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Original Article

EGFR, HER2, and MET gene amplification and protein expression profiles in biliary tract cancer and their prognostic significance

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Abstract

Background: There are limited conventional chemotherapy options for biliary tract cancers (BTCs), a heterogenous group of lethal, rare malignancies. The receptor tyrosine kinase (RTK) is closely associated with the progression of human malignancies through the regulation of cell cycle. Overexpression or amplification of RTKs has been investigated as a potential biomarker and therapeutic target in BTC; herein, we investigate the value of such interventions.

Materials and Methods: Overexpression of RTK proteins was examined by immunohistochemistry in 193 BTC samples, of which 137 were gallbladder carcinoma, 29 were perihilar cholangiocarcinoma, and 27 were intrahepatic cholangiocarcinoma. Silver in situ hybridization of *MET* and *HER2* was performed to assess gene amplification.

Results: In the entire cancer group, gallbladder, perihilar, and intrahepatic, MET amplification rates were 15.7%, 19.0%, 3.4%, and 14.8%, respectively, and of HER2 amplification rates were 22.4%, 27.2%, 17.2%, and 3.7%, respectively. MET and HER2 protein expressions were significantly correlated with their gene amplification status. RTKs were significantly associated with adverse clinicopathologic features such as advanced pT category and lymph node metastasis. Overall survival was significantly shorter in *MET*-amplified ($P = .024$) and EGFR-overexpressed cases ($P = .045$). Recurrence-free survival was significantly correlated with *HER2*-amplified ($P = .038$) and EGFR-overexpressed cases ($P = .046$) in all patient groups. Overall and recurrence-free survival were significantly shorter in patients who were double positive for HER2 and EGFR.

Conclusion: Our data suggested that MET, HER2, and EGFR might be potential therapeutic targets and that their co-expression is a strong prognostic factor for BTCs.

Key words: biliary tract cancer; gallbladder cancer; gene amplification; MET; HER2; EGFR.

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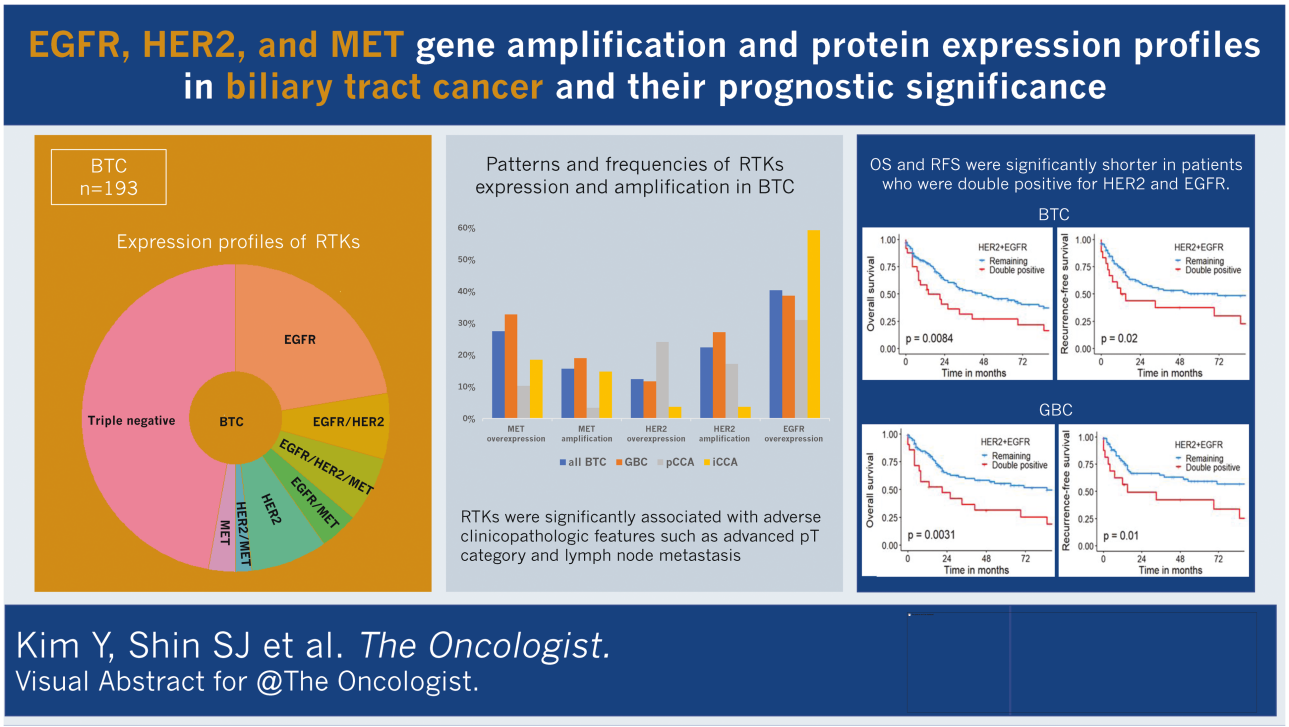
Graphical Abstract



Implications for practice

Conventional chemotherapy for biliary tract cancers (BTCs), a rare and lethal group of malignancies, is limited in its treatment options. Our findings highlight the potential of targeting receptor tyrosine kinases (RTKs), specifically MET, HER2, and EGFR, as promising avenues for intervention in BTCs. The observed correlation between RTK amplification/expression and adverse clinicopathologic features suggests the relevance of these biomarkers in guiding treatment decisions. Importantly, the shorter overall and recurrence-free survival in cases with specific RTK alterations emphasizes the prognostic significance of MET, HER2, and EGFR co-expression. These insights advocate for personalized treatment approaches targeting these RTKs in patients with BTC.

Introduction

Biliary tract cancers (BTCs), including cholangiocarcinoma (intrahepatic [iCCA] and perihilar [pCCA]) and gallbladder carcinoma (GBC), are relatively rare primary malignancies that carry a poor prognosis.[1] Over 50% of these tumors are diagnosed at an advanced stage and are non-resectable.[1] The 5-year survival rate of all patients is 5%-15%.[2,3] Conventional chemotherapy, consisting of a regimen of gemcitabine with cisplatin (GEMSIS), remains the recommended backbone treatment for patients with BTC as a first-line treatment.[4] However, in line with the current shift toward molecular-based therapy, genetic alterations in BTC are increasingly being recognized as potential targets for therapy.[5] Recent advancements in molecular profiling have revealed new opportunities for treating patients with BTC, who typically face a poor prognosis.

Recently, targeted therapies such as pemigatinib, infigratinib, and ivosidenib, which are aimed at FGFR and IDH1 mutations, have received FDA approval for their effectiveness in improving survival.[6-8] Despite the continuous evolution of precision medicine over the past decade, the treatment landscape for BTC requires further innovation. This need is particularly pronounced given the anatomical differences among iCCA, GBC, and pCCA, and the heterogeneity in their pathogenesis and genomic drivers.[9] Therefore, there has been an interest in the identification of new therapeutic targets in combination with a precision medicine approach for patients with BTC.

Receptor tyrosine kinase (RTK) signaling pathways play crucial roles in cell differentiation, proliferation, survival, and migration. The genetic alteration of RTKs has been associated with aggressive biologic behavior, poor prognosis, and resistance to therapy for several types of human malignancies,[10-12] including BTC.[13-16]

There have been reports of *HER2-EGFR* co-expression in several malignancies including lung cancer, breast cancer, prostate cancer, and urinary bladder cancer.[17-19] This suggests that the co-expression of multiple RTKs forms heterodimers and strengthens their oncogenic and synergistic effect.[20] Although these RTKs play a crucial role in oncogenic transformation and are a potential therapeutic target, there are few studies on EGFR, HER2, and MET in BTC and also few studies on the relationship between these RTKs.

Previous studies have reported amplification or mutation of *EGFR* in 6% and 15% of BTC, respectively.[21-23] Chang et al demonstrated that *EGFR* mutation is an independent prognostic marker in BTCs in addition to tumor stage and differentiation.[13]

Limited studies have investigated the prognosis associated with HER2 overexpression or amplification in BTCs. Vivaldi et al reported that HER2 overexpression represents an independent prognostic factor for disease recurrence in patients with BTC.[14]

Mesenchymal-epithelial transition (*MET*) is a proto-oncogene that encodes a heterodimeric transmembrane RTK for hepatocyte growth factor. While certain studies have not identified any clinicopathological significance associated with MET overexpression,[22,24] others have demonstrated a notable correlation with a poorer prognosis.[15,16]

Therefore, in this study, we evaluate the expression level and amplification of RTKs, MET, HER2, and EGFR in BTCs to determine whether they are reliable prognostic factors and potential therapeutic targets of BTC, with focus on the significance of co-expression of these RTKs.

Materials and methods

Patients and specimens

We retrospectively examined 193 BTCs samples obtained from surgical procedures performed at the Hanyang University Hospital between 1991 and 2016. The patients met the inclusion criterion of having histologically confirmed BTC, defined as tumors of the gallbladder and the intrahepatic, perihilar bile duct. The BTCs were assessed according to the staging system for primary tumor/regional lymph nodes/distant metastasis (TNM) described in the 8th AJCC cancer staging manual, and histological classification according to the World Health Organization classification of tumors. The clinical data were retrospectively obtained from electronic medical records and included age at diagnosis, sex, pathological profiles (size, stage, gross type, tumor location, histological grade, location, margin status, lymphovascular invasion, and perineural invasion), metastasis, date of recurrence, and date of death. Tumor recurrence was defined as tumor growth in any site of the body after the curative surgery, diagnosed clinically, radiologically, or pathologically, but mainly by computed tomography and ultrasonography. Among the 193 patients, 95 (49.2%) were men, and the median age was 65 years (range, 28-90 years). The BTC tumor types were: adenocarcinoma (n = 181), squamous and adenosquamous carcinoma (n = 8), and sarcomatoid and undifferentiated carcinoma (n = 4). The detailed clinicopathological features are described in Supplementary Table S1.

Tissue microarray construction

Hematoxylin-eosin slides were reviewed by S.J.S. and Y.K., and the most representative tumor regions without necrosis and hemorrhage were carefully selected and marked. A standard set of 2 tissue cores (3-mm diameter) was obtained from each of the 193 formalin-fixed paraffin-embedded primary tumors and assembled into a new recipient paraffin block using a manual tissue microarray (TMA) instrument (Quick-Ray, Unitma, Seoul, Korea).

Immunohistochemistry

Immunohistochemical staining (IHC) was performed on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's protocol. The primary antibodies used were anti-EGFR mouse monoclonal (clone 3C6, Ventana), anti-HER2 rabbit monoclonal (clone 4B5, Ventana) and anti-c-MET rabbit monoclonal (clone SP44, Ventana) antibodies.

Two pathologists (S.J.S. and Y.K.) independently evaluated MET, HER2, and EGFR IHC. The immunoreactivity of MET was scored using a 4-tier system, which was established in previous studies on MET IHC in non-small cell lung cancer.[25] This system was used to evaluate both proportion and staining intensity: 0, no staining or < 50% tumor cells with any intensity; 1+, ≥ 50% of tumor cells staining with weak intensity but < 50% with moderate or higher staining; 2+, ≥ 50% of tumor cells with moderate staining but < 50% with strong intensity; 3+, ≥ 50% of tumor cells staining with strong intensity.

HER2 scoring was conducted according to ASCO/CAP 2018: 0, no membrane staining in < 10% tumor cells; 1+, faint/barely perceptible or weak incomplete membrane staining in > 10% of tumor cells; 2+, weak to moderate complete membrane staining in > 10% of tumor cells or strong complete membrane staining in ≤ 10% of tumor cells; 3+, strong (intense and uniform) complete membrane staining in > 10% of tumor cells.

EGFR IHC was scored according to the EGFR pharmDx protocol: 0, no staining or membrane staining in ≤ 10% cancer cells; 1+, faint, incomplete staining of membranes and focal weak positivity in cytoplasm in > 10% cancer cells; 2+, complete and moderate staining of membranes and weak to moderate staining in the cytoplasm in > 10% cancer cells; 3+, complete and strong staining of membranes and moderate to strong staining of cytoplasm in > 10% cancer cells.

In this study, samples that demonstrated immunostaining levels of 2 + or 3 + for MET, HER2, and EGFR were classified as positive for overexpression (Figure 1).

In situ hybridization

MET and *HER2* silver in situ hybridization (SISH) was performed on an automated Ventana BenchMark XT platform (Ventana), according to the manufacturer's protocols.

The gene copy number was assessed independently by 2 pathologists (S.J.S. and Y.K.) and the number of *MET* or *HER2* and CEP7 or CEP17 signals was counted in at least 40 tumor cell nuclei (80 nuclei if there was no agreement). Then, the *MET*/CEP7 and *HER2*/CEP17 ratio were calculated. Cancer sites with very weak or no signals were recorded as insufficiently hybridized.

MET scoring was conducted according to a previous study.[26] High amplification was defined as a *MET*/CEP7 ratio ≥ 2.0, an average *MET* gene copy number per cell of ≥ 6.0, or ≥ 10% of tumor cells containing ≥ 15 MET signals. Intermediate amplification was defined as ≥ 50% of cells containing ≥ 5 *MET* signals, and the criteria for high amplification were not met. Low amplification was defined as ≥ 40% of tumor cells showing ≥ 4 *MET* signals, and the criteria for high or intermediate amplification were not met. All other tumors were classified as negative. Samples assessed as high, intermediate or low level were considered positive for *MET* amplification (Supplementary Fig. S1).

HER2 gene amplification status was classified according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP 2018) guidelines. When the ratio of *HER2*/CEP17 was ≥ 2.0 and the average *HER2* signals/cell were ≥ 4.0, or the ratio of *HER2*/CEP27 was < 2.0 and the average *HER2* signals/cell were ≥ 6.0, the *HER2* amplification status was classified as positive

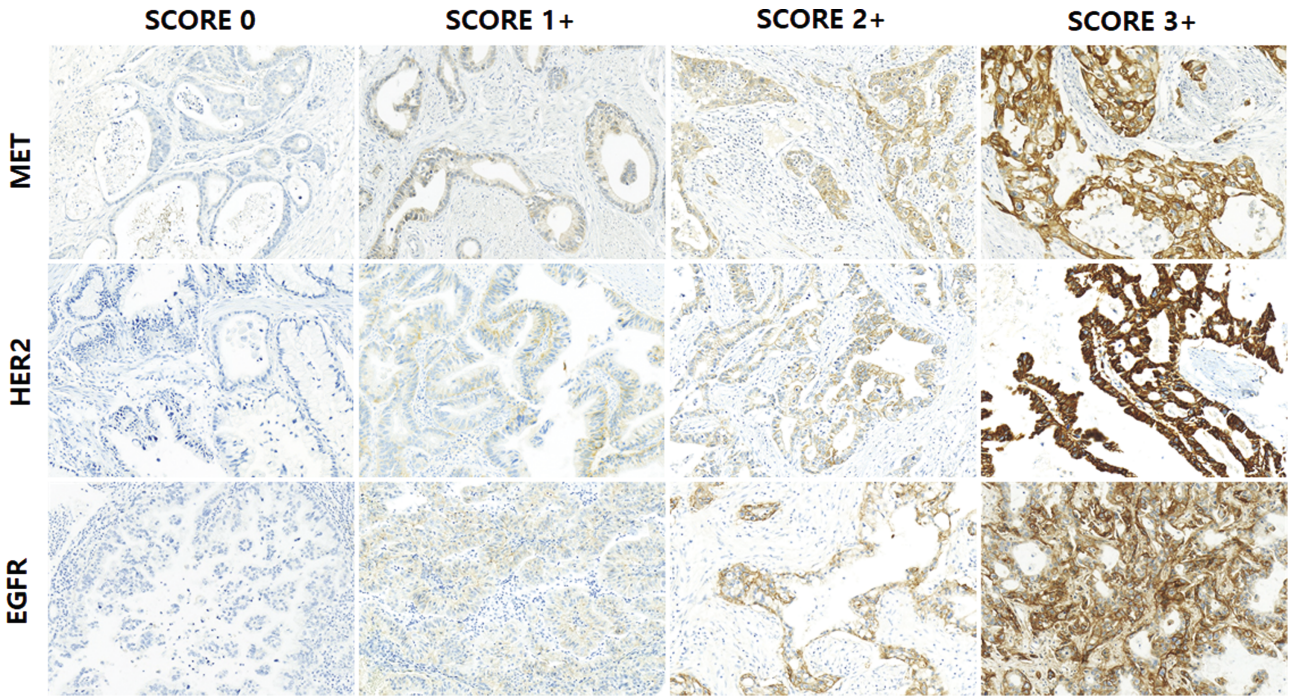


Figure. 1. Representative figures of immunohistochemistry (IHC) scores for MET, HER2, and EGFR expression in biliary tract cancers (original magnification 200×).

Statistical analysis

To analyze the association between the clinicopathological features and *MET* or *HER2* gene amplification as well as MET or HER2 protein expression, the chi-square test or Fisher's exact test were used when appropriate. Recurrence-free survival (RFS) and overall survival (OS) were determined using Kaplan-Meier survival curves, and the log-rank test was used to compare the differences. Univariate and multivariate analysis were conducted using the Cox regression model. The Cox proportional hazard regression model was used to evaluate the independent prognostic significance. All statistical analyses were carried out using R software, version 4.2.0. (https://www.R-project.org); $P < .05$ was considered as statistically significant.

RESULTS

Clinicopathological characteristics of series

A total of 193 BTCs, including 137 GBC, 29 pCCA, and 27 iCCA, were included in this analysis. Of these patients, 95 (49.2%) were male, 98 (50.8%) were female, with an even age distribution. The GBC group showed a slight female predominance (male vs. female, 43.0% vs. 57.0%), but the pCCA group (male vs. female, 65.5% vs. 34.5%) and iCCA group (male vs. female, 63.0% vs. 37.0%), showed male predominance (Supplementary Table S1).

Association of MET and HER2 protein expression with gene amplification

MET and HER2 IHC were successfully performed in 193/193 (100.0%) tissue cores. A MET IHC score of 0-3 was observed in 74 (38.3%), 66 (34.2%), 40 (20.7%), and 13 (6.7%) cases, respectively. MET overexpression, defined by IHC score 2 or higher, was found in 53/193 (27.5%), 45/137 (32.8%), 3/29 (10.3%), and 5/27 (18.5%) of all tumors, GBC, pCCA, and iCCA tumors, respectively. HER2 IHC scores of 0-3 were observed in 165 (85.5%), 4 (2.1%), 15 (7.8%), and 9 (4.7%) cases, respectively. HER2 overexpression, defined by IHC score 2 or higher, was found in 24/193 (12.4%), 16/137 (11.7%), 7/29 (24.1%), and 1/27 (3.7%) of all tumors, GBC, pCCA, and iCCA tumors, respectively (Supplementary Table S2).

MET SISH was successful in 172/193 (89.1%) tissue cores, and HER2 SISH in 192/193 (99.5%). *MET* amplification was found in 27/172 (15.7%), 22/116 (19.0%), 1/29 (3.4%), and 4/27 (14.8%) in all, GBC, pCCA, and iCCA tumors, respectively. *HER2* amplification was found in 43/192 (22.4%), 37/136 (27.2%), 5/29 (17.2%), and 1/27 (3.7%) in all, GBC, pCCA, and iCCA tumors, respectively (Supplementary Table S2).

The frequencies of *MET*, *HER2* amplification, and EGFR overexpression differed according to the site of the carcinoma, and these patterns were statistically significant (Supplementary Table S2): (1) Overexpression of MET was significantly more frequent in GBC (32.8%), followed by iCCA (18.5%) and pCCA (10.3%) ($P = .023$); (2) amplification of *HER2* was significantly more frequent in GBC (27.2%), followed by pCCA (17.2%) and iCCA (3.7%) ($P = .015$). (3) The frequencies of *MET* amplification and HER2 and EGFR overexpression in the 3 different locations were not significantly different. A comprehensive description of each gene is illustrated in Supplementary Figure S2.

The distribution of the correlation between expression of MET and HER2 and their gene amplification is shown in Supplementary Table S3. MET expression was significantly correlated with gene amplification in all patients ($P < .001$) and in each tumor type ($P < .001$, $P = .034$, and $P = .044$, for GBC, pCCA, and iCCA, respectively), and HER2 expression and its gene amplification were also associated ($P < .001$,

$P < .001$, $P = .008$, and $P = .037$ for all, GBC, pCCA, and iCCA, respectively).

Correlation between MET, HER2, and EGFR

When both *MET* and *HER2* amplification results were combined, 15 patients (7.8%) showed amplification of both *MET* and *HER2*. Nineteen patients showed amplification and overexpression of both *MET* and EGFR. Twenty-six patients showed amplification and overexpression of both *HER2* and EGFR. In all patients, *MET* amplification was significantly associated with *HER2* amplification ($P < .001$) and EGFR overexpression ($P = .001$). *HER2* amplification status was also significantly associated with EGFR overexpression ($P = .003$). In the GBC group, *MET* amplification was significantly correlated with *HER2* amplification ($P < .001$) and also with EGFR overexpression ($P < .001$). *HER2* amplification and EGFR overexpression also showed a significant correlation ($P = .001$). No significant association between *MET*, *HER2* amplification, and EGFR overexpression were found in the pCCA and iCCA groups (Supplementary Table S4).

The percentages of cases with co-expression of MET, HER2, and EGFR are shown in Supplementary Figure S3. Thirty-four (19%) of the 174 BTC cases showed co-expression for RTKs, with 12 cases (7%) of MET+/HER2+/EGFR+, 12 cases (7%) of MET–/HER2+/EGFR+, 7 cases (4%) of MET+/HER2–/EGFR+, and 3 cases (2%) of MET+/HER2+/EGFR–. In GBC groups, 12 cases (10%) were positive for all 3 RTKs, and 16 cases (14%) were positive for 2 RTKs. There were no triple positive cases in the pCCA and iCCA groups.

Correlation with clinicopathologic factors

In all patients, *MET* amplification and EGFR overexpression were significantly associated with large tumor size ($P = .029$ and $P = .014$), poor histological differentiation ($P = .015$ and $P = .023$), advanced pT stage ($P < .001$ and $P = .003$), nodal metastasis ($P = .029$ and $P = .012$), and advanced AJCC stage ($P < .001$ and $P = .004$). EGFR was also overexpressed in infiltrative or mass-forming gross histological type ($P = .025$). *HER2* amplification was significantly associated with large tumor size ($P = .031$), nodal metastasis ($P = .037$), and advanced AJCC stage ($P = .025$), but not with poor histological grade ($P = .514$). In addition, the amplification and overexpression of *MET*, *HER2*, and EGFR was significantly associated with other histological types, such as adenosquamous or sarcomatoid carcinoma ($P = .025$, .029, and <.001, respectively) (Table 1).

In the GBC group, *MET* amplification was also significantly associated with large tumor size, poor histological differentiation, advanced pT stage, nodal metastasis, advanced AJCC stage, and perineural invasion ($P < .05$ for all). *HER2* amplification was associated with histological type ($P = .024$), but not with tumor size, nodal metastasis, or advanced AJCC stage. EGFR overexpression was significantly associated with histological type, poor histological grade, advanced pT stage, nodal metastasis, and advanced AJCC stage ($P < .05$ for all) (Supplementary Table S5).

In the pCCA group, *HER2* amplification was significantly associated with nodal metastasis ($P = .034$) and advanced AJCC stage ($P = .010$), but lacked any association between other genes and clinicopathologic factors (Supplementary Table S6).

In the iCCA group, *MET* amplification was significantly associated with advanced pT stage ($P = .003$) and advanced AJCC stage ($P = .011$). There was no significant association between other genes and clinicopathologic factors (Supplementary Table S7).

Association between protein expression, gene amplification, and patient survival in BTCs

In all patients, EGFR expression was a prognostic factor for OS and RFS ($P = .045$ and $P = .047$). Additionally, *MET* amplification was significantly related to overall survival ($P = .024$). *HER2* amplification was significantly related to RFS ($P = .041$). Histological grade ($P = .001$ and $P = .002$), advanced pT stage (both, $P < .001$), nodal metastasis ($P < .001$ and $P = .002$), distant metastasis (both $P < .001$), advanced AJCC stage (both $P < .001$), lymphovascular invasion (both $P < .001$), and perineural invasion (both $P < .001$) were found to be prognostic of poor RFS and OS, respectively (Table 2; Supplementary Figure S4).

In the GBC group, similar results were observed in univariate analysis of general prognostic factors in all patients. *MET* amplification was significantly related to OS ($P = .029$). *HER2* amplification was significantly related to both OS and RFS ($P = .026$ and $P = .010$) (Table 2; Supplementary Fig. S5). No statistical significance was found between EGFR overexpression and survival in GBC by univariate analysis.

In the iCCA group, there was a significant correlation between *MET* amplification and OS ($P = .014$), but not RFS. Although EGFR overexpression did not show a significant association with OS, there was a tendency for the prognosis to be worse. There was no statistical significance for any of the gene expressions with OS or RFS in the pCCA group (Supplementary Table S8).

In the multivariate model, the prognostic role of histologic grade was confirmed by Kaplan-Meier analysis, which revealed a significantly shorter OS in all BTC. However, *MET* or *HER2* amplification did not show survival difference in multivariate analysis in all patients and the GBC group (Supplementary Table S9).

We additionally examined the associations between the co-expression of these RTKs in BTC with OS and RFS. OS and RFS were significantly shorter in patients who were double positive for HER2 and EGFR in all patients (Table 2; Figure 2A and B) and the GBC group (Table 2; Figure 2C and D). Multivariate analysis was done, but no statistically significant correlation was found (data not shown). No correlation was observed between survival rates (OS and RFS) and any other combination of RTK positivity (data not shown).

Discussion

Cancers of the biliary tract exhibit considerable intra-patient heterogeneity, making it challenging to predict a patient's response to treatment. Therefore, major efforts have been devoted to devising treatment strategies and improving the efficacy of non-surgical, adjuvant, or palliative treatment, including new therapeutic tools and targeted therapy.

The presence of RTKs has been reported to be associated with accelerated tumor progression and therapeutic resistance in various types of cancers, including breast, gastric, uterine, cervical, and lung cancer.[10,11,27,28] In BTC, RTKs have been studied, revealing significant variations in their expression rates across different tumor sites. Previous studies have shown that the prevalence of MET overexpression in BTC

Table 1. Correlation between *MET* amplification, *HER2* amplification, or EGFR expression and clinicopathologic factors of patients with biliary tract carcinoma (*n* = 193).

	MET amplification			*HER2* amplification			EGFR overexpression		
	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
Age			.836			1.000			1.000
< 65 years	66 (45.5%)	13 (48.1%)		71 (47.7%)	21 (48.8%)		55 (47.8%)	38 (48.7%)	
≥ 65 years	79 (54.5%)	14 (51.9%)		78 (52.3%)	22 (51.2%)		60 (52.2%)	40 (51.3%)	
Sex			1.000			.490			.769
Male	73 (50.3%)	13 (48.1%)		76 (51.0%)	19 (44.2%)		58 (50.4%)	37 (47.4%)	
Female	72 (49.7%)	14 (51.9%)		73 (49.0%)	24 (55.8%)		57 (49.6%)	41 (52.6%)	
Tumor size			.029			.031			.014
< 3 cm	78 (56.5%)	8 (32.0%)		77 (57.5%)	15 (37.5%)		62 (60.8%)	30 (41.1%)	
≥ 3 cm	59 (43.1%)	17 (68.0%)		57 (42.5%0	25 (62.5%)		40 (39.2%)	43 (58.9%)	
Histologic type			.025			.029			<.001
Adenocarcinoma	139 (95.9%)	22 (81.5%)		143 (96.0%)	37 (86.0%)		115 (100.0%)	66 (84.6%)	
Squamous/adenosquamous	4 (2.8%)	3 (11.1%)		3 (2.0%)	5 (11.6%)		0 (0%)	8 (10.3%)	
Sarcomatoid/undifferentiated	2 (1.4%)	2 (7.4%)		3 (2.0%)	1 (2.3%)		0 (0%)	4 (5.1%)	
Gross type			.664			.928			.025
Flat infiltrative	52 (37.1%)	12 (46.2%)		53 (37.3%)	17 (39.5%)		39 (35.5%)	32 (42.1%)	
Papillary	43 (30.7%)	6 (23.1%)		41 (28.9%)	13 (30.2%)		40 (36.4%)	14 (18.4%)	
Polypoid (mass-forming)	45 (32.1%)	8 (30.8%)		48 (33.8%)	13 (30.2%)		31 (28.2%)	30 (39.5%)	
Histologic grade			.015			.514			.023
Well differentiated	29 (22.0%)	1 (4.5%)		31 (22.6%)	5 (14.3%)		27 (24.1%)	9 (14.8%)	
Moderately differentiated	88 (66.7%)	14 (63.6%)		86 (62.8%)	23 (65.7%)		73 (65.2%)	36 (59.0%)	
Poorly differentiated	15 (11.4%)	7 (31.8%)		20 (14.6%)	7 (20.0%)		12 (10.7%)	16 (26.2%)	
pT category			<.001			.056			.003
T1	46 (33.8%)	1 (3.8%)		45 (31.7%)	6 (15.0%)		35 (31.8%)	16 (21.9%)	
T2	63 (46.3%)	8 (30.8%)		56 (39.4%)	18 (45.0%)		52 (47.3%)	23 (31.5%)	
T3	16 (11.8%)	15 (57.7%)		27 (19.0%)	14 (35.0%)		18 (16.4%)	23 (31.5%)	
T4	11 (8.1%)	2 (7.7%)		14 (9.9%)	2 (5.0%)		5 (4.5%)	11 (15.1%)	
Nodal status			.029			.037			.012
Negative	65 (60.7%)	7 (33.3%)		67 (62.0%)	16 (41.0%)		56 (65.1%)	27 (43.5%)	
Positive	42 (39.3%)	14 (66.7%)		41 (38.0%)	23 (59.0%)		30 (34.9%)	35 (56.5%)	
AJCC stage*			<.001			.025			.004
I	31 (22.8%)	0(0%)		32 (22.5%)	3 (7.3%)		24 (21.8%)	11 (14.9%)	
II	53 (39.0%)	5 (18.5%)		49 (34.5%)	10 (24.4%)		44 (40.0%)	15 (20.3%)	
III	38 (27.9%)	17 (63.0%)		45 (31.7%)	21 (51.2%)		32 (29.1%)	34 (45.9%)	
IV	14 (10.3%)	5 (18.5%)		16 (11.3%)	7 (17.1%)		10 (9.1%)	14 (18.9%)	
Perineural invasion			.284			1.000			.132
Absent	78 (55.7%)	11 (42.3%)		80 (55.6%)	22 (55.0%)		66 (60.0%)	36 (48.0%)	
Present	62 (44.3%)	15 (57.7%)		64 (44.4%)	18 (45.0%)		44 (40.0%)	39 (52.0%)	
Lymphovascular invasion			.195			.722			.374
Absent	73 (51.4%)	9 (36.0%)		72 (49.3%)	18 (45.0%)		57 (50.9%)	33 (44.0%)	
Present	69 (48.6%)	16 (64.0%)		74 (50.7%)	22 (55.0%)		55 (49.1%)	42 (56.0%)	

*AJCC 8th edition.
Abbreviation: AJCC, American Joint Committee on Cancer.

ranges from 5%-74% in GBC,[15,16,22,24,29] 12%-58% of intrahepatic cholangiocarcinoma,[22,30,31] and 0%-16% in extrahepatic cholangiocarcinoma.[22] HER2 overexpression has been found in 9.8%-12.8% in GBC,[32,33] and 0.9% and 8.5% in intra- and extrahepatic cholangiocarcinoma, respectively.[34] Overexpression of EGFR in GBC was observed in 44%-77% of patients.[35,36]

The prevalence of RTK overexpression and amplification observed in this study aligns with previous findings.[15,16,22,24,29-36] Notably, a significant association was observed between MET overexpression, *HER2* amplification, and the location of BTC. MET was frequently overexpressed in GBC, while *HER2* amplification was more common in pCCA and GBC compared to iCCA. Although the relationship between

Table 2. The univariate Cox regression analysis for overall survival (OS) and recurrence-free survival (RFS) in patients with biliary tract carcinoma (n = 193) and gallbladder carcinoma (n = 137).

Variables	Biliary tract carcinoma (BTC)					
	Overall survival (OS)			Recurrence-free survival (RFS)		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (< 65 vs. ≥ 65)	1.109	0.764-1.609	.585	1.010	0.641-1.593	.964
Sex (male vs. female)	1.153	0.793-1.677	.457	1.149	0.726-1.821	.553
Tumor size (< 3cm vs. ≥ 3cm)	1.045	0.699-1.563	.831	0.893	0.550-1.450	.647
Histologic grade* (G1 vs. G2, G3)	2.775	1.497-5.144	.001	3.106	1.500-6.429	.002
pT category (pT1, pT2 vs. pT3, pT4)	2.233	1.516-3.288	<.001	2.460	1.524-3.971	<.001
pN category (pN0 vs. pN1, pN2)	2.220	1.434-3.438	<.001	2.235	1.337-3.737	.002
pM category (pM0 vs. M1)	5.968	2.935-12.137	<.001	7.759	2.653-22.695	<.001
AJCC stage† (I, II vs. III, IV)	2.643	1.794-3.895	<.001	3.011	1.885-4.809	<.001
LVI (absent vs. present)	2.557	1.722-3.796	<.001	3.174	1.956-5.152	<.001
PNI (absent vs. present)	2.567	1.707-3.861	<.001	2.497	1.545-4.034	<.001
MET (not amplified vs. amplified)	1.771	1.079-2.908	.024	1.450	0.788-2.668	.232
HER2 (not amplified vs. amplified)	1.435	0.946-2.177	.089	1.691	1.020-2.804	.041
EGFR (not overexpressed vs. overexpressed)	1.463	1.008-2.123	.045	1.581	1.006-2.485	.047
HER2 + EGFR (remaining vs. double positive)	2.477	1.280-4.795	.007	2.839	1.213-6.645	.016

Variables	Gallbladder carcinoma (GBC)					
	Overall survival (OS)			Recurrence-free survival (RFS)		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (< 65 vs. ≥ 65)	1.151	0.735-1.803	.538	1.260	0.734-2.161	.402
Sex (male vs. female)	1.266	0.797-2.010	.318	1.171	0.678-2.022	.571
Tumor size (< 3cm vs. ≥ 3cm)	1.013	0.613-1.674	.959	0.899	0.505-1.603	.719
Histologic grade* (G1 vs. G2, G3)	2.216	1.134-4.314	.019	2.213	1.032-4.744	.041
pT category (pT1, pT2 vs. pT3, pT4)	2.662	1.683-4.210	<.001	2.598	1.492-4.524	<.001
pN category (pN0 vs. pN1, pN2)	2.540	1.482-4.351	<.001	2.570	1.392-4.744	<.001
pM category (pM0 vs. M1)	5.968	2.935-12.137	<.001	7.759	2.653-22.695	<.001
AJCC stage† (I, II vs. III, IV)	3.101	1.911-5.032	<.001	3.432	1.944-6.060	<.001
LVI (absent vs. present)	2.818	1.745-4.550	<.001	3.867	2.149-6.957	<.001
PNI (absent vs. present)	3.177	1.926-5.240	<.001	2.676	1.497-43785	<.001
MET (not amplified vs. amplified)	1.902	1.067-3.390	.029	1.647	0.830-3.272	.154
HER2 (not amplified vs. amplified)	1.734	1.069-2.815	.026	2.101	1.185-3.724	.010
EGFR (not overexpressed vs. overexpressed)	1.435	0.907-2.270	.123	1.513	0.880-2.600	.134
HER2 + EGFR (remaining vs. double positive)	2.326	1.321-4.094	.003	2.524	1.285-4.957	.007

*Histologic grade: G1: well differentiated; G2, moderately differentiated; G3, poorly differentiated.
†AJCC 8th edition.
Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; AJCC, American Joint Committee on Cancer; LVI, lymphovascular invasion; PNI, Perineural invasion.

expression/amplification of other genes and tumor site did not reach statistical significance, certain trends were observed. Specifically, EGFR expression was highest in iCCA, followed by GBC and pCCA.

Comparing IHC and SISH results, it was found that 43.5% of tumors in which there was MET overexpression also demonstrated gene amplification. Conversely, MET overexpression was observed in 74.1% of cases where MET amplification was present. While not all cases of overexpression and amplification were consistent with each other, they consistently exhibited statistically significant associations, with overexpression being more prevalent when amplification was present. Tumors with *MET* amplification were more likely to exhibit nuclear pleomorphism and hyperchromasia compared to those without amplification. Exploring the correlation between morphological features and genetic variations will provide valuable insights in future studies. Similar to MET, a significant correlation between protein overexpression and gene amplification was seen for HER2.

RTK amplification, particularly *MET* amplification, is associated with unfavorable clinicopathological features such as larger tumor size, higher histological grade, advanced cancer stage, nodal metastasis, and poor prognosis in BTC patients. However, the significance of MET overexpression

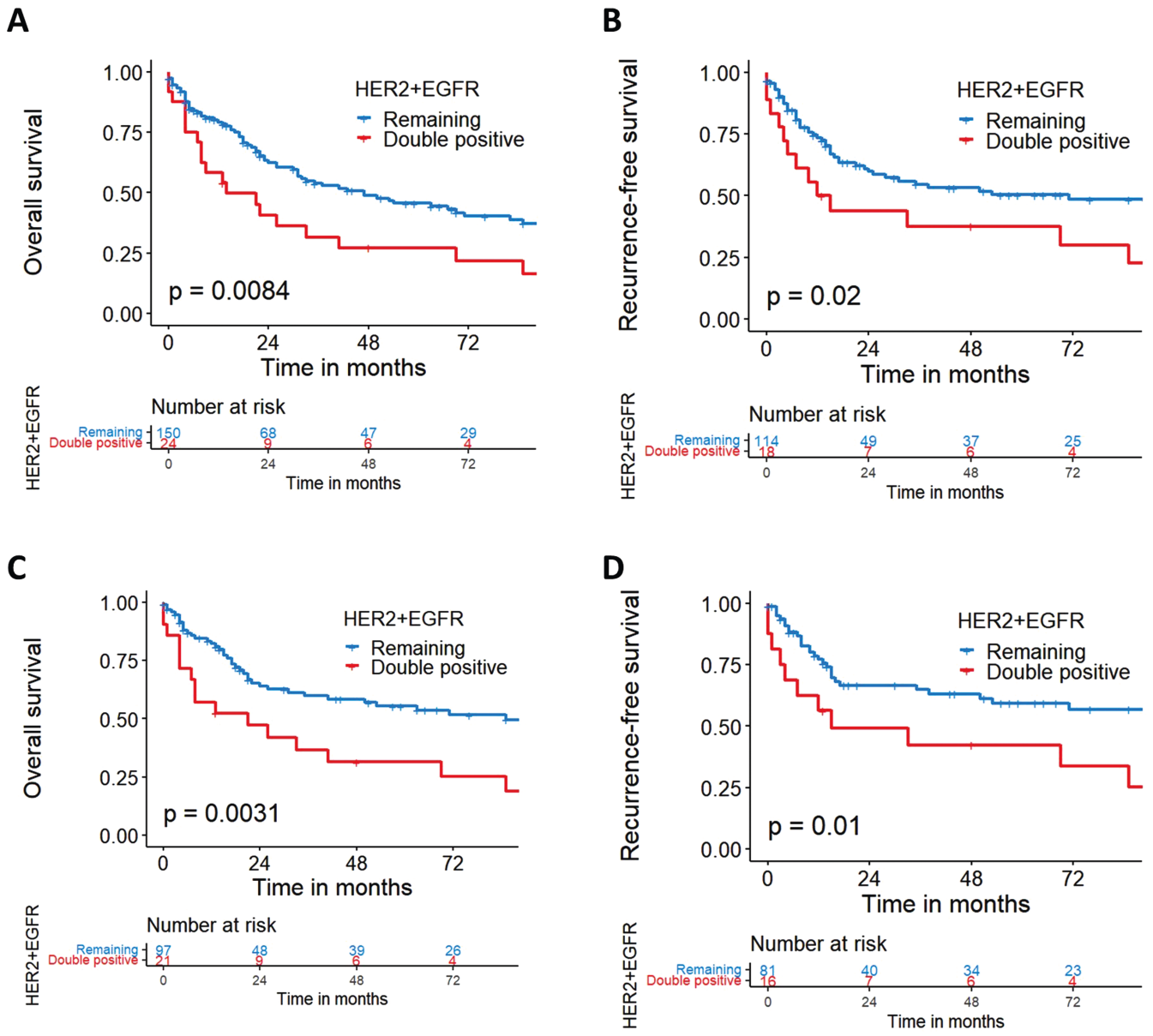


Figure. 2. Kaplan-Meier survival curves of all biliary tract carcinoma patients (A, B) and gallbladder carcinoma patients (C, D) forOS and RFS according to the co-expression of HER2 and EGFR.

is still debated, with previous studies showing inconsistent results.[15,16,22,24,37] The discrepancies in findings could be attributed to various factors, including differences in patient ethnicity, the different MET antibody used, variations in overexpression definition, and variations in sample sizes and types. Nonetheless, protein overexpression of MET has shown a significant correlation with *MET* amplification as detected by silver in situ hybridization (SISH), suggesting its potential as a screening test for amplification. *HER2* amplification and EGFR overexpression were also associated with poor prognostic factors in all patients.

In the GBC group, *HER2* amplification was shown to be significantly associated with histological type alone, which was in turn significantly associated with all RTKs. Interestingly, squamous and adenosquamous carcinoma showed multiple RTK amplification; in particular, EGFR was overexpressed in all squamous and undifferentiated carcinoma and sarcomatoid and undifferentiated carcinoma. However, the number of cases is small, and further studies are necessary to validate these findings.

A noteworthy finding is that double positivity for RTKs demonstrates a stronger correlation with OS compared to single positivity. Specifically, cases with simultaneous overexpression of EGFR and HER2 are significantly associated with the poorest prognosis, as revealed by univariate Cox-regression analysis and Kaplan-Meier survival analysis. Previous studies have indicated that co-expression of EGFR and HER2 is linked to increased tumor progression and worse prognosis in various cancers,[17-20] suggesting their collaborative role in tumor development. Heterodimerization between RTKs, particularly EGFR and HER2, can activate specific signaling pathways and promote prolonged and extensive cell growth and proliferation.[38,39] While lapatinib, a dual-specific inhibitor of EGFR and HER2, has shown promise in breast

cancer,[40,41] its effectiveness in advanced BTC is limited in unselected patient populations.[42,43] Ongoing clinical trials are investigating other targeted therapies, such as afatinib for BTC and GBC,[44] aiming to improve treatment options and explore novel therapeutic approaches.

Conclusions

Our study suggests that targeting cell surface RTKs could be a potential therapeutic strategy for BTC. RTK amplification or overexpression is associated with adverse clinicopathological characteristics, and patients with double positivity for EGFR and HER2 have a significantly shorter OS. These results suggest that RTKs could be actionable drug targets in BTC, emphasizing the importance of addressing multiple RTKs instead of relying on monotherapy, particularly in cases of co-expression. Overall, these findings have significant clinical implications for precision medicine in BTC.

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None declared.

Ethics approval

This study followed the World Medical Association Declaration of Helsinki recommendations and was approved by the Institutional Review Board of Hanyang University Hospital (IRB No. 2018-08-031-002). A waiver of informed consent for this study was obtained from IRB based on the retrospective analyses of archived tissues and clinical data.

Data availability

The data that support the findings of this study are available in the supplementary materials and from the corresponding author, upon reasonable request.

Conflicts of interest

The authors indicated no financial relationships.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Yeseul Kim, Seungyun Jee, Hyunsung Kim, Seung Sam Paik, Dongho Choi, Su Hyun Yoo, and Su-Jin Shin. The first draft of the manuscript was written by Yeseul Kim and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Supplementary material

Supplementary material is available at *The Oncologist* online.

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