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The Complete Loss of Tyrosine Kinase Receptors MET and RON Is a Poor Prognostic Factor in Patients with Extrahepatic Cholangiocarcinoma

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Abstract. *Background: Although the survival of patients with cholangiocarcinoma has improved, the prognosis remains unfavorable. The overexpression of mesenchymal–epithelial transition factor (MET) and recepteur d'origine nantais (RON) has been considered to be indicative of a poor prognosis in some types of cancer. On the other hand, some studies have shown that the expression of MET and RON is a favorable prognostic factor in certain types of tumors. Materials and Methods. Based on the immunohistochemical analysis of MET and RON, 290 patients who underwent resection for extrahepatic cholangiocarcinoma were divided into three groups: MET/RON-negative, -intermediate, and -positive. The associations between MET/RON expression and clinicopathological features, including prognosis, were analyzed. Results: MET/RON-negativity was associated with nodal metastasis and advanced pathological stage. The overall 5-year survival rates were significantly lower in the MET/RON-negative and MET/RON-positive groups than in the MET/RON-intermediate group (28.3%, 32.4% and 48.5%, respectively; p=0.01). Conclusion: The complete loss of one or both MET and RON, as well as their overexpression, is a poor prognostic factor in patients with extrahepatic cholangiocarcinoma, probably due to the high rate of lymph-node metastasis.*

Cholangiocarcinoma is a highly malignant invasive carcinoma arising from the ductal epithelium of the bile duct. Both intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma (EHCC) have a poor prognosis, partly because cholangiocarcinoma is difficult to diagnose at an early stage, and most patients have unresectable disease at first diagnosis. Surgical resection is the only curative therapy, but the recurrence rate is high, and prognosis is poor even after surgical resection (1-3). Although some studies demonstrated that gemcitabine-based chemotherapy improves prognosis (4, 5), the benefit of chemotherapy for cholangiocarcinoma has not been established. Currently, the development of individualized treatment based on patient characteristics is needed, and molecular-targeted agents might be a desirable option.

Mesenchymal–epithelial transition factor (MET) and recepteur d'origine nantais (RON) are cell surface receptor tyrosine kinases that bind hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP), respectively. MET and RON are involved in the epithelial to mesenchymal transition (EMT), the process by which epithelial cells obtain a mesenchymal phenotype. EMT is essential not only for tissue repair and organ regeneration but also embryonic development; however, cancer cells can take advantage of this process to acquire enhanced invasive and metastatic capabilities.

The enhanced expression of MET and RON has been reported in various tumor types (6). Missense mutations in *MET*, which are found in papillary renal cancer (7), childhood hepatocellular carcinoma (8) and gastric cancer (9), can result in the activation of MET signaling and are associated with increased aggressiveness and extensive metastasis of cancer. Increased MET expression is also found in the absence of mutation and is associated with metastasis and poor prognosis in carcinomas of the colon (10), liver (11), pancreas (12), stomach (13), prostate (14), ovary (15) and breast (16). *RON* overexpression has also been observed in various malignant tumors and is associated with more aggressive features and a poor prognosis (17-22). Cross-linking occurs between MET and RON *via* the formation of

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hetero-complexes, and transphosphorylation leads to the reciprocal regulation of kinase activity (23), suggesting that MET and RON expression can work cooperatively in cancer cells. Accordingly, our previous study revealed that among patients with advanced-stage perihilar cholangiocarcinoma, those with increased tumor expression of both MET and RON had a significantly worse prognosis (24).

On the other hand, some studies have shown that the expression of MET and RON is a favorable prognostic factor in certain types of cancer, such as diffuse large B-cell lymphoma (25, 26) and breast cancer (27, 28), indicating that both complete loss and overexpression of MET and RON may be factors associated with poor prognosis of patients with malignant tumors.

In this study, in order to clarify the true impact of MET or RON expression in EHCC, the expression of MET and RON were determined, and potential associations with clinicopathological features and prognosis were analyzed.

Patients and Methods

Patients. From 2001 to 2010, 91 patients with distal cholangiocarcinoma underwent resection at our Department. The addition of 248 patients with resected perihilar cholangiocarcinoma (previously described) (24) yielded a total of 339 enrolled patients. Patients with distant metastasis (M1) with/without noncurative resection (R2) were excluded (n=49); thus, 290 patients were analyzed (Figure 1). Clinicopathological findings were obtained from prospectively collected data. Factors related to tumor progression, such as T, N, and M stage and residual tumor classification, were determined in accordance with the TNM classification of the Union for International Cancer Control (UICC) (29).

Immunohistochemistry. Primary antibody total c-MET (clone SP44, rabbit monoclonal; Ventana Medical Systems, Inc., Tucson, AZ, USA) and anti-RON (clone EP1132Y, rabbit monoclonal; Abcam, Inc., Cambridge, UK) were purchased. Immunohistochemistry for MET and RON was performed using a Discovery XT automated slide preparation system (Ventana Medical Systems, Inc.) according to the manufacturer's protocol. The surgically resected specimens were embedded in paraffin. Before staining, the paraffin-embedded sections were blocked with 1% nonfat milk. For MET staining, the slides were incubated with the MET antibody with an amplification kit (Ventana Medical Systems, Inc.) for 60 min at room temperature. For RON staining, the slides were incubated with the RON antibody (1:100 dilution) for 12 h at room temperature. Primary antibody was detected using a diaminobenzidine (DAB) Map Detection Kit (Ventana Medical Systems, Inc.). Universal secondary antibody (Ventana Medical Systems, Inc.) was applied for 48 min at room temperature. The slides were counterstained with hematoxylin II (Ventana Medical Systems, Inc.) for 4 min.

To analyze MET and RON expression, a representative slide from the excised tumor was selected, and the entire tumor part in the slide was assessed. MET and RON expression was then categorized into three groups based on staining density according to previously published studies (30): no expression: −, complete absence of membranous staining; moderate expression: 1+, faint or partial membranous staining in at least 30% of cancer cells; or strong expression: 2+, strong and complete staining in at least 30% of cancer cells. Representative images of MET and RON expression are shown in Figure 2. In the current study, coexpression of MET and RON was also classified into three groups: MET/RON-positive: strong expression of both MET and RON; MET/RON-negative: no expression of either or neither MET and RON; or MET/RON-intermediate (neither strongly positive nor negative for MET or RON) (Table I). The tissue sections were evaluated by two observers without knowledge of the clinical data. Any disagreements in the grading of positivity were resolved by discussion between two observers.

Statistical analysis. Categorical variables were compared using the Chi-square test. Continuous variables are presented as the mean (±SD) and were compared using ANOVA. Patient survival was determined from the time of surgery to the time of death or most recent follow-up. Cumulative survival rates were analyzed by the Kaplan–Meier method using the log-rank test to compare groups. A multivariate analysis was performed with a Cox proportional hazards model. In all analyses, a value of $p<0.05$ was considered to indicate statistical significance. The data were analyzed using SPSS, version 11 (Chicago, IL, USA).

Results

Clinicopathological features and MET/RON expression. The numbers of patients with no expression, moderate expression and strong expression were 30 (10.3%), 127 (43.8%) and 133 (45.9%) for MET and 35 (12.1%), 124 (42.8%), and 131 (45.2%) for RON (Table I). In total, 76 (26.2%) patients had MET/RON-positive tumors, and 56 (19.3%) patients were in the MET/RON-negative group.

The demographic and clinical characteristics of patients are shown in Table II. The characteristics were compared among the groups with negative, intermediate and positive MET/RON expression. In terms of tumor location, patients with perihilar cholangiocarcinoma were more likely than those with distal disease to be in the MET/RON-negative group ($p<0.001$). Poorly differentiated tumors with advanced pathology (T and N grades) were frequently found in the MET/RON-negative group. The frequency of patients with advanced pathological stage was significantly higher in the MET/RON-negative group ($p=0.001$). Of note, when the N status was compared between the MET/RON-negative group and the other groups (comprising the intermediate and positive groups), advanced N status were found more frequently in the MET/RON-negative group [55.4% (n=31/56) and 40.2% (n=94/234), respectively; $p=0.039$]. Tumour N status was not significantly different in the MET/RON-positive group compared to a group comprising the other categories [44.4% (n=95/214) and 39.5% (n=30/76), respectively; $p=0.457$]. These data suggest that the complete loss of MET or RON, but not their overexpression, is associated with nodal metastasis and advanced pathological stage. Other characteristics, including age, sex, lymphatic invasion, venous invasion, histopathological classification and residual tumor classification, were not significantly different between these groups.

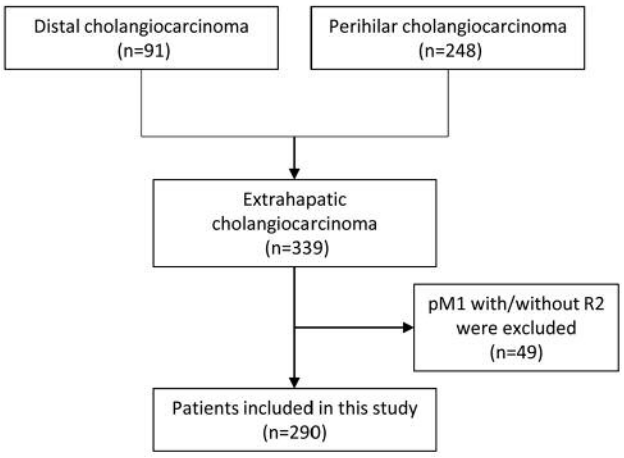


Figure 1. *Flow diagram of patients who were included or excluded from this study.*

Table I. *Frequency of immunohistochemical expression of mesenchymal–epithelial transition factor (MET) and recepteur d'origine nantais (RON).*

		MET score 0	1+	2+	Total
RON score	0	9 (3.1%)	18 (6.2%)	8 (2.8%)	35 (12.1%)
	1+	15 (5.2%)	60 (20.7%)	49 (16.9%)	124 (42.8%)
	2+	6 (2.1%)	49 (16.9%)	76 (26.2%)	131 (45.2%)
	total	30 (10.3%)	127 (43.8%)	133 (45.9%)	290

MET/RON-negative n=56 (19.3%)
MET/RON-intermediate n=158 (54.5%)
MET/RON-positive n=76 (26.2%)

Survival. The 5-year survival rate tended to be better for patients with moderate (1+) MET expression than for those with negative (0) or strong (2+) MET expression (47.1% *vs*. 30.0% and 36.3%, respectively; p=0.223) and was significantly better for the RON 1+ group compared with the RON 0 and the 2+ group (47.3% *vs*. 28.1% and 37.1%, respectively; p=0.042) (Figure 3A and B). Based on the hypothesis that the combined status of MET and RON expression represents a precise prognostic factor, MET/RON classification was employed. The 5-year survival rate was significantly higher for the MET/RON-intermediate group than for the groups with negative or positive MET/RON expression (48.5% *vs*. 28.3% and 32.4%, respectively; p=0.010), but there was no significant difference between the MET/RON-negative and MET/RON-positive groups (Figure 3C).

In the univariate analysis, RON expression, MET/RON expression, lymphatic vessel invasion, venous invasion, histopathological classification, pT, pN, pStage and resection margin (R status) were significantly associated with poor prognosis (Table III). Among these factors, seven (excluding RON expression alone and pStage) were included in the multivariate analysis. MET/RON positivity was identified as an independent prognostic factor (p=0.007), as were other factors such as venous invasion (p=0.006), pN (p<0.001), and resection margin (p=0.001). Unlike MET/RON positivity, negative MET/RON expression was not identified as an independent factor in the multivariate analysis, probably because complete loss of MET/RON is associated with advanced nodal status and pathological stage and thus cannot be an independent factor if these features are included in the analysis.

Discussion

In this study, we separated patients with EHCC into three groups according to the level of MET and RON coexpression: negative, intermediate or positive. We revealed that the 5-year survival rate was significantly lower among patients in the MET/RON-positive and MET/RON-negative groups compared with those in the MET/RON-intermediate group. These data suggest that both complete loss and overexpression of MET/RON are poor prognostic factors.

MET and RON are receptor tyrosine kinases that are associated with cancer progression, invasion and metastasis, and the expression of MET and RON has been reported as a poor prognostic factor in a variety of cancer types. Our previous study demonstrated that double positivity for MET and RON was an independent poor prognostic factor in patients with advanced stage perihilar cholangiocarcinoma (24). This result is consistent with those of other studies revealing that MET overexpression is a poor prognostic factor in cancer types such as cholangiocarcinoma (30, 31); however, the results of our previous study were obtained by limiting the analysis to patients with advanced-stage disease, and thus, the number of patients was relatively small (n=169). Therefore, it is unclear if MET/RON has a prognostic impact in early-stage cholangiocarcinoma and if this prognostic factor can be applied to all cases of EHCC, including distal bile duct carcinoma. In order to accurately and extensively investigate the impact of MET and RON expression on patients with EHCC, patients with early-stage cholangiocarcinoma with/without distal bile duct carcinoma were included in this study. Thus, a large number of patients (n=290) were analyzed, and we revealed that MET/RON expression is a general prognostic factor in EHCC.

While the overexpression of MET or RON is likely associated with aggressive tumor behavior, some reports have shown that MET and RON expression is a favorable prognostic factor in diffuse large B-cell lymphoma and

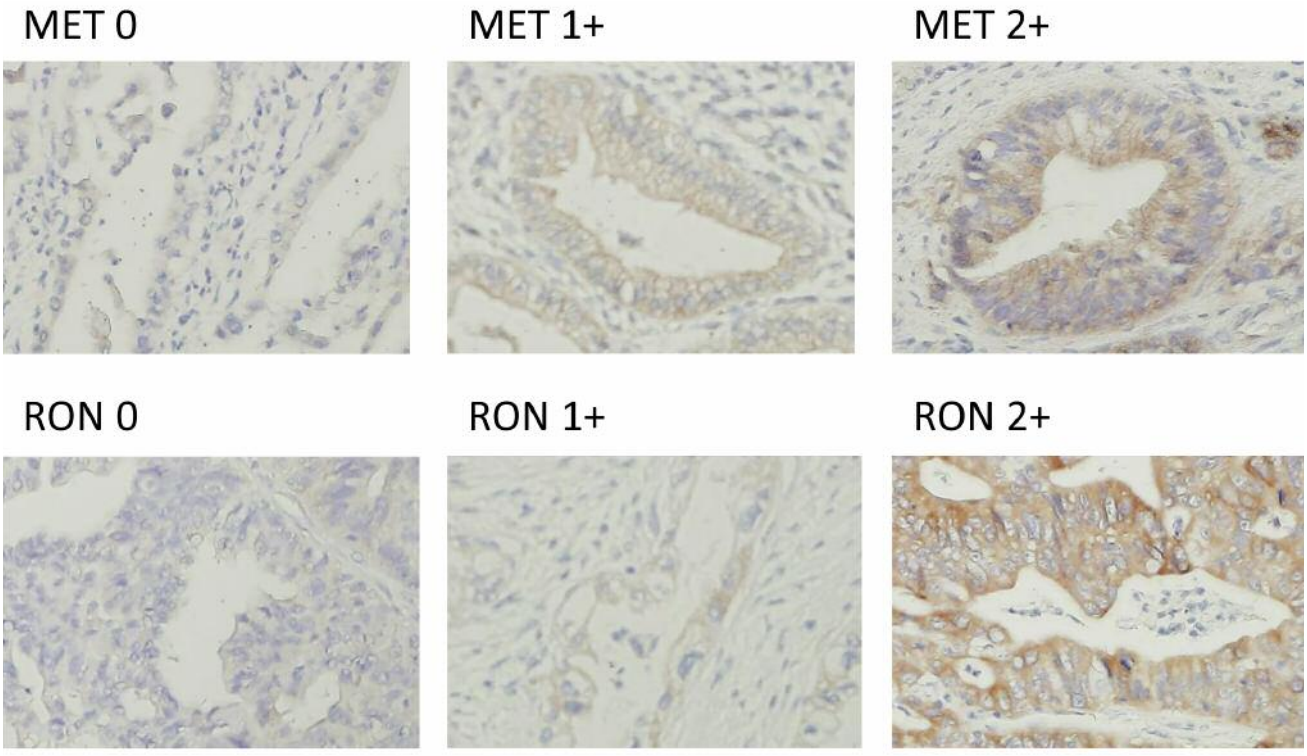
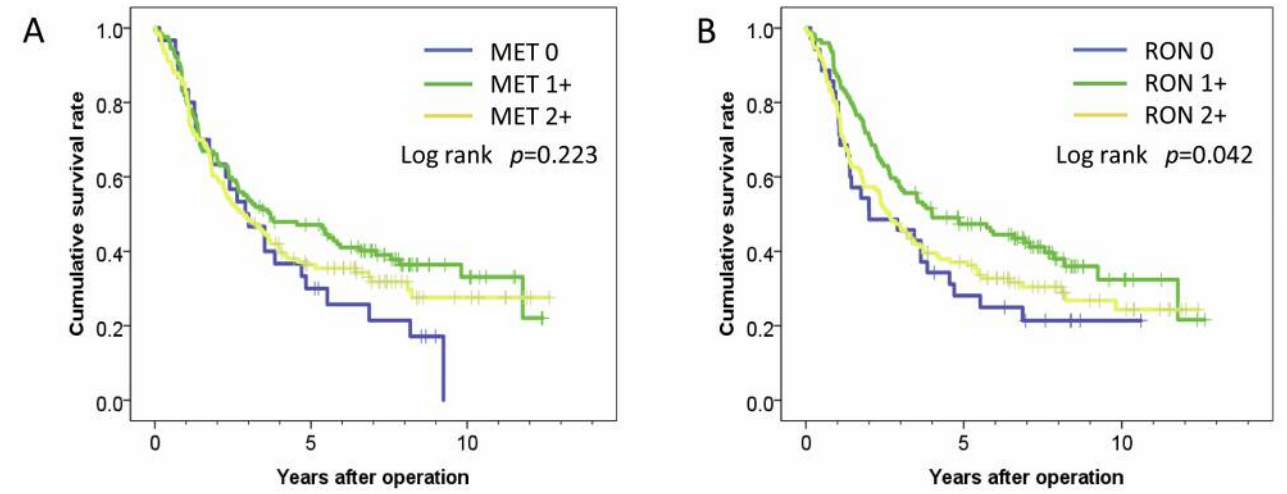


Figure 2. *Representative images of mesenchymal–epithelial transition factor and recepteur d'origine nantais expression.*

Table II. *Clinicopathological features according to expression of mesenchymal–epithelial transition factor (MET) and recepteur d'origine nantais (RON).*

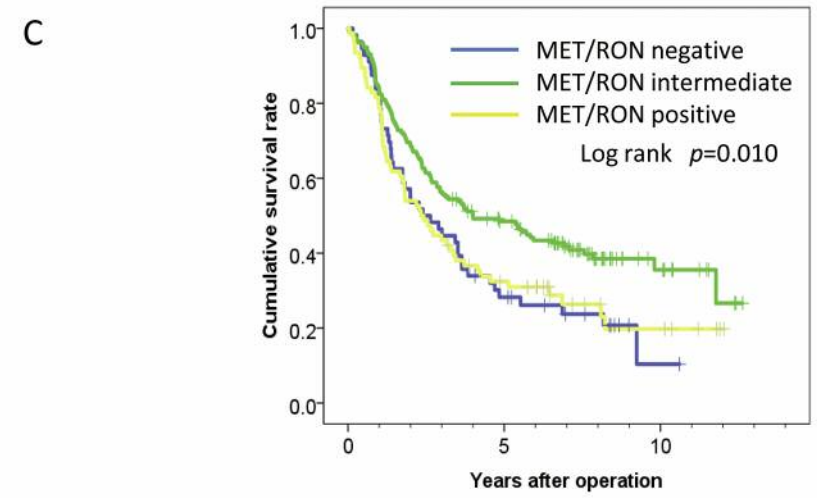
Characteristic		Total (n=290)	MET/RON			*p*-Value
			Negative (n=56)	Intermediate (n=158)	Positive (n=76)	
Age years	Mean±SD	65.5±9.6	64.2±9.0	65.0±10.3	67.6±8.2	0.070
Gender	Male	182 (62.8%)	41 (73.2%)	95 (60.1%)	46 (60.5%)	
	Female	108 (37.2%)	15 (26.8%)	63 (39.9%)	30 (39.5%)	0.197
Tumor location	Perihilar	204 (70.3%)	40 (71.4%)	127 (80.4%)	37 (48.7%)	
	Distal	86 (29.7%)	16 (28.6%)	31 (19.6%)	39 (51.3%)	<0.001
Lymphatic vessel invasion	Absent	80 (27.6%)	13 (23.2%)	45 (28.5%)	22 (28.9%)	
	Present	208 (71.7%)	41 (73.2%)	113 (71.5%)	54 (71.1%)	0.795
Venous invasion	Absent	192 (66.2%)	38 (67.9%)	102 (64.6%)	52 (68.4%)	
	Present	97 (33.4%)	17 (30.4%)	56 (35.4%)	24 (31.6%)	0.756
Histopatholoy	Pap/well	61 (21.0%)	6 (10.7%)	39 (24.7%)	16 (21.1%)	
	Other	229 (79.0%)	50 (89.3%)	119 (75.3%)	60 (78.9%)	0.088
UICC pT	T0-2	106 (36.6%)	16 (28.6%)	59 (37.3%)	31 (40.8%)	
	T3-4	184 (63.4%)	40 (71.4%)	99 (62.7%)	45 (59.2%)	0.338
UICC pN	N0	165 (56.9%)	25 (44.6%)	94 (59.5%)	46 (60.5%)	
	N1	125 (43.1%)	31 (55.4%)	64 (40.5%)	30 (39.5%)	0.118
UICC pStage	Stage 0-2	143 (49.3%)	21 (37.5%)	71 (44.9%)	51 (67.1%)	
	Stage 3-4	147 (50.7%)	35 (62.5%)	87 (55.1%)	25 (32.9%)	0.001
Residual tumor classification	R0	228 (78.6%)	43 (76.8%)	128 (81.0%)	57 (75.0%)	
	R1	62 (21.4%)	13 (23.2%)	30 (19.0%)	19 (25.0%)	0.537

Pap/well: Papillary/well-differentiated adenocarcinoma; other: moderately or poorly differentiated adenocarcinoma, and adenosquamous carcinoma; UICC: Union for International Cancer Control; R0: no residual tumor; R1: microscopic residual tumor.



Number at risk				
MET 0	30	15	9	0
MET 1+	127	69	56	10
MET 2+	133	64	41	9

Number at risk				
RON 0	35	16	9	1
RON 1+	124	71	53	8
RON 2+	131	61	44	10



Number at risk				
MET/RON negative	56	26	15	1
MET/RON intermediate	158	89	69	12
MET/RON positive	76	33	22	6

Figure 3. *Overall survival according to mesenchymal–epithelial transition factor (MET) (A), recepteur d'origine nantais (RON) (B) and MET/RON (C) expression.*

breast cancer (25-28). In patients with cholangiocarcinoma, Aishima *et al*. reported an inverse correlation between MET overexpression and the presence of lymph-node metastasis: MET positivity was frequent in papillary and well-differentiated cholangiocarcinomas, and patients with negative staining for c-MET had a significantly shorter survival than those with positive staining (32). These data are partly consistent with the results of this study. Basic research has shown that MET signaling can play a negative role in tumor growth. For instance, the antiproliferative and apoptosis-inducing effects of HGF on certain epithelial malignancies have been shown in previous studies (33-38),

Table III. *Univariate and multivariate analyses for prognostic factors.*

		No. of patients	Survival (%)		Median survival (years)	Univariate	Multivariate		
			5-Year	10-Year		Log-rank *p*-value	Hazard ratio	95% Confidence interval	*p*-Value
Age	<65 Years	126	47.5	32.6	3.7				
	≥65 Years	164	34.8	22.9	2.8	0.101			
Gender	Male	182	40.4	24.5	3.1				
	Female	108	40.3	32.2	3.0	0.551			
MET	0	30	30.0	0.0	2.9	0.078			
	1+	127	47.1	33.1	3.7				
	2+	133	36.3	27.6	2.8	0.281			
RON	0	35	28.1	21.4	2.0	0.025			
	1+	124	47.3	32.4	4.0				
	2+	131	37.1	24.3	2.6	0.041			
MET/RON	Negative	56	28.3	10.4	2.4	0.009	1.268	0.872-1.843	0.214
	Intermediate	158	48.5	35.5	4.0		1		
	Positive	76	32.4	19.8	2.3	0.015	1.601	1.140-2.246	0.007
Tumor location	Perihilar	204	41.2	26.9	3.2				
	Distal	86	38.5	32.1	2.7	0.935			
Lymphatic vessel invasion	Absent	80	57.2	40.4	7.6		1		
	Present	208	34.3	22.2	2.5	<0.001	0.946	0.629-1.423	0.789
Venous invasion	Absent	192	49.2	34.6	4.6		1		
	Present	97	23.3	12.3	1.8	<0.001	1.559	1.135-2.144	0.006
Histopathology	Pap/well	61	60.6	38.9	9.2		1		
	Other	229	35.0	24.8	2.6	0.001	1.450	0.946-2.222	0.088
UICC pT	T0-2	106	58.3	39.8	8.1		1		
	T3-4	184	29.9	21.7	2.3	<0.001	1.436	0.989-2.084	0.057
UICC pN	N0	165	54.9	37.5	6.6		1		
	N1	125	20.9	14.9	2.0	<0.001	1.797	1.300-2.483	<0.001
UICC pStage	Stage 0-2	143	51.3	38.0	5.4				
	Stage 3-4	147	29.9	17.5	2.3	<0.001			
Residual tumor classification	R0	228	46.2	32.0	3.7		1		
	R1	62	19.4	11.9	1.7	<0.001	1.762	1.264-2.455	0.001

MET: Mesenchymal–epithelial transition factor; RON: recepteur d'origine nantais; Pap/well: Papillary/well-differentiated adenocarcinoma; other: moderately or poorly differentiated adenocarcinoma, and adenosquamous carcinoma; UICC: Union for International Cancer Control; R0: no residual tumor; R1: microscopic residual tumor.

and Takami *et al*. reported that the loss of MET signaling in hepatocytes enhanced chemical hepatocarcinogenesis in a mouse model (39). Thus, it is conceivable that the complete loss of MET and RON might attenuate these antitumor effects and result in cancer progression and poor prognosis, as shown in this study.

Although the complete loss of MET/RON was a significant poor prognostic factor in the univariate analysis, it was not significant in the multivariate analysis, indicating that the loss of MET/RON is not an independent factor. As described in our previous study, MET/RON overexpression has no correlation with conventional pathological findings and is an independent factor. This is also true in this study; however, complete loss of MET/RON was significantly associated with lymph-node metastasis and higher pathological stage. These results suggest that while overexpression of MET/RON is an independent poor prognostic factor, complete loss of MET/RON can result in lymph-node metastasis, advanced-stage disease and poor prognosis. Given that MET and RON are important in EMT, one can speculate that the loss of MET/RON results in EMT in cancer cells, promoting lymph-node metastasis.

Dual MET and RON inhibitors are expected as new therapeutic agents for cancer, and their effects have been demonstrated both *in vitro* and *in vivo* (40-43). A dual inhibitor might represent a breakthrough in the treatment not only of patients with advanced cholangiocarcinoma but also of high-risk postoperative patients. It has been reported that lymph-node metastasis is a poor prognostic factor in cholangiocarcinoma (2, 3), and adjuvant chemotherapy might be beneficial in these cases; however, patients with lymph-

node metastasis frequently show complete loss of MET/RON, and dual inhibitors are potentially not effective in these patients. Even in patients with MET/RON overexpression, such an inhibitor might completely interrupt MET/RON signaling and thus have the opposite effect. Therefore, the indications for dual inhibitors should be precisely defined before administration, and overdose of such inhibitors should be avoided.

In conclusion, this study revealed that the cumulative survival rate was worse in patients with complete loss or overexpression of MET/RON than in those with intermediate MET/RON expression at all stages of EHCC. The expression status of MET/RON should be carefully evaluated not only to predict prognosis but also to define the indications for the use of MET/RON inhibitors.

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