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Original Article

Amplification and overexpression of the MET gene in intrahepatic cholangiocarcinoma correlate with adverse pathological features and worse clinical outcome

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Abstract: Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary hepatic malignancy, and remains a challenge to treat. At the molecular level, the hepatocyte growth factor/MET pathway is one of the most commonly activated signaling pathways in malignancies. Several studies have reported MET overexpression in human iCCAs, at varying frequencies. However, there is no consensus regarding the impact of MET overexpression on prognosis. The correlation between MET overexpression and gene amplification in iCCAs has not been explored. We obtained tissue samples from 86 patients with primary iCCAs, and performed immunohistochemical analysis of MET expression. Quantitative real-time polymerase chain reaction was also performed to examine *MET* amplification. MET overexpression was found in 39 of 86 cases (45.35%). It was significantly associated with older age, presence of hepatolithiasis, higher cancer stage, and more advanced primary tumor. MET overexpression was also a predictive factor for adverse outcome with shorter disease-free survival. *MET* amplification was detected in 10 cases of 84 cases (11.90%), and was significantly associated with larger tumor size (more than 5 cm) and shorter overall survival. There was no significant correlation between MET overexpression and gene amplification. Revealing the clinicopathological features of *MET* overexpression and amplification would assist in the development of personalized treatment for iCCAs.

Keywords: Copy number, immunohistochemical study, intrahepatic cholangiocarcinoma, MET

Introduction

As with other cancer types, multistep carcinogenesis in intrahepatic cholangiocarcinoma (iCCA) includes cumulative genetic and epigenetic changes that involve activation of oncogenes and inactivation of tumor suppressor genes. Several previous studies have identified mutations of proto-oncogenes in iCCA, including *KRAS* (22%; range 5-57%) [1-3], *BRAF* (7%; range 1-22%), and epidermal growth factor receptor (*EGFR*, 2%; range 0-20%) [1, 2, 4, 5]. Such activating mutations cause dysregulation of genes encoding growth receptors and signal transducers. These oncoproteins endow neoplastic cells with self-sufficiency in growth. Other activating mutations have also been reported, such as isocitrate dehydrogenase 1 (*IDH1*) and 2 (*IDH2*) (mean 14%; range 10-28%), although the functional relevance of these mutations in the development of iCCA currently remains unclear.

Loss-of-function mutations of *TP53* have also been identified, with an overall frequency of 15% [1, 6]. In the context of inactivating mutations of *TP53*, DNA damage goes unrepaired, and mutations accumulate in oncogenes, resulting in an increased risk for the development of malignant transformation. Epigenetic changes, resulting in promoter hypermethylation, have been found in iCCA. These include methylation of several tumor suppressor genes, *RASSF1A* (56%, range 47-64%), *p16INK4a/*

Table 1. Clinicopathological characteristics and associations with MET overexpression

Parameters	No. of patients	Protein overexpression (n = 86)		*P* value
		High expression	Low expression	
Age, years				
≥60	49	15	34	0.002*
>60	37	24	13	
Gender				
Male	48	21	27	NS
Female	38	18	20	
Hepatolithiasis				
Yes	19	15	4	0.003*
No	63	24	39	
Tumor number				
Solitary	69	32	37	NS
Multiple	16	6	10	
Tumor size				
≤5 cm	46	22	24	NS
>5 cm	40	17	23	
Necrosis				
No	62	31	31	NS
Yes	24	8	16	
VI				
No	52	28	24	NS
Yes	34	13	21	
NI				
No	55	24	31	NS
Yes	31	15	16	
Histologic grade				
I	26	16	10	NS
II + III	60	23	37	
pT				
pT1 + pT2	49	17	32	0.029*
pT3 + pT4	37	22	15	
Stage				
I + II	45	14	31	0.009*
III + IV	41	25	16	

NS: not significant; MF, mass-forming type; PI, periductal infiltrating type; VI, vascular invasion; NI, neural invasion; H, histology; pT: primary tumor; *Statistically significant.

CDKN2 (47%, range 11-83%), and APC (29%, range 21-46%) [7-9]. Epigenetic changes of SOCS-3 and RUNX3 have also been observed [9, 10].

Regarding carcinogenesis, the HGF/MET pathway is one of the most commonly activated signaling pathways in human malignancies. MET is a member of the family of tyrosine kinase growth factor receptors, identified as the receptor for hepatocyte growth factor (HGF) [11]. Previous studies have shown that activation of MET resulted in induction of angiogenesis, cell proliferation, and cell invasion [12]. It has also been reported that MET is deregulated in many types of human malignancies, including gastric cancer, non-small cell lung cancer (NSCLC), B-cell neoplasm (particularly diffuse large B-cell lymphoma), and multiple myeloma [13-16].

A review of the literature reveals that only a few studies have focused on MET expression in human iCCAs [17-21]. However, there is no consensus regarding the impact of MET overexpression on clinical outcome. In addition, no data have been reported in relation to the correlation between MET overexpression and gene amplification in iCCAs. To investigate the clinicopathologic role of MET overexpression and its relation to *MET* gene amplification, we obtained tissue samples from 86 patients with iCCAs, and performed immunohistochemistry (IHC) and polymerase chain reaction in each case.

Materials and methods

Specimens

The study was approved by the institutional review board, in accordance with the Declaration of Helsinki. All samples and medical data used in this study have been irreversibly anonymized. Formalin-fixed, paraffin-embedded tumor and non-tumor samples from 86 patients treated from 2003 to 2012 were obtained from the files of the Department of Pathology, Chang Gung Memorial Hospital at Kaohsiung, Taiwan. The medical records associated with the samples were available and were carefully reviewed. Survival time was defined as the time period between the date of diagnosis and the date of death or the patient's last follow-up. The hematoxylin and eosin-

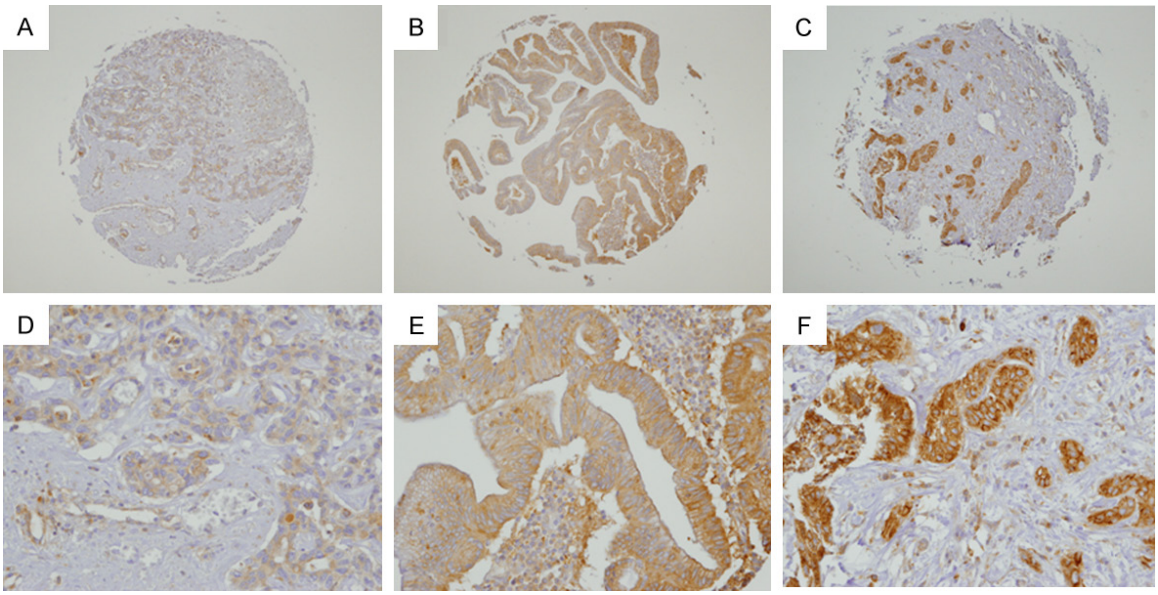

Figure 1. Immunohistochemistry (IHC) of MET expression in intrahepatic cholangiocarcinoma (iCCA). Representative photographs of expression of MET protein in iCCA. (A, C, and E) Represent tissue microarray (TMA) cores at magnification × 40; (B, D, and F) Represent selected areas from (A, C, and E) at higher magnification (× 200). Expression index was scored by multiplying the percentage of positive tumor cells by the average intensity. (A and B) Weak staining (1+) with 10% positive tumor cells. (C and D) Moderate staining (2+) with 60% positive tumor cells. (E and F) Strong staining (3+) with 75% positive tumor cells.

stained sections obtained at the time of diagnosis and repeats were reviewed. The American Joint Committee on Cancer (AJCC) 7th edition staging system was adopted for the staging of iCCA.

DNA extraction and copy number evaluation

Quantitative real-time polymerase chain reaction (qPCR) of target genes was performed on extracted DNA to determine copy number (CN) in test samples, as previously described [22]. Commercially available CN assays for the FAM-labeled probe MET (ABI assay ID: Hs02764674) and VIC-labeled probe RNase P (ABI Part Number: 4403326) were obtained from Applied Biosystems (Foster City, CA, USA). RNase P was used as the endogenous control. The CN (q) of the target gene was determined using the comparative quantitative threshold cycle (ΔΔCt) method, as previously described [22]. For statistical analysis, cases were classified into two groups according to the Q cutoff values, which were set at 3.0 to define the amplification.

IHC analysis

IHC was performed on tissue microarrays as previously described [23]. A polyclonal antibody against human MET (Santa Cruz Biotechnology, Santa Cruz, CA, USA; working dilution 1:100) was used. Positive controls were performed according to the manufacturer's instructions, and normal liver tissue, thyroid tissue, and placental tissue were used as negative controls. Slides were evaluated by a pathologist (SCW) blind to clinicopathological data. The labeling intensity was classified as negative, weak, moderate, and strong. The percentages of tumor cells with positive membranous immunoreactivity were recorded as the expression index in 5% increments. The labeling intensity, corresponding to the presence of negative, weak, moderate, and strong staining, was given a score from 0 to 3, respectively. An expression index was scored by multiplying the percentage of positive tumor cells by the labeling intensity. An index score between 0 and 300 was obtained, in which 300 was equal to 100% of tumor cells stained strongly (3+). The scores of multiple cores from the same patient were averaged to obtain a mean expression index. After testing a series of cutoff values, the cases were divided into two groups, high expression (expression index greater than or equal to 50) and low expression (expression index less than 50). High expression was considered as MET protein overexpression.

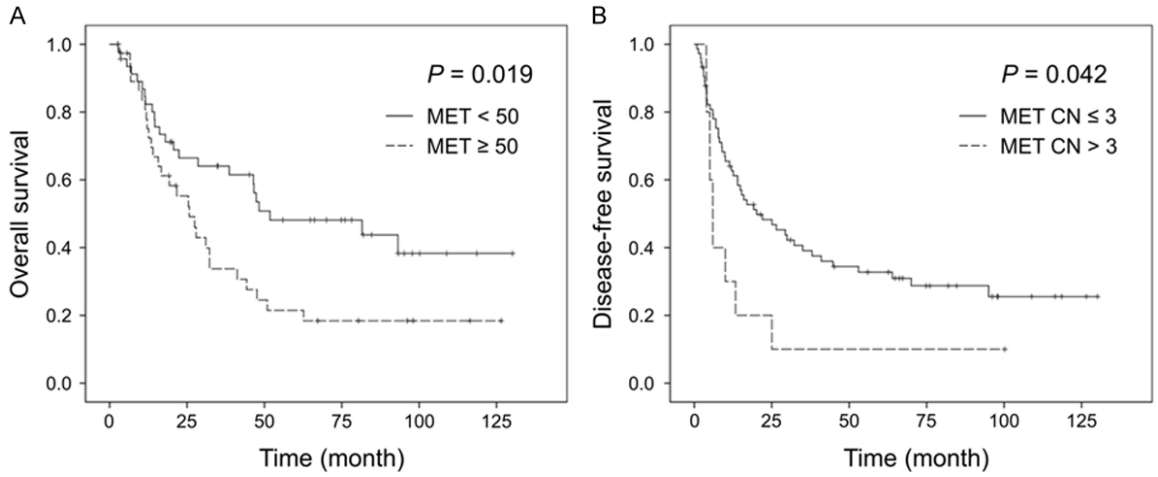

Figure 2. A. Cumulative survival shows a significantly poorer outcome in patients with MET expression index more than 50. Kaplan-Meier survival curves for patients categorized by MET expression index. Statistical significance was observed between groups. (MET<50: MET expression index less than 50; MET≥50: MET expression index greater than or equal to 50). B. Cumulative survival reveals a significantly poorer outcome in patients with MET gene copy number (CN) more than 3.0. Kaplan-Meier survival curves for patients categorized by MET gene CN. Statistical significance was observed between groups. (MET CN>3.0: MET CN more than 3.0; MET CN≤3.0: MET CN less than or equal to 3.0).

Statistical analysis

All statistical analyses were performed using SPSS for Windows 17.0 software (SPSS Inc., Chicago, IL, USA). The significance of association between histopathological variables and *MET* CN as well as MET expression was determined using the Chi-square and Fisher's exact tests. Overall survival (OS) was calculated from the date of diagnosis to death as a result of all causes. Disease-free survival (DFS) was computed from the time of surgery to cancer recurrence in the liver or distant metastasis. The Kaplan-Meier method was used for univariate survival analysis, and the difference between survival curves was tested by a log-rank test. For all analyses, two-sided tests of significance were used with a value of $P<0.05$ considered significant.

Results

Immunohistochemical analysis

Table 1 summarizes the associations between MET overexpression and the clinicopathological features. IHC was performed in all 86 cases, and MET overexpression was observed in 39 cases (45.35%) (**Figure 1**). In conjunction with the clinical factors, MET overexpression was significantly associated with older age (greater than 60 years, $P = .002$), presence of hepatolithiasis ($P = .003$), higher cancer stage (stage III and IV, $P = .009$), and more advanced primary tumor (pT3 and pT4, $P = .029$). MET overexpression was also a predictive factor for adverse outcome with shorter DFS (mean: 25.7 vs. 51.8 months, $P = .019$, **Figure 2A**).

qPCR study

Table 2 summarizes the associations between MET amplification and the clinicopathological features. qPCR was performed in 84 of 86 cases, with failure in two cases. Ten cases (11.9%) were found to have *MET* amplification (**Table 3**). *MET* amplification was significantly associated with larger primary tumor size (more than 5.0 cm in size, $P = .042$) and shorter OS (**Figure 2B**). There was no significant correlation between MET overexpression and *MET* gene amplification (**Figure 3**).

Discussion

In the present study, we investigated the clinicopathological role of the MET gene in human iCCAs. We found that overexpression and amplification of the target gene was correlated with the clinicopathological features of iCCAs, and implies an unfavorable outcome. In addition, MET expression does not correlate with *MET* gene amplification status, which suggests that another mechanism is responsible for its overexpression in this cancer type.

Table 2. Clinicopathological characteristics and associations with increased *MET* gene copy number

Parameters	No. of patients	Increased copy number (n = 84)		*P* value
		Positive	Negative	
Age, years				
≥60	49	7	42	NS
>60	35	3	32	
Gender				
Male	47	5	42	NS
Female	37	5	32	
Hepatolithiasis				
Yes	17	0	17	NS
No	63	10	53	
Tumor number				
Solitary	68	6	62	NS
Multiple	16	4	12	
Tumor size				
≤5 cm	44	2	42	0.042*
>5 cm	40	8	32	
Necrosis				
No	60	6	54	NS
Yes	24	4	20	
VI				
No	50	3	47	NS
Yes	34	7	27	
NI				
No	54	7	47	NS
Yes	30	3	27	
Histologic grade				
I	24	1	23	NS
II + III	60	9	51	
pT				
pT1 + pT2	49	7	42	NS
pT3 + pT4	35	3	32	
Stage				
I + II	45	5	40	NS
III + IV	39	5	34	

NS: not significant; MF, mass-forming type; PI, periductal infiltrating type; VI, vascular invasion; NI, neural invasion; H, histology; pT: primary tumor; *Statistically significant.

The MET protein is encoded by the *MET* proto-oncogene. It is a transmembrane protein, and serves as the receptor for HGF [11]. The binding of HGF to the MET protein results in the activation of downstream signaling targets, such as phosphatidylinositol 3-kinase (PI3K)-AKT, mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT) signaling pathways [24-26]. The activation evokes biological responses leading to increased cell growth, scattering and motility, invasion, protection from apoptosis, branching morphogenesis, and angiogenesis [24, 27]. It has been found that the MET protein is deregulated in many kinds of human malignancies, including gastric cancer, NSCLC, B-cell neoplasm (particularly diffuse large B-cell lymphoma), and multiple myeloma [13-16]. In such pathological conditions, MET overexpression promotes both tumor cell migration (hence contributing to invasive growth and metastasis) and proliferation and survival (resistance to apoptotic signals), and stimulates angiogenesis [12].

In previous studies, the observed range of frequency of MET overexpression has been relatively broad, ranging from 21.4% to 58% [17, 19, 20]. The studies of Terada et al. [17] and Aishima et al. [18] found that MET overexpression was correlated with a better prognosis, a finding contrary to our results. Nevertheless, the results of more recent studies, conducted by Miyamoto et al. [20] and Mao et al. [21], support our findings, suggesting an unfavorable prognosis and significantly shorter survival with MET overexpression. This discrepancy may be due to differences in the scoring system of immunostaining and the definition of overexpression. The relatively small case numbers may also contribute to discrepancies in the overexpression rate.

In the present study, the overexpression of MET in iCCAs was significantly associated with the presence of hepatolithiasis. Terada et al. [17] found that MET expression, which was not generally detected in the normal adult liver, was markedly increased in cases of hepatolithiasis without iCCA (26 of 31 cases, 81%). Aishima et al. [18] also reported similar results. The immunoreactivity was mainly detected in the biliary epithelial cells of hyperplastic large and septal bile ducts and proliferated peribiliary glands, including areas of dysplastic biliary epithelial cells in a few cases. Considering that heptolithiasis is also a primary risk factor for iCCA [28], the development of an iCCA in a condition of hepatolithiasis may be associated with MET overexpression. Further studies will be needed to confirm this association.

Some studies have investigated the relationship between MET expression and *MET* gene

Table 3. *MET* gene copy number change

Gene	Total case	CN	CN>2.5	CN>3.0
	n	Mean (range)	n (%)	n (%)
MET	84	1.953 (0.13-5.52)	18 (21.43%)	10 (11.90%)

CN: copy number; n: number.

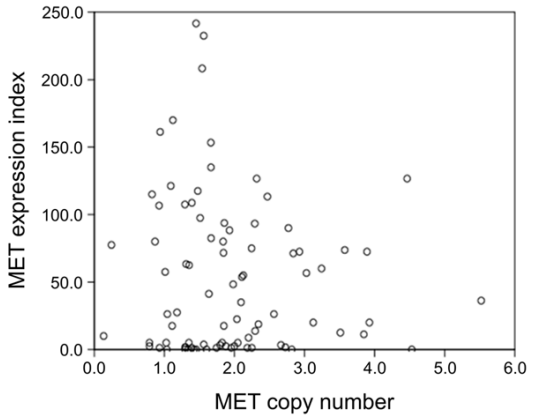

Figure 3. Two-dimensional Cartesian coordinates for MET expression index and MET copy number. No statistical correlation between MET expression index and MET copy number was observed.

amplification in human malignancies, including hepatocellular carcinoma, NSCLC, and gastric cancer [29-37], and the studies have yielded different results. Park et al. [32] and Lee et al. [36] suggested that MET overexpression was highly correlated with gene amplification in lung adenocarcinomas and gastric carcinomas, respectively. Lee et al. and Sun et al. also found a correlation between gene amplification and protein overexpression in hepatocellular carcinoma and NSCLC, respectively. However, both studies revealed that more than half of patients with MET overexpression had a neutral *MET* CN, suggesting that mechanisms other than gene amplification were causing MET protein overexpression. Other studies yielded an opposite result [30, 31, 34, 35, 37], suggesting no correlation between gene amplification and protein overexpression.

The conflicting findings between overexpression and amplification may result from the use of different methods and scoring systems for the evaluation of the *MET* gene CN. Although fluorescence in situ hybridization (FISH) is the gold standard technique for detecting gene CN, qPCR could provide an alternative and powerful method [38]. It is easy to perform, less expensive than FISH, and can be used to analyze a large area of tumors, thus minimizing CN deviation due to tumor heterogeneity. Data reviewing the correlation between MET overexpression and *MET* gene amplification in iCCAs is extremely scant. The present study found relatively higher MET overexpression frequency than the much lower *MET* amplification rate (45.35% vs. 11.90%). This finding indicated that the mechanism of regulation of MET expression could be much more complex in iCCAs. Different mechanisms are known to result in MET protein overexpression. Research has shown that the HGF/MET pathway may be deregulated by mutations or genomic amplification of the *MET* gene, increased ligand-mediated stimulation, increased expression of HGF activator, and interaction with other active cell-surface receptors [24, 39, 40]. In addition, activation of other oncogenes and transcription factors, inactivation of the *TP53* tumor suppressor, and hypoxia are known to increase *MET* transcription [41]. Some studies have also suggested that several microRNAs upregulate MET protein expression in a variety of cancers [42]. Further study to explore the HGF/MET pathway in iCCA is necessary.

The high mortality rate and poor prognosis of iCCA are associated with early invasion, widespread metastasis, and the lack of an effective therapy. The revelation of the association of the HGF/MET pathway with iCCA allows the development of novel targeted therapies for improved treatment of iCCAs. These targeted drugs, which include HGF activation inhibitors, HGF inhibitors, MET antagonists, and MET kinase inhibitors [43, 44], could be prescribed alone or combined with chemotherapy regimens.

Conclusion

The present study suggested that MET overexpression is associated with older patient age, presence of hepatolithiasis, higher cancer stage, and more advanced primary tumor. *MET* amplification is associated with larger primary tumor size. Both MET overexpression and *MET* amplification maybe useful biomarkers to predict disease progression. As it offers a potential therapeutic target in the HGF/MET signaling pathway, subsequent studies are required to clarify the mechanisms underlying MET overexpression in iCCA.

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Disclosure of conflict of interest

None.

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