary intraepithelial neoplasia (BilIN) in WHO classification. Chi-squared test is used to compare categorical data.
Abbreviations: ECC, extrahepatic cholangiocarcinoma; Bd, distal type; GBC, gallbladder cancer; AVC, ampullary cancer; HER2, human epidermal growth factor receptor 2.
P: Tendency is indicated and bold number indicates statistically significant. N.S.: not significant ($P > 1.0$).

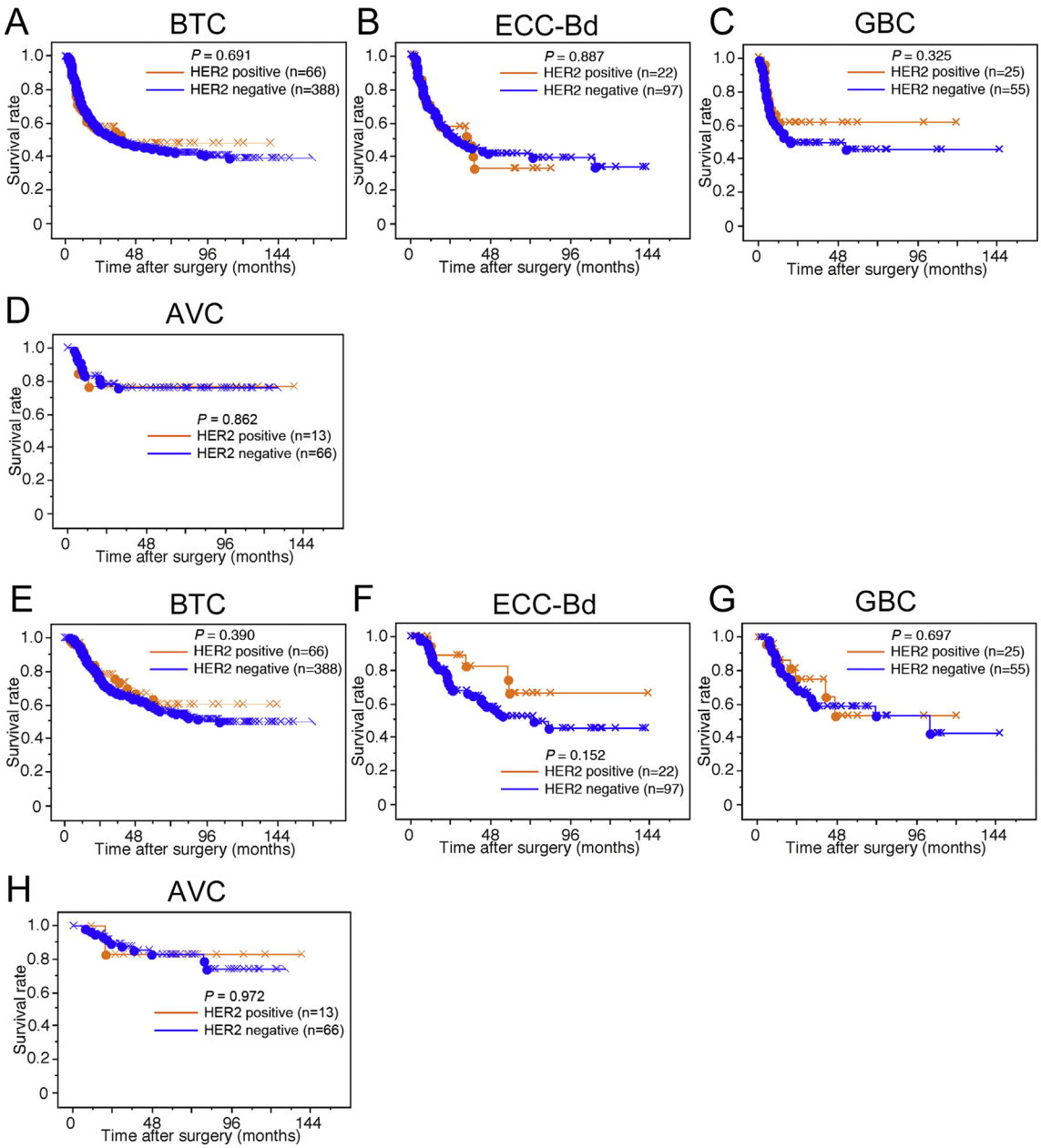

Fig. 1 Kaplan-Meier survival curves for DFS (A-D) and OS (E-H) in patients with biliary tract cancers (BTCs). The Kaplan-Meier survival curves for DFS and OS in the entire patients with BTCs are shown in A and E, respectively. ECC-Bd, distal type extrahepatic bile duct carcinoma; GBC, gallbladder cancer; AVC, ampullary carcinoma; DFS, disease-free survival; OS, overall survival; HER2, human epidermal growth factor receptor 2.

Table 4 Heterogeneity of HER2 status in BTCs with *HER2* gene amplification.

HER2 status heterogeneity	ICC	ECC-Bp	ECC-Bd	GBC	AVC
Homogeneous	0	0	3	6	4
Heterogeneous	4	3	25	21	12

Abbreviations: ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; Bp, perihilar type; Bd, distal type; GBC, gallbladder cancer; AVC, ampullary cancer; HER2, human epidermal growth factor receptor 2.

commonly found in these invasive directions (Fig. 2), it indicated dedifferentiation of cancer cells.

Next, we analyzed the relationship between the presence of heterogeneity of HER2 overexpression and other clinicopathological variables (compared the findings in type 1 to those in types 3, 4, and 5). Results showed that the presence of HER2 heterogeneity was significantly correlated with tumor histology in GBC and AVC (Supplementary Table S1). Papillary adenocarcinoma showed a higher homogeneous HER2-positive status than the other cancers. No

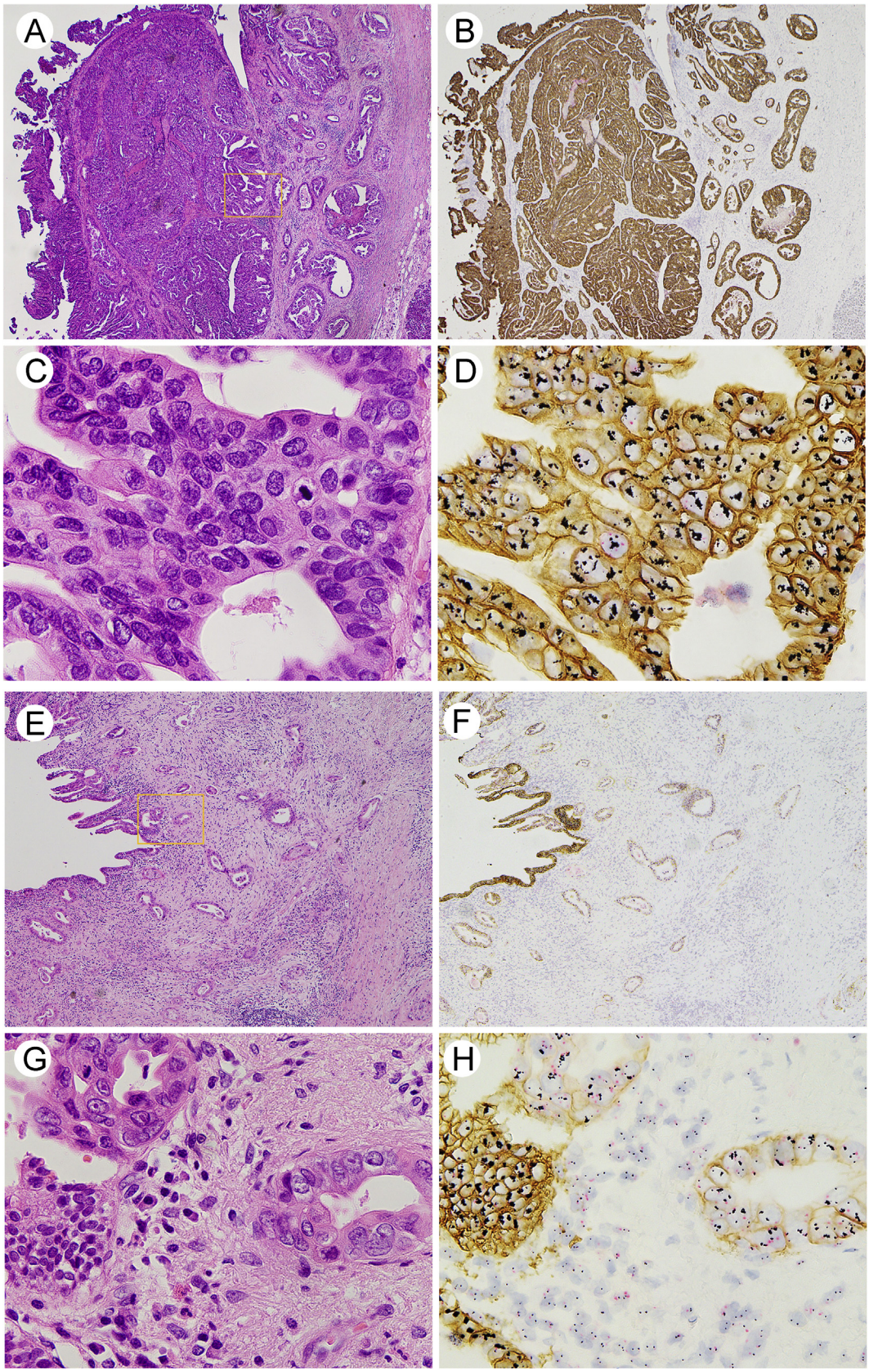

Fig. 2 Histologic and HER2 GPA features of homogeneous (A-D) and heterogeneous (E-H) HER2-positive extrahepatic cholangiocarcinomas. Low-power view of histologic (A, E) and GPA features (B, F); high-power view of histologic (C, G) and GPA features (D,

statistically significant difference in patient outcome was evaluated between homogeneous and heterogeneous HER2-positive cases in ECC-Bd, GBC, and AVC (Supplementary Fig. S3). All patients with BTCs having a homogenous HER2-positive status, except 1 GBC patient, were alive.

4. Discussion

The HER2-targeted therapy has been heading in the direction to use in clinical settings as a standard treatment for BTCs, although no details of the clinicopathological characteristics of HER2 status in BTCs are available. In this study, we evaluated details of HER2 status using whole tissue slides from 454 patients who underwent surgical resection for BTCs. Our findings are as follows: (1) the frequency of HER2 positivity differed among different BTCs (3.7% in ICC, 3.0% in ECC-Bp, 18.5% in ECC-Bd, 31.3% in GBC, and 16.4% in AVC); (2) the HER2-positive status was not significantly correlated with clinicopathological variables but tended to correlate with low histological grade, tumor histology, and macroscopic features in some tumors; (3) tumor histology of papillary adenocarcinoma tended to be HER2-positive, and HER2-positive papillary adenocarcinomas arose from BilIN or IPNB type 2 but not from IPNB type 1; and (4) HER2 heterogeneity was commonly and frequently (83%) detected among BTCs. The presence of heterogeneity of HER2 overexpression was not associated with patient outcome, although nearly all patients with a homogenous HER2-positive status were alive. To the best of our knowledge, this is the first report on the clinicopathological characteristics of HER2 heterogeneity in BTCs.

The frequency of HER2 heterogeneity in BTCs was high level in a comparison with that observed in the other cancers; a third or more of breast cancers [16] and 23−79% of gastric cancers [17]. In all BTCs except ICC, HER2-positive cancer cells were found in the mucosal layer, and HER2 protein overexpression was often reduced in the invasive areas, such as the deeper layer of the biliary tract, with simultaneous dedifferentiation of cancer cells. Albrecht et al. showed that HER2 positivity is also detected in intramucosal cancer in HER2-positive GBC (50%, 2/4) [23] and ECC (80%, 4/5) [18]. These results suggest that HER2 overexpression occurs in the early stages of carcinogenesis. There were 3 types of HER2 heterogeneities: 2 different heterogeneity patterns (types 3 and 4; Supplementary Fig. S1) and a mixed pattern of types 3 and 4 (type 5). Although a similar tendency was observed among different BTCs, the frequencies of these types were considerably different; 2%, 26%, and 72% of cases were of types 3, 4, and 5, respectively (Supplementary Fig. S1). Two different processes might be attributed in HER2 heterogeneity; first, HER2-positive cancer cells may lose *HER2* gene amplification or be replaced by cancer cells without *HER2* gene amplification; second, HER2 protein expression in HER2-positive cancer cells may be suppressed or inhibited. These two processes might be associated with HER2 heterogeneity. Both types of heterogeneity are also observed in breast and gastric cancers, and the first process is corresponded to a genetic heterogeneity [16].

The frequency of HER2 positivity has been shown to vary in the past reports, and the reasons were explained by potentially geographic and ethnic differences [23]. Meta-analysis using data from previous studies in which both IHC and FISH were used to examine HER2 status showed that the average frequency of HER2 positivity (95% confidential interval) was 4.8% (0−14.5%) in ICC, 17.4% (3.4−31.4%) in ECC, 19.1% (11.2−26.8%) in GBC, and 27.9% (0−60.7%) in AVC [33]. Although our findings are similar, the frequencies obtained in our study tended to be higher, probably because of the following: use of different antibodies, whole tissue sections (not tissue microarray), and GPA methods in addition to IHC and FISH. In other words, we could observe both HER2 protein overexpression and *HER2* gene amplification in cancer tissue widely, in addition to the different staining condition. We also found that HER2-positive cancer cells are commonly present in the mucosal and near mucosal areas, especially in BTCs with low frequency of HER2 positivity. Because BTCs exhibit frequent heterogeneous HER2 overexpression, large cancer tissues should be assessed to detect the status of HER2-positive cancer cells with a sensitive approach for avoiding false-negative cases.

The HER2 status is different in different BTCs, probably because each type of BTC has different clinicopathological features, biological characteristics, and carcinogenetic mechanisms [1−4]. Interestingly, in our study, the frequency of HER2 positivity in ECC was markedly different between ECC-Bp and ECC-Bd. In addition, both the HER2-positive status and correlation between HER2 status and other clinicopathological variables, including patient outcomes (data not shown) in ECC-Bp, were more similar to those in ICC than in ECC-Bd. ECC-Bp might share biological and clinicopathological characteristics with the large duct type of ICC, most of which are categorized as macroscopic periductal infiltrating (PI)-related types. However, no significant differences in HER2 status were found between the large duct type (macroscopic PI-related type) and small duct type (macroscopic mass forming-

H). The mucosal layer is positioned to the left (A, B, E, F) and in direction of the deeply invasive cancer cells to the right. The orange rectangles in A and E indicate C and G, respectively. Both *HER2* gene amplification and HER2 protein overexpression are apparent in the image (D). *HER2* gene amplification is apparent in all cancer cells observed (H). While HER2 protein overexpression is observable in mucosal cancer cells, HER2 protein expression is observably inhibited in the invasive area (H). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.) GPA, gene-protein assay; HER2, human epidermal growth factor receptor 2.

related type) of ICC in this study. To the best of our knowledge, this is the first study to show differences in the frequency of HER2 positivity in ECC based on localization. TNM classification recommends that ECC-Bp and ECC-Bd should be considered as separate clinical entities because of their differences in terms of patient outcomes, risk factors, and type of surgery performed [29,34]. Moreover, Ishida et al. reported that mucin-related protein profiles are different between ECC-Bp and ECC-Bd [35]. However, whether there are any differences in carcinogenic mechanisms between them remains unknown.

In this cohort, HER2 status was not associated with patient outcome in ECC-Bd, GBC, and AVC, which is consistent with previous findings [23,25,33]. In addition, no statistical differences between HER2 heterogeneity and homogeneity were found in ECC-Bd, GBC, and AVC cases, although all patients with homogenous HER2-positive status (except 1 GBC case) were alive. Hence, a homogenous HER2-positive status may act as a prognostic factor in these BTCs. The presence of HER2 heterogeneity is a poor predictor for HER2-targeted therapy response and a poor prognosticator for patients having breast cancers or gastric cancers with HER2-targeted therapy [6,14−17].

A multicenter phase II study of trastuzumab deruxtecan, an antibody-drug conjugated with an anti-HER2 antibody, has been conducted for HER2-positive unresectable or recurrent BTC (JMACCT ID: JMA-IIA00423). Using data from this clinical study, we aim to further analyze whether HER2 heterogeneity affects treatment effectiveness. Hence, in the near future, we will have a better understanding of the clinical effect of HER2 heterogeneity on BTCs.

In conclusion, we determined the HER2 status in 454 patients who underwent surgical resection for BTCs, using IHC, FISH, and GPA methods. HER2 positivity was detected in 14.5% of BTCs, although it varied among different BTCs. This indicates that a significant subgroup of HER2-positive BTC cases can be considered for HER2-targeted therapy. Because HER2 heterogeneity is common and frequent among patients with BTCs, it is recommended that their HER2 status be determined carefully in larger cancer tissues by using a sensitive approach. This will further help us evaluate the effect of HER2 heterogeneity on HER2-targeted therapy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2020.08.006.

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References

[1] Nakanuma Y, Klimstra DS, Komuta M, Zen Y. Intrahepatic cholangiocarcinoma. In: Carneiro F, Chan JKC, Cheung N-YA, Cree IA, Fitzgibbons PL, Gasson S, et al., editors. World Health organization classification of tumours 5th edition digestive System tumours. Lyon: IARC Press; 2019. p. 254−9.

[2] Roa JC, Adsay NV, Arola J, Tsui WM, Zen Y. Carcinoma of the gallbladder. In: Carneiro F, Chan JKC, Cheung N-YA, Cree IA, Fitzgibbons PL, Gasson S, et al., editors. World Health organization classification of tumours 5th edition digestive System tumours. Lyon: IARC Press; 2019. p. 283−8.

[3] Roa JC, Adsay NV, Arola J, Tsui WM, Zen Y. Carcinoma of the extrahepatic bile ducts. In: Carneiro F, Chan JKC, Cheung N-YA, Cree IA, Fitzgibbons PL, Gasson S, et al., editors. World Health organization classification of tumours 5th edition digestive System tumours. Lyon: IARC Press; 2019. p. 289−91.

[4] Adsay NV, Reid MD. Ampullary adenocarcinoma. In: Carneiro F, Chan JKC, Cheung N-YA, Cree IA, Fitzgibbons PL, Gasson S, et al., editors. World Health organization classification of tumours 5th edition digestive System tumours. Lyon: IARC Press; 2019. p. 127−30.

[5] Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007; 26:6469−87.

[6] Oh DY, Bang YJ. HER2-targeted therapies - a role beyond breast cancer. Nat Rev Clin Oncol 2020;17:33−48.

[7] Olson EM. Maximizing human epidermal growth factor receptor 2 inhibition: a new oncologic paradigm in the era of targeted therapy. J Clin Oncol 2012;30:1712−4.

[8] Bartley AN, Washington MK, Colasacco C, et al. HER2 testing and clinical decision making in gastroesophageal adenocarcinoma: guideline from the college of American pathologists, American society for clinical pathology, and the American society of clinical oncology. J Clin Oncol 2017;35:446−64.

[9] Bang YJ, Giaccone G, Im SA, et al. First-in-human phase 1 study of margetuximab (MGAH22), an Fc-modified chimeric monoclonal antibody, in patients with HER2-positive advanced solid tumors. Ann Oncol 2017;28:855−61.

[10] Javle M, Churi C, Kang HC, et al. HER2/neu-directed therapy for biliary tract cancer. J Hematol Oncol 2015;8:58.

[11] Nam AR, Kim JW, Cha Y, et al. Therapeutic implication of HER2 in advanced biliary tract cancer. Oncotarget 2016;7:58007−21.

[12] Hainsworth JD, Meric-Bernstam F, Swanton C, et al. Targeted therapy for advanced solid tumors on the basis of molecular profiles: results from MyPathway, an open-label, phase IIa multiple basket study. J Clin Oncol 2018;36:536−42.

[13] Hyman DM, Piha-Paul SA, Won H, et al. HER kinase inhibition in patients with HER2- and HER3-mutant cancers. Nature 2018;554: 189−94.

[14] Hou Y, Nitta H, Wei L, et al. HER2 intratumoral heterogeneity is independently associated with incomplete response to anti-HER2 neoadjuvant chemotherapy in HER2-positive breast carcinoma. Breast Canc Res Treat 2017;166:447−57.
[15] Kurozumi S, Padilla M, Kurosumi M, et al. HER2 intratumoral heterogeneity analyses by concurrent HER2 gene and protein assessment for the prognosis of HER2 negative invasive breast cancer patients. Breast Canc Res Treat 2016;158:99−111.
[16] Hanna WM, Ruschoff J, Bilous M, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol 2014;27:4−18.
[17] Kaito A, Kuwata T, Tokunaga M, et al. HER2 heterogeneity is a poor prognosticator for HER2-positive gastric cancer. World J Clin Cases 2019;7:1964−77.
[18] Albrecht T, Rausch M, Rossler S, et al. HER2 gene (ERBB2) amplification is a rare event in non-liver-fluke associated cholangiocarcinogenesis. BMC Canc 2019;19:1191.
[19] Yang X, Wang W, Wang C, et al. Characterization of EGFR family gene aberrations in cholangiocarcinoma. Oncol Rep 2014;32:700−8.
[20] Kawamoto T, Krishnamurthy S, Tarco E, et al. HER receptor family: novel candidate for targeted therapy for gallbladder and extrahepatic bile duct cancer. Gastrointest Cancer Res 2007;1:221−7.
[21] Kim HJ, Yoo TW, Park DI, et al. Gene amplification and protein overexpression of HER-2/neu in human extrahepatic cholangiocarcinoma as detected by chromogenic in situ hybridization and immunohistochemistry: its prognostic implication in node-positive patients. Ann Oncol 2007;18:892−7.
[22] Pignochino Y, Sarotto I, Peraldo-Neia C, et al. Targeting EGFR/HER2 pathways enhances the antiproliferative effect of gemcitabine in biliary tract and gallbladder carcinomas. BMC Canc 2010;10:631.
[23] Albrecht T, Rausch M, Roessler S, et al. HER2 gene (ERBB2) amplification is a low-frequency driver with potential predictive value in gallbladder carcinoma. Virchows Arch 2019;476:871−80.
[24] Harder J, Waiz O, Otto F, et al. EGFR and HER2 expression in advanced biliary tract cancer. World J Gastroenterol 2009;15:4511−7.
[25] Yoshida H, Shimada K, Kosuge T, Hiraoka N. A significant subgroup of resectable gallbladder cancer patients has an HER2 positive status. Virchows Arch 2016;468:431−9.
[26] Aloysius MM, Lobo DN, Rowlands BJ, Madhusudan S, Ilyas M, Zaitoun AM. HER-2/Neu overexpression is a rare event in periampullary cancer: assessment using the HercepTest. Histopathology 2009;55:236−7.
[27] Hechtman JF, Liu W, Sadowska J, et al. Sequencing of 279 cancer genes in ampullary carcinoma reveals trends relating to histologic subtypes and frequent amplification and overexpression of ERBB2 (HER2). Mod Pathol 2015;28:1123−9.
[28] Torbenson M, Zen Y, Yeh M. Intrahepatic cholangiocarcinoma and precursor lesions. In: Torbenson M, Zen Y, Yeh M, editors. AFIP atlas of tumor pathology series 4, tumors of teh liver. Washington, D.C.: American Registry of Pathology; 2018. p. 201−63.
[29] Brierley JD, Gospodarowicz MK, Wittekind C. TNM classification of malignant tumours. Hoboken, NJ: Wiley-Blackwell; 2017.
[30] Yonemaru J, Takahashi M, Nara S, et al. A yolk sac tumor of the pancreas and derived xenograft model effectively responded to VIP chemotherapy. Pancreatology 2020;20:551−7.
[31] Nitta H, Kelly BD, Padilla M, et al. A gene-protein assay for human epidermal growth factor receptor 2 (HER2): brightfield tricolor visualization of HER2 protein, the HER2 gene, and chromosome 17 centromere (CEN17) in formalin-fixed, paraffin-embedded breast cancer tissue sections. Diagn Pathol 2012;7:60.
[32] Hou Y, Nitta H, Li Z. HER2 gene protein assay is useful to determine HER2 status and evaluate HER2 heterogeneity in HER2 equivocal breast cancer. Am J Clin Pathol 2017;147:89−95.
[33] Galdy S, Lamarca A, McNamara MG, et al. HER2/HER3 pathway in biliary tract malignancies; systematic review and meta-analysis: a potential therapeutic target? Canc Metastasis Rev 2017;36:141−57.
[34] Wiltberger G, Krenzien F, Benzing C, et al. Prognostic accuracy of the seventh edition of the TNM classification compared with the fifth and sixth edition for distal cholangiocarcinoma. Ann Surg Oncol 2016;23:1320−6.
[35] Ishida K, Osakabe M, Eizuka M, et al. The expression of gastrointestinal differentiation markers in extrahepatic cholangiocarcinoma: clinicopathological significance based on tumor location. Hum Pathol 2019;92:91−100.