MET-RON dual inhibitor, BMS-777607, suppresses cholangiocarcinoma cell growth, and MET-RON upregulation indicates worse prognosis for intra-hepatic cholangiocarcinoma patients

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Received November 11, 2017; Accepted May 15, 2018

DOI: 10.3892/or.2018.6543

Abstract. Intra-hepatic cholangiocarcinoma (CCA) is an aggressive cancer with few effective therapeutic options. MET and RON have been found to be increased in a variety of tumors and to be associated with tumor progression and acquired resistance to therapy. The present study evaluated the efficacy of a MET-RON dual inhibitor (BMS-777607) for treating CCA and analyzed the prognostic significance of MET-RON upregulation. We treated CCA cell lines and rats with CCA with BMS-777607 to determine its effects on tumor growth and measured the MET-RON protein expression in samples obtained from 96 patients with CCA who previously underwent hepatectomies. A clonogenic assay revealed that BMS-777607 inhibited the growth of HuCCT1 and KKU-100 human CCA cells. It also decreased tumor growth in CCA rats. MET-RON upregulation independently predicted poor survival for CCA patients who previously underwent hepatectomies. In conclusion, MET-RON upregulation is a poor prognostic factor in CCA patients receiving hepatectomies and may be targeted using BMS-77760.

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Key words: MET-RON dual inhibitor (BMS-777607), cholangiocarcinoma, prognosis

Introduction

Cholangiocarcinoma (CCA) comprises 10-15% of all primary liver cancers, making it the second most common form of liver cancer. It is estimated that 1/100,000 people are annually diagnosed with CCA in Western countries (1-4). The worldwide incidence and mortality rates of the disease have also been increasing in recent years (5,6). Curative surgical resection remains the treatment of choice and, when feasible, provides some chance of a cure for CCA (7-9). However, due to the disease's high post-surgery recurrence rate and delays in diagnosing it, most patients are not good candidates for surgery and the prognosis is generally unfavorable (10). Overall, only 25-30% of CCA patients undergo surgery (11,12). For unresectable CCA, palliative chemotherapy with gemcitabine combined with cisplatin is the only standard first-line regimen, but the response is typically limited and patients receiving the regimen usually survive for less than one year (13). Furthermore, there is no effective drug for refractory CCA (14-16). Thus, it is important that a new means of treating this disease be developed.

MET proto-oncogene, receptor tyrosine kinase (MET) and Recepteur d'Origine Nantais (RON), which are structurally related transmembrane phosphotyrosine kinase receptors, are increased or show increased activity in a variety of tumors (including breast and small cell lung cancer, and others), and have been found to be associated with tumor progression and acquired resistance to therapy (17). Although MET and RON are often co-expressed, their distinct functional roles are not fully understood (17). Various inhibitors of MET and RON, including small molecular weight kinase inhibitors and

neutralizing antibodies, are being investigated in pre-clinical studies and clinical trials (17). It is not yet clear, however, whether some combination of MET and RON inhibitors can be used to treat CCA.

Therefore, the present study evaluated the effect of using BMS-777607 to treat CCA *in vitro* and *in vivo*. In addition, we investigated the impact of upregulation of MET-RON on the clinicopathological features and clinical outcomes of CCA patients.

Materials and methods

Cell culture. The CCA cell lines HuCCT1 and KKU-100, as well as MMNK-1 (a normal bile duct cell line), were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB; Osaka, Japan). All of the established cell lines used in this study were validated by short tandem repeat analysis performed by the Division of Transfusion Medicine, Taipei Veterans General Hospital. The HuCCT1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The KKU-100 and MMNK-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.). All the cells were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 μg/ml penicillin, and 2 mM L-glutamine in a humidified atmosphere containing 5% CO_2 at 37˚C.

Clonogenic assay. Cell growth was measured using a clonogenic assay. HUCCT1 or KKU-100 cells that were either treated or not treated with BMS-777607 (Bristol-Myers Squibb, Taiwan) were cultured for 10 days to allow clonogenic growth, as previously described (18).

Viability assay. Cell viability was determined using the TACS tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Trevigen, Gaithersburg, MD, USA), according to the manufacturer's instructions. MTT is used to determine cell viability in cell proliferation and cytotoxicity assays. Briefly, HUCCT1 or KKU-100 cells were seeded at a concentration of 1,000 cells/well in 100 μl culture medium into 96-well microplates. At 24 h post-seeding, the cells were treated with dimethyl sulphoxide (DMSO) or BMS-777607 for 6 days; the cells were then incubated in medium containing MTT for 2 h. The optical density at 570 nm was measured using a microplate reader (Spectral Max250; Molecular Devices, Sunnyvale, CA, USA).

Western blotting. Whole cell lysates from the CCA cell lines were obtained using Pierce radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Protein samples were separated on 6-8% gradient dodecyl sulfate-polyacrylamide gels and transferred to Immobilion-PVDF membranes (Millipore, Billerica, MA, USA). Antigen-antibody complexes were detected using an electrochemiluminescence blotting analysis system (Millipore). The following primary antibodies were used: RON (Abcam, Cambridge, UK), MET (Cell Signaling Technology Inc., Danvers, MA, USA), phospho-RON (Abcam) and β-actin (Abcam).

Rat orthotopic tumor model. Eighteen adult male Sprague-Dawley (SD) rats (310±14 g) were used in our animal experiments. They were equally divided (n=6) into the following 3 groups: i) A control group; ii) a gemcitabine/oxaliplatin treatment group; and iii) a BMS-777607 treatment group. The rats were housed in an animal room with lighting set to a 12:12 h light-dark cycle (lights on from 08:00 a.m. to 08:00 p.m.) and an ambient temperature set at 22˚C. Food and water were provided *ad libitum*. The rats were administered 300 mg/l thioacetamide (TAA) via drinking water daily for up to 20 weeks (19). TAA-induced rat CCA was induced as previously reported and followed the Animal Experimental Guidelines of Chang Gung Memorial Hospital, Chang Gung University (Taoyuan, Taiwan) with approval code: IACUC: 2010121409. The gemcitabine/oxaliplatin treatment group received gemcitabine [50 mg/kg, intraperitoneal injection (i.p.)] and oxaliplatin (2 mg/kg, i.p.) once every 2 weeks over a 4-week period starting at the 21st week. The MET-RON dual inhibitor treatment group received BMS-777607 [30 mg/kg, per os (p.o.)] 5 days/week (Monday through Friday) starting at the 21st week. The control group rats received i.p. injections of PBS following the same schedule.

Evaluation of treatment efficacy in rats by positron emission tomography. To evaluate the changes in glycolysis in live animals with liver tumors, we conducted 2-deoxy-2-[F-18] fluoro-d-glucose (FDG)-positron emission tomography (PET) studies of the rats at the Molecular Imaging Center of Chang Gung Memorial Hospital. In total, 18 rats were treated with TAA and received serial PET scanning at weeks 21, 23 and 25 using the Inveon™ system (Siemens Medical Solutions USA Inc., Knoxville, TN, USA). Equal numbers of animals were assigned to the control and treatment groups based on their baseline PET results, ensuring that the control and treatment groups possessed similar PET-positive rates. The details of the radioligand preparation, scanning protocols, and determination of optimal scanning time used in the present study have been previously described (20). Briefly, animals were fasted overnight prior to scanning. At 90 min post-^{18}F-FDG injection [intravenous injection (i.v.)], 30 min static scans were obtained for all of the animals. All imaging studies were performed using a temperature-(set to 37˚C) and anesthesia-(2% isoflurane vaporized in 100% oxygen) controlled imaging bed (Minerve System, Esternay, France). PET images were reconstructed using the 2D ordered subset expectation-maximization method (4 iterations and 16 subsets) without attenuation and scatter corrections. All imaging data were processed using the PMOD image analysis workstation (PMOD Technologies Ltd., Zurich, Switzerland). The largest liver tumor for each animal was identified by careful study of all 3 image sets for each rat. ^{18}F-FDG uptake into the largest liver tumor, as well as apparent normal liver tissue, was quantified by calculating the standardized uptake value (SUV). These values were calculated according to the recommendations made by the European Organization for Research and Treatment of Cancer (21). The tumor regions of interest (ROIs) were determined using transverse images of the selected tumors and measuring the largest diameter. The normal liver ROIs were also determined using the same transverse images. The mean SUV (SUV_{mean}) of the normal liver and tumor tissue

was calculated, and the tumor-to-liver radioactivity ratio was calculated for comparison.

Patient demographics. We analyzed the impact of expression of MET-RON on the characteristics and prognosis of 96 patients with mass-forming CCA (MF-CCA) who had received hepatectomies between 1989 and 2006 at the Department of Surgery, Chang Gung Memorial Hospital. The study was approved by the local Institutional Review Board of Chang Gung Memorial Hospital (clinical study nos. 99-2886B, 99-3810B and 102-5813B). Informed written consent for immunohistochemical tumor analysis was obtained from each patient.

MET and RON immunohistochemistry. MET and RON expression levels in the 96 MF-CCA patients were examined by immunohistochemical staining. Tissue sections (4 μm) were prepared from formalin-fixed, paraffin-embedded hepatectomy specimens, and incubated with anti-RON primary antibody (EP1132Y; 1:100 dilution; Abcam) and anti-MET primary antibody (8F11; 1:100 dilution; Abcam) overnight at 4˚C. After three 5-min washes with TBST, bound antibody signal was visualized using Dako Labelled Streptavidin-Biotin2 (LSAB2) System-HRP (Dako A/S, No. K0675; Dako, Glostrup, Denmark). Control slides were incubated with the secondary antibody only. For the assessment of immunohistochemical staining of MET-RON, the percentage of stained target cells was determined based on 10 random microscopic fields of view per tissue section (magnification, x400) using microscope Olympus BX51 and CCD capture for Olympus DP21 (Olympus Corp.). Their average scores were then calculated. Staining intensities were assigned scores of 1 (mild), 2 (moderate) or 3 (strong). H-scores were calculated as the percentage of positive staining (0-100) x the corresponding staining intensity (0-3). Specimens with H-scores of <160 or ≥160 were classified into those having low or high expression, respectively (range, 5-300; median, 160).

Follow-up study. The follow-up evaluation included physical examinations and blood chemistry tests during each visit. Additionally, serum levels of CEA and CA 19-9 were measured, and the remnant liver was examined by ultrasound (US) every 3 months. When a new lesion was detected by US or elevated levels of CEA/CA 19-9 were noted, the patients received abdominal CT or magnetic resonance cholangiopancreatography (MRCP) for confirmation. If the patients complained of bone pain, bone scans were performed to detect metastasis. Whether any of these procedures suggested recurrence, the patient in question was readmitted for a more comprehensive assessment, including angiographic evaluation or magnetic resonance imaging (MRI). The methods for treating recurrence included surgery, systemic chemotherapy, external beam radiotherapy, intraluminal radiotherapy, interventional radiological therapy and conservative treatment.

Statistical analysis. All data are presented as the mean ± SD. Differences between the experimental and control groups were calculated using the Student's t-test. Progression-free survival (PFS) and overall survival (OS) rates were evaluated with the Kaplan-Meier method. Several clinicopathological variables were considered for the initial univariate analysis, which was performed using the log-rank test. The Cox proportional hazards model was applied for multivariate regression. All statistical operations were performed using SPSS for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). A value of $P \leq 0.05$ derived from 2-tailed tests was considered significant.

Results

BMS-777607 inhibits the growth of human CCA cells. HuCCT1 and KKU-100 cells were selected as model cell lines since we found them to have higher levels of MET and RON than the normal bile duct cell line, MMNK-1, as determined by western blot analysis. We aimed to investigate the possible antiproliferative effects that BMS-777607 may have on CCA cells. To determine the potential effects, we used clonogenic assays to measure the growth of the two human intrahepatic CCA cell lines, HuCCT1 and KKU-100, in the presence of varying concentrations of BMS-777607. We found that BMS-777607 had a concentration-dependent antiproliferative effect on both the HuCCT1 and KKU-100 cell lines (Fig. 1A and B). In the HuCCT1 and KKU-100 cell lines, the IC_{50} values of MET-RON dual inhibitor (BMS-777607) 6 days after treatment were 11.4 and 5.9 μM, respectively (Fig. 1C). We also demonstrated that the expression of MET and RON protein in HuCCT1 and KKU-100 cells was higher than that observed in the MMNK-1 cells. In addition, we also demonstrated that the expression of phospho-RON was decreased in both HuCCT1 and KKU-100 cell lines after treatment with BMS-777607 (Fig. 1D).

BMS-777607 inhibits the in vivo growth of CCA in a rat animal model. We were also interested in assessing the possibility of using BMS-777607 to treat this cancer *in vivo,* as the rats with TAA-induced CCA displayed overexpression of MET and RON (Fig. 2A and B). To conduct this assessment, we compared the therapeutic efficacy of BMS-777607 to that of a combination of gemcitabine and oxaliplatin (e.g., a standard therapy for CCA) in treating the rats with TAA-induced CCA. As can be seen in Fig. 3A, animal PET-CT showed that the rats in each group had at least one FDG-avid tumor in the liver after 20 weeks of TAA treatment. These CCA rats were then treated with the vehicle alone, the MET-RON dual inhibitor alone (30 mg/kg, p.o., 5 days/week) (Monday through Friday), or gemcitabine plus oxaliplatin (gemcitabine 50 mg/kg + oxaliplatin 2 mg/kg, i.p., two times in 1 week) for 4 weeks. As of 2 to 4 weeks from the beginning of treatment, the group receiving the vehicle alone had steady increases in the mean tumor-to-liver (T/L) ratio of the SUV (that is, elevation from 33.0 to 50.0%). However, as of 2 weeks after beginning treatment, both the CCA rats receiving BMS-777607 and those receiving gemcitabine/oxaliplatin were found to have significant decreases in the T/L ratio of the SUV (P=0.041 for BMS-777607 and 0.006 for gemcitabine/oxaliplatin, respectively) (Fig. 3B). These findings indicate that MET-RON treatment significantly suppressed the *in vivo* growth of CCA tumors in our animal model.

MET-RON expression, clinicopathology and prognosis in 96 CCA patients. Forty-five of the 96 MF-CCA patient specimens (46.9%) were found to have high cytoplasmic

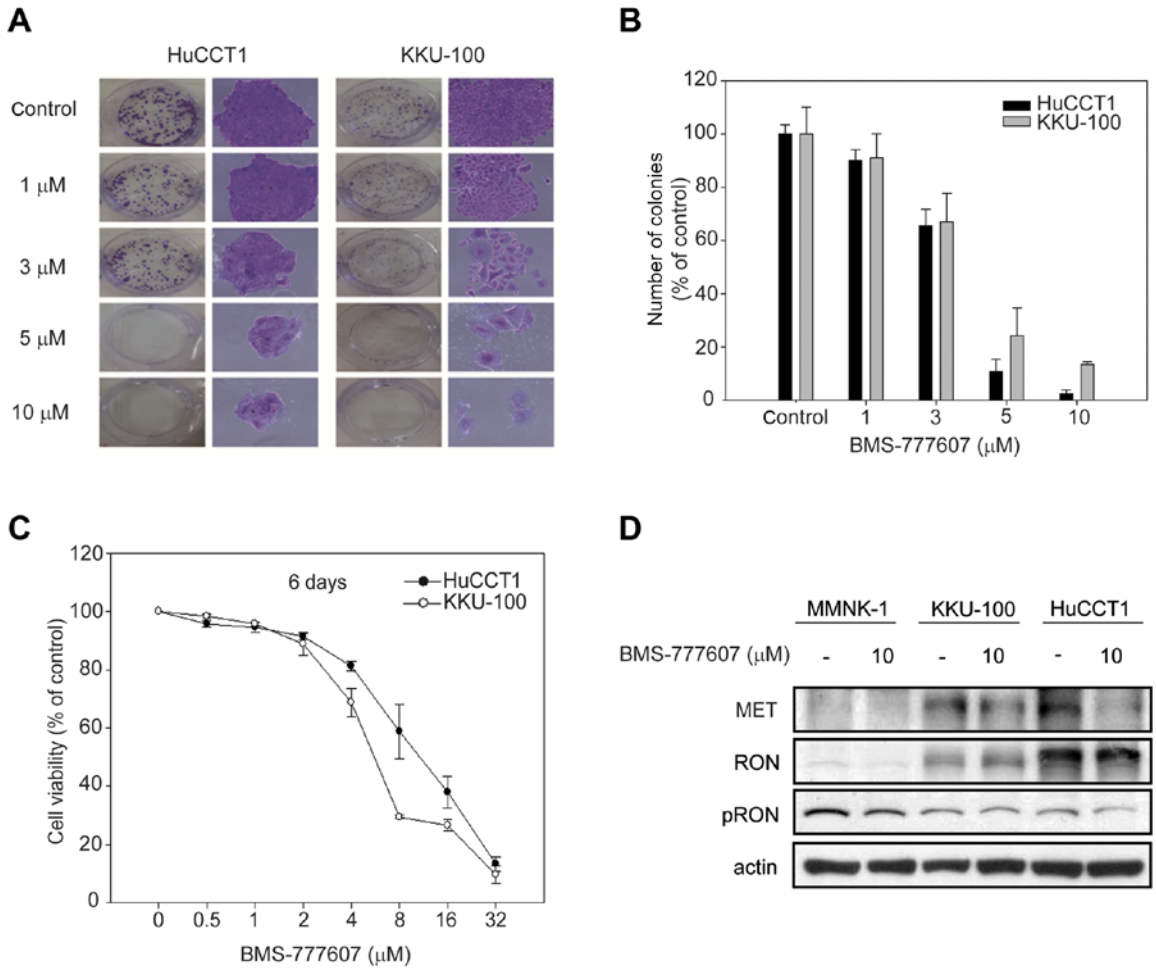


Figure 1. The antiproliferative effect of dual MET-RON inhibitor (BMS-777607) on HuCCT1 and KKU-100 cells was determined by colony formation assay. (A) Morphological changes in HuCCT1 and KKU-100 cells at 10 days following treatment with different amounts of dual MET-RON inhibitor (BMS-777607) at the indicated concentrations. (B) The antiproliferative effect of dual MET-RON inhibitor (BMS-777607) on HuCCT 1 and KKU-100 cells was analyzed by colony formation assay, as described above. Data shown are the average of 3 independent experiments. (C) The antiproliferative effect of BMS-77607 on HuCCT 1 and KKU-100 cells was determined by MTT assay. Cells in RPMI or DMEM with 5% FBS were cultured in 96-well plates and then treated with different amounts of BMS-77607 for 6 days. Data shown are the average of 3 independent experiments. (D) Total lysates prepared respectively from the MMNK-1, KKU-100 and HuCCT 1 cells (pre- and post-treatment with 10 μM BMS-77607) were subjected to MET, RON and phospho-RON markers. β-actin signals were used as the loading control.

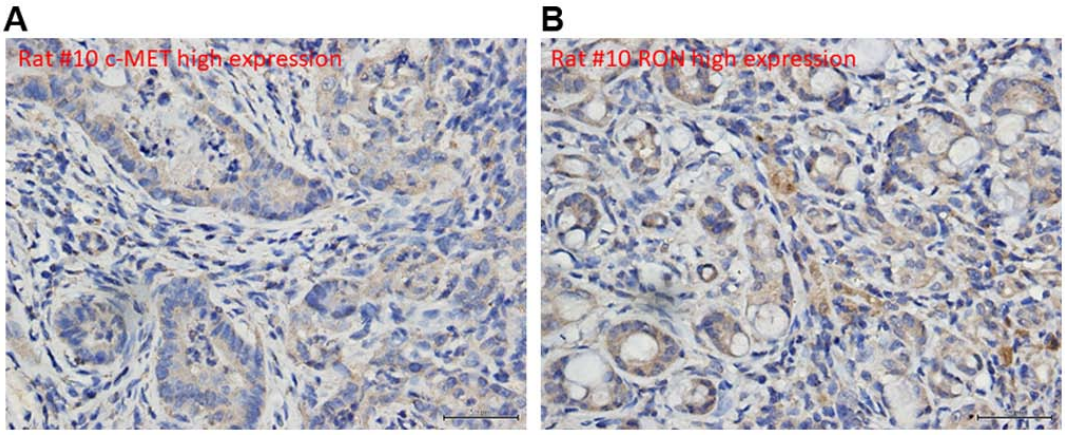


Figure 2. MET-RON overexpression is displayed in TAA-induced rat CCA. Representative slide showing strong immunostaining of (A) MET and (B) RON in rat CCA. TAA, thioacetamide; CCA, cholangiocarcinoma.

immunostaining for MET-RON (H score ≥160, Fig. 4). The overexpression of MET-RON was associated with elevated alkaline phosphatase, elevated CEA, tumor size larger than 5 cm, and positive resection margin, but only elevated

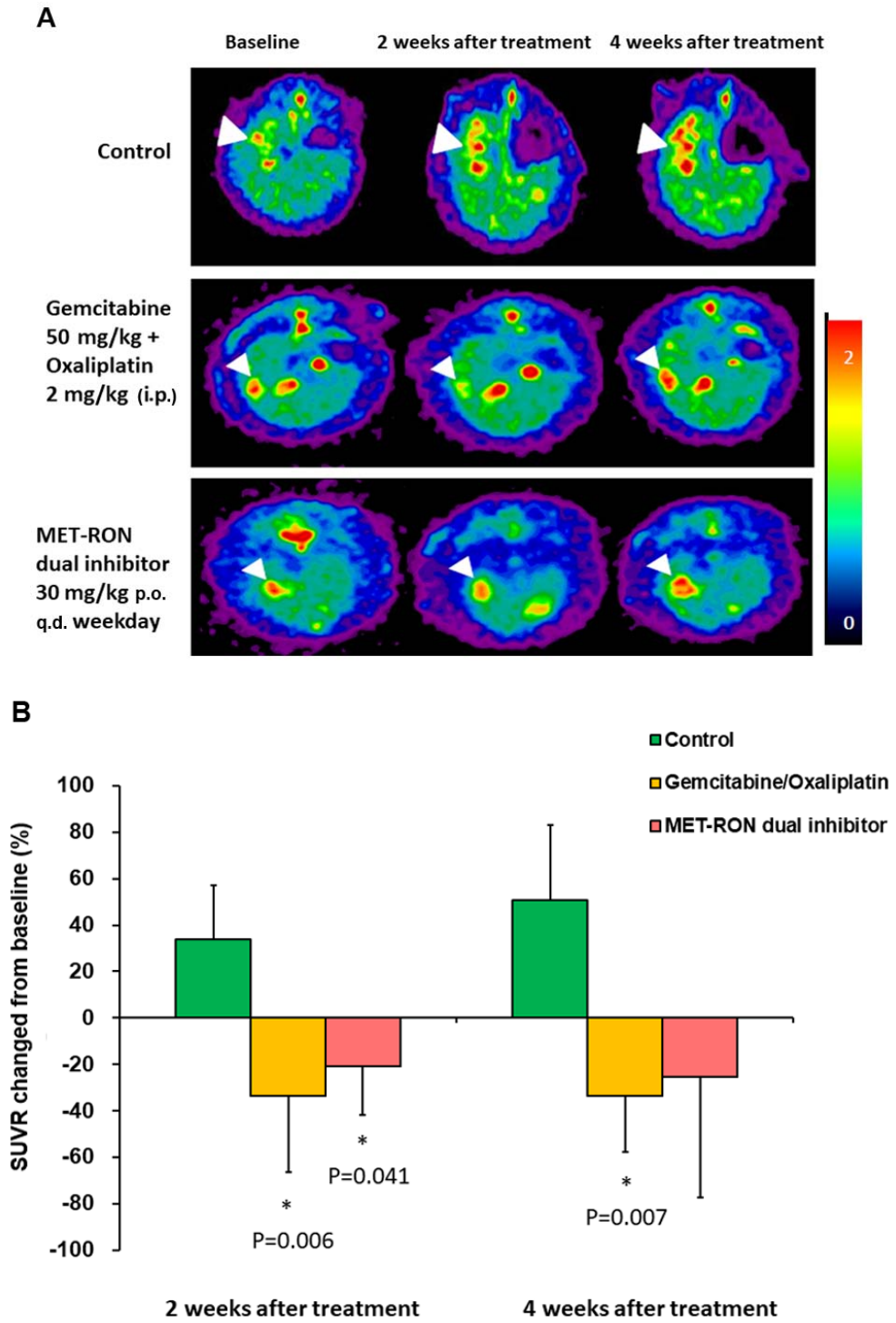


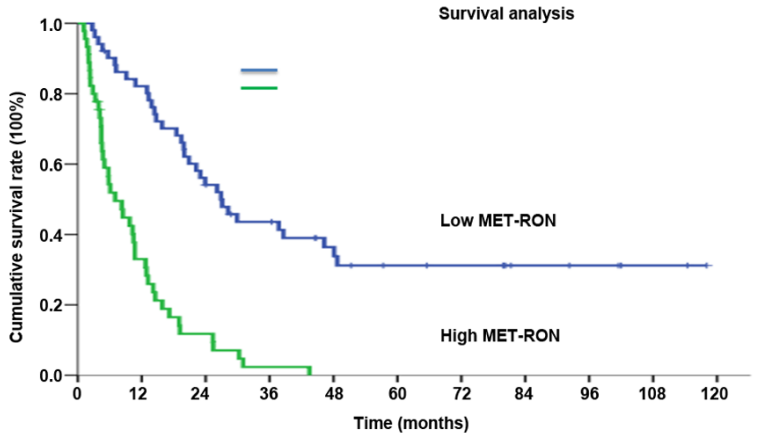
Figure 3. Dual MET-RON inhibitor (BMS-777607) suppresses cholangiocarcinoma tumorigenicity *in vivo*. Detection of rat CCA by animal PET and changes in tumor/liver SUV ratio. (A) Coronal views of fused CT and PET scans of representative control and experimental rats revealed CCA-expressing areas of the liver in which the ^{18}F-FDG uptake was increased from the baseline and as of 2-4 weeks after the experiment (i.e., at weeks 20, 22 and 24). (B) The change of tumor-to-liver (T/L) ratio of SUV in the control and experimental groups at 2 and 4 weeks after the experiment (i.e., at weeks 22 and 24). As can be seen, the mean tumor-to-liver (T/L) ratio of the SUV showed a steady elevation in the vehicle group (from 33.0 to 50.0% from 2 to 4 weeks after treatment). In contrast, there were significant decreases in the T/L ratio of the SUV after two weeks of the MET-RON dual inhibitor (BMS-777607) and gemcitabine/oxaliplatin treatments (P=0.041 and 0.006, respectively). CCA, cholangiocarcinoma; SUV, standardized uptake value.

alkaline phosphatase and elevated CEA were independently associated with it (Table I). With regard to survival, univariate log-rank analysis of the 96-post hepatectomy patients with MF-CCA identified the following factors as having adverse influences on OS: presence of symptoms, elevated alkaline phosphatase, elevated CEA, decreased albumin, tumor size >5 cm, positive surgical margin and lymph node status, and higher MET-RON immunostaining (Table II). Multivariate Cox proportional hazard analysis, however, revealed that both positive symptoms and higher MET-RON immunostaining scores independently predicted worse OS (Table II and Fig. 4).

A

MET-RON expression	N	OS,median (months)	Survival rate (%) 1-year	3-year
Low	51	26.9	54.1	31.2
High	45	7.0	33.0	2.4

Log-rank test, p<0.001



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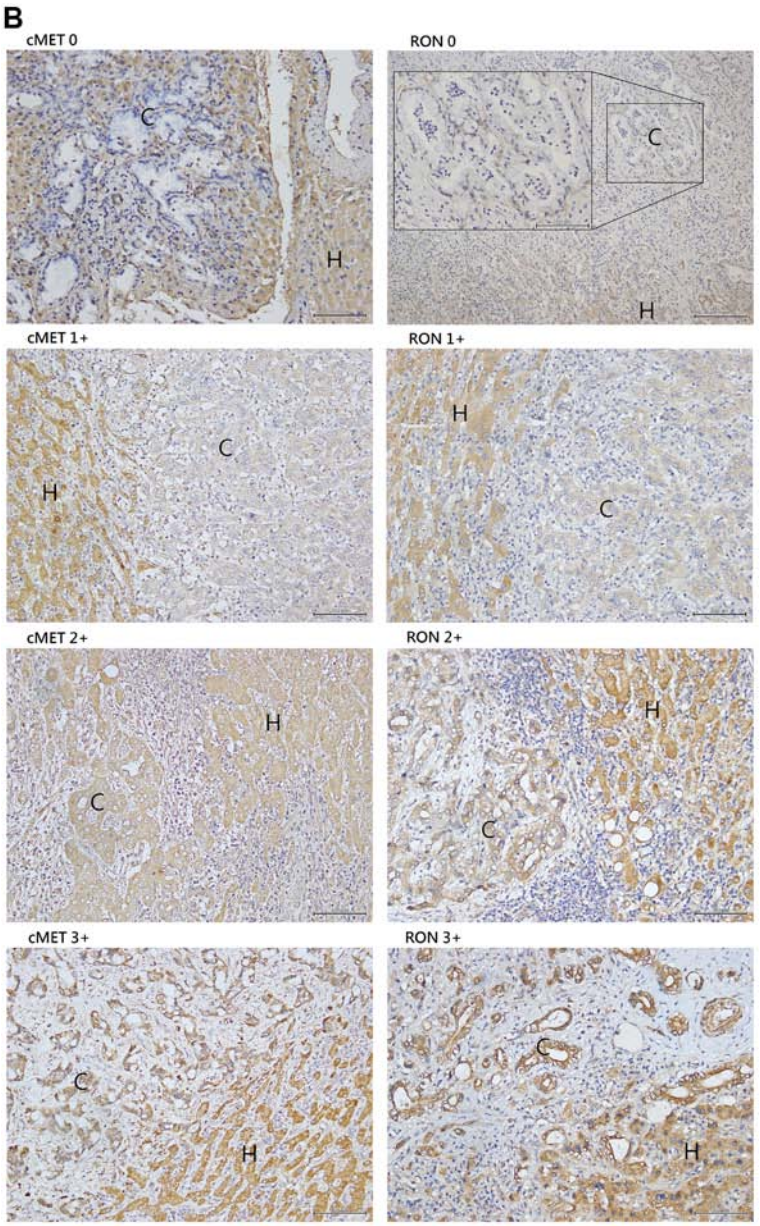

Figure 4. MET-RON overexpression is correlated with worse survival in patients with resectable CCA. (A) Kaplan-Meier plot of overall survival (OS) in patients with resectable intrahepatic CCA based on their MET-RON expression levels. (B) Representative no (0+), low (1+), intermediate (2+) and high (3+) MET and RON immunohistochemical staining intensities, respectively. H, hepatocyte; C, cholangiocyte. Scale bar, 50 μm. CCA, cholangiocarcinoma.

Table I. Association between the clinicopathological features and MET-RON expression in 96 patients with cholangiocarcinoma undergoing hepatectomy.

	MET-RON low expression (N=51)	MET-RON high expression (N=45)	P-value
Age (years)	60.61±12.95	59.93±10.45	
Sex, n (%)			0.734
Male	21 (41.2)	17 (37.8)	
Female	30 (58.8)	28 (62.2)	
Symptoms, n (%)			0.055
No	12 (23.5)	4 (8.9)	
Yes	39 (76.5)	41 (91.1)	
AST (IU/l), n (%)			0.437
≦34	29 (58.0)	22 (50.0)	
>34	21 (42.0)	22 (50.0)	
ALT (U/l), n (%)			0.636
≦36	29 (63.0)	25 (58.1)	
>36	17 (37.0)	18 (41.9)	
ALP (U/l), n (%)			**0.006**[a]
≦94	25 (53.2)	11 (25.0)	
>94	22 (46.8)	33 (75.0)	
Bilirubin (total), n (%) (mg/dl)			0.158
≦1.3	46 (90.2)	36 (80.0)	
>1.3	5 (9.8)	9 (20.0)	
Albumin (g/dl), n (%)			0.393
≦3.5	10 (22.2)	13 (30.2)	
>3.5	35 (77.8)	30 (69.8)	
Serum CEA (ng/ml), n (%)			**0.019**[a]
≦5	24 (64.9%)	13 (37.1)	
>5	13 (35.1)	22 (62.9)	
Size (cm), n (%)			**0.015**
≦5	27 (56.3)	14 (31.1)	
>5	21 (43.8)	31 (68.9)	
Lymph node, n (%)			0.423
Negative	36 (70.6)	27 (62.8)	
Positive	15 (29.4)	16 (37.2)	
Differentiated, n (%)			0.403
Well	2 (3.9)	1 (2.2)	
Moderate	29 (56.9)	19 (42.2)	
Poorly	19 (37.3)	24 (53.3)	
Other	1 (2.0)	1 (2.2)	
Surgical margin, n (%)			**0.003**
Negative	44 (86.3)	27 (60.0)	
Positive	7 (13.7)	18 (40.0)	
Post chemotherapy, n (%)			0.306
Without (n=48)	28 (54.9)	20 (44.4)	
With (n=48)	23 (45.1)	25 (55.6)	
Post radiotherapy, n (%)			0.817
With (n=84)	45 (88.2)	39 (86.7)	
Without (n=12)	6 (11.8)	6 (13.3)	

[a]Statistically significant by multi-logistic regression analysis: P=0.007 for alkaline phosphatase with relative risk [95% confidence interval: 6.68 (1.70-26.23)]; P=0.038 for CEA with relative risk [95% confidence interval: 3.58 (1.07-11.91)]. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CEA, *carcinoembryonic antigen*. Bold print indicates statistical significance in univariant logistic regression analysis.

Table II. Survival analysis of factors influencing the overall survival of 96 patients with cholangiocarcinoma undergoing hepatectomy.

	Survival time (months)				
Factors	Median	95% CI of median	3-year (%)	5-year (%)	P-value
Sex					0.589
Male (n=38)	18.51	11.88-25.14	23.9	20.9	
Female (n=58)	14.14	10.39-17.89	24.7	13.7	
Age (years)					0.670
≤60 (n=46)	14.70	7.46-21.93	25.2	20.4	
>60 (n=50)	14.53	7.87-21.20	23.7	12.3	
Symptoms					**0.002**[a]
Negative (n=16)	46.26	13.92-78.60	58.9	43.0	
Positive (n=80)	12.99	9.82-16.15	17.8	11.6	
AST (IU/l)					0.243
≤34 (n=51)	14.40	6.89-21.91	29.5	22.2	
>34 (n=43)	15.85	5.83-25.86	20.3	11.6	
ALT (IU/l)					0.426
≤36 (n=54)	14.53	7.79-21.27	27.1	19.4	
>36 (n=35)	15.85	8.72-22.97	19.6	9.8	
ALP (IU/l)					**0.001**
≤94 (n=36)	26.86	14.77-38.95	40.3	27.5	
>94 (n=55)	10.72	6.29-15.15	14.5	9.1	
Bilirubin (total) (mg/dl)					0.392
≤1.3 (n=82)	15.81	10.07-21.56	26.2	16.8	
>1.3 (n=14)	10.72	0.00-22.23	14.3	14.3	
Albumin (g/dl)					**0.023**
≤3.5 (n=23)	5.75	4.06-7.45	17.4	13	
>3.5 (n=65)	19.89	14.35-25.43	25.1	15	
Serum CEA (ng/dl)					**0.030**
≤5 (n=37)	19.17	10.82-27.51	37.3	22.6	
>5 (n=35)	12.72	8.24-17.20	8.9	8.9	
Surgical margin					**<0.001**
Negative (n=71)	19.89	14.80-24.98	33.6	22.8	
Positive (n=25)	4.70	2.69-6.71	0	0	
Size (cm)					**0.006**
≤5 (n=41)	20.88	12.58-29.17	38.8	29.1	
>5 (n=52)	12.89	8.76-17.01	14.8	7.4	
Lymph node					**0.016**
Negative (n=63)	20.88	14.11-27.64	31.1	18.4	
Positive (n=31)	12.72	4.83-20.61	12.9	12.9	
Histological differentiation					0.960
Well (n=3)	6.08	0.72-11.45	33.3	33.3	
Moderate (n=48)	15.81	8.89-22.73	26.6	18.6	
Poor (n=43)	14.40	10.92-17.88	22.9	14.6	
Others (n=2)	10.72	NA	0	0	
MET-RON expression					**<0.001**[a]
Low (n=51)	26.86	19.22-34.50	43.6	31.2	
High (n=45)	7.00	3.59-10.41	2.4	0	
Post-op chemotherapy					0.383
Without (n=48)	14.40	0.00-30.88	33.4	23.8	
With (n=48)	14.70	10.34-19.05	16.7	10.4	

Table II. Continued.

Factors	Survival time (months)				P-value
	Median	95% CI of median	3-year (%)	5-year (%)	
Post-op radiotherapy					0.075
Without (n=84)	14.53	10.66-18.40	28.2	19.2	
With (n=12)	7.17	0.00-27.65	0	0	

[a]Statistically significant in Cox's proportional hazards analysis; relative risks (P-value) for symptoms and MET-RON expression are 4.02 (0.038) and 4.95 (<0.001), respectively. CI, confidence interval; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; IU, international unit; op, operation. Bold prints indicates statistical significance in univariant survival analysis.

Discussion

The present study found the upregulation of MET-RON to be a poor prognostic factor for patients with intra-hepatic CCA who had received hepatectomies. BMS-777607 inhibited the growth of two human CCA cell lines and *in vivo* in the treatment of TAA-induced CCA rats (18). These findings suggest that the treatment of this malignancy may be improved by targeting MET-RON upregulation, which we achieved in this study using the MET-RON dual inhibitor (BMS-777607).

This study also found a significant correlation between MET-RON expression, CEA and alkaline phosphatase, suggesting that MET-RON expression is involved in the aggressive tumor behavior of intra-hepatic CCA. MET and RON belong to the same proto-oncogene family and have been reported to form a noncovalent complex on the cell surface, synergistically affecting the development of an invasive-metastatic phenotype in pathologic conditions (22). Diagnostic adjuncts for CCA (e.g., serum markers, including CA 19-9 and CEA measurements) may be usefully employed for the clinical management of this disease. Serum CA 19-9 values, which have been positively correlated with tumor burden, are elevated in patients with unresectable CCA. Several studies have reported that elevated CEA and CA19-9 are predictive of a poor prognosis (23-25). Although the cause is still unclear, one of our prior studies also reported elevated CEA levels to be an independent predictor of poor prognosis in patients with CCA who had received hepatectomies (26).

With regard to alkaline phosphatase although research still needs to be performed to explain the complex interaction between the clinical parameters, laboratory data, and pathologic factors and to elucidate their impact on HCC prognosis, our previous studies consistently found that elevated ALP levels negatively influence long-term OS and disease-free survival for patients with hepatocellular carcinoma (HCC) (27-29) and negatively affect OS in CCA patients following hepatectomies. Elevated ALP levels may indicate the presence of liver disease and bile duct obstruction. It should be noted that high preoperative ALP levels have also been correlated with increased mortality after hepatectomy for metastatic diseases (30).

Previous studies have found MET-RON expression to be associated with the progression, invasion and metastasis of malignant cells both in *in vivo* and *in vitro* (31-33). The recruitment and binding of substrates/adaptor proteins to the phosphorylated carboxy-terminal docking sites of activated c-Met and RON provide the platform to activate signaling cascades. PI3K and MAPK activation are major signaling molecules that are activated through c-Met and RON signaling. Numerous cellular responses are attributed to c-Met and RON signaling, including cytoskeletal changes, EMT, migration and invasion, stemness, resistance to apoptosis, angiogenesis and proliferation (17). MET-RON overexpression has also been associated with a poorer prognosis in urothelial carcinoma, bladder cancer, HCC, breast, colorectal and ovarian cancer (34-39). Consistent with peri-hilar CCA (40), the co-expression of MET and RON was associated with a poor prognosis in our intra-hepatic CCA patients. These results suggest that assessments of MET and RON expression may enable a tailored biological classification of intra-hepatic CCA patients who cannot otherwise be delineated using conventional pathologic methods. Moreover, the preoperative analysis of MET and RON expression in biopsy samples may support clinical decision making (i.e., whether or not a given patient is a suitable candidate for neoadjuvant therapy), because patients with positive MET and RON expression tend to have a poor prognosis even after undergoing curative resection.

Dual inhibitors of MET and RON have been developed and investigated in several *in vitro* and *in vivo* models (41). However, there are only a few clinical trials using oral multikinase inhibitors targeting MET, RON and other receptors that are currently ongoing, with those trials investigating papillary renal cell carcinoma and unresectable solid tumors (42,43). Based on our findings, dual inhibitors for MET and RON may hold therapeutic potential in the treatment of intra-hepatic CCA, particularly in patients with poor prognosis despite curative resection. However, it is unclear whether the signaling pathways that are associated with MET and RON are intensively activated by hepatocyte growth factor and hepatocyte growth factor-like protein in CCA. Therefore, there is a need for more clinical data and further mechanistic investigations.

The present study had some limitations. One major limitation is that the underlying reason to the poor prognosis of patients with overexpressed MET and RON remains unknown.

Another limitation is that only preclinical studies have found that MET-RON dual inhibitors may hold therapeutic potential. Clinical trials are thus still needed to study their value in treating patients with advanced or metastatic intra-hepatic CCA in whom MET-RON is upregulated.

In conclusion, MET and RON positivity predicts worse OS rates than either MET or RON negativity in patients with intra-hepatic CCA. BMS-777607 may thus potentially be used to treat certain patients with intra-hepatic CCA.

Acknowledgements

The authors thank the Laboratory Animal Center, Chang Gung Memorial Hospital, Linkou for animal care, the Center for Advanced Molecular Imaging and Translation, Chang Gung Memorial Hospital, Linkou and the Tissue Bank, Chang Gung Memorial Hospital, Linkou for technical support. We also thank Miss Wen-Chi Chiang for PET imaging analysis.

Funding

The present study was supported in part by grants from the Chang Gung Medical Foundation, Chang Gung Memorial Hospital, Linkou (nos. CMRPG3B0363, CMRPG3B0533, NMRPG5D6031~2, CMRPG3E1611~2, CRRPG3F0031~2 and NMRPG3F6021~2 to C.N.Y. and no. CMRPG8F1771 to Y.Y.C.) and by grants from the Ministry of Science and Technology (nos. MOST103-2314-B-182A-081-MY2 and MOST105-2314-B-182A-041-MY2 to C.N.Y.).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

CTC and YYC contributed equally to this manuscript and wrote the manuscript. RCW performed the pathological examination and interpreted the results. CYT, KCC and TSY helped to perform the experiments and interpreted the results. MHC and CNY reviewed and edited the manuscript and finally approved the publication of the manuscript. CTC, YYC, MHC and CNY were also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the local Institutional Review Board of Chang Gung Memorial Hospital (clinical study nos. 99-2886B, 99-3810B and 102-5813B). Informed written consent for immunohistochemical tumor analysis was obtained from each patient. The animal study followed the Animal Experimental Guidelines of Chang Gung Memorial Hospital, Chang Gung University (Taoyuan, Taiwan) with approval code: IACUC: 2010121409.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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