The prognostic value of hepatocyte growth factor receptor expression in patients with perihilar cholangiocarcinoma.

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Synopsis

This study determined the expression of MET and RON in the resected surgical specimens of perihilar cholangiocarcinoma. In patients with stage III and IVa, the overall survival rates in both the MET- and RON-positive patients were worse than the others.

ABSTRACT

Background: Although an aggressive surgical approach to perihilar cholangiocarcinoma (PHC) has improved survival, a prognosis of advanced PHC remains unsatisfactory. The overexpression of mesenchymal-epithelial transition factor (MET) and Recepteur d'Origine Nantais (RON) has been shown to be associated with poor prognosis in some types of cancer.

Methods: One hundred and sixty-nine patients who underwent histologically curative resection for PHC were subjected to immunohistochemical analysis for MET and RON. The association between a positive expression of MET or RON and clinicopathological features as well as the patients' prognosis were analyzed.

Results: There were 27 patients (16%) who revealed a positive expression for both MET and RON. Although clinicopathological features in the either MET- or RON-negative group were not significantly different compared to the both MET and RON-positive group, the prognosis tended to be worse in the patients with both MET and RON positivity. When the analysis was limited to the advanced stage patients (stage III and IVa), a multivariate analysis revealed that both MET and RON positivity and lymph node metastasis were identified as independent poor prognostic factors.

Conclusions: This study demonstrated that the overall survival rate with both MET and RON positivity was worse than that with either MET or RON negativity in the patients with advanced PHC. The poor prognosis in these patients was not associated with unfavorable clinicopathological features. The examination of MET and RON expression in PHC may enable a tailored method for patient classification that could not otherwise be achieved using the conventional pathological classification system.

Introduction

Perihilar cholangiocarcinoma (PHC) is an intractable disease because of the anatomical complexity of the tumor location and the capacity to invade adjacent tissues such as portal veins, hepatic arteries, and liver parenchyma [1,2]. Although an aggressive surgical approach to PHC has improved survival, a prognosis of advanced PHC remains poor even after curative resection [3,4]. To improve the prognosis of PHC, a tailored patient management including the estimation of the malignant potential of the tumor and the establishment of effective molecular target therapy are necessary.

In patients with PHC, previous studies have shown that histological grade, microscopic lymphatic or venous invasion, microscopic perineural invasion, lymph node metastasis, and curability (R status) are significantly correlated with poor prognosis after resection [4,5]. However, even with favorable pathological findings, some patients recur and die in the early postoperative period.

Mesenchymal-epithelial transition factor (MET) and Recepteur d'Origine Nantais (RON) proto-oncogenes encode the cell surface receptors for hepatocyte growth factor (HGF) [6,7] and hepatocyte growth factor-like protein (HLP) [8-11], respectively. MET and RON have been demonstrated to induce mitogenesis and morphogenesis in epithelial cells and have been associated with tissue regeneration and development [12,13]. The activation of MET by HGF and RON by HLP in carcinoma cells can initiate many signaling pathways that are implicated in tumor progression and metastasis [14-16]. The overexpression of MET or RON in some carcinomas has been demonstrated to be associated with poor prognosis [17-22]. Furthermore, cooperative signaling by MET and RON may result in more aggressive

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phenotypes [20]. However, the co-expression profile of MET and RON in PHC specimens has not been studied. It is also unknown whether the expression of MET or RON has any association with the malignant potential of PHC.

The aim of this study was to determine the expression profile of MET and RON in curatively resected surgical specimens of PHC. The strength of MET or RON expression and its association with patient prognosis were also analyzed.

Methods

Patients

From 2001 to 2007, 248 patients with PHC underwent resection at the First Department of Surgery, Nagoya University Hospital. The resected specimens were histologically examined and approved as having carcinoma of the perihilar bile duct. Patients with distant metastasis (pM1) and/or non-curative resection (R1 or 2) were excluded (n=79), and 169 patients (including 55 patients with stage I or II and 114 patients with stage III or IVa; according to the UICC 7th edition) who had undergone histologically curative resection were finally analyzed. Clinical and pathological data were obtained from prospectively collected data.

Chemicals

CONFIRM anti-Total c-MET (SP44) Rabbit Monoclonal Primary Antibody (rabbit monoclonal, Ventana Medical Systems Inc., Tucson, AZ,) [23] and Anti-RON antibody [EP1132Y] (rabbit monoclonal, Abcam Inc., Cambridge, UK) [24] were purchased.

Immunohistochemistry for MET and RON

Immunohistochemistry was performed on 169 formalin-fixed, paraffin-embedded tissue sections (4 μm in thickness). Staining for MET and RON were performed using a DISCOVERY XT automated slide preparation system (Ventana Medical Systems Inc.). Prior to staining, the paraffin-embedded sections were blocked with 1% non-fat milk. The staining procedure was performed according to the manufacturer's instructions (Ventana Medical Systems Inc.). For MET staining, the slides were incubated with the MET antibody with an amplification kit (Ventana Medical Systems Inc.) for 60 minutes at room temperature. For RON staining, the slides were incubated with 1:100 of the RON antibody for 12 hours at room temperature. Primary antibody was detected using the DAB Map Detection Kit (Ventana Medical Systems Inc.). Universal Secondary Antibody (Ventana Medical Systems Inc.) was applied for 48 minutes at room temperature. The slides were counterstained with Hematoxylin II (Ventana Medical Systems Inc.) for 4 minutes.

Scoring system used in this study basically followed the method used in a previously published study that demonstrated the expression of MET in cholangiocarcinoma specimens [17]. Briefly, the immunoexpressions of MET and RON were categorized into three groups according to their dyeing density: no expression [−] (complete absence of membrane staining), moderate expression [+] (faint and partial membrane staining in at least 30% of cancer cells), or strong expression [++] (strong and complete staining in at least 30% of cancer cells). The representative images of MET and RON expression are depicted in Figure 2. The tissue sections were evaluated by two observers without knowledge of the clinical data. Two observers had a discussion to make a final decision when the grading of positivity was not matched among them. In this study, only the cases with strong expression either for MET

or RON were considered to have positive expression because there was more discrepant grading in the group of no or moderate expression.

Statistical analysis

Statistical analysis was performed using Student's *t* test, the χ^2 test, or Fisher's exact probability test, as appropriate. Patient survival was determined from the time of surgery to the time of death or most recent follow-up. The median follow-up periods were 1405 days (range, 15-4845 days). Nineteen of the 169 PHC patients (11.2%) died from other causes. Four of the 169 PHC patients (2.4%) died from postoperative complications. Patients who died from other causes during follow-up with no evidence of recurrence were treated as censored cases. Postoperative survival was calculated by means of the Kaplan–Meier method, and differences in the survival curves were compared with the log rank test. The Cox proportional hazards model was used for multivariate analysis. Analyses were performed using the SPSS[R] statistical package, version 11 (IBM, Armonk, NY). *P* <0.050 was considered statistically significant.

Results

Immunohistochemical analysis of MET and RON in PHC specimens

MET and RON staining was localized in both the cell membrane and cytoplasm of PHC cells (Figure 2). Positive staining for MET was demonstrated in 56 (33.1%) of the 169 cases, whereas that for RON was demonstrated in 66 (39.1%) of the 169 cases (Table 1).

Co-expression of MET and RON and clinicopathological factors

There were 27 patients who revealed a positive expression for both MET and RON.

The average age of the patients in the both MET- and RON-positive group was significantly higher than that in the either MET- or RON-negative group (the OTHERS group) (Table 2). Invasion to the portal vein was more frequently observed in the OTHERS group (55 of 142, 38.7%) than the both MET- and RON-positive group (2 of 27, 7.4%) (P=0.002) (Table 2). Invasion to other organ was also more frequently observed in the OTHERS group (124 of 142, 87.3%) than the both MET- and RON-positive group (18 of 27, 66.7%) (P=0.018). Nevertheless, the overall survival rate tended to be lower in the both MET- and RON-positive group than the OTHERS group, although it did not reach to a significant difference (P=0.234) (Figure 3A). The five-year survival rate in the both MET- and RON-positive group was 37.0%, whereas that in the OTHERS group was 47.9%.

Subclass analysis for stage III and IVa patients

In the subsequent analysis, we performed a subclass analysis in patients with stage IIIa, IIIb, and IVa PHC according to the UICC 7th edition (n=114). In the univariate analysis, 3 of 14 possible clinicopathological prognostic factors, such as microscopic venous invasion, lymph node metastasis (pN), and both MET and RON positivity, were significantly associated with poor prognosis (Table 3). A multivariate analysis using the 3 significant factors identified via univariate analysis revealed that both MET and RON positivity and lymph node metastasis were identified as independent prognostic factors (Table 3). The overall survival rate was significantly lower in the both MET- and RON-positive group than the OTHERS group (P=0.021) (Figure 3B). The five-year survival rate in the both MET- and RON-positive group was 16.7%, whereas that in the OTHERS group was 39.8%. In contrast to the results in patients with advanced stage PHC, there was no clinical impact of MET and RON expression

in patients with low-stage PHC (stage I or II) (data not shown).

Discussion

In this study, the expression levels of MET and RON, two homologous receptor tyrosine kinases that are associated with cancer progression [25], were examined in a highly uniform patient population: curatively resected cases of PHC. After an immunohistochemical analysis, the rates for the strong expression of MET and RON were 33.1% and 39.1%, respectively. Although clinicopathological features in the OTHERS group (either MET- or RON-negative group) were not significantly different or even worse (more portal vein and other organ invasions) compared to the both MET and RON-positive group, the prognosis tended to be worse in the patients with both MET and RON positivity than in the patients with either MET or RON negativity. When the analysis was limited to the advanced stage patients (stage III and IVa), a multivariate analysis revealed that both MET and RON positivity and lymph node metastasis were identified as independent poor prognostic factors (Table 3). The hazard ration for MET and RON positivity (1.809) was close to that for pN1 (2.039), which is known as the strongest risk factor for poor prognosis in PHC (Table 3). These results indicated that the expression of MET and RON represents the malignant potential of PHC, which cannot be predicted using conventional pathological findings. Moreover, the results in this study implied a therapeutic potential for combined MET and RON inhibitors, which are available in the pre-clinical setting [26-28] as an adjuvant molecular targeting chemotherapy for advanced PHC.

Miyamoto et al. examined the expression of MET and demonstrated its prognostic impact in patients with cholangiocarcinoma [17]. In their study, the positive expression of MET was significantly correlated with histopathological classification in extrahepatic cholangiocarcinoma. However, this correlation was not applied to the cases of intrahepatic cholangiocarcinoma. Additionally, in patients with intrahepatic cholangiocarcinoma, the positive expression of MET was significantly associated with poor prognosis, whereas it was not associated with poor prognosis in patients with extrahepatic cholangiocarcinoma [17]. In the current study, there was no correlation between MET expression and histopathological classification (data not shown). The previous study included patients who underwent non-curative resection. It also included patients in whom the tumor varied in location (e.g., patients with intrahepatic and extrahepatic cholangiocarcinoma). These heterogeneities in the patient population may partly explain the divergence from the results of the current study.

MET and RON are members of the same proto-oncogene family and were reported to form a non-covalent complex on the cell surface and to cooperate in intracellular signaling. The concomitant activation of these two receptors could lead to a synergistic effect and may favor, in pathological conditions, the invasive-metastatic phenotype [29]. The activation of MET and RON receptors promotes the progression, invasion, and metastasis of malignant cells both in *in vivo* and *in vitro* experiments [16, 30, 31]. Therefore, it is important to investigate the co-expression of MET and RON in cancer specimens. The overexpression of MET and RON and their association with a poorer prognosis have been confirmed in urothelial carcinoma [32], bladder cancer [33], hepatocellular carcinoma [34], breast cancer [35], colorectal cancer [36], and ovarian cancer [37]. However, the co-expression of MET and RON has never been investigated

in cholangiocarcinoma. As is consistent with other types of solid cancer, the co-expression of MET and RON may be associated with a poor prognosis in advanced PHC patients. In our previous report reviewing 34 years of experience with 574 consecutive resections for PHC, a multivariate analysis revealed that the presence of lymph node metastasis was the strongest prognostic indicator [4]. In this study, in the subclass analysis for stage III and IVa PHC, the rate of lymph node metastasis in the patients with both MET and RON positivity was not higher than that in other patients (50% vs. 59.4%). Nevertheless, the overall prognosis was significantly worse in the patients with both MET and RON positivity. These results indicated that the examination of MET and RON expression may enable a tailored biological classification of PHC patients that cannot otherwise be delineated using conventional pathological methods. Moreover, preoperative analysis for MET and RON expression in the biopsy samples may support clinical decision making (i.e., candidate for neoadjuvant therapy), because patients with positive MET and RON expression tend to have a poor prognosis even after the curative resection.

Dual inhibitors of MET and RON have been developed and investigated in multiple *in vitro* and *in vivo* models [26-28, 38]. However, only a few clinical trials using oral multi-kinase inhibitor targeting MET, RON, and other receptors are currently ongoing for papillary renal cell carcinoma [39] and unresectable solid tumors [40]. Based on the observations in this study, dual inhibitors for MET and RON may have a promising therapeutic potential in PHC, especially in patients with poor prognosis despite curative resection. To promote translational research for molecular targeting therapy against MET and RON, further mechanistic studies

that precisely elucidate the therapeutic value of dual inhibitors for biologically malignant cholangiocarcinoma are required.

The major limitation of this study was the small sample size (n=169). The present study also revealed no clinical importance of MET and RON expression in patients with low-stage PHC (stage I or II). We suspect that the major reasons for insignificant findings may be related to the small number of patients (n=55) and highly favorable postoperative prognosis in these patients (80.5% 5-year survival rate) [41, 42]. Nevertheless, further large scale study is necessary to determine whether the positive expression for both MET and RON has a negative prognostic impact even at low-stage PHC. Additionally, the mechanistic reason behind the poor prognosis of patients with the co-expression of MET and RON remains unknown. Among those patients, the recurrence pattern was variable for local recurrence, liver metastasis, peritoneal dissemination, and lymph node metastases (data not shown). There was no clear biological property that explained poor prognosis in the patients with co-expression of MET and RON. It is also unclear whether signaling pathways that are associated with MET and RON are intensively activated by HGF and HLP in cholangiocarcinoma. Further clinical data and mechanistic investigations are necessary to clarify these issues.

In conclusion, this study demonstrated that the overall survival rate with both MET and RON positivity was worse than that with either MET or RON negativity in patients with advanced PHC. The poor prognosis in these patients was not associated with unfavorable clinicopathological features. The examination of MET and RON expression in PHC may enable a tailored patient classification that cannot otherwise be achieved using the

conventional pathological classification. Moreover, the results in this study implied promising

therapeutic potential for dual inhibitors of MET and RON for biologically malignant PHC.

Figure Legends

Figure 1.

A flow diagram of the patients included and excluded from the study.

Figure 2.

Representative images for the expression of MET and RON in PHC specimens. MET and RON were localized in both the cell membrane and cytoplasm in PHC cells.

Figure 3.

Overall survival in PHC patients with both MET and RON positivity and OTHERS (either MET or RON negativity). (A) all patients (n=169), (B) patients with stage III and IVA (n=114).

 * log-rank test.

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Table 1. The number of patients with the immunohistochemical expression of MET or RON. (Stage I, II, III, and IVa; n=169)

	no expression (-) n (%)	moderate expression (+) n (%)	strong expression (++) n (%)
MET	16 (9.5)	97 (57.4)	56 (33.1)
RON	20 (11.8)	83 (49.1)	66 (39.1)

Table 2. Clinicopathological features (Stage I, II, III, and IVa; n=169): MET and RON positivity vs. OTHERS (either MET or RON negativity)

	OTHERS (n=142)	(%)	MET and RON positivity (n=27)	(%)	P value †	
Age　Mean±SD	63.5±9.8		68.9±8.2		0.007	‡
Gender					0.728	
male	84	(59.2)	15	(55.6)		
female	58	(40.8)	12	(44.4)		
Bismuth type					0.977	
I, II, III	89	(62.7)	17	(63.0)		
IV	53	(37.3)	10	(37.0)		
Combined resection						
With PV and/or HA	52	(36.6)	8	(29.6)	0.487	
With PD	15	(10.6)	4	(14.8)	0.521	
Histopathological classification					0.189	
well/pap	50	(35.2)	6	(22.2)		
mod, por, asq	92	(64.8)	21	(77.8)		
Lymphatic vessel invasion (present) *	97	(68.8)	16	(59.3)	0.333	
Venous invasion (present)	49	(34.5)	9	(33.3)	0.906	
Perineural invasion (present) *	118	(83.7)	24	(88.9)	0.771	§
Invasion to portal vein (present)	55	(38.7)	2	(7.4)	0.002	
Invasion to hepatic artery (present)	23	(16.2)	3	(11.1)	0.502	
Invasion to other organ (present)	124	(87.3)	18	(66.7)	0.018	§
UICC pT					0.779	
T1, T2	62	(43.7)	11	(40.7)		
T3, T4	80	(56.3)	16	(59.3)		
UICC pN					0.506	
N0	85	(59.9)	18	(66.7)		
N1	57	(40.1)	9	(33.3)		

SD, standard deviation; PV, portal vein resection and reconstruction; HA, hepatic artery resection and reconstruction; PD, pancreatoduodenectomy.

well, well differentiated adenocarcinoma; pap, papillary adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; asq, adenosquamous carcinoma.

*Lymphatic vessel invasion and perineural invasion were not determined in one patient, respectively.

† χ^2 test, except ‡ Student's t test and § Fisher's exact test.

Table 3. Univariate and multivariate analyses for prognostic factors in patients with stage III and IVa (n=114)

		Survival (%)		Univariate	Multivariate	
	n	3-year	5-year	*P*	Hazard Ratio (95% Confidence Interval)	*P*
Age				0.529		
<65	55	51.6	45.6			
≥65	59	45.4	27.2			
Sex				0.763		
male	64	49.4	36.3			
female	50	46.7	35.6			
Combined PD				0.715		
Absent	104	48.1	35.5			
Present	10	50	40			
Combined PV and/or HA				0.362		
Absent	58	50.2	37.6			
Present	56	44.4	34.3			
Histology				0.446		
well/pap	27	59.3	40.7			
others	87	44.7	34.5			
Lymphatic vessel invasion*				0.277		
Absent	19	55.6	38.9			
Present	94	45.1	35			
Venous invasion				0.017		0.071
Absent	62	50	44.8		1.00	
Present	52	42.1	25.2		1.506 (0.966-2.349)	
Perineural invasion*				0.399		
Absent	5	80	40			
Present	108	46.3	35.4			
Invasion to portal vein				0.588		
Absent	57	50	34.6			
Present	57	46.7	37.3			
Invasion to hepatic artery				0.506		
Absent	89	51.5	37.1			
Present	25	36.4	31.8			

Invasion to other organs				0.146			
Absent	6	66.7	44.4				
Present	108	47.2	34.2				
UICC pT				0.694			
T3	31	41.9	32.3				
T4	83	44.6	33.7				
UICC pN				0.001			0.003
0	48	63.2	54.5			1.00	
1	66	35.8	22.2			2.039 (1.269-3.277)	
MET and RON				0.021			0.036
Either negative	96	51	40			1.00	
Both positive	18	28	17			1.809 (1.040-3.147)	

HA, hepatic artery resection and reconstruction; PV, portal vein resection and reconstruction;

PD, pancreatoduodenectomy

well, well differentiated adenocarcinoma; pap, papillary adenocarcinoma; others, moderately

differentiated adenocarcinoma, poorly differentiated adenocarcinoma or adenosquamous carcinoma.
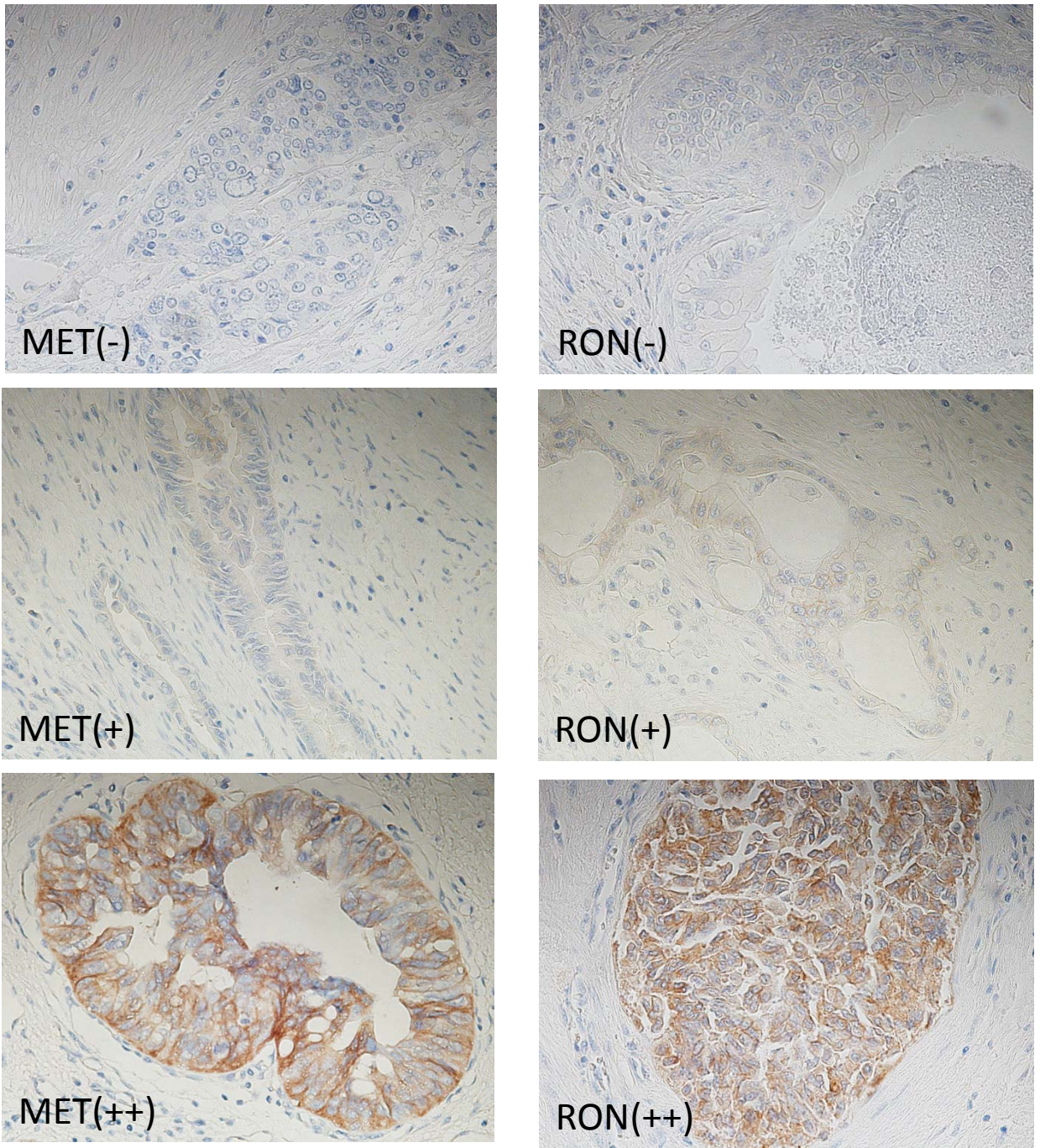
*Lymphatic vessel invasion and perineural invasion were not determined in one case, respectively.

Figure 1
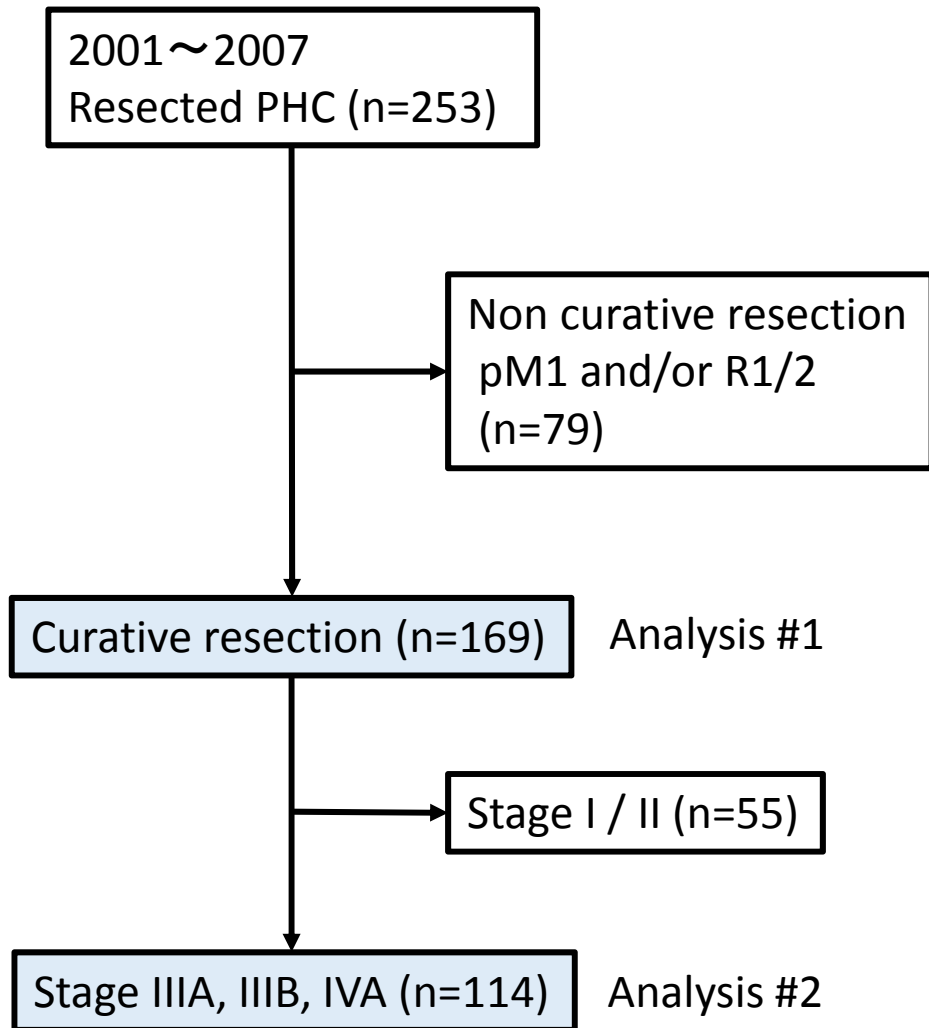
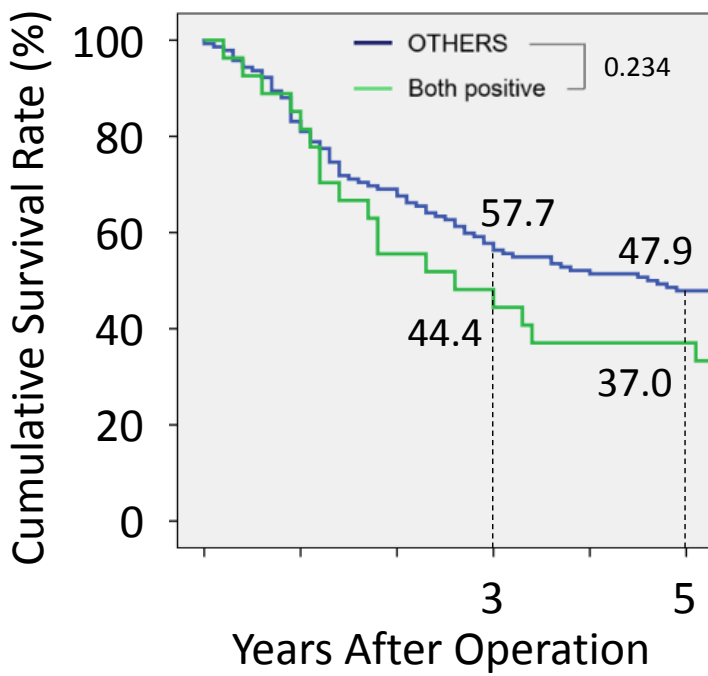A flow diagram of the patients included and excluded from the study.

Figure 2

Representative images for the expression of MET and RON in PHC specimens. MET and RON were localized in both the cell membrane and cytoplasm in PHC cells.
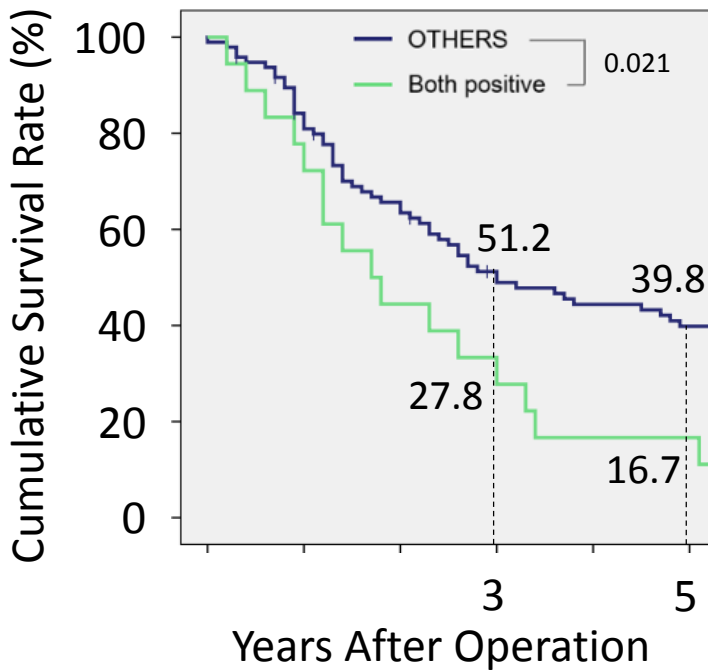
Figure 3

(A)

No at risk			
OTHERS	142	82	68
BOTH positive	27	12	10

(B)

No at risk			
OTHERS	96	45	35
BOTH positive	18	5	3

Overall survival in PHC patients with both MET and RON positivity and OTHERS (either MET or RON negativity). (A) all patients (n=169), (B) patients with stage III and IVA (n=114).
* log-rank test.