

Prevalence and Clinicopathological Significance of MET Overexpression and Gene Amplification in Patients with Gallbladder Carcinoma

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Purpose

Mesenchymal epithelial transition (MET) is a proto-oncogene that encodes a heterodimeric transmembrane receptor tyrosine kinase for the hepatocyte growth factor. Aberrant MET signaling has been described in several solid tumors—especially non-small cell lung cancer—and is associated with tumor progression and adverse prognosis. As MET is a potential therapeutic target, information regarding its prevalence and clinicopathological relevance is crucial.

Materials and Methods

We investigated MET expression and gene amplification in 113 gallbladder cancers using tissue microarray. Immunohistochemistry was used to evaluate MET overexpression, and silver/fluorescence *in situ* hybridization (ISH) was used to assess gene copy number.

Results

MET overexpression was found in 37 cases of gallbladder carcinoma (39.8%), and gene amplification was present in 17 cases (18.3%). MET protein expression did not correlate with MET amplification. MET amplification was significantly associated with aggressive clinicopathological features, including high histological grade, advanced pT category, lymph node metastasis, and advanced American Joint Committee on Cancer stage. There was no significant correlation between any clinicopathological factors and MET overexpression. No difference in survival was found with respect to MET overexpression and amplification status.

Conclusion

Our data suggested that MET might be a potential therapeutic target for targeted therapy in gallbladder cancer, because MET amplification was found in a subset of tumors associated with adverse prognostic factors. Detection of MET amplification by ISH might be a useful predictive biomarker test for anti-MET therapy.

Key words

Gallbladder cancer, Gene amplification, Gene copy number, *In situ* hybridization, Immunohistochemistry, MET

Introduction

Gallbladder cancer (GBC) is a relatively rare primary malignancy, but it is the most common malignant neoplasm of the biliary tract, and one of the most aggressive cancers of the biliary tract with poor prognosis [1]. A variety of genetic and epigenetic alterations are associated with GBC, involving tumor suppressor genes, oncogenes, and DNA repair genes.

Previous studies have focused on the importance of oncogenes (epidermal growth factor receptor [EGFR], K-Ras, and ERBB2), tumor suppressor genes (p53), cell cycle regulators (cyclin D1, cyclin E), and micro-RNAs in the development and prognosis of GBC. However, the precise information regarding genetic and molecular alterations in GBC is still poorly understood [2]. Li et al. [3] investigated genetic alterations in 57 GBC cases by whole exome and targeted sequencing, and found frequent mutations of TP53, KRAS, and

ERBB3. Moreover, ErbB signaling, including EGFR, ERBB2, ERBB3, ERBB4, and their downstream genes is the most extensively mutated pathway, affecting 36.8% of patients with GBC. Although cytotoxic chemotherapy with the combination of gemcitabine and cisplatin has been accepted as the first-line therapy, the patient outcome is still disappointing. After ErbB signaling pathway was found to be one of the pathways contributing to gallbladder carcinoma, target-oriented agents, including monoclonal antibodies and tyrosine kinase inhibitors against EGFR and vascular endothelial growth factor have entered clinical trials [4,5]. However, as those clinical trials revealed no, or marginal benefits, novel targets are needed to improve the patient outcomes.

The mesenchymal epithelial transition (MET) receptor tyrosine kinase pathway is one of the most commonly activated signaling pathways in primary human cancers. MET has a heterodimeric structure composed of an extracellular α subunit and a β chain that contains an extracellular domain, a transmembrane segment, and an intracellular tyrosine kinase region [6]. Activation of MET signaling has a critical role not only in tumor formation, progression, angiogenesis, and metastasis, but is also involved in mediating resistance against other targeted treatments [7,8]. Hepatocyte growth factor (HGF), a ligand of MET, stimulates the tyrosine kinase activity of MET and activates multiple downstream signaling pathways, including the Grb2-Ras-mitogen-activated protein kinase (MAPK) cascade, the phosphatidylinositol-3 kinase (PI3K) pathway, the Wnt/ β -catenin pathway, and the signal transducer and activator of transcription (STAT) pathway [9]. Dysregulation of MET protein has been reported in a variety of human malignancies and premalignant lesions, including hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC) [10,11]. Accordingly, the possibility of targeting the *MET* gene in different cancer types is now being evaluated in clinical trials. Several drugs have been developed that target MET or HGF. These agents are divided into small molecule inhibitors and monoclonal antibodies. The small molecule tyrosine kinase inhibitors are further subdivided into multiple kinases and selective MET inhibitors. Crizotinib and cabozantinib are examples of multikinase MET inhibitors. Selective MET inhibitors include capmatinib and tepotinib. Monoclonal antibody therapy is divided into anti-MET antibodies, such as onartuzumab and emibetuzumab, and anti-HGF antibodies, including ficlatuzumab and rilotumumab [12].

As MET is a potential therapeutic target, information regarding its prevalence and clinicopathological relevance is important. However, the prevalence of MET overexpression and gene amplification in GBCs and its clinicopathological significance is not yet known precisely. Therefore, this study evaluated the expression level and gene copy number of MET in GBC tissues and their correlations with various clin-

icopathological characteristics, including patient's prognosis.

Materials and Methods

1. Patients and specimens

We examined 116 GBC samples (113 patients) obtained from consecutive surgical procedures performed at the Hanyang University Hospital between 1991 and 2016. Three patients had synchronous tumors in the gallbladder. The GBCs were assessed according to the system for staging primary tumor/regional lymph nodes/distant metastasis (TNM) described in the 8th American Joint Committee on Cancer (AJCC) cancer staging manual and histological classification according to the World Health Organization classification of tumors. The clinical data were retrospectively obtained from electronic medical records and included age at diagnosis, sex, pathological profiles (size, stage, histological grade, location, margin status, lymphovascular invasion, and perineural invasion), metastasis, recurrence, and date of death. Tumor recurrence was defined as tumor growth in any site of the body after the surgery, which was diagnosed clinically, radiologically, or pathologically, but mainly by computed tomography and ultrasonography. Among the 113 patients, 44 (37.9%) were men, and the median age was 63.5 years (range, 28 to 90 years). The tumor types of gallbladder were as follows: adenocarcinoma (n=109), squamous carcinoma (n=2), adenosquamous carcinoma (n=4), and small cell carcinoma (n=1). The detailed clinicopathological features are described in Table 1.

2. Tissue microarray construction

Hematoxylin and eosin slides made from formalin-fixed paraffin-embedded tissue blocks were reviewed, and the most representative tumor regions without necrosis and hemorrhage were carefully selected and marked. Single tissue cores, each 3.0 mm in diameter, were punched from each paraffin block and assembled into a new recipient paraffin block using a manual tissue microarray (TMA) instrument (Quick-Ray, Unitma, Seoul, Korea). A total of four TMA blocks were constructed.

3. Immunohistochemistry

Immunohistochemical staining (IHC) was performed on Ventana BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ), according to the manufacturer's protocol.

Table 1. Histological and clinical characteristics of gallbladder cancer patients

Clinicopathological characteristic	No. (%)
Age (yr)	
< 65	60 (53.1)
≥ 65	53 (46.9)
Sex	
Male	44 (38.9)
Female	69 (61.1)
Histological type	
Adenocarcinoma	109 (94.0)
Squamous	2 (1.7)
Adenosquamous	4 (3.4)
Small cell neuroendocrine	1 (0.9)
Histological grade	
<i>In situ</i>	6 (5.5)
Well	26 (23.9)
Moderate	50 (45.9)
Poor	27 (24.8)
T category	
Tis	6 (5.3)
T1a	8 (7.1)
T1b	8 (7.1)
T2	46 (40.7)
T3	37 (32.7)
T4	8 (7.1)
AJCC stage	
0	6 (5.3)
I	16 (14.2)
II	31 (27.4)
III	44 (38.9)
IV	16 (14.2)
Lymph node metastasis	
N0	56 (57.1)
N1	31 (31.6)
N2	11 (11.2)
Distant metastasis	
M0	103 (91.2)
M1	10 (8.8)
Lympho-vascular invasion	
Absent	58 (64.3)
Present	55 (35.7)
Perineural invasion	
Absent	72 (63.7)
Present	41 (36.3)

AJCC, American Joint Committee on Cancer.

The primary antibody used was anti-MET (clone sp44) rabbit monoclonal antibody (Ventana Medical Systems).

Two pathologists (K.J. and Y.K.) independently evaluated MET IHC. The immunoreactivity was scored using a four-

tier system, which has been established in previous studies on MET IHC scoring system in NSCLC [13]. This system was used to evaluate both proportion and staining intensity: 0, no staining or < 50% tumor cells with any intensity; 1+, ≥ 50% of tumor cells staining with weak or higher staining but < 50% with moderate or higher strong intensity; 2+, ≥ 50% of tumor cells with moderate or higher staining but < 50% with strong intensity; 3+, ≥ 50% of tumor cells staining with strong intensity. Samples exhibiting 2+ or 3+ immunostaining were considered positive for MET overexpression.

4. *In situ* hybridization

MET silver *in situ* hybridization (SISH) was performed on an automated Ventana BenchMark XT platform (Ventana Medical Systems), according to the manufacturer's protocols and using both MET-specific and centromere 7 (CEP7)-specific probes. Fluorescence *in situ* hybridization (FISH) was used to confirm *MET*-amplified cases by SISH. FISH was performed using Zytolight SPEC MET / CEN7 Dual color probe (Zytovision, Bremerhaven, Germany), according to the manufacturer's protocol.

The gene copy number was assessed independently by two pathologists (K.J. and Y.K.) and the number of MET and CEP7 signals was counted in at least 40 tumor cell nuclei in "hot spot" area (80 nuclei if there was no agreement). Then, the mean number of MET and CEP7 copies per nuclei and the MET/CEP7 ratio was calculated. We used the four different scoring systems for *in situ* hybridization (ISH) interpretation.

- Method A: High-level amplification was defined in tumors with a MET/CEP7 ratio ≥ 2.0, an average *MET* gene copy number per cell of ≥ 6.0, or ≥ 10% of tumor cells containing ≥ 15 MET signals. Intermediate level of gene copy number gain being defined as ≥ 50% of cells containing ≥ 5 MET signals and criteria for high-level amplification are not fulfilled. Low level of gene copy number gain was defined as ≥ 40% of tumor cells showing ≥ 4 MET signals and criteria for high-level amplification or intermediate level of gene copy number gain are not fulfilled. All other tumors were classified as negative [14].
- Method B: Tumors with a mean gene copy number of ≥ 5 MET signals per tumor cell were classified as *MET* amplification [15].
- Method C: Tumors with a MET/CEP7 ratio ≥ 2 were defined as *MET* amplification by PathVysion [16,17].
- Method D: High-level amplification (presence of loose or tight clusters of MET signals too numerous to count) was defined in tumors with MET/CEP7 ratio more than 5.0. Low-level amplification was defined in tumors with MET/CEP7 ratio ≥ 2.2 and ≤ 5.0 [18].

5. Targeted sequencing by OncoPrint comprehensive assay

DNA and total RNA were extracted by using RecoverAll Multi-Sample RNA/DNA Isolation Workflow (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Library preparation for each sample was performed using the multiplex PCR-based Ion Torrent AmpliSeq technology (Thermo Fisher Scientific, Waltham, MA), together with the OncoPrint comprehensive assay (OCA) v1 panel (Thermo Fisher Scientific) according to the manufacturer's protocol. OCA v1 panel covers multiple exons in 143 genes with 156 amplicons. Next generation sequencing (NGS) was performed on the Ion Torrent S5XL using an Ion 540 chip (PGM, Life Technologies, Grand Island, NE). Results were analyzed using the Variant caller by aligning the reads to the hg19 reference genome, calling the variants, and generating an interactive report for visualization and quality control. Data analysis was performed using Ion Reporter Server hosting informatic tools (Ion Reporter Software v5.0) for variant analysis, filtering, and annotations.

6. Statistical analysis

To analyze the association between the clinicopathological features and *MET* gene copy number status as well as *MET* protein expression, chi-square test was employed. Disease-free survival and overall survival (OS) were determined using Kaplan-Meier survival curves, and the log-rank test was used to compare the differences. The Cox proportional hazard regression model was used to evaluate the independent prognostic significance. All statistical analyses were carried out using SPSS for Windows ver. 19.0 software (SPSS Inc., Chicago, IL), and $p < 0.05$ was considered as statistically significant.

7. Ethical statement

This study was approved by the Institutional Review Board of Hanyang University Hospital (IRB No. 2018-08-031-002). Waiver of informed consent for this study was obtained from IRB based on the retrospective analyses of archived tissues and clinical data.

Results

1. *MET* overexpression and gene amplification in GBCs

MET IHC was successfully performed in 93/116 tissue cores (80.2%). The unsuccessful staining was mainly due to empty TMA cores or absence of tumor cells. Many tissue cores showed focal or diffuse cytoplasmic/membranous staining pattern with varying intensity. *MET* IHC score 0-3 was observed in 26 (28.0%), 30 (32.3%), 22 (23.7%), and 15 (16.1%) tumors, respectively. *MET* overexpression, defined by IHC score 2 or more, was found in 37/93 (39.8%) GBC tumor tissues (Fig. 1).

SISH was successful in 93/116 tissue cores (80.2%), and 17/93 (18.3%), 9 (9.7%), 12 (12.9%), and 11 (13.4%) showed *MET* gene amplification according to four different interpretation methods (Table 2). FISH was performed in same TMA sections and all cases showed concordant results with SISH. The representative images of SISH and FISH are shown in Fig. 2.

To identify the intratumoral heterogeneity of *MET* copy number, we performed ISH on whole tissue sections in subset of amplified cases (6 out of 17 cases). Because the assessment of heterogeneity is difficult on FISH slide due to dark field, we performed bright field SISH to observe morpho-

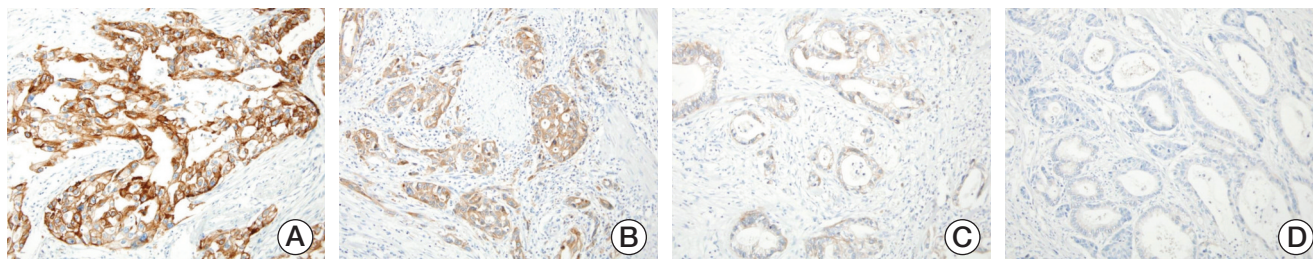


Fig. 1. Representative sections of immunohistochemical (IHC) scores for *MET* expression in gallbladder cancers ($\times 400$). (A) IHC score 3, strong cytoplasmic/membranous staining in almost all tumor cells. (B) IHC score 2, more than 50% of tumor cells with moderate staining intensity but $< 50\%$ strong intensity. (C) IHC score 1, weak staining in more than 50% of tumor cells. (D) IHC score 0, no staining observed in invasive tumor cells.

Table 2. Prevalence of *MET* amplification in gallbladder cancers according to different interpretation criteria

	Reference	Classification	No.	No. of amplified cases (%)
Method A	Schildhaus et al. [14]	High	12	17 (18.3)
		Intermediate	0	
		Low	5	
		Negative	76	
Method B	Cappuzzo et al. [15]	Positive	9	9 (9.7)
		Negative	84	
Method C	PathVision [16,17]	Positive	12	12 (12.9)
		Negative	81	
Method D	Ou et al. [18]	High	0	11 (13.4)
		Low	11	
		Negative	82	

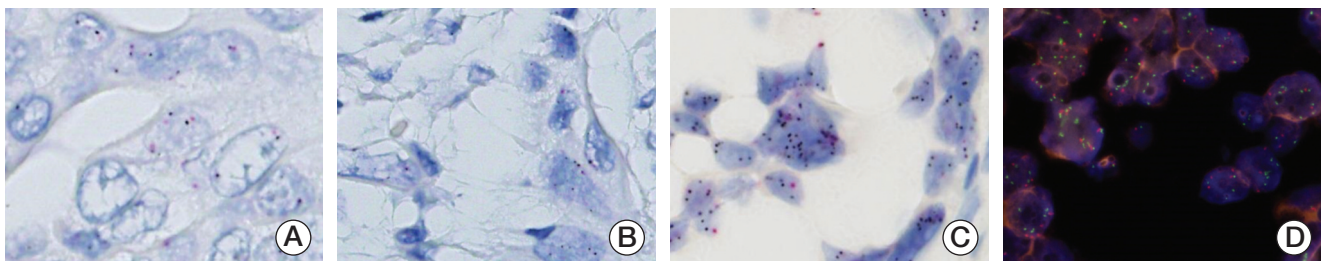


Fig. 2. Representative sections of silver *in situ* hybridization (SISH) and fluorescence *in situ* hybridization (FISH) analyses of *MET*/centromere 7 (CEP7) ($\times 1,000$). (A) SISH, no *MET* amplification, 1-2 *MET* gene signals (black), and 1-2 CEP7 signals (red) were present in each nucleus. (B) SISH, low-grade *MET* copy number gain, 2-7 *MET* gene signals (black), and 1-2 CEP7 (red) were present in each nucleus. (C) SISH, high-level amplification, 7-8 or more *MET* gene signals (black), and 1-5 CEP7 (red) were present in each nucleus. (D) FISH, high-level amplification, 5-10 or more *MET* gene signals (green), and 4-5 CEP7 (red) were present in each nucleus.

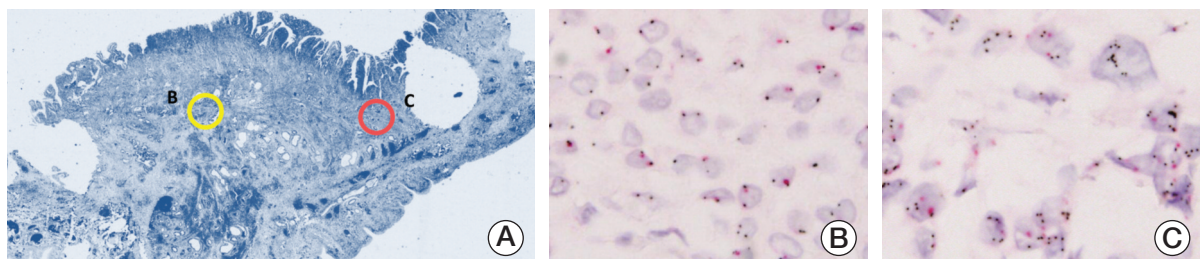


Fig. 3. Representative section of silver *in situ* hybridization on whole section. (A) Yellow and red circle highlight the non-amplified lesion and amplified lesion, respectively. (B) The tumor cells exhibit 1-2 *c-MET* gene signals (black), and 1-2 centromere 7 signals (red) in each nucleus. (C) In the amplified lesion, tumor cells exhibit 4-12 *c-MET* gene signals ($\times 1,000$).

logic detail. As we expected, intratumor heterogeneity of *MET* copy number was found within the tumors (Fig. 3).

Tumors with high-level *MET* amplification tended to express higher *MET* protein expression than those with low-

level copy number gain or no copy number gain (S1 Table). *MET* gene copy number was marginally higher for *MET* overexpressed tumors. (mean *MET* gene copy number, 2.2 vs. 1.9). However, no statistically significant correlation was

Table 3. Correlations between *MET* amplification and various clinicopathological parameters of gallbladder cancer patients

Characteristic	No.	Method A			Method B			Method C			Method D		
		Negative	Positive	p-value	Negative	Positive	p-value	Negative	Positive	p-value	Negative	Positive	p-value
Age (yr)													
< 65	47	40 (85.1)	7 (14.9)	0.432	42 (89.4)	5 (10.6)	> 0.99	41 (87.2)	6 (12.8)	> 0.99	42 (89.4)	5 (10.6)	0.759
≥ 65	46	36 (78.3)	10 (21.7)		42 (91.3)	4 (8.7)		40 (87.0)	6 (13.0)		40 (87.0)	6 (13.0)	
Sex													
Male	36	30 (83.3)	6 (16.7)	0.791	31 (86.1)	5 (13.9)	0.301	31 (86.1)	5 (13.9)	> 0.99	31 (86.1)	5 (13.9)	0.745
Female	57	46 (80.7)	11 (19.3)		53 (93.0)	4 (7.0)		50 (87.7)	7 (12.3)		51 (89.5)	6 (10.5)	
Histological grade													
G1, G2	69	60 (87.0)	9 (13.0)	0.038	66 (95.7)	3 (4.3)	0.011	64 (92.8)	5 (7.2)	0.011	65 (94.2)	4 (5.8)	0.006
G3	19	12 (63.2)	7 (36.8)		14 (73.7)	5 (26.3)		13 (68.4)	6 (31.6)		13 (68.4)	6 (31.6)	
T category													
Tis, T1, T2	61	56 (91.8)	5 (8.2)	0.001	59 (96.7)	2 (3.3)	0.007	58 (95.1)	3 (4.9)	0.003	58 (95.1)	3 (4.9)	0.007
T3, T4	32	20 (62.5)	12 (37.5)		25 (78.1)	7 (21.9)		23 (71.9)	9 (28.1)		24 (75.0)	8 (25.0)	
N category													
N0	46	41 (89.1)	5 (10.9)	0.011	45 (97.8)	1 (2.2)	0.003	44 (95.7)	2 (4.3)	0.003	44 (95.7)	2 (4.3)	0.006
N1, N2	33	21 (63.6)	12 (36.4)		25 (75.8)	8 (24.2)		23 (69.7)	10 (30.3)		24 (72.7)	9 (27.3)	
M category													
M0	86	69 (80.2)	17 (19.8)	0.342	77 (89.5)	9 (10.5)	> 0.99	74 (86.0)	12 (14.0)	0.367	75 (87.2)	11 (12.8)	0.593
M1	7	7 (100)	0		7 (100)	0		7 (100)	0		7 (100)	0	
AJCC stage													
I, II	48	46 (95.8)	2 (4.2)	< 0.001	47 (97.9)	1 (2.1)	0.013	47 (97.9)	1 (2.1)	0.001	47 (97.9)	1 (2.1)	0.003
III, IV	45	30 (66.7)	15 (33.3)		37 (82.2)	8 (17.8)		34 (75.6)	11 (24.4)		35 (77.8)	10 (22.2)	
Lymphovascular invasion													
Absent	49	43 (87.8)	6 (12.2)	0.181	46 (93.9)	3 (6.1)	0.466	45 (91.8)	4 (8.2)	0.336	46 (93.9)	3 (6.1)	0.180
Present	43	33 (76.7)	10 (23.3)		38 (88.4)	5 (11.6)		36 (83.7)	7 (16.3)		36 (83.7)	7 (16.3)	
Perineural invasion													
Absent	58	50 (86.2)	8 (13.8)	0.161	55 (94.8)	3 (5.2)	0.067	54 (93.1)	4 (6.9)	0.026	55 (94.8)	3 (5.2)	0.015
Present	33	24 (72.7)	9 (27.3)		27 (81.8)	6 (18.2)		25 (75.8)	8 (24.2)		25 (75.8)	8 (24.2)	

Values are presented as number (%). AJCC, American Joint Committee on Cancer.

Table 4. Univariate Cox regression analysis of variables related to the prognosis of gallbladder cancer

Variable	Recurrence-free survival			Overall survival		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (< 65 yr vs. ≥ 65 yr)	1.075	0.687-1.682	0.750	1.388	0.870-2.214	0.169
Sex (male vs. female)	1.324	0.830-2.112	0.239	1.206	0.745-1.953	0.447
Histologic grade (G1, G2 vs. G3)	1.698	0.923-3.122	0.088	0.813	1.263-2.604	0.001
T category (Tis, T1, T2 vs. T3, T4)	2.464	1.565-3.880	< 0.001	2.539	1.575-4.093	< 0.001
Lymph node metastasis (N0 vs. N1, N2)	2.462	1.470-4.124	0.001	2.749	1.584-4.769	< 0.001
Distant metastasis (M0 vs. M1)	5.639	2.733-11.639	< 0.001	6.157	2.984-12.703	< 0.001
AJCC stage (I, II vs. III, IV)	2.705	1.681-4.353	< 0.001	2.870	1.731-4.759	< 0.001
Lymphovascular invasion (absent vs. present)	2.457	1.545-3.909	< 0.001	2.753	1.698-4.464	< 0.001
Perineural invasion (absent vs. present)	3.045	1.856-4.994	< 0.001	3.643	2.185-6.071	< 0.001
<i>MET</i> amplification (absent vs. present) ^{a)}	1.299	0.668-2.525	0.299	1.667	0.868-3.202	0.125
<i>MET</i> overexpression (score 0-1 vs. score 2-3)	0.935	0.587-1.490	0.777	1.040	0.641-1.688	0.873

AJCC, American Joint Committee on Cancer. ^{a)}By the criteria of Schildhaus et al. [14] (method A).

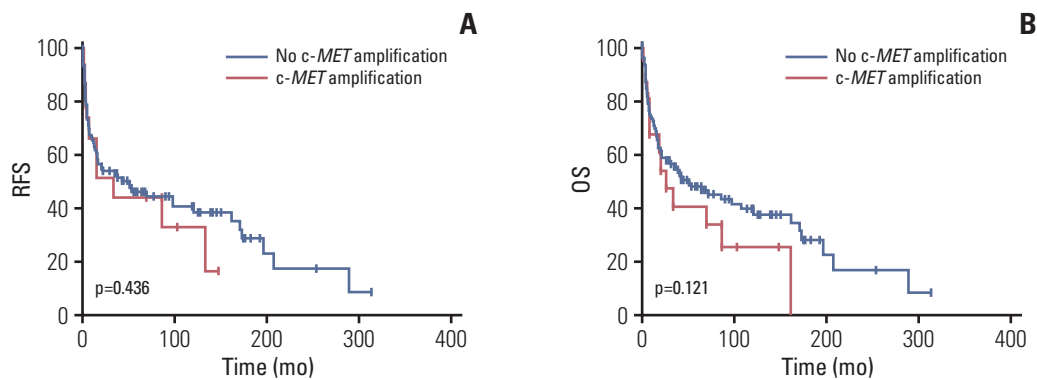


Fig. 4. Kaplan-Meier survival curves of gallbladder cancer patients stratified on the basis of *MET* amplification status. (A) Recurrence-free survival (RFS) according to *MET* amplification (log-rank test, $p=0.436$). (B) Overall survival (OS) according to *MET* amplification (log-rank test, $p=0.121$).

found between *MET* protein overexpression and gene copy number changes.

Targeted sequencing data was available in 17 cases. *MET* copy number ranged from 0 to 9.9 (mean, 2.53). No *MET*-amplified case was detected by NGS method in all 17 cases (S2 Table).

2. Correlation between *MET* overexpression/amplification and clinicopathological characteristics in GBCs

MET protein expression by immunohistochemistry, including the proportion of positive tumor cells, the staining intensity, and IHC scores, were not correlated with any clinicopathological characteristics (S3 Table).

MET amplification by the criteria of Schildhaus et al. [14]

(method A) significantly correlated with higher histological grade ($p=0.038$), advanced T category ($p=0.001$), frequent lymph node metastasis ($p=0.011$), and advanced AJCC stage ($p < 0.001$). *MET*-amplified tumors by the criteria of Capuzzo et al. [15] (method B) showed higher histological grade ($p=0.011$), advanced T category ($p=0.007$), frequent lymph node metastasis ($p=0.003$), and advanced AJCC stage ($p=0.013$). *MET* amplification by the criteria of PathVysion [16,17] (method C) correlated with higher histological grade ($p=0.011$), advanced T category ($p=0.003$), frequent lymph node metastasis ($p=0.003$), presence of perineural invasion ($p=0.026$), and advanced AJCC stage ($p=0.001$). *MET* amplification by the criteria of Ou et al. [18] (method D) correlated with higher histological grade ($p=0.006$), advanced T category ($p=0.007$), frequent lymph node metastasis ($p=0.006$), presence of per-

Table 5. Prevalence of MET overexpression and amplification in previous studies

Study	No. of patients	Detection	Cut-off for MET overexpression	Prevalence of MET overexpression (%)	Clinicopathological correlation
Moon et al. [28]	28 Invasive GBC 8 <i>In situ</i> carcinoma 7 Adenoma	IHC	Staining intensity with 1+, 2+ or 3+, more than 10% of tumor cells	74 of GBCs, especially in the invasive front	Associated with depth of invasion
Nakazawa et al. [25]	89 GBC	IHC FISH	Staining intensity with 2+ or 3+	5.6 No MET amplification detected	No correlation
Sanada et al. [27]	14 Biliary cancers, including 5 GBCs	IHC	Diffuse membranous or cytoplasmic staining in greater than 30%	28.6 of biliary cancers	N/A
Yang et al. [26]	108 GBCs	IHC	Percentage of positive tumor cells with 25% or more	55.6	Associated with differentiation, lymph node metastasis, depth of invasion, poor overall survival
Heo et al. [29]	12 GBC	IHC	Staining intensity with 2+ or 3+, more than 10% of tumor cells	41.7	No correlation

GBC, gallbladder cancer; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; N/A, not available.

ineural invasion (p=0.015), and advanced AJCC stage (p=0.003) (Table 3).

3. Survival analysis of MET expression and gene amplification in GBCs

The median recurrence-free survival (RFS) and OS for all patients were 38.6 months and 64.7 months, respectively. Survival analyses were performed to evaluate the conventional pathological prognostic factors of our cohort. Histological grade (p=0.088 and p=0.001), advanced T stage (both p < 0.001), lymph node metastasis (p=0.001 and p < 0.001), distant metastasis (both p < 0.001), advanced AJCC stage (both p < 0.001), lymphovascular invasion (both p < 0.001), and perineural invasion (both p < 0.001) were revealed as predictors of poor RFS and OS, respectively (Table 4).

Neither MET protein expression nor MET amplification status showed a significant association with patient's survival (Table 4, Fig. 4). However, patients with MET-amplified tumor, defined by criteria of Schildhaus et al. [14] (method A), exhibited shorter OS compared to those with the non-amplified tumor (median OS, 26.8 months vs. 72.3 months). The median RFS of the patients with MET-amplified tumor was also shorter than those with the non-amplified tumor (median RFS, 16.0 months vs. 51.7 months). Similar results were observed with other criteria for MET amplification.

Discussion

MET is a transmembrane protein, which acts as a receptor of HGF. The binding of HGF to the MET protein triggers several downstream signaling pathways, including PI3K/AKT, MAPK, Wnt/ β -catenin, and STAT signaling pathways [19]. Activation of these pathways can drive various biological processes related to cancer, such as cell proliferation, migration, invasion, inhibition of apoptosis, epithelial to mesenchymal transition, and angiogenesis [8,19]. MET was first identified in a human osteosarcoma as an oncogene, in the form of TPR-MET fusion [20]. Then, dysregulation of MET protein and amplification had been found in several human malignancies including gastric [21], colorectal [22], breast [23], and NSCLC [10].

The oncogenic role of MET was also investigated in biliary tumors, including intra- and extrahepatic cholangiocarcinomas and GBCs. MET overexpression has been observed in the neoplastic glandular epithelium of furan-induced rat cholangiocarcinomas [24]. Previous studies with clinical samples have investigated MET expression by IHC and demonstrated that the prevalence of MET overexpression in GBCs

was variable and ranged from 5% to 74% (Table 5) [25-29]. This variability might be due to the difference in primary antibodies used in IHC and interpretation criteria for overexpression. Moon et al. [28] revealed that *MET* overexpression was observed in 74% of the GBC cases and associated with invasion depth and increased staining intensity of *MET* at the invasive front of GBCs, which suggested their important role in tumor invasion [28]. However, Sanada et al. [27] found diffuse membranous staining of *MET* in the intraductal neoplastic components and no staining in the invasive components, suggesting that *MET* overexpression might be related to early events of carcinogenesis rather than tumor progression [27]. Yang et al. [26] found that *MET* overexpression correlated with adverse clinicopathological factors, such as lymph node metastasis, invasion, and decreased OS. In our study, *MET* overexpression was observed in 39.8% of GBCs. The interpretation criteria of overexpression used in this study was adopted from a previous clinical trial, which revealed that dual *MET*/EGFR inhibition was clinically beneficial in NSCLC only in the *MET* overexpression group. However, no significant correlation was observed between *MET* overexpression and clinicopathological features in our GBC cohort. We performed IHC and SISH on whole tissue sections in subset of cases. Although the different *MET* immunoreactivity and gene copy number is observed at different areas within tumor, there is no tendency of increased *MET* immunoreactivity or gene copy number at invasive front as compared to tumor center. Although some previous studies have found no clinicopathological significance of *MET* overexpression [25,29], other studies have shown significant correlation with worse prognostic factors [26,28]. These discrepancies might be due to different ethnicities of cohorts, the definition of overexpression used, and relatively small number of tumor samples.

Only one ISH study on the *MET* gene copy number in GBCs was found in the literature, and it revealed that no amplification was detected in 89 cases of GBCs [25]. The previous studies using NGS data reported that *MET* amplification was rare event in GBCs [30,31]. In our study, no *MET* amplification was detected by NGS method in 17 available cases. However, we found that 18.3% of GBCs showed *MET* amplification by ISH. This discrepancy between ISH and NGS methods for *MET* copy number evaluation may be resulted from intratumoral heterogeneity, contamination of non-neoplastic stromal and immune cells, and different threshold for detecting copy number alteration in high throughput data. However, the most important strength of tissue section based ISH method is that we can discriminate tumor cells from other non-neoplastic components. The tumor cellularity of GBC used in sequencing is generally low and usually less than 50%. In the previous study, there have been reported intratumoral heterogeneity of *MET* expression

in solid tumors [32,33]. We also identified intratumoral heterogeneity of *MET* expression and amplification on whole tissue sections.

MET amplification, regardless of interpretation criteria, showed significant association with the adverse clinicopathological features, including higher histological grade, advanced pT category, frequent lymph node metastasis, and advanced AJCC stage. Moreover, *MET* amplification showed a tendency to shorten OS and RFS, which were not statistically significant.

We investigated the correlation between *MET* protein expression and gene amplification. However, there was no correlation between *MET* protein overexpression and amplification, although the incidence of *MET* overexpression tended to be higher in tumors from patients with *MET* amplification than that in tumors from patients with no amplification. Some studies have shown lack of correlation between *MET* overexpression and gene amplification in HCC and NSCLC [34,35]. These studies and our results suggested that other than gene amplification, there are different mechanisms of protein overexpression. It can be inferred that the other mechanisms, including autocrine and paracrine HGF, increased expression of HGF activator, ligand-independent interactions with other receptors, interactions with other active cell-surface receptors and/or epigenetic regulation might play an important role in *MET* expression without genomic amplification [19,36,37]. Additionally, activation of other oncogenic factors, inactivation of tumor suppressors, such as TP53, and upregulated microRNAs have been known to enhance *MET* transcription [38].

In conclusion, the present study found that *MET* amplification was observed in approximately 20% of the GBCs, and that it was associated with adverse clinicopathological characteristics, such as histological grade, T category, lymph node metastasis, and AJCC stage. Further studies are needed to elucidate the significance of *MET* amplification as a potential therapeutic target and predictive biomarker for anti-*MET*-targeted therapy in GBC patients.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (<https://www.e-crt.org>).

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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